Hypochlorite modified LDL are a stronger agonist for platelets than copper oxidized LDL

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Abstract Experimental low density lipoprotein (LDL) oxidation is usually performed using trace copper, although the in vivo relevance of this method has been called into question. Such LDL augment adenosine 5′-diphosphate (ADP) induced platelet aggregation, presumably by the action of lipid derived compounds. In striking contrast, we find that LDL oxidized to a comparable extent by hypochlorite, an in vivo occurring oxidant, reveal themselves to be potent promoters of platelet aggregation. Interestingly, hypochlorite modified LDL seem to mediate their influence on human platelets by means of the modified apolipoprotein B-100 (apoB) moiety. Also, the finding that hypochlorite modified albumin is able to trigger platelet aggregation suggests an essential role for hypochlorite modified protein(s) in the process of platelet activation.

Key words: Oxidized low density lipoprotein; Hypochlorous acid; Trace metal; Platelet aggregation; Atherosclerosis; Thrombosis

1. Introduction

Oxidative modification dramatically changes the biologic properties of low density lipoprotein (LDL), converting them into a form that is both atherogenic and pro-thrombotic [1–3].

A hallmark of LDL oxidation is the loss of specificity to the apolipoprotein B-100 (apoB) receptor and the emergence of specificity to the so-called scavenger receptor (SR) [4]. Contrary to the expression of the apoB receptor, this receptor is not down-regulated by increased intracellular cholesterol levels. Thus incubation of macrophages with modified LDL leads to an unregulated cellular uptake of lipoproteins and results in massive cholesterol deposition within the cells. As macrophages incubated in this way demonstrate a striking similarity to foam cells, which are characteristic of atherosclerotic lesions, it was concluded at an early date that SRs might play a decisive role in the development of atherosclerosis (for review see [5]).

The formation of high uptake LDL is generally held to be preceded by, and to some extent caused by, peroxidation of the LDL lipid moiety, a process which also results in the formation of numerous lipid derived bioactive compounds. In fact, of the numerous effects attributed to oxidized LDL (OxLDL) (e.g. cytotoxicity [6], chemotaxis [7] or the impairment of different nitric oxide synthase isoforms [8,9]), many seem to be mediated by such lipid derived compounds rather than by direct SR binding of the apoB moiety and subsequent downstream signaling.

Experimentally, LDL oxidation in vitro is usually performed by incubating the lipoproteins with trace copper. Although copper (as well as iron) has been shown to be present in atherosclerotic lesions [10], the significance of trace metal mediated LDL oxidation in vivo has been called into question (recently discussed in review [11]).

In fact, there is little knowledge about the prevalent oxidation mechanism(s) taking place in vivo. At present, direct evidence for the involvement of particular oxidants in (subendothelial) LDL modification is limited to lipoxygenase [12,13], peroxynitrite [14], oxygen centered radicals [15] and hypochlorite [15–17]. Among these, hypochlorite is of special interest as LDLs modified by this agent elementarily differ from LDL oxidized via other pathways. In particular, it has been shown that LDL modification by hypochlorite results in almost exclusive modification of the apoB moiety without inducing the formation of significant amounts of lipid peroxides [18]. Beside semi-stable chloramines, chlorinated derivatives of tyrosine are formed as a specific consequence of hypochlorite interaction with proteins and the identification of chlorinated tyrosine in atherosclerotic lesions provides clear evidence for the in vivo existence of hypochlorite modified (lipo)proteins (reviewed in [19]). Furthermore, monoclonal antibodies against hyp-OxLDL (LDL oxidized by HOCl/OCl^-) have been raised that show specificity for parts of human atherosclerotic lesions; nevertheless, these antibodies *do not* cross-react with copper oxidized LDL [16]. Therefore, LDL modification by hypochlorite results in lipoproteins that elementarily differ from conventional experimentally used copper oxidized LDL and results obtained with this lipoprotein species might have higher in vivo relevance than those obtained with conventionally used copper oxidized LDL.

2. Materials and methods

Carrier-free $Na^{125}I$ was purchased from ICN (Costa Mesa, CA, USA). Maleylated human serum albumin (M-HSA) was prepared as originally described for bovine albumin [20]. Essentially fatty acid-free human albumin was purchased from Sigma (Vienna, Austria). Electrophoretic mobility of native and modified LDL under non-denaturing conditions was assessed by agarose electrophoresis [21]. Thiobarbituric acid reactive substances (TBARS) were assessed as described

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Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized low density lipoprotein; M-HSA, maleylated human serum albumin; hyp-OxLDL, low density lipoprotein oxidized by HOCl/OCl^-; Cu-OxLDL, copper oxidized low density lipoprotein; ADP, adenosine 5′-diphosphate; apoB, apolipoprotein B-100; f.c., final concentration

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[22]. NaOCl was from Sigma-Aldrich (Vienna, Austria) and was standardized at 290 nm using a molar extinction coefficient of 350 M^{-1} cm^{-1} [23] and freshly diluted in phosphate buffered saline.

2.1. Lipoproteins

Each step was performed at 4°C and ethylenediaminetetraacetic acid (EDTA) was added to all solutions (except preceding copper oxidation), resulting in a final concentration (f.c.) of 0.1 mM, to prevent inadvertent oxidation by trace metal ions. Lipoproteins were used immediately after preparation. Native LDL were rebuffered immediately before use (100 mM borate buffer, 0.1 mM EDTA, 50 mM NaCl, pH 7.3). Unless otherwise stated, rebuffering was performed by gel filtration (Econo-Pac 10DG Columns, Bio-Rad, CA, USA).

Concentrations of lipoproteins are expressed in terms of their protein content as determined by the Lowry method [24].

2.2. Preparation of native LDL

LDL were isolated from normal human plasma (anticoagulated with 1/10 volume of 3.8% (w/v) trisodium citrate) immediately after blood sampling by sequential centrifugation at $100\,000 \times g$ in the density range $d = 1.019$–1.063 g/ml [25]. After flotation, the lipoproteins were filtered (0.45 μm).

2.3. Oxidation of LDL

LDL oxidation by copper ions was performed according to a commonly used protocol. After exhaustive dialysis of LDL against borate buffer (100 mM, pH = 7.2), the LDL (at a concentration of 200–250 μg/ml) were incubated with $CuSO_4$ (f.c. 5 μM) at 37°C in the same buffer. After 24 h the incubation was stopped and the lipoproteins were concentrated to approximately 2 mg/ml using ultrafree-15 units with a molecular weight cut-off of 100 000 (Millipore, Vienna, Austria). Subsequently, OxLDL were rebuffered (100 mM borate buffer, 0.1 mM EDTA, 50 mM NaCl, pH 7.3). REM of copper oxidized LDL was 1.95 ± 0.2 ($n = 5$). Preliminary studies showed that the electrophoretic mobility increased over the 24 h of incubation at 37°C, although the resulting increase in REM remained the same even when copper concentrations were varied between 1 μM and 20 μM.

Hypochlorite modification of LDL was basically performed according to Arnhold et al. [26]. In brief, native LDL were transferred to sodium borate buffer (100 mM, containing 0.1 mM EDTA, pH = 7.2). At 0°C, HOCl/NaOCl was added to LDL while rapidly and gently mixing. There was a direct linear correlation between the amount of oxidant used and the electrophoretic mobility of the resulting hypochlorite modified LDL. For the comparative studies described herein, the amount of hypochlorite for LDL oxidation was chosen such that the same electrophoretic mobility was achieved as LDL from the same donor oxidized by copper. As a result, an approximately 300-fold molar excess of hypochlorite over LDL was employed. After 15 min, the possibly remaining oxidant was removed by gel filtration (100 mM borate buffer, 0.1 mM EDTA, 50 mM NaCl, pH 7.3). Hyp-OxLDL were always stored on ice and used within 1 week.

2.4. Reversibility of LDL modification

Two hours after hypochlorite modification, the lipoproteins were treated with the indicated amounts of ascorbate. After 10 min at room temperature, the lipoproteins were rebuffered.

2.5. Radioiodination

Radioiodination of LDL was performed by the Iodo Bead method using *N*-chloro-benzenesulfonamide conjugated polystyrene beads (Pierce, Rockford, IL, USA) following the manufacturers instructions. The specific radioactivity of the labeled OxLDL species ranged from 150 to 250 cpm/ng protein.

2.6. Isolation of human platelets

Human platelets were isolated from freshly drawn blood as previously described [27]. Briefly, blood was anticoagulated with 1/10 volume of 3.8% (w/v) trisodium citrate and centrifuged immediately at $120 \times g$ for 20 min to yield platelet-rich plasma. Prostacyclin (PGI_2) was added at a f.c. of 25 μg/l. Platelets were pelleted at $800 \times g$ for 5 min and resuspended with Tyrode buffer without Ca^{2+} (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5.5 mM D-glucose, 3.5 g/l human serum albumin, 25 μg/l PGI_2, pH 6.5). This washing procedure was repeated twice and final resuspension of the platelet pellet was performed with Tyrode buffer containing 2 mM $CaCl_2$, pH 7.35, without PGI_2. After isolation, the platelet suspension was immediately chilled on ice for at least 30 min before performing the binding assays or kept at room temperature for aggregation studies.

2.7. Aggregation studies

Platelet aggregation was determined in a 490-4D four-channel aggregometer (Chronolog Corp., Havertown, PA, USA). Transmission was measured between 0% and 100% based on values obtained with platelet suspension and suspension medium, respectively. Platelet suspensions (adjusted to 2×10^5 cells/μl in a final volume of 0.5 ml) were supplemented with purified human fibrinogen (0.2 g/l, f.c.) and human albumin (3.5 g/l, f.c.). Suspensions were stirred for 3 min in siliconized glass cuvettes in the aggregometer (without further additions or with M-HSA) before adding the aggregating agents. As a control, adenosine 5′-diphosphate (ADP) induced aggregation (5 to 30 μM) was performed and the experiments were discontinued after the first decline of reactivity was observed.

2.8. Binding and competition studies

Binding and competition studies were performed on ice due to the extreme potency of hyp-OxLDL in triggering platelet aggregation. Albumin was present throughout the incubation procedure (2 h) at a concentration of 0.1%. After incubation with the labeled lipoproteins, platelet suspensions were centrifuged through a prechilled 1:3 dilution of Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) in saline. The supernatants (containing the unbound lipoprotein fraction) were aspirated and the tips containing the pellets (containing the platelet bound LDL fraction) were cut off using a hot wire [27].

Bound and unbound fractions were counted in a Cobra II gamma counter (Canberra Industries, Meriden, CT, USA).

Non-specific binding was determined by adding a 20-fold excess of the respective unlabeled LDL to the incubation mixture and was usually between 10% and 20% of total binding.

All experiments were performed with LDL from at least three different donors. Results are expressed as mean values ± standard deviation (S.D.) of at least four experiments. Aggregation studies were performed with platelets from five different donors the results of which were basically identical. Aggregation tracings shown in Figs. 3 and 4 represent the results of two such experiments.

3. Results and discussion

Oxidatively modified LDL have been implicated in the development of atherosclerosis, a disease that is accompanied by platelet hyperreactivity and severe thrombotic disorders. These effects on platelets could also in part be the result of a direct interaction between oxidized LDL and human platelets, as OxLDL have been shown to stimulate human platelets in vitro [28–37]. As most studies dealing with the interaction of OxLDL and human platelets have been performed with copper oxidized LDL, it was the aim of this work to study the influence on human platelets of hyp-OxLDL, a presumably naturally occurring form of LDL modification, and to directly compare the results with those obtained with copper oxidized LDL.

Incubation of native LDL with trace copper following a commonly used protocol (see Section 2) resulted in an approximately 2-fold increase of the electrophoretic mobility of the lipoproteins (REM = 1.95 ± 0.2, $n = 5$) and significant accumulation of TBARS (9.25 ± 2.5 vs. 0.45 ± 0.15 nmol/mg for native LDL, $n = 5$). Treatment of LDL with reagent hypochlorite resulted in an increase of the electrophoretic mobility of hyp-OxLDL that was strictly dependent on the amount of hypochlorite added (not shown). In contrast to oxidation by trace copper, LDL modification by hypochlorite did not result in the formation of TBARS, which is in accordance with

recently published results [26]. In order to obtain adequate data for the comparison of Cu-OxLDL (copper oxidized LDL) and hyp-OxLDL, hypochlorite was added to native LDL such that the REM values were virtually identical to those obtained with Cu-OxLDL (see Section 2).

As shown in Fig. 1, apoB modification (measured as REM) of hyp-OxLDL [18,38], but not that of Cu-OxLDL, can be reversed in a dose dependent way by the subsequent addition of ascorbate, although high levels of antioxidant are needed for the reversal of such a high degree of modification. The time frame in which the reversibility of hypochlorite modification of LDL was possible covered a period of several days from the moment of modification onward, provided that the hyp-OxLDL were constantly stored at 4°C.

Binding experiments revealed that both hyp-OxLDL and Cu-OxLDL show specific and saturable high affinity binding to the membrane of human platelets with virtually identical affinities ($K_d = 5.75 \pm 0.45$ μg/ml and 6.04 ± 0.2 μg/ml for Cu-OxLDL and hyp-OxLDL, respectively). As depicted in Fig. 2, binding of both modified LDL species is not influenced by either native LDL or fucoidan (a selective inhibitor of OxLDL binding to class A SRs). In contrast, M-HSA, an inhibitor of OxLDL binding to all currently classified SRs, reduces lipoprotein binding to background values. These results indicate an exclusive involvement of receptors with properties of class B SRs in the binding of both lipoprotein species, which is in accordance with previously published results obtained with Cu-OxLDL [39].

Despite similar binding and competition patterns, hyp-OxLDL and Cu-OxLDL dramatically differ with regard to their impact on platelet function. As shown in Fig. 3, Cu-OxLDL, despite being able to augment ADP induced platelet aggregation, are not capable of independently triggering an aggregation response (investigated to f.c. of 1 mg/ml). In contrast, hyp-OxLDL modified to a comparable extent, reveal themselves to be potent promoters of platelet aggregation (Fig. 3, tracing (f)). In fact, the impact of hyp-OxLDL on platelet function turns out to be far stronger than that of any other LDL modification reported thus far, as final lipoprotein concentrations of approx. 20 μg/ml are sufficient to achieve a maximal aggregation response (not shown). The cyclooxygenase pathway is obviously not involved in platelet aggregation triggered by hyp-OxLDL, as acetylsalicylic acid is not able to reduce this effect. In contrast, both PGI_2 and theophylline are able to impair this type of aggregation re-

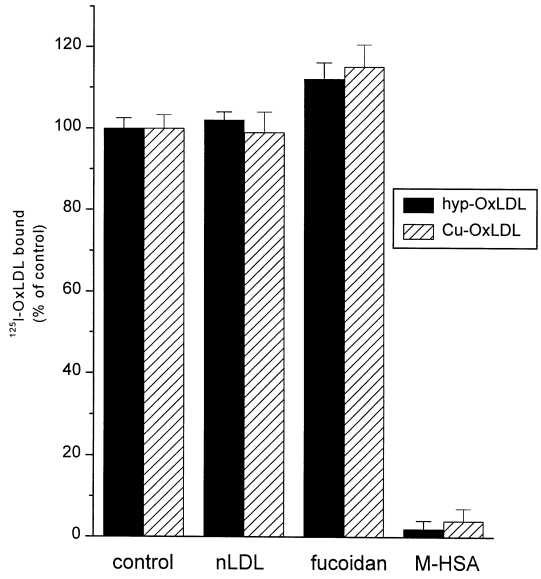

Fig. 2. Specific binding of radioactive labeled oxidized LDL (10 μg/ml) to human platelets in the presence of native LDL (200 μg/ml), fucoidan and M-HSA (50 μg/ml each). Data represent the mean of four experiments in duplicate ± S.D.

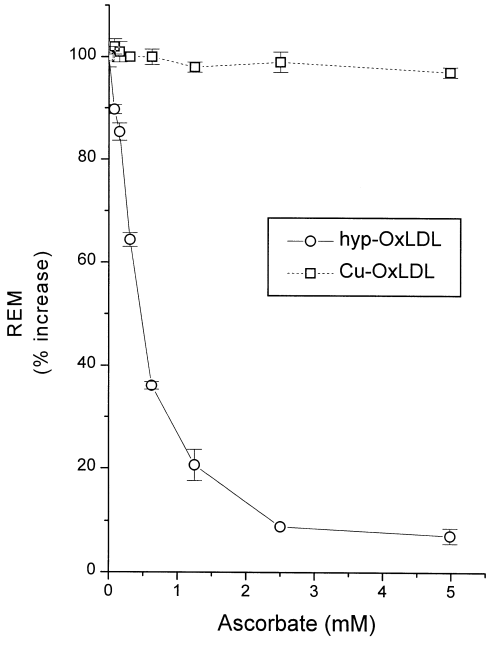

Fig. 1. Relative electrophoretic mobility of hyp-OxLDL and Cu-OxLDL after treatment with the indicated amounts of ascorbate. Each data point represents the mean of five experiments (with LDL from different donors) ± S.D.

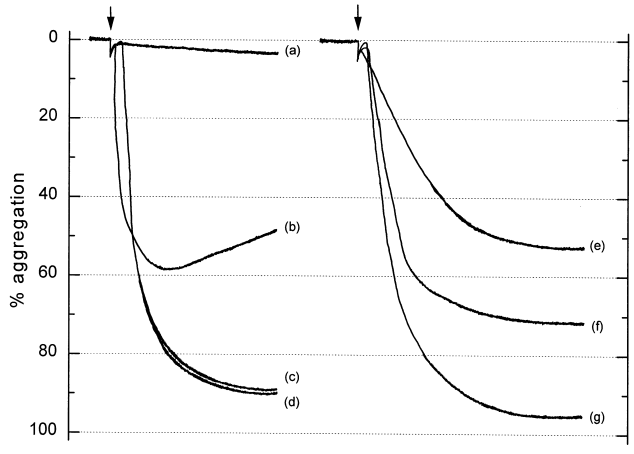

Fig. 3. Influence of hyp-OxLDL and Cu-OxLDL on platelet aggregation: (a) Cu-OxLDL, (b) ADP, (c) Cu-OxLDL+ADP, (d) Cu-OxLDL+ADP in the presence of M-HSA (50 μg/ml); f.c. for Cu-OxLDL and ADP were 100 μg/ml and 10 μM, respectively. (e) Hypochlorite modified albumin (f.c. 100 μg/ml, 100-fold molar excess of NaOCl over albumin), (f) hyp-OxLDL (15 μg/ml, 300-fold molar excess of NaOCl over LDL), (g) hyp-OxLDL (10 μg/ml, 400-fold molar excess of NaOCl over LDL).

sponse in a dose dependent manner (not shown), therefore ruling out the possibility that the observed effects result from agglutination of platelets.

Regarding LDL modified by copper and other means, sensitization of human platelets by oxidatively modified LDL has been reported in most cases but contradictory reports exist as to whether these lipoproteins are able to independently trigger platelet aggregation [28–31] or not [32–37].

In cases where a self-aggregatory effect has been attributed to oxidatively modified LDL, lipoprotein modification was achieved by oxidation with copper ions [28–30] and peroxynitrite [30], respectively, or by autooxidation [31]. In any case, the threshold lipoprotein concentration used in the above cited works for triggering platelet aggregation was at least 160 μg/ml (f.c.) and up to 2 mg/ml of lipoprotein were required to achieve maximal aggregation response, which is some two orders of magnitude higher than the concentrations obtained here for hyp-OxLDL.

Reports exist that platelet aggregation can be evoked by minimally modified LDL (presumably due to lipid mediators capable of stimulating human platelets) and that lipoproteins do not possess this biologic property when fully oxidized [30,31]. Interestingly, such an inverse correlation between the degree of lipoprotein oxidation and platelet stimulatory effects obviously is not true for LDL modification by hypochlorite, as the modified lipoproteins used in the present work clearly represent heavily oxidized LDL (displaying an approx. 2-fold increase in their REM). A further increase of the molar excess of hypochlorite over LDL results in greater electrophoretic mobility of the resulting LDL and this is accompanied by a higher potency for stimulating human platelets (see Fig. 3, tracing (g)). On the other hand, lipoproteins displaying a lower degree of apoB modification – as a result of using lower amounts of hypochlorite (not shown) or following subsequent treatment with ascorbate – are consequently less potent in triggering platelet aggregation (shown in Fig. 4, tracings (a)–(e)).

Thus, the close relationship between the extent of apoB modification and the capability of hyp-OxLDL to trigger platelet aggregation might be an argument for an essential role of the hypochlorite modified apoB moiety in this process of platelet activation. The hypothesis that protein modification by hypochlorite can result in the acquisition of platelet stimulatory properties is also strengthened by the finding that even hypochlorite modified albumin is able to trigger platelet aggregation (depicted in Fig. 3, tracing (e)). Furthermore, addition of M-HSA in concentrations that are able to block specific hyp-OxLDL binding to the platelet surface results in a dose dependent inhibition of platelet aggregation induced by these lipoproteins (shown in Fig. 4, tracings (f)–(j)). Therefore, we consider it likely that the effects of hyp-OxLDL on platelet function arise from modification of apoB by hypochlorite and that these effects are a direct consequence of specific binding of the modified apoB moiety to the platelet surface. Nevertheless, as seemingly unrelated ligands such as albumin and LDL acquire platelet stimulatory effects after modification by hypochlorite, it should be taken into account that the resulting effects might arise from the interaction of semi-stable chloramines with the platelet surface. As the decomposition of chloramines is known to result in the formation of free radicals [40], such decomposition might be accelerated by exoenzymes located on the platelet surface and might yield

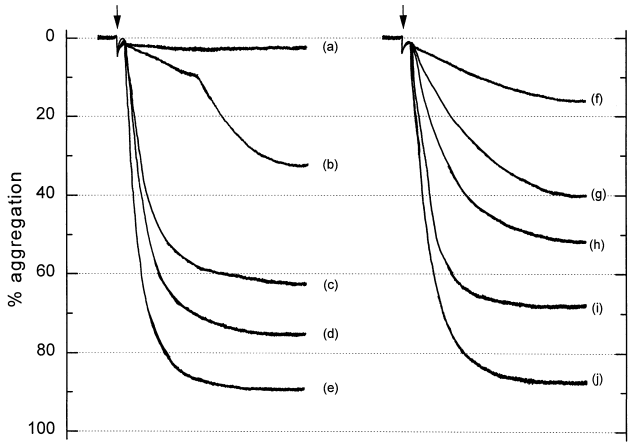

Fig. 4. Platelet aggregation induced by: hyp-OxLDL (20 μg/ml, 300-fold molar excess of NaOCl over LDL) pretreated with ascorbate: (a) 1.25 mM, (b) 625 μM, (c) 156 μM, (d) 78 μM, (e) 0 μM; hyp-OxLDL (20 μg/ml, 300-fold molar excess of NaOCl over LDL) in the presence of M-HSA: (f) 40 μg/ml, (g) 20 μg/ml, (h) 10 μg/ml, (i) 3 μg/ml, (j) without M-HSA. Both ascorbate and M-HSA alone did not influence ADP induced platelet aggregation.

radicals that exert direct influence on the reactivity of human blood platelets.

Apart from the fact that Cu-OxLDL modified to a comparable extent are not able to trigger an aggregation response, there are also fundamental differences to hyp-OxLDL regarding the results obtained with ascorbate and M-HSA: treatment of Cu-OxLDL with ascorbate fails to show any effect on aggregation tracings obtained in the presence of both ADP and these lipoproteins (not shown). While this lack of influence on the aggregation response might be expected as the electrophoretic mobility of Cu-OxLDL is not influenced by prior treatment with ascorbate, blocking of specific binding of Cu-OxLDL to the platelet surface by M-HSA also fails to show any normalizing effect on the observed augmented aggregation response in the presence of ADP (previously shown in [37] and depicted in Fig. 3, tracing (d)). These results are in accordance with the assumption that some types of oxidized LDL mediate their influence on human platelets independently of specific binding of the apoB moiety, i.e. probably by means of one or more oxidation derived lipid mediators capable of stimulating human platelets [31,41].

Nevertheless, the finding that the apoB moiety of Cu-OxLDL apparently fails to exert any effect on platelet function poses the intriguing possibility that the in vivo occurring oxidant hypochlorite (but *not* trace copper) is able to modify apoB such that upon binding to distinct receptors on the platelet surface it is able to trigger specific post binding events, which are responsible for the observed effects.

Although in vivo conditions might result in more complex modification of the LDL particle than that obtained with reagent hypochlorite (e.g. due to the combined action of $HOCl/OCl^-$ and H_2O_2 [42] or the formation of peroxynitrite and free radicals), the results shown here in any case demand a critical reassessment of the method of choice for experimental LDL oxidation.

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