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7,8 DIHYDRONEOPTERIN INHIBITS LOW DENSITY LIPOPROTEIN OXIDATION IN VITRO. EVIDENCE THAT THIS MACROPHAGE SECRETED PTERIDINE IS AN ANTI-OXIDANT

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Neopterin and its reduced form, 7,8 dihydroneopterin are pteridines released from macrophages and monocytes when stimulated with interferon gamma *in vivo*. The function of this response is unknown though there is an enormous amount of information available on the use of these compounds as clinical markers of monocyte/macrophage activation. We have found that in vitro 7,8-dihydroneopterin dramatically increases, in a dose dependent manner, the lag time of low density lipoprotein oxidation mediated by Cu^{++} ions or the peroxyl radical generator 2,2'-azobis (2-amidino propane) dihydrochloride (AAPH). 7,8-Dihydroneopterin also inhibits AAPH mediated oxidation of linoleate. The kinetic of the inhibition suggests that 7,8-dihydroneopterin is a potent chain breaking antioxidant which functions by scavenging lipid peroxyl radicals. No anti-oxidant activity was observed in any of the oxidation systems studied with the related compounds neopterin and pterin.

KEY WORDS: neopterin, 7,8 dihydroneopterin, low-density lipoprotein, copper oxidation, antioxidant, peroxyl radical.

Abbreviations: 7,8NP, 7,8 dihydroneopterin; AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; BHT, 2,6-Di-tert-butyl-4-methylphenol; EDTA, ethylenediaminetetraacetic acid disodium salt; LDL, low-density lipoprotein; PBS, phosphate buffered saline; SDS, sodium dodecylsulphate.

INTRODUCTION

7,8 dihydroneopterin (7,8NP) and its oxidised form neopterin are pteridines (figure 1) released from primate and human monocytes/macrophages when activated by gamma interferon.[1,2,3] The measurement of neopterin concentration in patient blood and urine samples has been used for a number of years as a clinical marker of immune cell activation. There is a wealth of clinical information available on the use of neopterin as a clinical marker in the study of allograft rejection,[6] autoimmune diseases,[10] malignant diseases,[11] viral infections,[7] intracellular bacteria[8] and parasites.[9] However, the actual physiological function of neopterin and 7,8NP during immune cell activation has remained uncertain.

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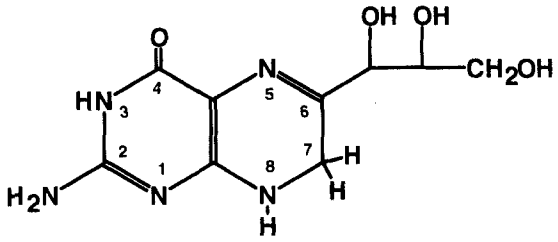
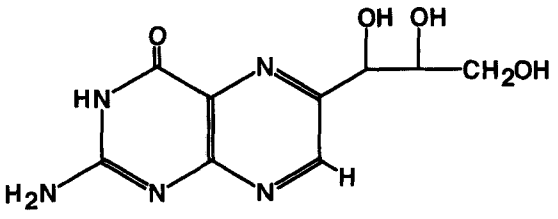
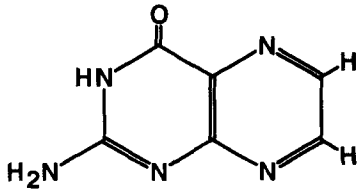


7,8-dihydroneopterin



neopterin



pterin

FIGURE 1 Structure of pteridines examined.

Biochemically, pteridines are synthesized by the enzymatic degradation of guanosine-5'-triphosphate (GTP) by GTP cyclohydrolase I to 7,8-dihydroneopterin-triphosphate. The action of 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase[4] converts the 7,8-dihydroneopterin-triphosphate to 5,6,7,8-tetrahydrobiopterin. This compound serves as a cofactor for mammalian mono-oxygenase enzymes responsible for hydroxylation of the aromatic amino acids, phenylalanine, tyrosine and tryptophan for neurotransmitter biosynthesis.[5] In primate and human monocytes/macrophages the GTP cyclohydolase I enzyme is cytokine inducible, but these cells contain very little of the subsequent enzymes required for 5,6,7,8-tetrahydrobiopterin biosynthesis.[4] This results in a build up of 7,8-dihydroneopterin triphosphate in gamma interferon stimulated monocyte/macrophages. The action of cellular phosphatases converts the triphosphate to 7,8NP which can leak from the cells into the circulation. Partial oxidation converts approximately 1/3 of the 7,8NP to neopterin which can then be measured in serum and urine using high performance liquid chromatography with fluorescence detection.[3]

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Recently, *in vitro* experiments using the xanthine/xanthine oxidase system or PMA (phorbol myristate acetate) stimulated macrophages have shown that 7,8NP may be a potent scavenger of the superoxide radical.[12,13,14] Similarly, 7,8NP was found to be a strong suppressant of luminol-dependent chemiluminescence induced by chloramines or by hydrogen peroxide.[15]

These findings may have significant implications for the oxidation of LDL by macrophages in the artery wall, a process which according to current theories is an important step in the pathogenesis of atherosclerosis in humans.[16] Atherosclerotic lesions appear to be sites of low level inflammation as oxidised LDL stimulates recruitment of monocytes to the intima and their differentiation into macrophages. The balance between antioxidant defense and free radical generating systems appears to affect the progression of this disease. Therefore the release of 7,8 dihydroneopterin by activated macrophages may have a significant effect on the extent of LDL oxidation during atherogenesis. We have previously reported that in advanced stages of atherosclerosis, plasma neopterin levels may become elevated, possibly as a result of macrophage activation within the atherosclerotic plaque.[17]

We report here on our studies on the effect of neopterin, 7,8NP and pterin on *in vitro* oxidation of LDL and linoleate. We also present an extensive characterization of the anti-oxidative properties of 7,8NP.

MATERIALS AND METHOD

The reagents used were of AR grade or better and obtained from Merck (Darmstadt, F.R. Germany). α-tocopherol and sodium dodecylsulphate (SDS) were also supplied by Merck. The linoleic acid was supplied by Sigma (St. Louis, U.S.A.). 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Poly Science (Warrington, U.S.A) and the trolox was supplied by Aldrich Chemical Company, (Milwaukee, USA). All solutions were prepared from ion exchanged laboratory water filtered through a Millipore 'inorganic' cartridge (Bedford, USA). All solutions of phosphate buffered saline (PBS) (160 mM sodium chloride, 10 mM sodium phosphate buffer pH 7.4) were stirred with chelex-100 resin supplied by BioRad (Richmond, USA) to remove heavy metal ions usually contaminating even phosphate salts of high purity.

Neopterin, 7,8 dihydroneopterin and pterin were generous gifts from Dr B. Schirck's laboratory in Jona, Switzerland. Stock solutions of 200 μM neopterin, 7,8 dihydroneopterin or pterin were made up in chelex treated argon gassed PBS no more than 10 minutes prior to use. Pterin was the most difficult of these compounds to dissolve, requiring up to 4 minutes of sonication under argon gas in a 'econ-clean' sonicating water bath supplied by Spirig, (Rapperswil, Switzerland).

The inhibition of AAPH mediated oxidation of linoleate by 7,8NP and other compounds was examined in principle by using the method of Pryor *et al.*.[18] In brief, a solution of 1 mM linoleic acid in PBS containing 250 μM SDS was prepared, 2.4 ml of this solution plus 5–10 μl of the test compound was added to each cuvette, after 5 minutes preincubation at 30°C oxidation was initiated by addition of 5 μl AAPH (100 mM). The rate of oxidation was followed by monitoring the increase of the absorption at 234 nm. Uninhibited oxidation was followed in a cuvette containing all components except the test compound. Lag times were obtained by graphical interpolation and rates oxidation were calculated using a molar extinction coefficient of 26100 $M^{-1}cm^{-1}$.[18]

A pool of EDTA plasma was prepared from blood drawn from ten healthy male and female normolipidemic donors (age 25–35 years). The plasma was frozen in

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aliquots at −80°C in 0.6% sucrose for up to two months.[19] The LDL used for the study of the 7,8NP concentration effect on LDL oxidation was prepared from a pool of plasma different to that used in the experiments on tocopherol loss and oxidation products.

The LDL was prepared from the EDTA-plasma by a single 2 hour ultracentrifugation using a one step discontinuous gradient in a Beckman NVT65 rotor as previously described.[19] The prepared LDL was stored under argon gas at 4°C for no more than two weeks before use.

For experiments with LDL prepared from individual donors, the LDL samples were prepared on the same day as the blood was drawn and subsequently studied (i.e. oxidised) over the following two days. These LDL samples were prepared from four healthy normolipidemic male volunteers after an overnight fast.

The LDL was desalted 30 minutes prior to oxidation by gel filtration using 'econ columns' supplied by BioRad (Richmond, USA) which were equilibrated in argon gassed PBS. All oxidations were performed with an LDL concentration of 0.1 μM (250 μg total mass/ml) in PBS with 1.6 μM copper sulphate at 30°C except for the study of LDL prepared from individuals which was oxidised at 37°C. The LDL molar concentration was determined by enzymatic cholesterol determination using the 'Chol MPR 2' kit supplied by Boehringer Mannheim GmbH (Vienna, Austria) assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6%. The lag time for LDL peroxidation was determined by measuring the increase in absorbance at 234 nm due to the formation of conjugated dienes using a Shimadzu temperature controlled UV-1202 UV/Vis spectrophotometer equipped with a six cuvette holder as previously described.[19,20] The 234 nm absorbance of the cuvettes was automatically recorded every 5 minutes. For AAPH oxidation a solution of ice cold AAPH in PBS was added to the LDL solution to give a final concentration of 1 mM. In all experiments involving Cu^{++} oxidation, the 7,8NP was added to the LDL solution and left to stand in the dark at room temperature for 5 minutes before the initiation of oxidation. For AAPH oxidation, this time was extended to 15 minutes.

α-Tocopherol loss was measured by taking 0.5 ml samples at 20 minutes intervals from a solution of LDL in a 25 ml flask. At the same time the 234 nm absorbance of the sample was also measured. The α-tocopherol concentration was determined by first stopping the reaction by adding 10 μL of 0.1 g/ml (270 mM) EDTA and 0.5 ml ethanol containing 1 mg/ml (4.5 mM) BHT to the sample and then extracting with 1 ml hexane by vortexing. After centrifugation 0.7 ml of the hexane was dried down under dry nitrogen gas and the residue was solubilised in 60 μL of methanol, 20 μL of which was used for α-tocopherol determination by reverse phase high performance liquid chromatography with fluorescence detection as previously described.[21]

The development of the neopterin-like fluorescence was monitored by oxidising a 6 ml solution of 0.1 μM LDL containing 2 μM 7,8NP and 1.6 μM copper sulphate. Half of this solution was placed in a cuvette and the formation of conjugated dienes was monitored measuring 234 nm absorbance in a temperature controlled spectrophotometer. The other three ml was placed in a Shimadzu temperature controlled RF-5001PC spectrofluorophotometer and the development of the 353 nm excitation / 450 nm emission fluorescence was measured every 20 minutes. Between fluorescence scans the lamp shutter was closed to prevent photo-oxidation of the LDL and 7,8NP.

Linear regression analysis was performed using the Excel spreadsheet computer program (Microsoft, USA). Shown in the figures are specific experiments which are representative of several.

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TABLE 1

Effect of selected pteridines on the lag time of Cu^{++} medicited LDL oxidation. 10 μM pteridine was added to the LDL and allowed to incubate at room temperature in the dark for 5 minutes. The lag times were determined by monitoring the diene formation at 37°C with 1.6 μM copper sulphate. The lag times were reproducible plus or minus 5%. The control was Cu^{++} mediated LDL oxidation with no added pteridine.

Sample	Lag time (minutes)
control	83
neopterin	88
7,8 dihydroneopterin	495
pterin	90

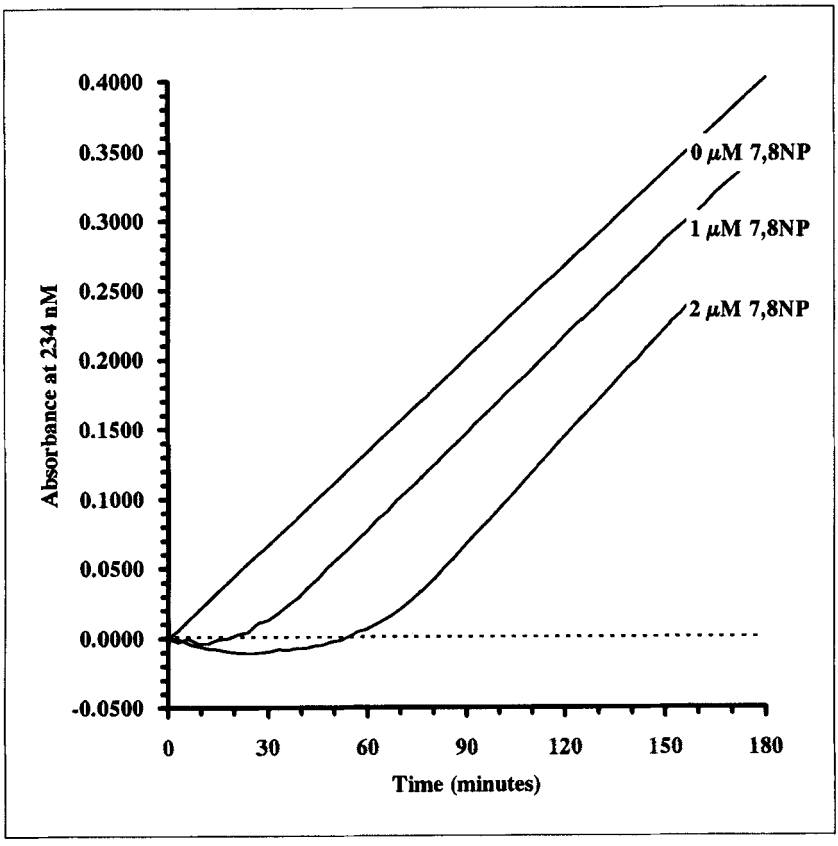

FIGURE 2 Inhibition of AA PH oxidation of linoleate by 7,8 NP at 30°C. 7,8 NP was added to a phosphate buffered (pH 7.4) solution of linoleic acid in SDS to give the indicated final concentrations. After five minutes an AAPH solution was added to give a final concentration of 1 mM and the oxidation was monitored by the increase in 234 nm absorbance due to the formation of conjugated dienes.

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RESULTS

A) 7,8 Dihydroneopterin Inhibits Oxidation of LDL and Linoleate

7,8 Dihydroneopterin, caused a dramatic six fold increase in the length of the Cu^{++} mediated LDL peroxidation lag time at a concentration of 10 μM, (Table 1) whereas neopterin and pterin had no significant effects on the oxidation, even at concentrations of 20 μM.

To examine whether 7,8NP is an inhibitor of lipid peroxidation its effect in a model system consisting of linoleate and AAPH was studied. Figure 2 shows that in absence of 7,8NP, linoleate is oxidised without any inhibition period (lag time) at a constant rate equal to 85 nM diene/min. A concentration of 1 and 2 μM 7,8NP caused a 27 and 67 minute inhibition of linoleate oxidation. At the end of the inhibition period, the rate of oxidation increased to the same rate as observed in the uninhibited control. α-Tocopherol and trolox examined in the same model system inhibited at a concentration of 1 μM oxidation for 40 and 24 minutes respectively. The rate of diene formation during the inhibited period was 40 nM diene/min (α-tocopherol) and 19 nM diene/min (trolox). For the 7,8NP experiment no rate for the inhibited period can be given, since the 234 nm absorption slightly decreased, most likely due to consumption of 7,8NP (see below). No inhibition of AAPH mediated oxidation of linoleate was observed with pterin or neopterin at 2 μM concentration.

B) Dose Dependence of Inhibition of Cu^{++} and AAPH Mediated LDL Oxidation

The addition of 7,8NP to Cu^{++} mediated LDL oxidation was found to cause a direct concentration dependent increase in the measured lag time ($r^2 = 0.99$) without causing a change in the rate of propagation (period of oxidation where a rapid rise in 234 nm absorbance occurs) as measured by the slope of the diene curves, (figure 3). This is characteristic of a chain breaking anti-oxidant which is consumed during the inhibition period. If 7,8NP or its oxidation products would act as Cu++ chelators the rate of propagation should decrease with increasing 7,8NP concentration.

To further distinguish between a peroxyl radical scavenging activity of 7,8NP and a possible copper ion complexing effect, the effect of 7,8NP on AAPH mediated LDL oxidation was examined. AAPH is a water soluble, thermolabile compound which breaks down to give two peroxyl radicals capable of abstracting H atoms from amino acids and polyunsaturated fatty acids in the LDL particles. The rate of radical generation is therefore dependent on the AAPH concentration and the incubation temperature. In this oxidation system the 7,8NP also showed a concentration dependent increase in lag time ($r^2 = 0.98$) with no effect on the propagation rate up to 5 μM 7,8NP as measured by the increase in 234 nm absorbance (figure 4), indicating that in these systems, the 7,8NP was probably acting as a lipid peroxyl radical scavenger. The slightly lower rate of 234 nm absorbance increase in the progartation phase of the 8 and 10 μM 7,8NP incubations maybe due to optical interference by the AAPH and 7,8NP breakdown products.

In Cu^{++} and AAPH oxidation of LDL and AAPH linoleate oxidation, the diene curves in the presence of 7,8NP decrease at the start of the oxidation. The exact cause of this absorption change is difficult to fully describe as both 7,8NP and its possible oxidative product, neopterin, both absorb UV light with maxima around 234 nm. This absorbance is seen as a very slight shoulder to the much stronger absorbance of the

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protein moiety of the LDL. As a result the absorbance spectrum changes observed are difficult to quantify.

In our preliminary experiments, 7,8NP tended to produce rather erratic results with considerable variations in lag times. It was subsequently found that to observe a reproducible, concentration dependent inhibition of Cu^{++} or AAPH mediated LDL oxidation it was necessary to pre-incubate the LDL and 7,8NP at room temperature in the dark before the addition of the pro-oxidant (data not shown). For Cu^{++} oxidation the preincubation required was 5 minutes while AAPH oxidation required 15 minutes for consistent results.

C) Effect of 7,8NP on α-tocopherol Loss

Figure 5 shows an oxidation experiment of an LDL sample in the presence and absence of 7,8NP. It is clearly evident that beside prolonging the lag time, 7,8NP slows the rate

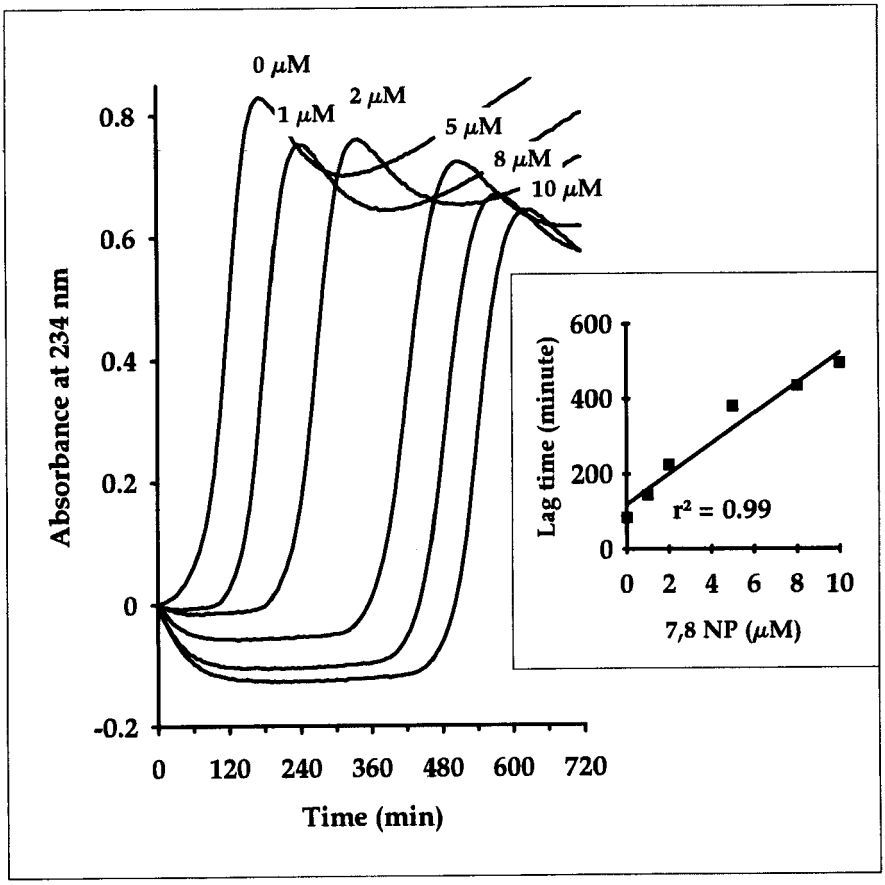

FIGURE 3 Concentration dependence of 7,8 dihydroneopterin inhibition of Cu^{++} medicated LDL oxidation. LDL in PBS, prepared from pooled plasma, was incubated for 5 minutes with 1–10 μM 7,8NP in the dark at room temperature before being oxidised with 1.6 μM copper sulphate at 30°C. The data shown have been standardised by subtracting the initial absorbance for each individual incubation. The insert shows the correlation between 7,8NP concentration and lag time.

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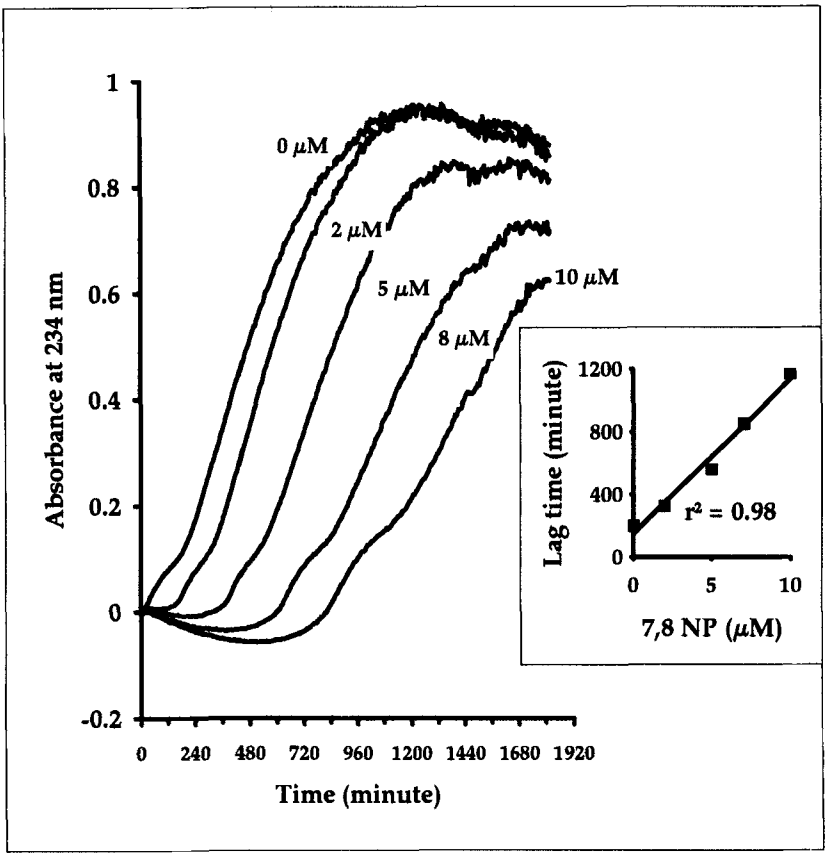

FIGURE 4 Concentration dependence of 7,8 dihydroneopterin inhibition of AA PH mediated LDL oxidation.

LDL prepared from pooled plasma, in PBS, was incubated for 15 minutes with 1–10 μM 7,8NP in the dark at room temperature before being oxidised with 1 mM AAPH at 30°C. The data shown have been standardised by subtracting the initial absorbance for each individual incubation. The insert shows the correlation between 7,8NP concentration and lag time.

of α-tocopherol loss. In the absence of 7,8NP the α-tocopherol half life was 10 minutes where as in the presence of 7,8NP the half life was increased to 40 minutes. The rate of α-tocopherol consumption was 0.5 $nM.min^{-1}$ in the absence of 7,8NP and in the presence of 7,8NP the rate decreased to 0.17 $nM.min^{-1}$. This observation, that 7,8NP reduces the rate of α-tocopherol consumption suggests a competition between the 7,8NP and the α-tocopherol, most likely for the lipid peroxyl radical.

We suspected that the major breakdown product of 7,8NP would be neopterin which fluoresces strongly at 450 nm when excited at 353 nm in PBS buffer at pH 7.4. Fluorescent analysis of Cu^{++} oxidised LDL in the presence of 7,8NP showed a 353/450 nm fluorescence which appeared to be neopterin. This fluorescence was not observed with LDL oxidised in the absence of 7,8NP LDL (results not shown). This fluorescent compound appeared to be stable and resistant to further oxidization. Neopterin was also found to be stable during LDL oxidation.

We were therefore able to obtain a time curve of the 7,8NP oxidation by monitoring

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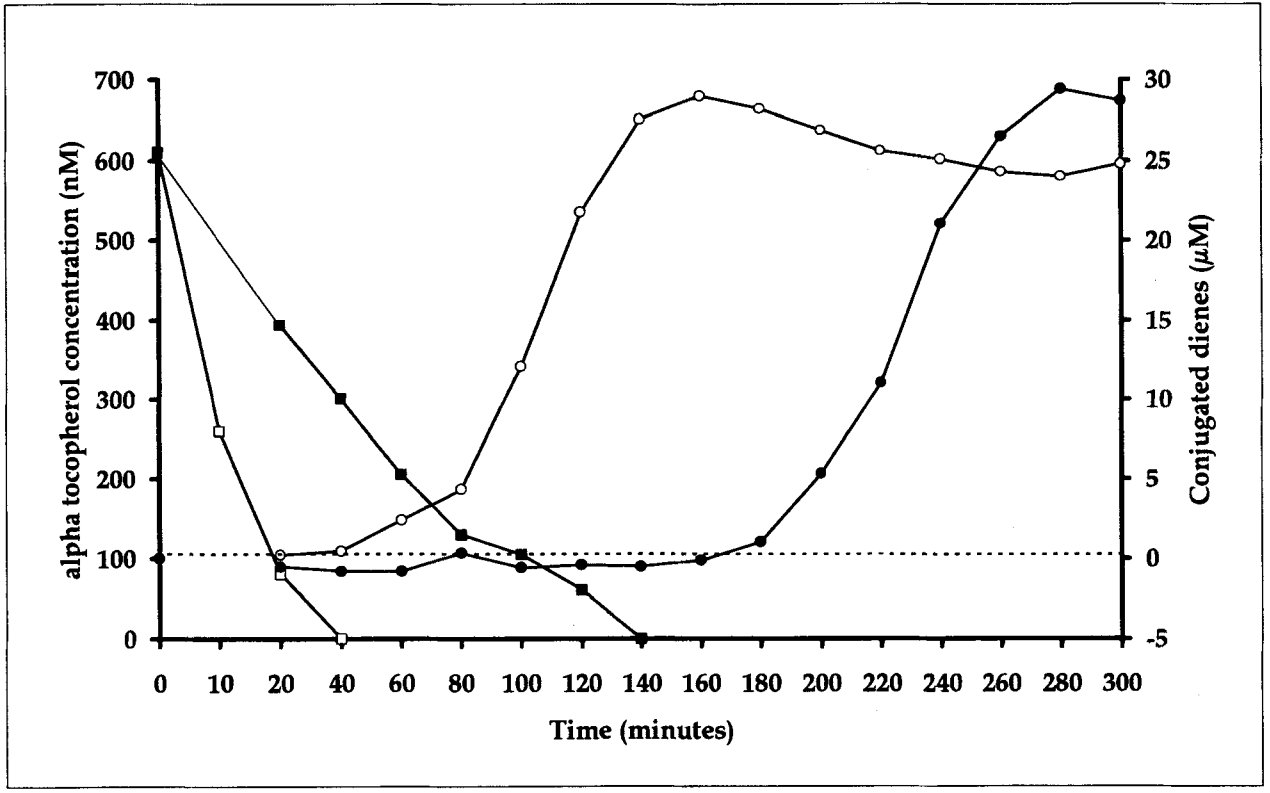

FIGURE 5 Effect of 2 μM 7,8 dihydroneopterin on α-tocopherol oxidation during 1.6 μM Cu^{++} mediated LDL oxidation at 30°C.

The loss of tocopherol and the formation of conjugated dienes was measured as described in methods. The diene concentration was calculated by using the diene molar extinction coefficient of 29500 $M^{-1}cm^{-1}$. The LDL used was from a different pool than that used in figures 1 & 2. The data show; α-tocopherol loss without 7,8NP, (□); α-tocopherol loss with 7,8NP, (■); conjugated diene formation without 7,8NP, (○); conjugated diene formation with 7,8NP, (●);

(via repeated spectrofluorescence scans) the formation of the 430 nm fluorescent product, along with the conjugated diene formation, (figure 6). As the same conditions and LDL preparation were used in this experiment as that used for the tocopherol loss study we are able to directly compare the 7,8NP oxidation (figure 6) with the α-tocopherol loss (figure 5). This assumption appears reasonably sound as identical conjugated diene curves were obtained for LDL containing 7,8NP in figures 5 and 6. We found that formation of the 450 fluorescent product appears to be reasonably linear with time except for a 20 minute period at the start where the fluorescence did not change. The reason for this is unclear but it is possible that some type of quenching mechanism of the fluorescence may be occurring. By 140 minutes 92% of the 7,8NP and 100% of the α-tocopherol has been oxidised (assuming the relative fluorescence of the product relates directly to the amount of 7,8NP remaining). It is at this time that diene formation becomes significant as would be expected if the α-tocopherol and 7,8NP were competing for the lipid peroxyl radicals and so preventing chain propagation.

D) Other Observations

Table 2 shows that 7,8NP inhibitory effects are not due to properties of LDL prepared from frozen pooled plasma. The LDL samples prepared from fresh, non-frozen plasma from 4 individuals displayed lag times between 69 and 100 minutes at 37°C with 1.6 μM Cu^{++}. The addition of 2 μM 7,8NP lead to a 150 to 250% increase in the lag time depending on the donor.

Most of the experiments described in this study were performed with LDL samples prepared from a pooled plasma stored in presence of 0.6% sucrose at −80°C. Kleinveld *et al.*[22] reported that sucrose supplemented plasma can be stored at −80°C for a period of 5 weeks without change in oxidisability indices. In a previous study[19] we observed no change upon storage for 15 days. In the present study, we have repeatedly measured the oxidation resistance (i.e. diene curves in Cu^{++} mediated oxidation) of LDL samples prepared from one frozen plasma pool and found no change over a period almost 2 months. Lag times of 65, 58, 59, 64, 58, were observed after 0, 17, 31, 37, and 45 days of storage at −80°C.

Finally, we examined whether neopterin which is also secreted by macrophage might be a pro-oxidant in presence of hydrogen peroxides, another product released by

TABLE 2

The Increase in lag time of LDL preparations due to the addition of 2 μM 7,8NP to the reaction mixture. The lag times were determined by monitoring the diene formation at 37°C with 1.6 μM copper sulphate. Donors 1–4 were health normolipidemic males aged between 25 and 34 years.

LDL sample	lag time without 7,8NP (minutes)	Lag time with 2 μM 7,8NP, (minutes)	Percentage Increase[1]
Pool plasma[2]	57 ± 3[3]	218 ± 11	280
Donor 1	69 ± 3	229 ± 2	230
Donor 2	77 ± 1	237 ± 9	210
Donor 3	90 ± 4	277 ± 4	210
Donor 4	100 ± 9	252 ± 5	150

1) Calculated as the difference between the lag times divided by the control lag time and multiplied by 100 to give percentage increase.
2) The LDL was prepared from a pool of plasma prepared from ten donors. This pool was a different preparation to that used in figures 3 & 4.
3) Mean ± standard deviation of the three oxidations.

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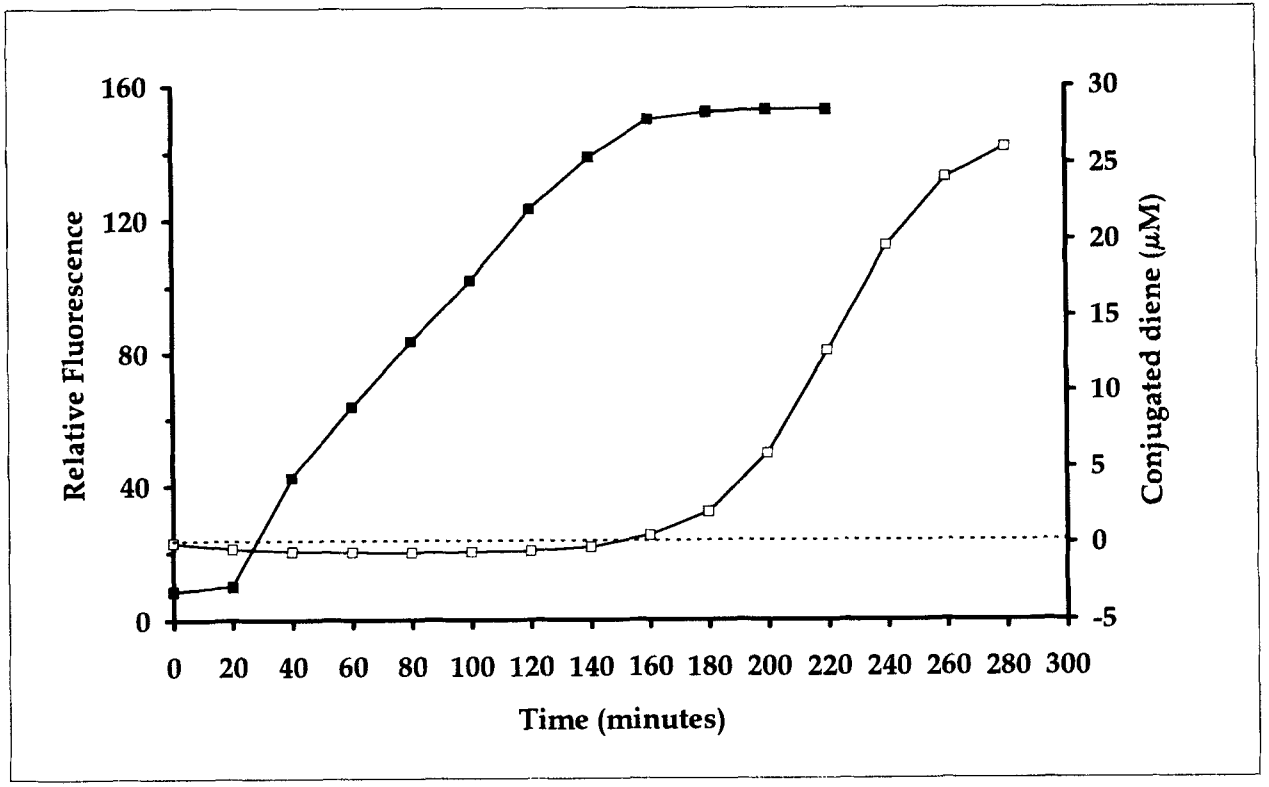

FIGURE 6 The formation of a neopterin-like fluorescent compound from 7,8 dihydroneopterin during Cu^{++} mediated LDL oxidation.

A 6 ml incubation of LDL in PBS with 2 μM 7,8NP and 1.6 μM copper sulphate was equally split between two cuvettes so 3 ml of the mixture was monitored for 234 nm absorbance due to the formation of conjugated dienes, (o), and the remaining volume was monitored for an increase in 450 nm emission fluorescence, (n) (excitation 353 nm). The cuvettes holder of both machines was maintained at 30°C during the oxidation. The diene concentration was calculated as in figure 5. The LDL preparation was exactly the same as that used in figure 4.

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activated macrophages. Previously it has been shown that neopterin enhances toxicity of hydrogen peroxide[15] and luminol dependent chemiluminescence.[23] In the presence of 50 μM hydrogen peroxide no pro-oxidant activity was observed with 10 μM neopterin during Cu^{++} mediated LDL oxidation.

DISCUSSION

With the present data it is of course not possible to definitely suggest a mechanism for the inhibition of Cu^{++} and AAPH mediated LDL oxidation by 7,8NP. Provisionally we would propose that 7,8NP acts as a chain breaking antioxidant by scavenging lipid peroxyl radicals. This assumption is supported by the following observations; the inhibition by 7,8NP of AAPH mediated linoleate oxidation, the increase in lag time by 7,8NP with both pro-oxidants, the absence of an effect on the rate of propagation after 7,8NP and α-tocopherol consumption, with both pro-oxidants the antioxidant activity is directly proportional to the 7,8NP concentration, and the kinetics of α-tocopherol consumption suggest a competition between 7,8NP and α-tocopherol where they are both scavenging the lipid peroxyl radicals at comparative rates, (figure 5).

Pterin and neopterin had no detectable anti-oxidant activity in any of the oxidation systems examined.

As a pre-incubation period was essential for maximum activity it is also clearly evident that 7,8NP acts in or on the lipid environment of the LDL particle where the lipid peroxyl radicals will be present, as opposed to interacting with aqueous radicals. The longer incubation time required for AAPH oxidation compared to Cu^{++} mediated oxidation may relate to the localization of these two pro-oxidants within the LDL particle. The pro-oxidant copper ions appear to become localized to the apolipoprotein B moiety of the LDL[19] whereas AAPH is known to form an equilibrium between the lipid and aqueous environment with radicals being generated in both phases.[18] Therefore time is required for the 7,8NP to become more integrated into the LDL particle to achieve maximum protective effect during AAPH mediated oxidation.

It is unlikely that 7,8NP acts as a preventative radical scavenger by reacting directly with the AAPH derived $ROO^{\cdot}$ radical since a similar efficiency in preventing LDL oxidation was observed with both pro-oxidants.

The fact that neopterin and pterin showed no anti-oxidant activity suggests that the hydrogens on nitrogen-8 and carbon-7 (figure 1) may be involved in the hydrogen transfer reactions. We also propose one of the possible products of 7,8NP radical scavenging may be neopterin. The 353/450nm fluorescent compound monitored during 7,8NP inhibited LDL oxidation had a similar fluorescence to neopterin, and like neopterin was also stable during Cu^{++} mediated LDL oxidation. This does not however exclude the possibility of other reactions products. Preliminary HPLC studies (results not shown) show the presence of a second compound slightly more hydrophobic than neopterin in samples of LDL oxidised in the presence of Cu^{++} with 7,8NP.

It was interesting to note that for LDL prepared from individual donors there was a correlation between lag time and the prolongation of the lag time suggesting that the shorter initial lag time the greater protective effect of the 7,8NP (Table 2). As this study involved only 4 donors and a sample prepared from pooled plasma it is not clear whether this observation can be generalised.

These results and those of others[13,14,15] strongly suggest that an important *in vivo* role of 7,8NP is that of an intracellular and possibly extracellular anti-oxidant. *In vitro* studies on phorbol myristate acetate activated neutrophils have shown that they suffer

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from free radical damage to a number of key enzymes and overall the level of protein oxidative damage as measured by carbonyl group formation increases.[24] The concentration of both ceruloplasmin (a copper ion binding and iron ion oxidising protein)[25] and α-1-anti-chymotrypsin[26] (an inhibitor of NADPH oxidase enzyme complex) increase during oxidative burst. 7,8NP may be yet another defence mechanism but aimed at scavenging lipid peroxyl radicals and so protecting cellular membranes. The leakage of 7,8NP into the extracellular space may reduce host tissue damage which occurs during neutrophil respiratory burst activity.[27]

As to the role of 7,8NP in preventing atherogenesis, *in vitro* data suggest cell activation is not required for LDL oxidation,[28,29] therefore it is unlikely 7,8NP is involved in the inhibition of LDL oxidation during the early stages of plaque formation. The correlations reported between neopterin levels in plasma of atherosclerotic patients and the severity of their disease[17,30] is probably an indication of the onset of a more advanced level of stage of plaque development where 7,8NP anti-oxidant activity may be important. This does not however exclude the possibility of using this compound as a therapeutic agent in the treatment of critically ill patients with free radical mediated diseases.

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