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REVIEW

Potential to inhibit growth of atherosclerotic plaque development through modulation of macrophage neopterin/7,8-dihydroneopterin synthesis

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The rise in plasma neopterin observed with increasing severity of vascular disease is a strong indicator of the inflammatory nature of atherosclerosis. Plasma neopterin originates as the oxidation product of 7,8-dihydroneopterin secreted by γ-interferon stimulated macrophages within atherosclerotic plaques. Neopterin is increasingly being used as a marker of inflammation during clinical management of patients with a range of disorders including atherosclerosis. Yet the role of 7,8-dihydroneopterin/neopterin synthesis during the inflammatory process and plaque formation remains poorly understood and controversial. This is partially due to the unresolved role oxidants play in atherosclerosis and the opposing roles of 7,8-dihydroneopterin/neopterin. Neopterin can act as pro-oxidant, enhancing oxidant damage and triggering apoptosis in a number of different cell types. Neopterin appears to have some cellular signalling properties as well as being able to chelate and enhance the reactivity of transition metal ions during Fenton reactions. In contrast, 7,8-dihydroneopterin is also a radical scavenger, reacting with and neutralizing a range of reactive oxygen species including hypochlorite, nitric oxide and peroxyl radicals, thus protecting lipoproteins and various cell types including macrophages. This has led to the suggestion that 7,8-dihydroneopterin is synthesized to protect macrophages from the oxidants released during inflammation. The oxidant/antioxidant activity observed *in vitro* appears to be determined both by the relative concentration of these compounds and the specific chemistry of the *in vitro* system under study. How these activities might influence or modulate the development of atherosclerotic plaque *in vivo* will be explored in this review.
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Abbreviations: AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; GM-CSF, colony-stimulation factor; GTP, guanosine 5′-triphosphate; 3HAA, 3-hydroxyanthranilic acid; HMDM, human monocyte-derived macrophages; HPLC, high-performance liquid chromatography; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; TNF-α, tumour necrosis factor-α

Introduction

Atherosclerotic plaques are sites of chronic inflammation (Libby *et al*., 2002). This is clearly shown by the presence of large numbers of immune cells including macrophages, and various inflammatory markers within the plaque and plasma of patients. Although much attention has been given to the elevation of C-reactive protein in the plasma of heart disease patients, the inflammation marker neopterin is also significantly elevated in patients with vascular disease (Tatzber *et al*., 1991; Schumacher *et al*., 1992; Rudzite *et al*., 2005). Neopterin is synthesized and released from γ-interferon activated macrophages as part of the inflammation process. Neopterin has been investigated as a marker of immune cell activation in a wide range of diseases as it is relatively easy to analyse by high-performance liquid chromatography (HPLC) and is generated by one of the key inflammatory cells, the macrophage, during inflammation.

The macrophage is considered to be the key cell in the development and growth of atherosclerotic plaques (Carpenter *et al*., 1995; Steinberg, 1995). Both fatty streaks and advance plaques are rich in macrophages. Macrophage cells release a range of proteolytic and oxidizing agents including superoxide, hydrogen peroxide, lipid peroxides, lipoxygenases and possibly hypochlorite (Ylaherttuala *et al*., 1989; Schewe and Kuhn, 1991; Chisolm *et al*., 1999). All these agents have been shown *in vitro* to alter the low-density lipoprotein (LDL) found within the tissue bed of the intima (innermost layer of the artery wall) to oxidized LDL (oxLDL).

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OxLDL is readily taken up by macrophages via scavenger receptors in a relatively non-regulated process. The uptake of oxLDL causes the macrophages to differentiate into cholesterol-loaded macrophages often referred to as 'foam cells' due to their foamy appearance (Goldstein *et al*., 1979; Hoff *et al*., 1989; Steinbrecher *et al*., 1989). A number of the oxidized fatty acids within oxLDL are chemotactic to monocytes while the oxysterols and fatty acid peroxides are toxic to macrophages, fibroblasts, smooth muscle cells and endothelium, all of which make up advanced plaques (Lassila, 1993; Hegyi *et al*., 1996). The death and lysis of all these cells, especially lipid-loaded macrophages, results in the formation of a lipid-rich necrotic core region within atherosclerotic plaque. Atherosclerotic plaques containing lipid-rich necrotic cores are prone to rupture and thrombus formation (Van Der Wal and Becker, 1999). The finding that plasma neopterin levels increase with atherosclerotic plaque formation shows a direct link between macrophage activation and plaque development.

This oxidative model of heart disease has been heavily criticized in recent years due to the failure of various antioxidant intervention trials to demonstrate a significant level of protection. This criticism ignores the fact that ascorbate and tocopherol levels are tightly controlled *in vivo* as both are potent pro-oxidants in elevated concentrations (Bowry and Stocker, 1993; Clement *et al*., 2001). Therefore, neither are good therapeutic agents from a pharmacological point of view. The criticism also ignores the fact that oxidized lipids and proteins are both found within plaques and these agents have reasonably well-defined inflammatory effects on cells (Carpenter *et al*., 1995; Woods *et al*., 2003). LDL oxidation may not initiate plaque formation, but its presence will significantly and detrimentally affect the functioning of cells within the artery wall. The rise of C-reactive protein and neopterin clearly show atherosclerosis has a significant inflammatory component. It is therefore possible that agents which alter this inflammatory process will slow or prevent plaque growth, or the formation of more complex plaques. *In vitro* both neopterin and its reduced form 7,8-dihydroneopterin have been shown to have significant effects on oxLDL formation and cell death. Unlike ascorbate, tocopherol or dietary flavonoids, neopterin and 7,8-dihydroneopterin are generated within the plaque and therefore do not need to be delivered to the site of artherogenesis.

Neopterin and 7,8-dihydroneopterin synthesis

Neopterin is the oxidized product of 7,8-dihydroneopterin, a pterin synthesized by primate macrophages when stimulated with γ-interferon (Muller *et al*., 1991; Wachter *et al*., 1992). GTP-cyclohydrolase is one of the many enzymes upregulated by γ-interferon. The enzyme catalyses the breakdown of GTP to 7,8-dihydroneopterin triphosphate. In primate macrophages, low levels of the enzyme 6-pyruvoyltetrahydropterin synthetase causes a build up of 7,8-dihydroneopterin triphosphate which is then released as 7,8-dihydroneopterin due to the action of intracellular phosphatases (Schoedon *et al*., 1987). 7,8-Dihydroneopterin diffuses out of the activated

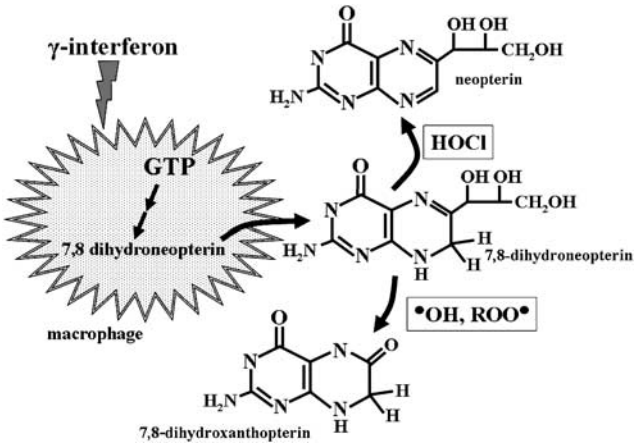

Figure 1 γ-Interferon stimulation of macrophages causes the enzymatic breakdown of intracellular GTP to dihydroneopterin which can diffuse from the cell and either be oxidized to the high fluorescent neopterin by HOCl or to 7,8-dihydroxanthopterin by reactive oxygen species.

macrophages into the intracellular spaces and finally the plasma (Figure 1). Some of the 7,8-dihydroneopterin is oxidized to the highly fluorescent neopterin. The release of 7,8-dihydroneopterin and neopterin is specific to monocytes, macrophages and dendritic cells (Wirleitner *et al*., 2002) although kidney epithelial cells have also been observed to release neopterin (Moutabarrik *et al*., 1994).

Primate macrophages (including human) are unique in this response, as non-primate macrophages convert 7,8-dihydroneopterin-triphosphate into inducible nitric oxide synthase (iNOS) cofactor 5,6,7,8-tetrahydrobiopterin. As a result primate macrophages, unlike mouse macrophages, do not generate significant levels of nitric oxide when stimulated with interferon but release the highly fluorescent neopterin.

Interestingly, the main reaction generating neopterin from 7,8-dihydroneopterin is oxidation by hypohalous acids such as HOCl (Widner *et al*., 2000). Proton abstraction from carbon-7 and nitrogen-8 of 7,8-dihydroneopterin generates neopterin. Neutrophils and possibly macrophages release significant amounts of HOCl during inflammation (Schraufstatter *et al*., 1990) suggesting much of the neopterin measured in plasma has come from sites of inflammation where HOCl is being released. The presence of neopterin within plasma is further indication of the inflammatory origin of these pterins.

Clinical measurement

The central role of γ-interferon communicating between T cells and macrophages with the subsequent release of neopterin make plasma neopterin measurements an ideal method for gauging immune activation within a patient (Wachter *et al*., 1989, 1992). The injection of γ-interferon causes a rapid and sustained rise in plasma neopterin levels (Muller *et al*., 1991). Neopterin is easily measured in plasma and urine by HPLC due to its extremely high fluorescence

(Werner *et al*., 1987; Rippin, 1992) although many clinical laboratories also use immuno-based methods such as enzyme-linked immunosorbent assay to measure neopterin (Westermann *et al*., 2000).

In response to infection, plasma neopterin levels rise rapidly in parallel with C-reactive protein levels, well before a patient becomes sero-positive. Measurements of plasma neopterin levels are used as a clinical tool to assess efficacy of treatments for a range of infections including malaria (Reibnegger *et al*., 1984; Awandare *et al*., 2006), tuberculosis (Fuchs *et al*., 1984; Yuksekol *et al*., 2003) and human immunodeficiency virus (Fuchs *et al*., 1988) to name a few. As elevated neopterin levels appear to occur with most inflammatory conditions, some hospitals measure plasma neopterin as a primary screen for blood donations (Strohmaier *et al*., 1996). The monitoring of plasma neopterin has also been used in the study and management of cancer (Reibnegger *et al*., 1991), autoimmune disease (Reibnegger *et al*., 1986; Schroecksnadel *et al*., 2003) and transplant patients (Margreiter *et al*., 1983; Yokoyama *et al*., 2002) where the rise in plasma or urine neopterin levels can give clinicians adequate warning of allograft rejection enabling them to alter immunosuppressant treatment.

Although plasma neopterin is not generally used in the management of vascular disease, there is a growing amount of knowledge on its value. Serum neopterin is elevated in patients with unstable angina and acute myocardial infarction (Tatzber *et al*., 1991; Schumacher *et al*., 1992, 1997). There is also a strong correlation between serum neopterin and the thrombolysis in myocardial infarction risk score in patients with unstable angina, or acute myocardial infarction (Johnston *et al*., 2006). The anti-inflammatory affects of HMG-CoA inhibiting statin drugs is also demonstrated by the lowering of serum neopterin levels (Neurauter *et al*., 2003; Walter *et al*., 2003).

Surprisingly, although the Web of Science lists over 2000 references relating to neopterin, the exact role of neopterin synthesis and release by monocyte-derived cells is not understood. It has been suggested that neopterin and 7,8-dihydroneopterin are synthesized as pro-oxidants, enhancing oxidant production and cell death in combination with tumour necrosis factor (TNF). In contrast, 7,8-dihydroneopterin has also been reported to act as an antioxidant, protecting biomolecules and macrophages from oxidants released during inflammation. Neopterin release has also been suggested to provide a feedback to T cells on the level of immune activation occurring. There is good experimental evidence supporting all these mechanisms and all have the potential to alter atherosclerotic plaque development.

Inhibition of LDL oxidation

Like many reducing agents, 7,8-dihydroneopterin rapidly reacts with free radical and oxidizing species. This was first noted with chemiluminescence-based assays where reduced pterins, including 7,8-dihydroneopterin, were found to inhibit the luminescence signal from superoxide and hydrogen peroxide (Shen, 1994). When 7,8-dihydroneopterin was sent to Esterbauer's laboratory in Graz, Austria, it was soon found that 7,8-dihydroneopterin was a potent inhibitor of metal ion and aqueous peroxyl radical (2,2′-azobis(amidinopropane) dihydrochloride (AAPH))-mediated LDL oxidation (Gieseg *et al*., 1995). A few years earlier, tetrahydroneopterin had been shown to inhibit xanthine/xanthine oxidase and phorbol myristate acetate stimulated macrophage superoxide production (Kojima *et al*., 1992) and inhibiting linoleic acid oxidation, so in hindsight it was not a surprising finding. What was unexpected was that 7,8-dihydroneopterin could out-compete the LDL tocopherol (vitamin E) for the primary propagating lipid radical. 7,8-Dihydroneopterin had a high reaction rate with peroxyl radicals, but this did not explain how a water-soluble compound could react with the lipid radicals within the LDL particle (Oettl *et al*., 1997). This mechanism remains unresolved but it has been suggested that 7,8-dihydroneopterin may bind or become compartmentalized on the LDL (Gieseg *et al*., 1995, 2003). Studies on the inhibition of peroxynitrite oxidation of LDL by 7,8-dihydroneopterin suggested that 7,8-dihydroneopterin might diffuse into the phospholipid layer of the LDL particle (Herpfer *et al*., 2002). With both peroxynitrite and copper-mediated LDL oxidation, the protective effect of 7,8-dihydroneopterin was enhanced by preincubation before addition of the oxidant. Nitric oxide and peroxynitrite have been implicated in modification of LDL within plaques, especially the nitration of some amino-acid side chains like tyrosine which is effectively blocked by 7,8-dihydroneopterin (Widner *et al*., 1998; Oettl *et al*., 2004). Protein hydroperoxides and their decay product carbonyls make a large and significant contribution to the oxidative damage occurring on the LDL particle (Yan *et al*., 1997; Gieseg *et al*., 2003). This protein oxidation on the ApoB100 moiety of the LDL particle is effectively inhibited by 7,8-dihydroneopterin through scavenging of lipid peroxyl radicals.

Although oxidative levels of transition metals and peroxyl radicals appear to exist within atherosclerotic plaques, copper ion and AAPH-peroxyl radical-mediated LDL oxidation appears relatively artificial when carried out in dilute buffers. There is evidence that ceruloplasmin-bound copper ions alone maybe able to oxidize LDL within atherosclerotic plaques (Shukla *et al*., 2006). Macrophages and other cells are considered by many to be the key mediators of oxLDL formation within atherosclerotic plaques. *In vitro* cell-mediated oxLDL formation is either superoxide dependent or independent depending on the cell type and condition used (Jessup *et al*., 1993; Aviram *et al*., 1996). With monocyte-like THP-1 cells and human monocyte-derived macrophages (HMDM), oxLDL formation is totally inhibited by micromolar concentrations of 7,8-dihydroneopterin (Gieseg *et al*., 2003; Gieseg and Cato, 2003). As THP-1 cell-mediated oxidation is independent of superoxide formation, it is likely inhibition is due to scavenging the lipid peroxyl radicals in the LDL.

However, 7,8-dihydroneopterin can also accelerate LDL oxidation if added after initiation of the oxidation process due to its role as a reducing agent (Herpfer *et al*., 2002; Greilberger *et al*., 2004). 7,8-Dihydroneopterin reduces oxidized metal ions, which increases the pool of reduced copper ions available to react with polyunsaturated lipids and peroxyl radicals within the LDL. The same effect was

reported in phosphate-buffered solution containing low levels of iron where 7,8-dihydroneopterin enhanced the rate of hydroxyl radical generation (Oettl *et al*., 1999). So like tocopherol, under the right conditions, 7,8-dihydroneopterin may actually accelerate oxLDL formation within plaque (Bowry *et al*., 1992; Niu *et al*., 1999).

It is interesting to note that 7,8-dihydroneopterin is not the only compound generated by γ-interferon-stimulated macrophages, which can inhibit LDL oxidation. The enzyme indoleamine 2,3-dioxygenase (IDO) is also upregulated by interferon stimulation. IDO catalyses the degradation of the amino-acid tryptophan to a range of products including kynurenine and 3-hydroxyanthranilic acid (3HAA) (Werner-Felmayer *et al*., 1990). Like 7,8-dihydroneopterin, 3HAA inhibits copper and AAPH-peroxyl radical-mediated LDL oxidation at micromolar concentrations. At high tryptophan concentrations, interferon-stimulated macrophages generated enough 3HAA to inhibit macrophage-mediated LDL oxidation (Christen *et al*., 1994; Thomas *et al*., 1996).

In vitro, interferon stimulation of macrophages fails to generate enough 7,8-dihydroneopterin to inhibit the LDL oxidation. Tissue culture levels are in the low nanomolar range although can be elevated to over 100 nM with the addition of either colony-stimulation factor (GM-CSF), phorbal esters, or IL-6 to the interferon-containing media. Yet our studies have shown the level of neopterin within atherosclerotic plaques is in the micromolar range (Figure 2). This suggests that additional factors are involved within plaques to generate these elevated levels of neopterin. The fact that the plaques appear to be the source of serum neopterin also suggests a high level of 7,8-dihydroneopterin synthesis within the plaque.

On this basis 7,8-dihydroneopterin and 3HAA, generated by the interferon-stimulated macrophages should inhibit plaque formation and LDL oxidation. Yet oxidized lipids and proteins can be detected within atherosclerotic plaques and high levels of neopterin. This suggests there is some sort of balance between oxidant and antioxidant, which becomes disturbed in regions of the artery wall. The correction of this balance through the control of macrophage antioxidant/oxidant generation may slow or inhibit plaque growth.

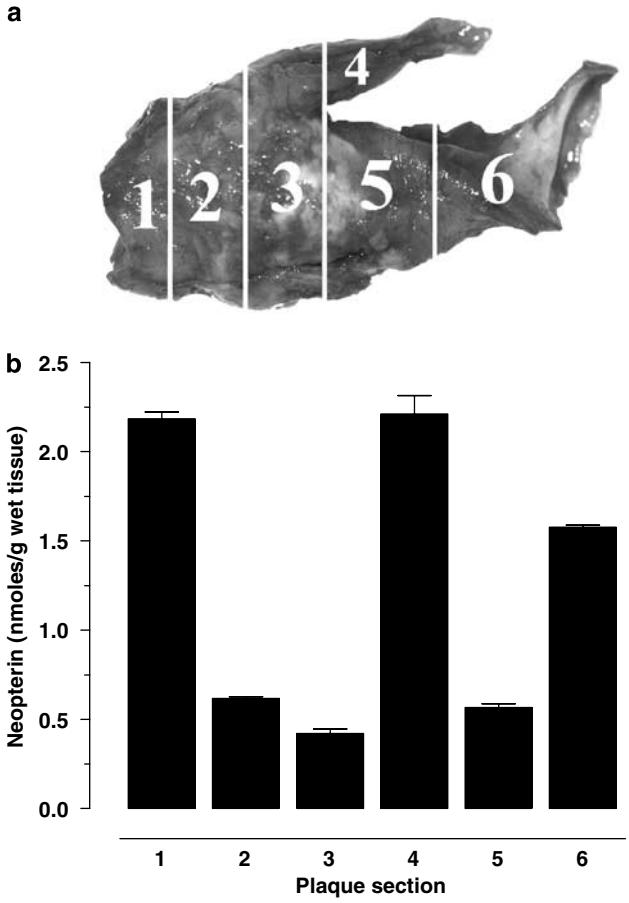

Figure 2 Concentration of neopterin across the length of a carotid atherosclerotic plaque. The plaque was removed via carotid endarterectomy from the left common carotid artery. The plaque was sectioned longitudinally into six slices, 2–3 mm thick, from the proximal to the distal end (**a**). Sections 1–2 represent the pre-bifurcation region, sections 3 and 5 the bifurcation, section 4 the secondary branch and section 6 the post-bifurcation of the primary branch. The sections were homogenized and neopterin levels measured using HPLC (high-performance liquid chromatography) with fluorescence detection (**b**). The plaque sample was highly calcified and caused a 90% stenosis in the artery.

Protection from cellular toxicity

Sites of inflammation, including atherosclerotic plaques, contain a range of reactive oxidants which can trigger apoptotic and necrotic death within cells (Martinet and Kockx, 2001). OxLDL is particularly cytotoxic to a range of cells including macrophages (Clare *et al*., 1995; Marchant *et al*., 1995). The finding that reduced pterins such as 7,8-dihydroneopterin can protect LDL and other biomolecules leads to the hypothesis that 7,8-dihydroneopterin was synthesized by interferon-stimulated macrophages to protect these antigen-presenting cells from the oxidants encountered within an inflammatory site (Schroder *et al*., 1987; Kojima *et al*., 1992; Gieseg *et al*., 1995, 2002). The fact that 3HAA is also generated by macrophages during inflammation further supports this hypothesis (Christen *et al*., 1990; Werner-Felmayer *et al*., 1990). Micromolar concentrations of 7,8-dihydroneopterin do inhibit cellular damage to red blood cells and the monocyte like human-derived U937 cells, from a range of oxidants including hydrogen peroxide, hypochlorite, aqueous peroxyl and direct plasma membrane oxidation by ferrous ions (Gieseg *et al*., 2000, 2001a, b). There is evidence that all these oxidants occur within sites of inflammation and atherosclerotic plaques (Brown *et al*., 1997; Leeuwenburgh *et al*., 1997; Hazen *et al*., 2000; Stadler *et al*., 2004).

OxLDL-induced necrosis in monocyte like U937 cells is inhibited by micromolar concentrations of 7,8-dihydroneopterin (Baird *et al*., 2005). The mechanism appears to involve the protection of the intracellular glutathione pool thus maintaining the redox status of the cell. The same mechanism also appears to occur with 7,8-dihydroneopterin protection of HMDM cells where necrosis is triggered by the loss of glutathione by oxLDL exposure (unpublished data).

Surprisingly, 7,8-dihydroneopterin does not protect THP-1 cells, another human-derived monocyte-like cell line, from

oxLDL or AAPH-peroxyl radical-induced cell death (Baird *et al*., 2005). Although 7,8-dihydroneopterin protects HMDM cells from oxLDL, it provides little or no protection against AAPH-peroxyl radicals (Firth *et al*., 2007). Serum albumin oxidation by AAPH-peroxyl radicals is completely inhibited by 7,8-dihydroneopterin through scavenging of aqueous peroxyl radicals (Platt and Gieseg, 2003), yet with THP-1 cells and HMDM cells this inhibition did not appear to occur. This suggests that 7,8-dihydroneopterin protects cells through either a specific intracellular radical scavenger mechanism, or by acting as a form of signal molecule, which triggers various anti-necrotic processes within the cell.

Although neopterin is described as the oxidation product of 7,8-dihydroneopterin, the scavenging of hydrogen peroxide, superoxide and peroxyl radicals generates 7,8-dihydroxanthopterin (Gieseg *et al*., 2002). This compound forms through the loss of the trihydroxypropyl side chain attached to carbon-6 of 7,8-dihydroneopterin (Figure 1). The reaction appears to occur through a retro–aldol reaction initiated by the abstraction of an atom of hydrogen from the middle carbon hydroxyl group on the 7,8-dihydroneopterin side chain. Unfortunately, 7,8-dihydroxanthopterin is not fluorescent and is difficult to detect in plasma. Currently, the only mechanism to describe the generation of neopterin from 7,8-dihydroneopterin *in vivo* is the reaction with hypochlorite. Hypochlorite is released from activated neutrophils and possibly macrophages during inflammation (Chisolm *et al*., 1999). This highly reactive oxidant has been implicated in the modification of LDL within plaques and killing cells (Daugherty *et al*., 1994; Fabjan *et al*., 2001; Whiteman *et al*., 2005). 7,8-Dihydroneopterin protects cells from hypochlorite by rapidly reacting with it (Gieseg *et al*., 2001a, b). 7,8-Dihydroneopterin also appears to inactivate myeloperoxidase, the enzyme responsible for generating hypochlorite *in vivo* (Widner *et al*., 2000; Razumovitch *et al*., 2004).

Pro-oxidant effects and cell signalling

With macrophages, neopterin has no apparent effect on cell survival whereas 7,8-dihydroneopterin protects HMDM and U37 cells from oxLDL and some oxidants (Gieseg *et al*., 2002; Baird *et al*., 2005). At the extremely high concentration of 5 mM, 7,8-dihydroneopterin causes sufficient oxidative stress to kill monocyte-like U937 cells (Baier-Bitterlich *et al*., 1995), neuronal NT2/HNT cells (Spottl *et al*., 2000) and the rat pheochromocytoma cell line PC12 (Enzinger *et al*., 2002b) but only in the presence of TNF-α. Loss of mitochondrial dehydrogenase activity only occurs in ovarian carcinoma cell lines at 7,8-dihydroneopterin concentrations of 1 mM and higher (Rieder *et al*., 2006). Likewise, with Jurkart T cells, 7,8-dihydroneopterin only induces apoptosis above 1 mM (Baier-Bitterlich *et al*., 1996; Wirleitner *et al*., 1998, 2001) via the redox-sensitive Bcl-2 pathway (Enzinger *et al*., 2002a). All these mechanisms seem to involve the direct generation of oxidants within the tissue culture media due to the reducing activity of 7,8-dihydroneopterin as first observed in chemiluminescence assays (Oettl *et al*., 1999). 7,8-Dihydroneopterin, like ascorbate (Buettner, 1988), may cause the reduction of redox-active metal ions within the buffers, which will increase the formation of various reactive oxygen species. This oxidant generation only becomes significant at extremely high millimolar 7,8-dihydroneopterin concentrations and in protein-free media. Proteins are very effective radical scavengers so the high concentration of soluble protein within plaque may make these reactions unlikely though still possible.

Neopterin had no effect on cell death in these experiments but did cause apoptosis at lower micromolar concentrations with vascular smooth muscle (Schobersterger *et al*., 1996). The combination of interferon, TNF-α and between 10 and 100 μM neopterin caused iNOS activation and enhanced oxidative stress-triggering apoptosis (Hoffmann *et al*., 1996, 1998). The effect was also seen with 100 μM 7,8-dihydroneopterin (Schobersterger *et al*., 1996). This suggests that neopterin generated from oxidation of 7,8-dihydroneopterin during inflammation could trigger the death of the plaque smooth muscle cells, especially if there was sufficient levels of TNF-α.

Intracellular calcium in human-derived monocyte-like THP-1 cells is affected by micromolar levels of both neopterin and 7,8-dihydroneopterin and effectively inhibits ATP-induced calcium release from alveolar epithelial cells (Woll *et al*., 1993; Hoffmann *et al*., 2002). At similar concentrations, neopterin was also reported to cause cardiac contractile dysfunction in isolated perfused rat hearts (Margreiter *et al*., 2000; Balogh *et al*., 2005). The mechanism proposed behind this activity was oxidative stress but the reactive chemistry, and low oxidant yield observed with neopterin suggests a more direct mechanism in the cells and the intracellular calcium pools. The study clearly shows infusion of micromolar levels of pterin may have adverse clinical outcomes. The capacity of neopterin and 7,8-dihydroneopterin to cause calcium release may be important in plaque development where the formation of calcium deposits represents a serious deterioration in patient prognosis due to the increasing complexity of the plaque tissue.

Atherosclerotic plaques

From the current literature, a hypothetical model can be drawn where interferon-stimulated macrophages release 7,8-dihydroneopterin to inhibit oxidation and cell death by scavenging oxidants generated by metal ions and superoxide released by cells. The 7,8-dihydroneopterin scavenges the neutrophil-released hypochlorite-producing neopterin, which inhibits further hypochlorite release via inhibition of myeloperoxidase. The neopterin also stimulates cell death in combination with TNF-α released by the various immune cells present. In experimental models of sepsis where healthy volunteers are infused with endotoxin, the peak in neopterin levels occurs 20 h after the peak in TNF-α (Fijen *et al*., 2000) showing that 7,8-dihydroneopterin activity occurs late in the inflammatory process. But what happens during the chronic inflammatory process of atherosclerosis? Low levels of 7,8-dihydroneopterin and possibly 3HAA would shift the redox balance to oxidation causing oxLDL formation and cell necrosis/apoptosis. High hypochlorite formation would

generate elevated neopterin levels shutting off myeloperoxidase activity and possibly triggering neutrophil apoptosis. However, excess 7,8-dihydroneopterin synthesis with low levels of hypochlorite would give a high localized 7,8-dihydroneopterin concentration which would enhance the cell stability. Manipulation of this system could prevent the growth of plaques and their development to complex, unstable plaques through inhibiting oxLDL formation and cell apoptosis/necrosis.

The hypothesis that 7,8-dihydroneopterin is generated as an antioxidant also suggests a reason for the development of this response. The enzyme IDO which is also upregulated by γ-interferon is rapidly inhibited by nitric oxide (Thomas *et al*., 1994). So it is possible that primate macrophages have evolved to suppress nitric oxide production by macrophages to preserve the activity of IDO. The result of this is that interferon stimulation of macrophages causes the synthesis of two potent antioxidants, 3HAA and 7,8-dihydroneopterin. This combination of antioxidants may allow primate macrophages to survive longer within sites of inflammation although this has yet to be shown. The down side of this mechanism may mean human macrophages survive longer within atherosclerotic plaques so enhancing plaque formation.

The key question is then, what is the *in vivo* concentration of neopterin and 7,8-dihydroneopterin? The *in vitro* studies clearly show that both 7,8-dihydroneopterin and neopterin could have significant effects on plaque growth. Our own studies on atherosclerotic plaques have shown that neopterin levels can be as high as 2.5 μM within some sections of the plaque (Figure 2). The labile nature of 7,8-dihydroneopterin makes it difficult to accurately measure 7,8-dihydroneopterin levels within plaques but it is possible that the concentration greatly exceeds the levels of neopterin measured. We feel it is likely that 7,8-dihydroneopterin/neopterin is being generated within plaques at concentrations that influence LDL oxidation and cell survival. The literature also suggests that where hypochlorite is oxidizing 7,8-dihydroneopterin to neopterin, the level of neopterin will be sufficiently high to promote cell death and plaque instability, especially where elevated levels of TNF-α occur. In support of this hypothesis, elevated levels of neopterin have been observed within unstable plaques (Garcia-Moll *et al*., 2000a, b) and some correlation between plasma neopterin and TNF-α has been reported in atherosclerosis patients (Anwaar *et al*., 1998). Also the lowering of neopterin levels with statin treatment is associated with increased patient survival (Neurauter *et al*., 2003; Walter *et al*., 2003). The control of the neopterin/7,8-dihydroneopterin system through specific anti-inflammatory agents may prevent the formation of complex plaques. γ-Interferon, GM-CSF and 1,25-dihydroxyvitamin D3 all increase 7,8-dihydroneopterin synthesis within macrophage cells (Schwende *et al*., 1996) while histamine inhibits 7,8-dihydroneopterin synthesis (Gruber *et al*., 2000). The difference in concentrations we have measured in plaques and those observed in tissue culture suggests there are other agents, which promote 7,8-dihydroneopterin/neopterin synthesis which may help control oxidative stress within plaques. The early observation that treatment of hepatitis C patients with γ-interferon decreased the level of serum lipid peroxides suggests that modulation of the 7,8-dihydroneopterin/neopterin system to the benefit of the patient might be possible (Higuras *et al*., 1994). To achieve this, a greater understanding of the plaque concentration of 7,8-dihydroneopterin and how this relates to the other antioxidants and oxidants present is required. The role of neopterin and TNF-α induced death within plaques also needs to be further quantified.

Conclusion

In vitro studies have demonstrated that neopterin and 7,8-dihydroneopterin could alter the redox balance within atherosclerotic plaques. 7,8-Dihydroneopterin promotes cell stability while inhibiting oxidative damage. Neopterin promotes cell apoptosis and changes in intracellular calcium. The balance between these two pterins *in vivo* is dependent on the rate of pterin synthesis in the macrophage and the level of hypochlorite and other oxidants within the plaque. Anti-inflammatory agents which promote 7,8-dihydroneopterin synthesis while inhibiting hypochlorite production may move the plaque oxidative environment to a less oxidative state so limiting oxLDL formation and cell death.

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Conflicts of interest

The authors state no conflict of interest.

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