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Modulation of LDL Oxidation by 7,8-Dihydroneopterin

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Human macrophages stimulated with interferon-γ generate neopterin and 7,8-dihydroneopterin which interfere with reactive species involved in LDL oxidation. While neopterin was found to have pro-oxidative effects on copper-mediated LDL oxidation, the influence of 7,8-dihydroneopterin is more complex. This study provides detailed information that 7,8-dihydroneopterin reveals both pro-oxidative and anti-oxidative effects on copper mediated LDL oxidation. 7,8-dihydroneopterin inhibited the oxidation of native LDL effectively monitored by (i) formation of conjugated dienes, (ii) relative electrophoretic mobility (EM) and (iii) specific oxidized epitopes. Using minimally oxidized LDL (mi-LDL) or moderately oxidized LDL (mo-LDL) 7,8-dihydroneopterin changed its antioxidative behavior to a strongly pro-oxidative. Incubation of 7,8-dihydroneopterin with native LDL, mi-LDL or mo-LDL in the absence of copper ions showed that formation of conjugated dienes was more increased in mo-LDL than in mi-LDL while no diene formation was observed with native LDL.

We suggest that 7,8-dihydroneopterin is a modulator for LDL oxidation in the presence of copper ions depending on the "oxidative status" of this lipoprotein.

Keywords: 7,8-Dihydroneopterin; Lipid hydroperoxides (LPO); Lipid peroxidation; Electrophoretic mobility (EM); Free radicals; Atherosclerosis

INTRODUCTION

Oxidation of LDL has been proposed as a biological process that initiates and accelerates development of atherosclerotic lesions. Oxidized LDL accumulates in lesions and may form at other inflammatory sites and stimulate thrombotic processes.[1–5] LDL oxidation can be initiated by endothelial cells, smooth muscle cells and monocytes/macrophages in the presence of trace amounts of free metal ions like copper generating peroxyl radicals in the LDL core.[6] Peroxyl radicals react immediately with polyunsaturated fatty acids to form hydroperoxides which will be decomposed to aldehydes, like 4-hydroxynonenal (HNE), malondialdehyde (MDA), and others.[7,8] HNE and MDA were shown to interfere with the apolipoprotein B part of LDL resulting in oxidatively modified LDL (oxLDL).[9] OxLDL itself has chemotactic properties acting on monocytes and macrophages which generate scavenger receptors recognizing oxLDL.[10–11] This leads to uncontrolled uptake of oxLDL by macrophages via the scavenger receptors resulting in the formation of foam cells, a hallmark in early atherosclerosis.[3] It is well known that cupric ions catalyse lipid peroxidation *in vitro* but the presence of free copper or iron ions *in vivo* is unlikely. A recent review described ceruloplasmin (Cp), a copper-containing acute phase plasma protein, as a potent oxidant in monocyte-macrophage mediated LDL oxidation.[12] Zymosan, endotoxin and interferon-γ are well known activators stimulating monocyte-macrophage Cp production but they also stimulate synthesis of cell derived factors like peroxynitrite, lipoxygenase or superoxide radical which reduce Cp bound copper to copper ions.[13–15] Superoxide radicals alone have been shown not to be sufficient enough for LDL oxidation by monocytes-macrophages.[16,17] Therefore, in some studies the authors speculate that unknown cell derived activators are necessary for monocyte-macrophage-mediated LDL oxidation.[12]

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In humans and primates, 7,8-dihydroneopterin and neopterin are compounds generated also by interferon-γ stimulated macrophages.[18] While elevated neopterin was found in human blood and urine during viral infections, various malignant disorders, autoimmune diseases and allograft rejection episodes,[18–24] little is known about the physiological role of 7,8-dihydroneopterin. Two studies showed a linkage between copper and/or peroxynitrite mediated lipid peroxidation of LDL and neopterin derivatives.[25,26] While for neopterin a slightly pro-oxidative effect on copper mediated LDL oxidation was found, the role of 7,8-dihydroneopterin in the metal-dependent oxidation of LDL is still unclear. A pro- and an anti-oxidative effect of 7,8-dihydroneopterin on copper mediated LDL oxidation was described recently.[26] Here we show for the first time that in the presence or absence of copper ions 7,8-dihydroneopterin protects native LDL against oxidation but in the case of minimally and moderately oxidized LDL (mi-LDL and mo-LDL), the anti-oxidative role of this pteridine is converted into a pro-oxidative role.

MATERIALS AND METHODS

Isolation of LDL from Human Plasma

Lipoproteins were isolated from plasma of healthy fasting young human donors, with serum lipoprotein (a) levels lower than 1 mg/dl.[27] Kallikrein inactivator (Aprotinin; 100.000 U/l; Bayer, Germany), Pefabloc (11.2 mg/l; Merck, Germany) and DTPA [diethylen-triamine-pentaacetic acid] (1 g/l; Merck, Darmstadt, Germany) were present during all steps of lipoprotein preparation to prevent lipid peroxidation and apo B cleavage by contaminating bacteria or proteinases. LDL (1.019–1.063 g/ml) was obtained by sequential ultracentrifugation by adjusting density with KBr. LDL protein was measured by the method of Lowry *et al.*, with BSA (Sigma) as standard and stored sterile-filtered at 4°C.

Neopterin and 7,8-dihydroneopterin

Neopterin and 7,8-dihydroneopterin (Dr Schircks Laboratories, Jona, Switzerland) stock solutions (1 mM) were prepared in 0.1 mol/l phosphate buffer (pH 7.4, containing 20 μM EDTA) by sonication.

Oxidation of LDL

During storage of native LDL lipid peroxidation could take place. Therefore, we estimated the amount of lipid hydroperoxides (LPO) of each LDL sample before use.[28] Freshly prepared LDL contained 0–2 nmol LPO/mg LDL (native LDL), minimally oxidized LDL (mi-LDL) 6–15 nmol LPO/mg LDL and mo-LDL $>$ 15 nmol LPO/mg LDL.

Prior to oxidation, native LDL, mi-LDL and mo-LDL were dialysed against 0.1 mol/l phosphate buffer (pH 7.4, containing 20 μM EDTA), which was carefully degassed and then saturated with nitrogen. Neopterin and 7,8-dihydroneopterin (100 μM) or 0.1 mol/l phosphate buffer (pH 7.4, containing 20 μM EDTA) were added to the LDL solutions by gentle mixing. Cu^{2+}-mediated oxidation of LDL (44 μg/ml) was started at 37°C with 50 μM $CuSO_4$ recording the conjugated dienes at a mean absorption maximum of 234 nm on a U2000 spectrophotometer (Hitachi, Tokyo, Japan). The absorption values were corrected by the respective specimen without LDL at each time point.[26]

At intervals between 0 and 140 min LDL aliquots were taken and oxidation was terminated by adding a stop solution to achieve a final concentration of EDTA of 0.27 mM. The samples were saturated with nitrogen and stored at 4°C for the estimation of the electrophoretic mobility (EM) and for the determination of oxidation specific epitopes with the solid phase fluorescence immunoassay.[29]

The degree of modification of these copper oxidized LDL aliquots was estimated as the relative EM, i.e. relative to native LDL, on 1% agarose gel (Owl Easycast electrophoresis chamber, Portsmouth, U.S.A.) for 60 min at 100 V in a barbital buffer of pH 8 (9 g/l diethylbarbituric acid-sodium salt, 4.23 g/l sodiumacetate, 1.25 g/l citric acid monohydrate, 0.08 g/l sodiumazide). Gels were fixed and stained with phosphotungstic acid.[26]

Determination of Oxidation Specific Epitopes

To investigate the influence of neopterin and 7,8-dihydroneopterin on the formation of oxidation-specific epitopes on Cu(II) oxidized LDL an assay was performed as follows.[29] For the binding assay, microtitration plates (Nunc, Roskilde, Denmark) were coated with 200 μl of copper oxidized LDL aliquots (2.2 μg/ml) in 10 mM phosphate buffer (pH 7.4) containing 1 g/l EDTA at 4°C overnight. After washing three times with washing buffer (10 mM phosphate buffer, pH 7.4, containing 9 g/l NaCl, 0.2 g/l Tween 20) using a microtitration plate washer (Wallac Oy, Turku, Finland), 300 μl of blocking buffer (10 mM phosphate buffer, 1 g/l EDTA, 10 g/l BSA pH 7.4) was added to the wells to block the remaining binding sites for 90 min at 25°C. The wells were then washed three times and 200 μl of OB/04—a monoclonal antibody specifically recognizing epitopes newly formed

on apo B containing lipoproteins upon oxidation (1:200 dilution) or rabbit-anti-human-apo B (1:10000 dilution) in assay buffer pH 7.4 (10 mM phosphate buffer, 1 g/l EDTA) were incubated for 1 h at 25°C. After three washes the amount of bound antibodies was detected by adding 200 ng/ml anti-mouse IgG or anti-rabbit-IgG labelled with europium (DELFIA, Eu^{3+}-labeling kit 1244-302, Pharmacia) according to the manufacturer's description. After 1 h incubation at 25°C plates were washed for six times and 200 μl of enhancement solution (Pharmacia) per well was added to measure the fluorescence counts with a Victor (Wallac Oy). Results are expressed with the parameter OB/04/apoB (counts/counts).

Oxidation of LDL in Presence of Metal Chelators

Formation of conjugated dienes of native LDL, mi-LDL and mo-LDL incubated with 7,8-dihydroneopterin was measured in absence of metal ions by chelating with 20 μM EDTA or 20 μM EDTA and 20 μM DTPA. Incubation of native LDL, mi-LDL and mo-LDL (44 μg/ml) was started at 37°C by adding 100 μM 7,8-dihydroneopterin either in 10 mM phosphate buffer pH 7.4 containing 20 μM EDTA or 10 mM phosphate buffer containing 20 μM EDTA and 20 μM DTPA and conjugated dienes were measured at 234 nm as described above.

Statistical Analysis

A two-sided Student's *t*-test was performed for testing the significance of differences with $p < 0.01$ as the criterion for significance and $p < 0.001$ for very high significance.

RESULTS

Influence of Neopterin Derivatives on the Formation of Conjugated Dienes in Native LDL, mi-LDL and mo-LDL during Copper Mediated Oxidation

Recording the generation of conjugated dienes we found an antioxidative effect of 7,8-dihydroneopterin during a copper mediated oxidation of native LDL (Fig. 1a). On the other hand neopterin exerted a slight pro-oxidative effect. While neopterin had a slight pro-oxidative effect in mi-LDL, too, 7,8-dihydroneopterin showed a high pro-oxidative effect as shown in the formation of conjugated dienes (Fig. 1b). In the presence of 7,8-dihydroneopterin no lag time of mi-LDL oxidation was measured. Formation of conjugated dienes reached maximal absorption within 45 min during oxidation in the presence of 7,8-dihydroneopterin.

Using mo-LDL shorter lag times were found in the absence of any additive (Fig. 1c) compared to native and mi-LDL. 7,8-dihydroneopterin again strongly enhanced the formation of conjugated dienes (Fig. 1c) resulting in maximal absorption after 30 min; neopterin acted slightly pro-oxidative.

Influence of Neopterin Derivatives on the EM of Native LDL, mi-LDL and mo-LDL during Copper Mediated Oxidation

The EM of native LDL increased during copper mediated oxidation without further additives, as expected (Fig. 2a). The presence of neopterin led to a moderate but significant increase of the EM at least after 90 min incubation. In contrast, in the presence of 7,8-dihydroneopterin the increase of the EM induced by Cu^{2+} was completely inhibited. Using mi-LDL for copper mediated oxidation the EM was increasing during time, too. Neopterin was again slightly pro-oxidative, but this effect was not statistically significant. 7,8-dihydroneopterin, however, exerted a strong enhancing effect concerning EM (Fig. 2b). After 140 min incubation the EM of mi-LDL was 45% higher in the presence compared to the absence of 7,8-dihydroneopterin. The EM of mo-LDL dramatically increased within the first 35 min in the presence of 7,8-dihydroneopterin, while no further increase was obtained after 90 and 140 min (Fig. 2c). Neopterin also showed a slight increase in the EM of mo-LDL after 35 and 90 min compared to the control. At 140 min the EM of mo-LDL oxidized in the presence of neopterin was comparable to the control incubation.

Influence of Neopterin Derivatives on the Formation of Oxidatively Modified Epitopes of Native LDL, mi-LDL and mo-LDL during Copper Mediated Oxidation

Specific epitopes on native LDL were generated after 90 min and increased within 140 min (Fig. 3a). Neopterin showed a slight pro-oxidative effect on the generation of specific epitopes during copper mediated oxidation of native LDL between 90 and 140 min, while the formation of epitopes was completely inhibited in the presence of 7,8-dihydroneopterin at all time points.

Using mi-LDL 7,8-dihydroneopterin amplified the generation of epitopes of mi-LDL after 35 min and reached maximal OB/04/apoB ratio within 140 min as shown in Fig. 3b. The OB/04/apoB ratio of mi-LDL oxidized in the presence of 7,8-dihydroneopterin for 35 min was four times higher than the ratio for mi-LDL with neopterin. The formation of epitopes on mi-LDL during copper initiated oxidation in the presence of neopterin was slightly enhanced compared to the control without neopterin.

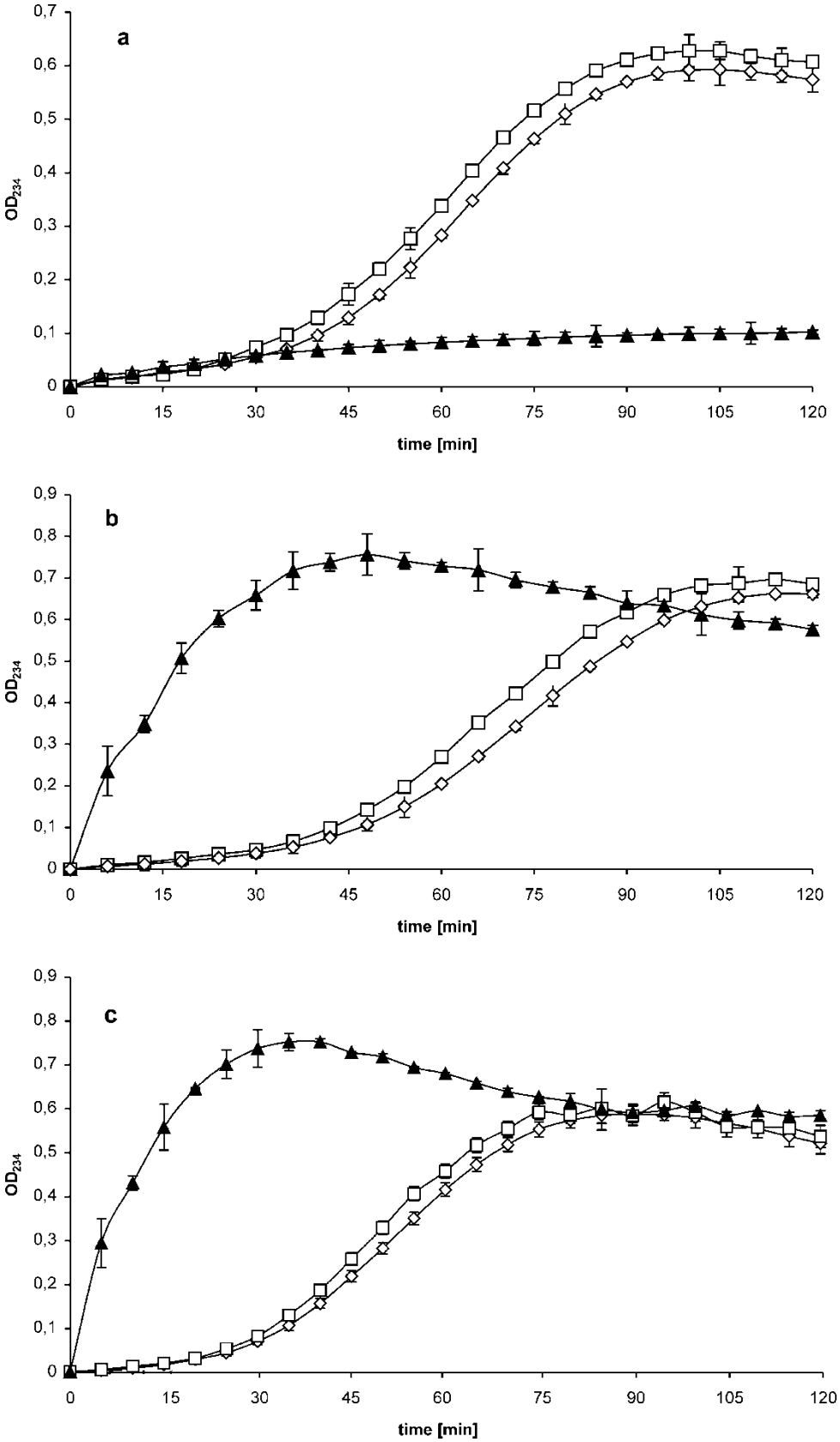

FIGURE 1 Influence of neopterin (100 μMi; □), 7,8-dihydroneopterin (100 μMi; ▲) and no additives (◇) on Cu(II)-mediated (50 μM Cu(II)/20 μM EDTA) LDL oxidation (44 μg/ml LDL protein) of (a) native LDL, (b) mi-LDL and (c) mo-LDL. Formation of conjugated dienes was monitored by measuring $OD_{234\,nm}$. Values are the means and S.E.M. of three experiments in triplicate.

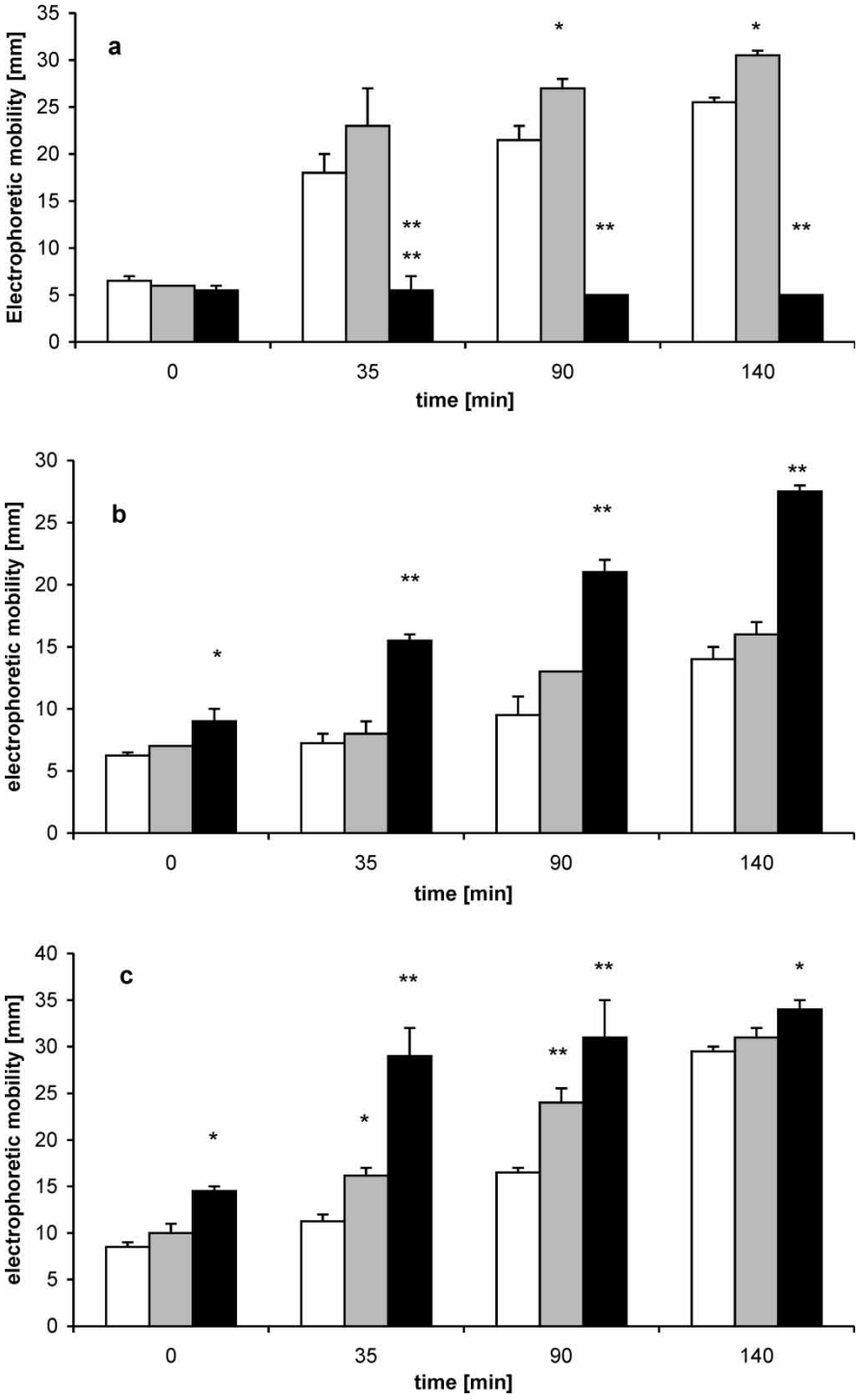

FIGURE 2 The EM during Cu(II)-mediated (50 μM Cu(II)/20 μM EDTA) LDL oxidation (44 μg/ml LDL protein) of (a) native LDL, (b) mi-LDL and (c) mo-LDL in the presence of neopterin (100 μM; grey bars) or 7,8-dihydroneopterin (100 μM; black bars) or absence of neopterin derivatives (open bars). Values are the means ± S.E.M. of three experiments in duplicate. Values marked with asterisks differ significantly (*$p < 0.01$) or very high significantly (**$p < 0.001$) from the corresponding control value without neopterin derivatives.

Figure 3c shows the formation of epitopes on mo-LDL during copper mediated oxidation. In the presence of 7,8-dihydroneopterin epitope formation starts immediately and reaches its maximum already after 35 min. On the other hand, using neopterin the formation of epitopes is similar to the control incubation.

Influence of 7,8-dihydroneopterin on Native LDL, mi-LDL and mo-LDL in the Absence of Copper Ions

7,8-dihydroneopterin was described to have radical promoting activity without addition of free metal ions.[30] Therefore, we investigated the influence of

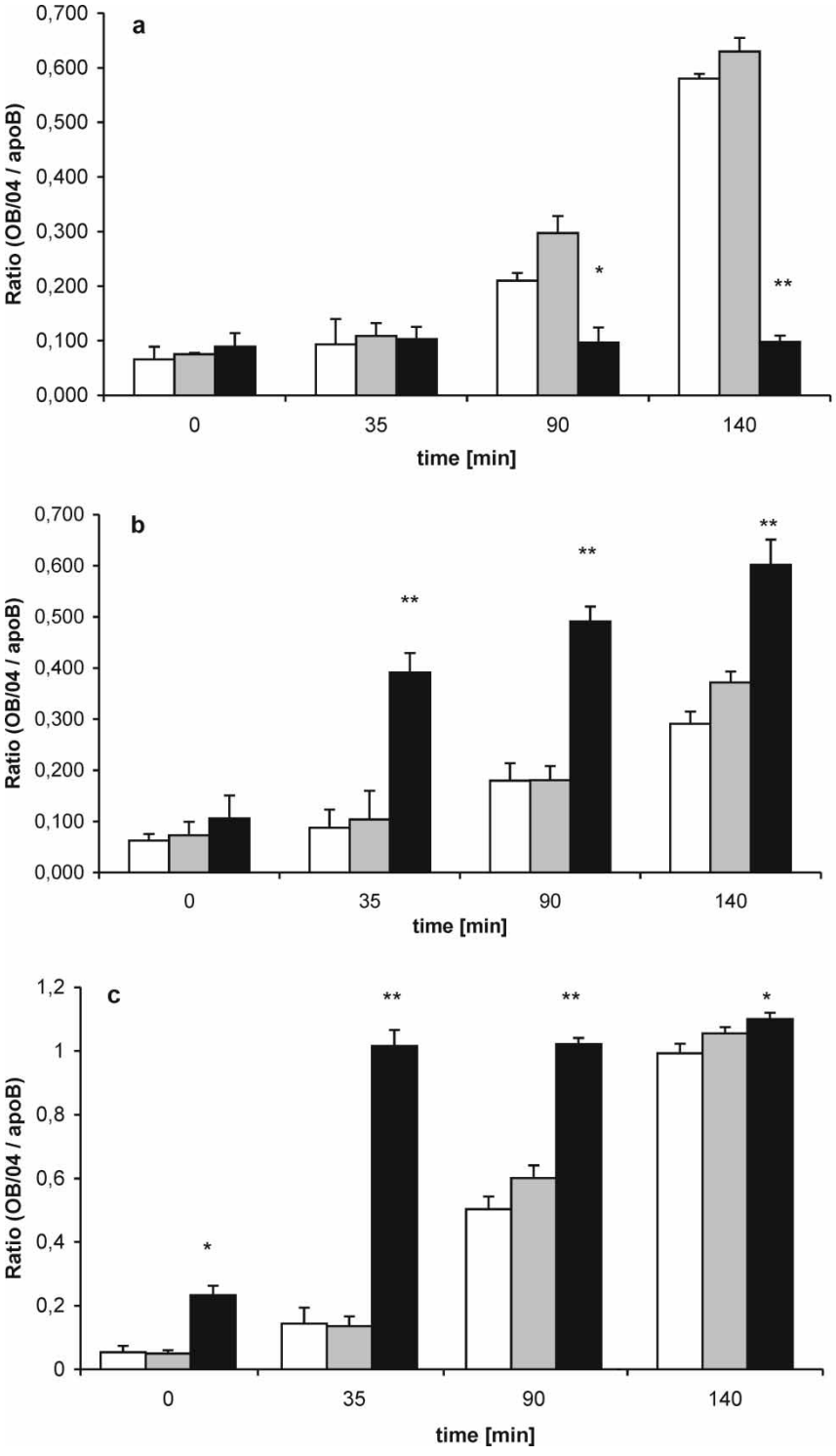

FIGURE 3 Effect of neopterin and 7,8-dihydroneopterin upon copper mediated oxidation of LDL. The ratio of immunoreactivity against monoclonal antibody OB/04 to polyclonal anti-apolipoprotein B (OB/04/apoB) was measured during copper (II) (50 μM Cu(II)/20 μM EDTA) mediated LDL oxidation (44 μg/ml LDL protein) of (a) native LDL, (b) mi-LDL and (c) mo-LDL in the presence of neopterin (100 μM; grey bars) or 7,8-dihydroneopterin (100 μM; black bars) or in absence of neopterin derivatives (open bars). Values are the means ± S.E.M. of three experiments in triplicate. Values marked with asterisks differ significantly (*$p < 0.01$) or very high significantly (**$p < 0.001$) from the corresponding control value without neopterin derivatives.

7,8-dihydroneopterin on native LDL, mi-LDL and mo-LDL measuring the formation of conjugated dienes in presence of DTPA/EDTA or EDTA. The time dependent formation of conjugated dienes in native LDL, mi-LDL and mo-LDL initiated by 7,8-dihydroneopterin is shown in Fig. 4. Native LDL showed no increase in the formation of conjugated dienes in the presence of 7,8-dihydroneopterin either

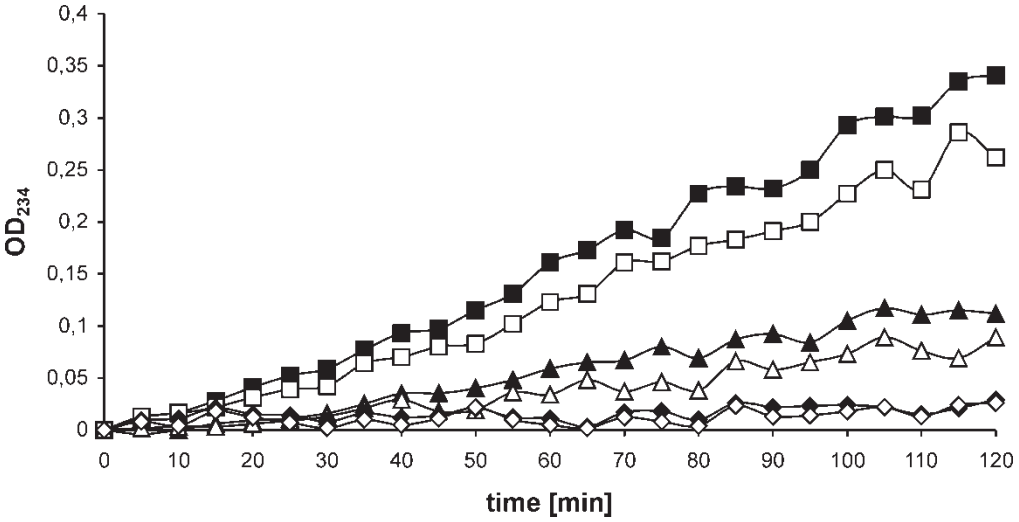

FIGURE 4 Effect of 7,8-dihydroneopterin (100 μM) on LDL oxidation (44 μg/ml protein). Formation of conjugated dienes was measured during incubation of 7,8-dihydroneopterin with native LDL (◇), mi-LDL (△) and mo-LDL (□) containing either 0.1 M PBS pH 7.4 + 20 μM EDTA (filled symbols) or 0.1 M PBS pH 7.4 + 20 μM EDTA and 20 μM DTPA (open symbols). Values are the means ± S.E.M. of two experiments in duplicate.

with EDTA/DTPA or EDTA alone. Absorbance of conjugated dienes of both mi-LDL and mo-LDL was higher in the presence of EDTA compared to EDTA/DTPA.

DISCUSSION

7,8-dihydroneopterin was shown to inhibit both, Cu(II) and AAPH [2,2′-azobis(2-amidinopropane)-hydrochloride] mediated LDL oxidation presumably by scavenging peroxyl radicals.[25,26] Our data completely agree with these findings using native LDL. The use of pre-oxidized LDL, like mi-LDL and mo-LDL, reversed the protective effect of 7,8-dihydroneopterin completely resulting in a highly pro-oxidative action. In contrast, neopterin remains slightly prooxidative as it is with native LDL. The kinetic curve of the formation of conjugated dienes in presence of 7,8-dihydroneopterin of mi-LDL and mo-LDL is different from the "classic" curve as obtained with native LDL: there is no lagtime at all and the increase of diene formation during the propagation phase is more pronounced compared to the control oxidation. Gieseg *et al.*,[25] described a direct interaction of 7,8-dihydroneopterin with lipid peroxyl radicals during copper or AAPH mediated oxidation of LDL acting as a scavenger of peroxyl radicals. These findings correlate with our observations as far as native LDL is concerned. Previous studies described the effect of metal ions and 7,8-dihydroneopterin in promoting hydroxyl radical formation in the absence of other radical sources.[30] In solutions containing copper and 7,8-dihydroneopterin the generation of hydroxyl radicals within few minutes was detected.[31,32] These findings could well explain the pro-oxidative effect 7,8-dihydroneopterin exerts on pre-oxidized LDL. An anti-oxidative or pro-oxidative effect of 7,8-dihydroneopterin on copper mediated oxidation of LDL was also observed when using different ratios of 7,8-dihydroneopterin to Cu(II) [7,8-dihydroneopterin/Cu(II)].[26] This could be the key to explain our observations: after reducing Cu(II) to Cu(I) 7,8-dihydroneopterin prevents the re-oxidation of Cu(I) to Cu(II) by small amounts of LPO, which are necessary in the mechanism of LDL oxidation.[10] The amount of LPO in native LDL used in our experiments was between 0–2 nmol LPO/mg LDL, which is not able to initiate further oxidation of native LDL. After increasing the content of LPO in LDL using mi-LDL (6–15 nmol LPO/mg LDL) or mo-LDL (>15 nmol LPO/mg LDL) re-oxidation of Cu(I) to Cu(II) obviously takes place resulting in the formation of additional LPO in the "LDL particle". Again 7,8-dihydroneopterin reduces Cu(II) to Cu(I) for further lipid peroxidation.

Under these conditions 7,8-dihydroneopterin obviously supports lipid peroxidation of mi-LDL or mo-LDL, until the amount of 7,8-dihydroneopterin is completely consumed to 7,8-dihydroxanthopterin. Addition of 7,8-dihydroneopterin to such an oxidation mixture again started the formation of conjugated dienes (data not shown). Therefore, 7,8-dihydroneopterin, but not 7,8-dihydroxanthopterin or other reaction products, is able to amplify lipid peroxidation of mi-LDL or

mo-LDL in the presence of copper. Neopterin will not be consumed during copper mediated LDL oxidation, which in contrast to 7,8-dihydroneopterin is not a reducing agent. The slightly pro-oxidative effect of neopterin is caused by its potential in oxidizing Cu(I) to Cu(II) as it was discussed earlier.[26]

We also have to include the possibility of the reduction of protein and peptide hydroperoxides to reactive free radicals by 7,8-dihydroneopterin. Headlam *et al.*,[33] described the reduction of protein or peptide LPO via trace transition metal ions and cell-surface reduction systems, such as trans-plasma membrane electron transport chain, which are present in a wide range of mammalian cell types including monocytes or macrophages. Ascorbate loaded THP-1 cells showed an increase of protein hydroperoxides and protein radicals compared to THP-1 cells in presence of trace metal ions. Similar to ascorbic acid 7,8-dihydroneopterin is a strong reducing agent and therefore, a possible source for inducing protein hydroperoxides in cells but also in mi-LDL and mo-LDL.

The concentrations of 7,8-dihydroneopterin found in body fluids were much lower than used in our experiments. Thus, we can only speculate that either the local 7,8-dihydroneopterin concentration in the vicinity of stimulated macrophages may be much higher than the levels in the circulating blood or 7,8-dihydroneopterin is consumed after it's secretion by monocytes/macrophages by reaction with copper ions revealing 7,8-dihydroxanthopterin or other derivatives. However, little is known about their *in vivo* concentrations so far.

We suggest a possible *in vivo* role of 7,8-dihydroneopterin as one of the discussed cell-derived factors for macrophage-monocyte LDL oxidation in the presence of copper ions.[16,34] On the other hand, 7,8-dihydroneopterin seems to "distinguish" between native and circulating minimally oxidatively modified LDL.[35,36] It is known that binding and uptake of oxidized LDL by the various scavenger receptors on monocytes/macrophages is much higher for moderately or heavily oxidized LDL compared to mi-LDL. Therefore, 7,8-dihydroneopterin may have a regulatory effect on the oxidation of LDL by monocyte-macrophage outside the cells thereby enhancing oxidation of mi-LDL to a form which can be recognized more easily by the scavenger receptor.

Acknowledgements

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