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Neopterin and 7,8-Dihydroneopterin Interfere With Low Density Lipoprotein Oxidation Mediated by Peroxynitrite and/or Copper

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Low density lipoprotein (LDL) oxidation within the artery wall likely represents a key event in the formation of atherosclerotic lesions. Oxidatively modified LDL particles exert chemotactic properties on macrophages, and the uncontrolled uptake of modified LDL by macrophages leads to the formation of lipid-loaded foam cells, a hallmark of early stage atherosclerosis. Human macrophages stimulated by interferon-γ generate reactive oxygen species (ROS), neopterin, and 7,8-dihydroneopterin. Higher concentrations of neopterin were found in atherosclerosis, and earlier studies have provided evidence that these neopterin derivatives are able to interfere with reactive species. We therefore investigated whether they also modulate LDL oxidation mediated by Cu(II) and/or peroxynitrite ($ONOO^-$). By means of UV-absorption recording the formation of conjugated dienes in the course of lipid oxidation as well as by measuring the relative electrophoretic mobility of oxidized LDL, we found that neopterin is capable of enhancing $ONOO^-$- as well as Cu(II)-mediated LDL oxidation, whereas 7,8-dihydroneopterin mainly protects LDL from oxidation. However, in case of Cu(II)-mediated LDL oxidation, an initial prooxidative effect of 7,8-dihydroneopterin could be observed. We hypothesize that 7,8-dihydroneopterin may chemically reduce Cu(II) to Cu(I) thereby increasing its oxidative capacity. After total reduction of Cu(II), excess 7,8-dihydroneopterin may block the oxidative potential of Cu(I) and thus decrease the oxidation of LDL.

These findings confirm the general behavior of pteridines in redox processes and suggest an *in vivo* contribution to the process of LDL oxidation.

Keywords: Neopterin; 7,8-Dihydroneopterin; Copper; Peroxynitrite; Lipid oxidation; Atherosclerosis

INTRODUCTION

Oxidation of low density lipoprotein (LDL) is considered to be one of the key events in the pathogenesis of atherosclerosis.[1] Oxidatively modified LDL particles are found in atherosclerotic lesions.[2,3] They are cytotoxic to endothelial cells,[4] and they stimulate inflammatory[5] and thrombotic processes.[6] Mainly two types of chemical reactions play an essential role in LDL oxidation: (1) lipid peroxidation generating peroxyl radicals[7,8] which will destroy in a chain reaction a great number of unsaturated lipids involving molecular oxygen. Oxidation products are a various pattern of hydroperoxides and aldehydes as to be malondialdehyde. (2) Concomitant with lipid peroxidation, chemical modification of the apolipoprotein (apo) B moiety occurs[9,10] converting LDL particles to a ligand which has chemotactic properties to monocytes and macrophages.[10,11] Scavenger receptors[11] of macrophages recognize oxidatively modified LDL particles and mediate their uncontrolled uptake, resulting in the formation of foam cells, a cellular hallmark of the early onset of atherosclerosis.[12] LDL oxidation can be initiated by the major cells of the artery wall including endothelial cells, smooth muscle cells and macrophages,[13] and it depends on the oxidation state of LDL which again is influenced by e.g. the ratio poly-/mono-unsaturated fatty acids[5] or by its

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intrinsic antioxidative capacity.[7,10,14,15] Macrophages originating from the differentiation of monocytes in the intima play a key role in LDL oxidation,[16] and oxidized LDL, in turn, is immunogenic.[17] Therefore, activation of the immune system seems to be closely associated with LDL oxidation, and immune activation is usually associated with oxidative stress. LDL oxidation can be induced by free radicals and/or by transition metal ions. Mainly peroxynitrite[18] and, respectively, its decomposition products, e.g. hydroxy radical, have been discussed to be responsible for LDL modification,[19] but it is also initiated by iron or copper (Cu(II))-ions[20] alone or in combination with peroxynitrite ($ONOO^-$).[21] Little is known about the *in vivo* relevance of free Cu(II), yet it could be shown that peroxynitrite mediates its release from caeruloplasmin.[22] However, also intact caeruloplasmin is able to oxidize LDL.[23] Peroxynitrite is probably generated by monocytic cells as part of their cytotoxic repertoire.[24] Inducible nitric oxide synthase (iNOS) as well as nitrotyrosine could be identified within human atherosclerotic lesions.[25–27] Thus, the formation of $ONOO^-$ in atherosclerotic lesions is likely, especially in later stages of atherogenesis when immunocompetent cells are crucially involved. Besides that, it could be shown that other reactive nitrogen species such as nitrogen dioxide radicals, derived from the myeloperoxidase reaction (which is also present and active in atherosclerotic lesions), may also contribute to the atherogenic process.[28] In addition, interferon-γ-activated monocytic cells produce large amounts of neopterin and 7,8-dihydroneopterin.[29] The influence of these neopterin derivatives on oxidation processes and signal transduction pathways mediated by reactive species has been demonstrated.[30–33] 7,8-Dihydroneopterin has also been found to interfere with LDL oxidation.[34] In atherosclerosis, increased serum neopterin concentrations were actually measured,[35,36] indicating an involvement of immunological mechanisms.

In this study, we further investigated whether these neopterin derivatives are able to influence peroxynitrite- and/or copper-mediated LDL oxidation. Finally, a possible *in vivo* contribution of neopterin derivatives to the process of LDL oxidation will be discussed.

MATERIALS AND METHODS

Isolation of LDL from Human Plasma

Only blood with Lp(a) < 5 mg/dl was selected. Subjects fasted for at least 12 h before 300 ml blood was collected in 1 M Tris-buffer (Merck, Darmstadt, Germany), and plasma was obtained by 15 min centrifugation at 3000 rpm. Plasma was subjected to a second centrifugation at 4000 rpm for 15 min. Afterwards the plasma was adjusted with sodium bromide to a density of 1.063 g/ml and centrifuged for 24 h at 57,000 rpm. The upper layer consisting of VLDL and LDL was removed and filled up to 160 ml with 0.1 M Tris-buffer (pH 7.4, +0.9% NaCl and 12 mg/l Pefabloc (Merck), a proteinase inhibitor). The density was adjusted to 1.050 g/ml with NaBr and the suspension was re-centrifuged for 24 h (57,000 rpm). Again the upper layer was isolated and filled up to 160 ml with 0.1 M Tris-buffer (pH 7.4 + 12 mg/l Pefabloc), thus yielding a density of 1.020 g/ml. Then it was centrifuged for 24 h (57.000 rpm) in order to separate VLDL from LDL. The lower layer consisting of LDL was dialyzed against 0.1 M Tris-buffered saline (pH 7.4, +0.9% NaCl, 20 μM ascorbic acid). The LDL solution was sterile filtered and stored at 4°C. The LDL content was determined by protein assay according to Lowry,[37] assuming that protein was 22% (w/w) from total LDL mass. All Tris-buffered solutions contained 2 mM EDTA. Immediately before use, the LDL solution was finally dialyzed against 0.1 M phosphate buffer (pH 7.4, +20 μM EDTA).

Three LDL preparations from three donors have been used either separately or pooled (1:1:1).

Solutions for LDL Oxidation

Isolated LDL was diluted to a concentration of 44 μg/ml LDL protein (for measuring the formation of conjugated dienes as well as for measuring the relative electrophoretic mobility (REM)) with 0.1 M phosphate buffer containing 20 μM EDTA, pH 7.1 for peroxynitrite incubation, and pH 7.4 for copper-mediated oxidation. Neopterin and 7,8-dihydroneopterin (Dr. Schircks Laboratories, Jona, Switzerland) stock solutions (1 mM) were prepared in phosphate buffer by sonication. For incubation, their concentrations ranged from 20 to 150 μM. For preparation of Cu(II)-stock solution, 20 mM $CuCl_2$ was dissolved in bidistilled water. $ONOO^-$ was prepared according to Hughes and Nicklin[38] by incipient mixing of equal volumes of 0.7 M H_2O_2-solution in 0.6 M HCl and of 0.6 M $NaNO_2$ on ice and immediate termination of the reaction with 1.5 M NaOH. H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. $ONOO^-$ concentration was determined spectrophotometrically in 1 M NaOH at 302 nm (extinction coefficient: 1670 $M^{-1}\,cm^{-1}$).[38]

Oxidation of LDL was initialized by gentle mixing of the respective solutions. For volume compensations of controls, the proper buffer solutions were used. All concentrations used for $ONOO^-$- and Cu(II)-mediated oxidation were optimized in

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foregoing experiments to yield reliably measurable oxidation products.

Assessment of LDL Oxidation and the Impact of Neopterin and 7,8-Dihydroneopterin by Measuring the Formation Rate of Conjugated Dienes

During LDL oxidation, lipid oxidation products with conjugated dienes develop,[39] having a mean absorption maximum around 234 nm (extinction coefficient: 29,500 $M^{-1} cm^{-1}$). The time-dependent generation of dienes was recorded on a U2000 spectrophotometer (Hitachi, Tokyo, Japan). At each time point of every experiment the absorption values $E_{234\,nm}$ were measured also without LDL (but with all the other parameters being the same), and these values were subtracted from the absorption values obtained with LDL. In some experiments, a preincubation of LDL with neopterin or with 7,8-dihydroneopterin for 20 min at room temperature under light protection was undertaken before addition of the oxidants in order to enable the diffusion of pteridines into the LDL particles. During incubation, the cuvettes were not covered to ensure a sufficient oxygen supply along with LDL oxidation. The incubation temperature was 37°C.

Conjugated diene formation begins with a lag phase where no or only a slow rate oxidation occurs due to the intrinsic antioxidants scavenging the oxidative assault. The following propagation phase is characterized by a rapid generation of conjugated dienes reaching a maximum of formed conjugated dienes. Thereafter a decrease of absorption is obtained followed by a second increase. The latter goes along with an increase of degradation products (e.g. unsaturated carbonyls) of lipid peroxides absorbing in the 234 nm range.[10] The LDL oxidation rate is recorded by the length of the lag phase, the slope of the propagation phase and the height of the maximum.

Assessment of LDL Oxidation and the Impact of Neopterin and 7,8-Dihydroneopterin by Measuring the Relative Electrophoretic Mobility (REM)

In later stages of LDL oxidation, the positive charges of the apo B moiety, especially the ε-amino group of lysine, are continuously compensated by oxidation products. Hence, since the positive charge of the ε-amino group of lysine is abolished, the net charge negatively increases in dependence of the oxidation rate. Therefore, the quotient (migration distance of oxidized LDL/migration distance of untreated LDL) is a proportional measure for oxidation rate.[10]

During the measurement of conjugated dienes of LDL at 37°C, aliquots of the samples were withdrawn. In those aliquots, the reaction was stopped by incubation on ice and freezing at −20°C, and the

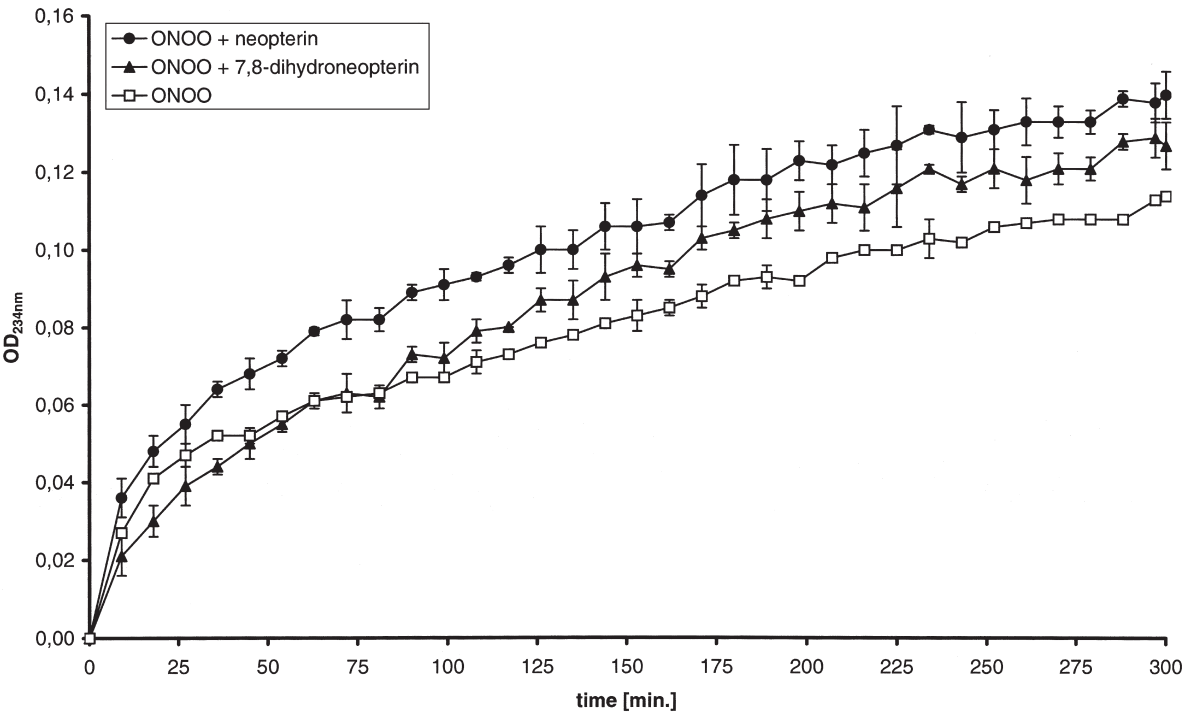

FIGURE 1 Influence of neopterin and 7,8-dihydroneopterin on $ONOO^-$-mediated LDL oxidation monitored by the formation of conjugated dienes. LDL was pooled from preparations of three donors (1:1:1) at a concentration of 44 μg/ml LDL protein. The concentration of neopterin and of 7,8-dihydroneopterin was 20 μM, the concentration of $ONOO^-$ was 1 mM. Absorption values were normalized by subtracting the values obtained without LDL ($ONOO^-$ and/or its decomposition products with or without pteridines) at each time point measured. Values are the means and the bars represent the standard deviations of three experiments.

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electrophoretic mobility was determined immediately by electrophoresis using 1% agarose gels. Electrophoretic runs were performed at room temperature for 90 min at 200 V in a barbital buffer pH 8. Gels were fixed and stained with polyphosphowolframate. All results were related to the mobility of the non-oxidized control sample in each electrophoretic run.

RESULTS

Influence of Pteridines on LDL Oxidation Monitored by the Formation of Conjugated Dienes

LDL Oxidation Induced by $ONOO^-$

Figure 1 demonstrates the effects of neopterin and 7,8-dihydroneopterin on LDL oxidation induced by

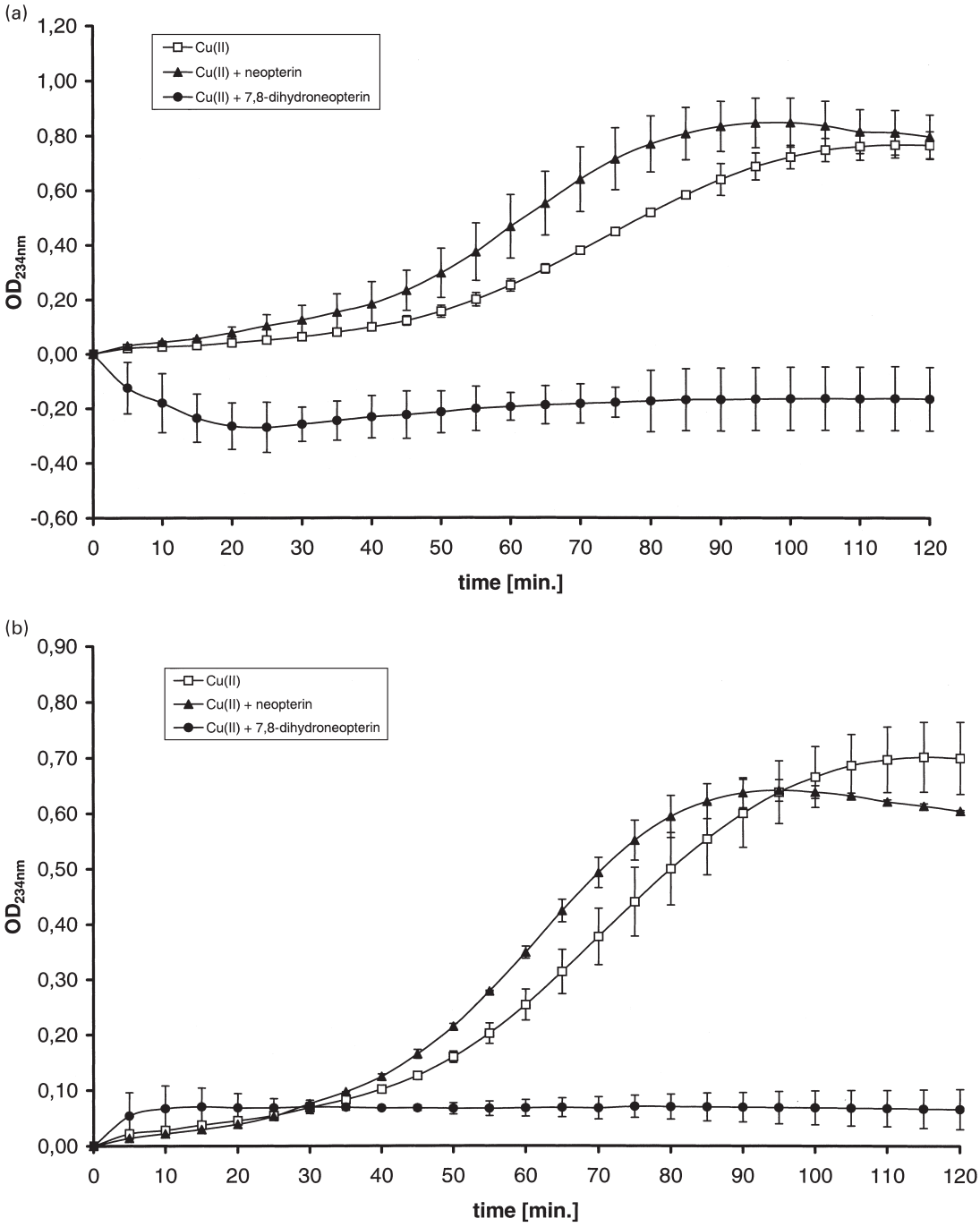

FIGURE 2 Influence of neopterin and 7,8-dihydroneopterin on copper-mediated LDL oxidation monitored by the formation of conjugated dienes. LDL was pooled from preparations of three donors (1:1:1) at a concentration of 44 μg/ml LDL protein. The concentration of neopterin and of 7,8-dihydroneopterin was 100 μM. Copper was added as 50 μM Cu(II)/20 μM EDTA. Absorption values were normalized by subtracting the values obtained without LDL (50 μM Cu(II)/20 μM EDTA with or without pteridines) at each time point measured. Experiments with coincubation of pteridines are shown in (a), experiments with preincubation of pteridines for 20 min are shown in (b). Values are the means and the bars represent the standard deviations of three experiments.

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$ONOO^-$. LDL was pooled from preparations of three donors (1:1:1), and each experiment was performed three times. Immediately after addition of $ONOO^-$, absorption of the incubation mixture increased due to the formation of conjugated dienes. This was true for all incubations. No relevant lag phase was observed. Neopterin had a prooxidative effect on LDL. The absorption curve, indicative for conjugated diene formation, was constantly higher for the experiments with $ONOO^-$ + neopterin compared to $ONOO^-$ alone. In contrast, 7,8-dihydroneopterin decreased the formation of conjugated dienes within the first 60 min of incubation. However, this antioxidative effect turned into a prooxidative one after 90 min.

Cu(II)-mediated LDL Oxidation

The time dependent formation of conjugated dienes in LDL during a copper-mediated (50 μM Cu(II)/20 μM EDTA) oxidation is shown in Fig. 2. Like in the experiments shown in Fig. 1, LDL was pooled from preparations of three donors (1:1:1), and each experiment was performed three times. Figure 2(a) depicts the data obtained with coincubation, and Fig. 2(b) shows the results obtained with preincubation of the pteridines with LDL. In both cases, neopterin accelerated the formation of conjugated dienes. The prooxidative effect of neopterin can be read by both, a steeper slope (Fig. 2(a) and (b)) and a higher absorption maximum (Fig. 2(a)) in comparison to the control incubations without neopterin. However, in case of preincubation, 7,8-dihydroneopterin showed a slight increase in conjugated dienes for the first 30 min before preventing very efficiently the formation of conjugated dienes (Fig. 2(b)). In the experiments with coincubation, 7,8-dihydroneopterin effectively suppressed the formation of conjugated dienes at any measured time point (Fig. 2(a)).

LDL Oxidation by Cu(II)/$ONOO^-$

The Cu(II)-mediated LDL oxidation described above was enhanced by the presence of $ONOO^-$, and neopterin further increased the formation of conjugated dienes, whereas 7,8-dihydroneopterin again was inhibitory (data not shown). In this set of experiments, three LDL preparations from three donors were investigated (Fig. 3(a)–(c), Table I). The prooxidative effect of coincubation of LDL with neopterin (100 μM) was displayed at several concentrations of $ONOO^-$. The rate of conjugated diene formation for $ONOO^-$ was concentration dependent, the lag time decreased with increasing $ONOO^-$-concentrations, though in two of three cases (Fig. 3(a) and (b)) the maximum absorption for 30 μM $ONOO^-$ was lower than for 10 and for 3 μM $ONOO^-$. In general, neopterin had a slight promoting effect on the formation of conjugated dienes, but it showed variations depending on individual LDL preparations and on $ONOO^-$-concentrations. In Fig. 3(a), the enhancement of LDL oxidation by neopterin was most strongly pronounced at a 10 μM $ONOO^-$-concentration, in Fig. 3(c) at a 30 μM $ONOO^-$-concentration, whereas in Fig. 3(b), the effect of neopterin was rather weakly expressed at all $ONOO^-$-concentrations.

All results were essentially the same when the experiments were carried out with preincubations of LDL with neopterin (data not shown).

The Influence of Pteridines on LDL Oxidation Indicated by the Electrophoretic Mobility

Measurements of the enhanced electrophoretic mobility of oxidized LDL relative to native LDL (REM) (indicating a higher negative net charge of the lipoprotein) are another way of following oxidative modifications of LDL. In all these experiments, the results obtained with three LDL preparations from three donors as well with the pooled LDL (1:1:1) were combined. The LDL concentration in these experiments was the same as in the experiments where conjugated dienes were measured.

TABLE I Characteristics of the formation of conjugated dienes concomitant with Cu(II)-mediated LDL oxidation under influence of $ONOO^-$ and neopterin. Values are derived from a representative experiment illustrated in Fig. 3(a). Cu(II)-concentrations were 50 μM, neopterin concentrations were 100 μM

Incubation	Lag-phase time (min)	Max. of CD formation rate (nM/min)	Maximum of CD formation (μM)
Cu(II) + 0 μM $ONOO^-$	42.7	2.02	19.1
Cu(II) + 3 μM $ONOO^-$	17.4	1.76	19.7
Cu(II) + 3 μM $ONOO^-$ + Neo	17.6	2.24	24.0
Cu(II) + 10 μM $ONOO^-$	3.8	2.04	19.2
Cu(II) + 10 μM $ONOO^-$ + Neo	0	2.36	21.7
Cu(II) + 30 μM $ONOO^-$	1.6	2.67	16.6
Cu(II) + 30 μM $ONOO^-$ + Neo	1.0	2.98	16.9

CD: conjugated dienes; Neo: neopterin

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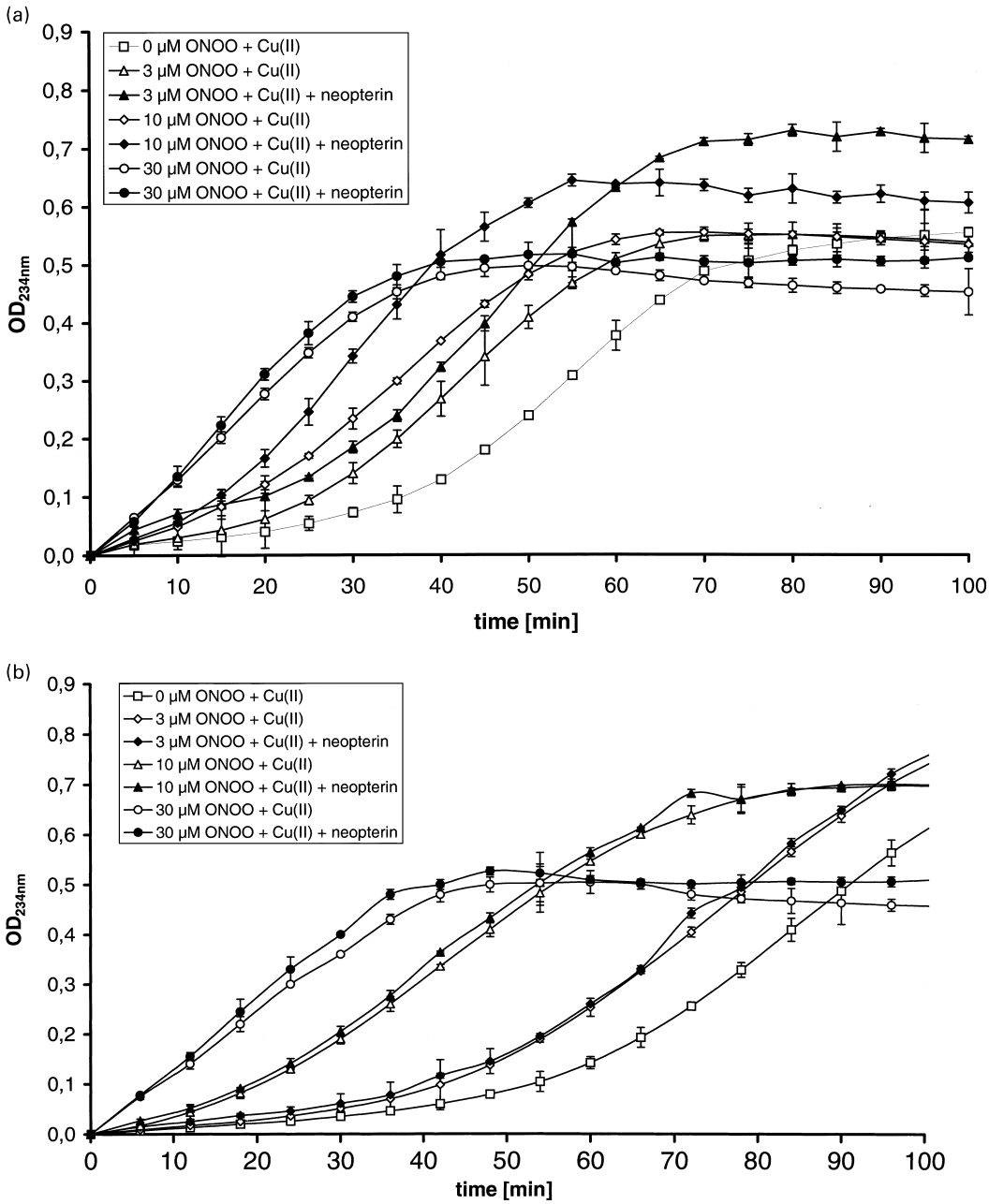

FIGURE 3 Influence of neopterin on Cu(II)-mediated LDL oxidation at coincubation with different concentrations of $ONOO^-$ monitored by the formation of conjugated dienes. LDL from three LDL preparations from three donors (a, b, c) were used at a concentration of 44 µg/ml LDL protein. Neopterin was present at a concentration of 100 µM. Copper was added as 50 µM Cu(II)/20 µM EDTA. The concentrations of $ONOO^-$ ranged between 0 and 30 µM. Absorption values were normalized by subtracting the values obtained without LDL (50 µM Cu(II)/20 µM EDTA with or without $ONOO^-$ and/or its decomposition products with or without neopterin) at each time point measured. Values are the means and the bars represent the standard deviations of three experiments.

LDL Oxidation Induced by $ONOO^-$

The increase in the REM in dependence of the concentration of $ONOO^-$ (50, 100, 200 and 500 µM) and of the incubation time (45, 90 and 135 min) is shown in Fig. 4.

When LDL was incubated with neopterin or with 7,8-dihydroneopterin in the absence of oxidants, no modulation of the REM of the lipoprotein could be detected (data not shown). In combination with $ONOO^-$, neopterin, at concentrations of 100 and 150 µM, led to an increase in the REM. This effect became weaker with the duration of the incubation time. However, 7,8-dihydroneopterin caused a marked decrease of the REM which was stronger with the higher concentration of 7,8-dihydroneopterin (Fig. 5). Thus, 7,8-dihydroneopterin had a concentration-dependent pronounced protective effect on LDL during oxidation caused by peroxynitrite.

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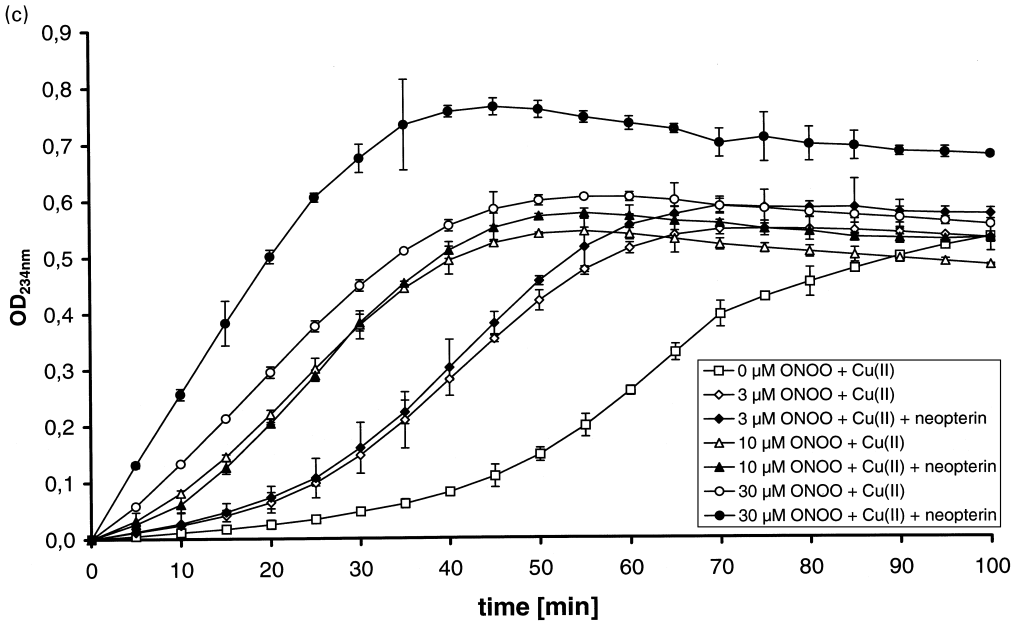

Figure 3 continued

Cu(II)-mediated LDL Oxidation

The copper-mediated (50 μM Cu(II)/20 μM EDTA) oxidation of LDL was markedly increased with 100 μM neopterin when the incubation time was 140 min. Co- or preincubation with this pteridine had little influence (Fig. 6(a)).

In contrast, the presence of 7,8-dihydroneopterin during the Cu(II)-mediated LDL oxidation clearly reduced the REM of oxidized LDL at all incubation times beyond 35 min (95, 140, 1320 min) no matter if LDL was co- or preincubated with this pteridine, as shown in Fig. 6(b). The protective action of 7,8-dihydroneopterin was paradoxically weaker when a higher concentration (150 μM as opposed to 100 μM) of this pteridine was tested. However, at an incubation time of 35 min and especially at a concentration of 150 μM, 7,8-dihydroneopterin seemed to exert some prooxidative effect.

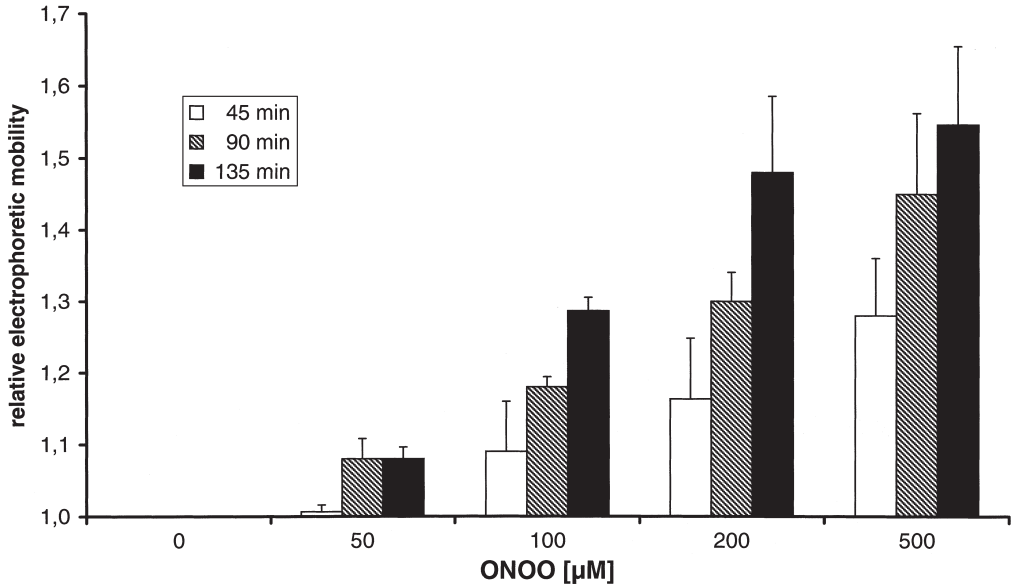

FIGURE 4 The relative electrophoretic mobility of oxidized LDL in dependence of the incubation time and of the $ONOO^-$ concentration. The LDL protein concentration was 44 μg/ml. The $ONOO^-$ concentration ranged between 0 and 500 μM. The incubation time varied between 45 and 135 min. Values are the means and bars represent the standard deviations of the combined results obtained with three LDL preparations from three donors as well with LDL pooled from these three preparations (1:1:1).

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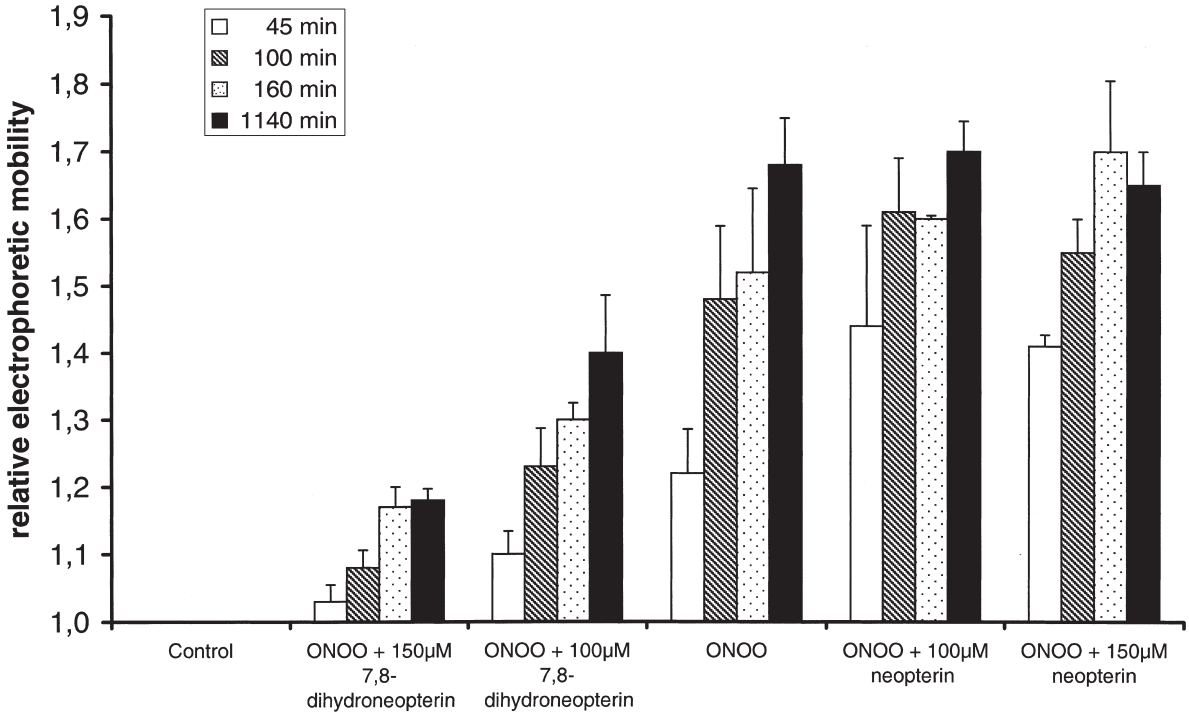

FIGURE 5 Effects of 7,8-dihydroneopterin and of neopterin on $ONOO^-$-mediated LDL oxidation monitored by the relative electrophoretic mobility. The LDL protein concentration was 44 μg/ml. The $ONOO^-$ concentration was 400 μM. The concentration of 7,8-dihydroneopterin and of neopterin was 100 and 150 μM, respectively. The incubation time varied between 45 and 1140 min. Values are the means and bars represent the standard deviations of the combined results obtained with three LDL preparations from three donors as well with LDL pooled from these three preparations (1:1:1).

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DISCUSSION

Two different methods have been applied to investigate the effects of neopterin and 7,8-dihydroneopterin on LDL oxidation. Monitoring the UV-absorption at 234 nm due to diene formation is a suitable method for following LDL oxidation in the early phase of this process. It is indicative for changes taking place at the lipid moiety of the LDL particle. Measuring the REM is more appropriate for investigating stages of LDL oxidation which are basically caused by the changes of the apo B moiety and which become more expressed with the progress of the oxidative modification.

In our experiments, neopterin was found to be capable of enhancing $ONOO^-$- as well as Cu(II)-mediated LDL oxidation, whereas it had no effect on its own. In contrast, 7,8-dihydroneopterin, with some exceptions, mainly protected LDL from oxidation.

These observations resemble the general behavior of neopterin and 7,8-dihydroneopterin in other model reactions for oxidative damage where neopterin showed prooxidative effects, whereas 7,8-dihydroneopterin was rather capable of inhibiting oxidative damage.[31,32,40] In these experiments, neopterin is unaffected (no change of concentration nor chemical modification), whereas 7,8-dihydroneopterin is oxidized to 7,8-dihydroxanthopterin and xanthopterin. These findings suggest a catalytic mechanism by which neopterin enhances the production of reactive intermediates, in contrast to 7,8-dihydroneopterin, whose antioxidative capacity is concurrent with stoichiometric consumption.[41]

Our study is in agreement with the results of an earlier LDL investigation by Gieseg *et al.*[34] which addressed 7,8-dihydroneopterin as a potent antioxidative chain-breaking agent in copper-mediated LDL oxidation.

Our experiments showed that a more or less prooxidative effect of neopterin on LDL oxidation was obtained no matter how this oxidation was mediated. However, the origin of the respective LDL sample seemed to be of importance. This might be due to the varying intrinsic antioxidative capacity and ratio of poly-/mono-unsaturated fatty acids. In addition, freshly prepared LDL may also contain different portions of lipidhydroperoxides. In following the formation of conjugated dienes, the prooxidative effect of neopterin becomes evident especially by accelerating the propagation phase but also by shortening lag phases compared to control oxidations.

Compared to neopterin, the measured effects of 7,8-dihydroneopterin seemed to be more sensitive to the prevailing conditions, like e.g. the type of oxidant ($ONOO^-$ vs. Cu(II)), the concentration of 7,8-dihydroneopterin or better the [7,8-dihydroneopterin/oxidant] ratio, the origin of the LDL preparation, the

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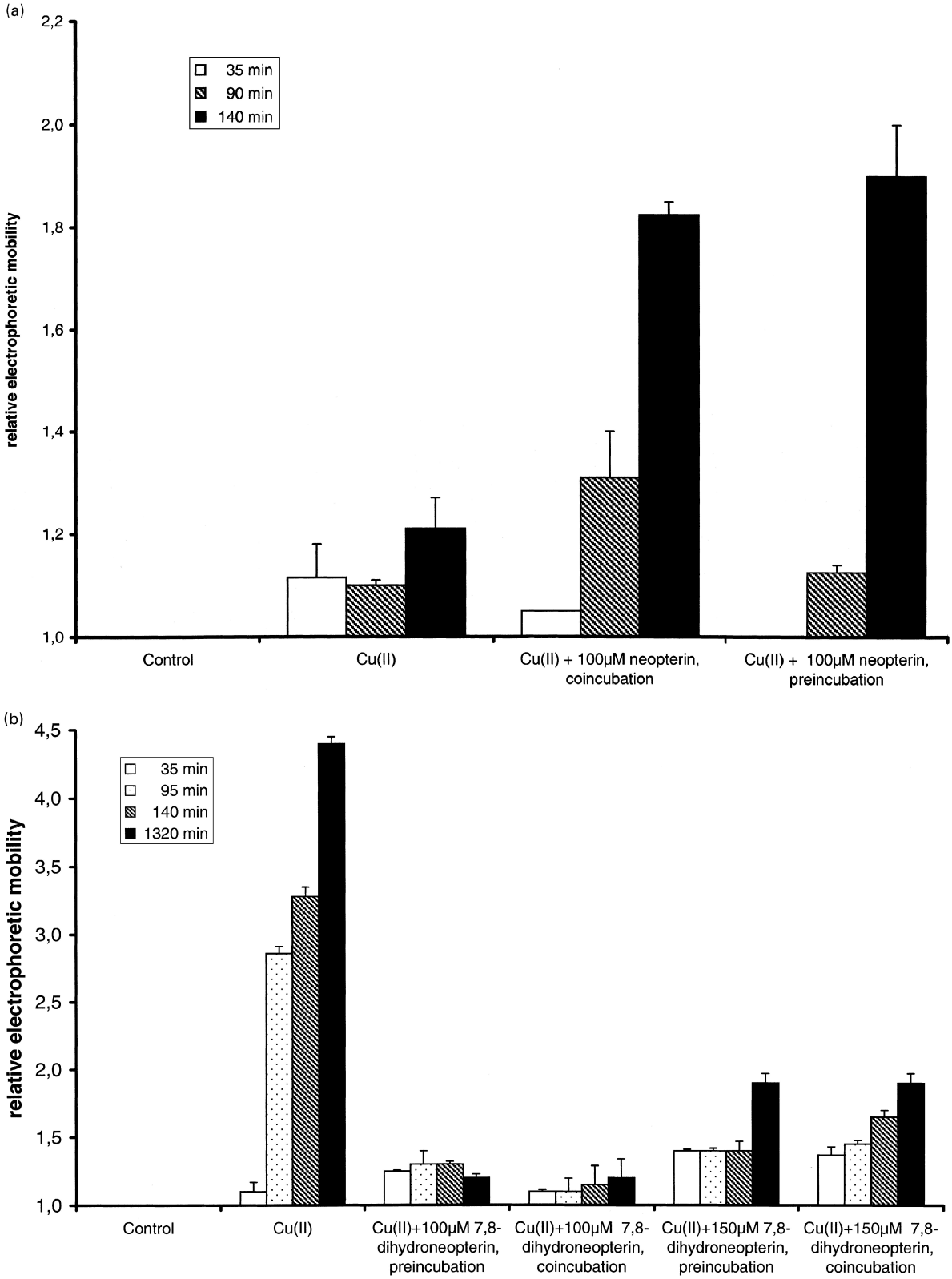

FIGURE 6 Effects of neopterin (a) and of 7,8-dihydroneopterin (b) on copper-mediated LDL oxidation monitored by the relative electrophoretic mobility. The LDL protein concentration was 44 μg/ml. The concentration of neopterin was 100 μM. The concentration of 7,8-dihydroneopterin was 100 and 150 μM, respectively. Copper was added as 50 μM Cu(II)/20 μM EDTA. The incubation time varied between 35 and 1320 min. Values are the means and bars represent the standard deviations of the combined results obtained with three LDL preparations from three donors as well with LDL pooled from these three preparations (1:1:1).

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incubation conditions (preincubation vs. coincubation), the incubation time, and last but not least the method of assessing the LDL oxidation (conjugates dienes vs. REM).

That means, in most of the experiments performed, 7,8-dihydroneopterin acts as an antioxidant but our experiments also showed that under certain circumstances, 7,8-dihydroneopterin may act additionally deleterious on LDL. It appears that the ratio [7,8-dihydroneopterin/$ONOO^-$] or the ratio [7,8-dihydroneopterin/Cu(II)] is most crucial whether 7,8-dihydroneopterin acts as a pro- or as an antioxidant. In ONOO-induced LDL oxidation, 7,8-dihydroneopterin usually acts as an antioxidant in a dose-dependent manner unless the ratio [7,8-dihydroneopterin/$ONOO^-$] is as low as 0.02 (Fig. 1). In this case, the antioxidative power seems to be very limited and only lasts for about 1 h. In Cu(II)-induced LDL oxidation, an initial prooxidative effect of 7,8-dihydroneopterin could be observed. In diene measurements ($[7,8-\text{dihydroneopterin/Cu(II)}] = 5$), this effect could only be seen after preincubation of 7,8-dihydroneopterin with LDL and only for the first 30 min. In REM measurements ([7,8-dihydroneopterin/Cu(II)] = 5 or 7.5) the prooxidative effect of dihydroneopterin can also be observed for the first 35 min but it is only very weakly expressed. In addition, it is more pronounced with preincubation and even more expressed at a ratio [7,8-dihydroneopterin/Cu(II)] of 7.5. Thus, we hypothesize that under certain circumstances 7,8-dihydroneopterin initially reduces Cu(II) to Cu(I), thereby initiating its oxidative potential in a stoichiometric relationship. After total reduction of Cu(II) to Cu(I) (in our case: after about 35 min), higher concentrations of 7,8-dihydroneopterin leading to an excess of this neopterin derivative are able to block the oxidative potential of Cu(I). Gieseg *et al.*[34] did not yield evidence for a prooxidative behavior of 7,8-dihydroneopterin on LDL oxidation, recording the formation of conjugated dienes. In that former study, however, lower [7,8-dihydroneopterin/Cu(II)] ratios were applied, i.e. 0.63–6.3, and 7,8-dihydroneopterin was preincubated for only 5 min before the initiation of oxidation. The whole issue certainly requires further clarification with respect to the role of lipoprotein–copper complexes in copper-catalyzed peroxidation of LDL.[42]

Besides, there is data in literature,[43,44] describing the formation of free radicals when O_2 reacts with reduced pteridines. In this context, superoxide formed by large amounts of 7,8-dihydroneopterin may preferentially contribute to toxicity.

The effect of preincubation of LDL with neopterin derivatives might be explained by diffusion of the pteridines into the phospholipid layer of LDL leading to a higher capacity of affecting lipid peroxidation than in the aqueous phase.

So far, the physiological role of neopterin production by monocytes/macrophages has not been completely clarified. Data imply an impact of neopterin derivatives on redox-controlled processes. However, it remains unclear by which extent neopterin derivatives may actually influence redox-sensitive reactions *in vivo*, because of the complex interactions during immune response, involving cytokines, reactive oxygen species (ROS), and antioxidative defense mechanisms. It has to be kept in mind that neopterin and 7,8-dihydroneopterin may increase or decrease ROS-mediated effects during immune response since both compounds are always released in parallel. The environmental conditions may decide whether the enhancing or scavenging impact of neopterin derivatives will become more important.

Reactive prooxidative molecules for LDL oxidation may originate from immunocompetent cells like, for example, macrophages, which give rise to the generation of foam cells in early pathogenesis of atherosclerosis. When a specific immune response is activated, interferon-γ induces monocytic cells to produce large amounts of neopterin and 7,8-dihydroneopterin. Thus, *in vivo* the situation may occur that macrophages, LDL and high concentrations of neopterin derivatives as well as $ONOO^-$ and/or transition metal ions accumulate in a local vicinity, increasing the probability that LDL oxidation is influenced in a way, similar to the model experiments investigated here. As explained above, it remains unclear whether pro- or antioxidative effects prevail in the *in vivo* situation. It may depend on the conditions whether pteridines inhibit or accelerate LDL oxidation. Since atherosclerosis is associated with increased serum neopterin concentrations,[35,36] an *in vivo* effect of neopterin to accelerate atherogenesis seems to be possible.

To conclude, our study may broaden the view of a potential role of neopterin derivatives to modulate redox processes involved in LDL oxidation by macrophages.

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