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OxLDL induced cell death is inhibited by the macrophage synthesised pterin, 7,8-dihydroneopterin, in U937 cells but not THP-1 cells

Sarah K. Baird[a], Linzi Reid[a], Mark B. Hampton[b], Steven P. Gieseg[a,*]

[a]*Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch, New Zealand*
[b]*Department of Pathology, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand*

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Abstract

The atherosclerotic plaque is an inflammatory site where macrophage cells are exposed to cytotoxic oxidised low density lipoprotein (oxLDL). Interferon-γ released from T-cells results in macrophage synthesis of 7,8-dihydroneopterin which has antioxidant and cytoprotective activity. Using the human derived monocyte-like U937 and THP-1 cell lines, we examined whether 7,8-dihydroneopterin could inhibit the cytotoxic effect of oxLDL. In U937 cells, oxLDL caused a dramatic loss of cellular glutathione and caspase independent cell death associated with phosphatidylserine exposure on the plasma membrane. 7,8-Dihydroneopterin completely blocked the cytotoxic effect of oxLDL. In contrast, oxLDL initiated THP-1 cell apoptosis with reduction in cellular thiols, caspase-3 activation and plasma membrane phosphatidylserine exposure. 7,8-Dihydroneopterin was unable to alter these processes or restore the THP-1 cellular thiol content. 7,8-Dihydroneopterin did provide some protection to both THP-1 cells and U937 cells from AAPH derived peroxyl radicals. The preincubation of oxLDL with 7,8-dihydroneopterin did not reduce cytotoxicity, suggesting that 7,8-dihydroneopterin may be acting in U937 cells by scavenging intracellular oxidants generated by the oxLDL. The data show that μM levels of 7,8-dihydroneopterin may prevent oxLDL mediated cellular death within atherosclerotic plaques.

Keywords: Neopterin; Oxidised-low-density-lipoprotein; Macrophage; Caspase; Apoptosis; Glutathione

1. Introduction

The presence of immune cells and markers of immune cell activation strongly support the view that atherosclerotic plaques are sites of localised chronic inflammation [1–3]. One of the immune markers reported to correlate with atherosclerotic plaque development is plasma neopterin [4,5]. Neopterin can be formed through the oxidation of 7,8-dihydroneopterin by HOCl [6,7]. The presence of chloro-tyrosine indicates the generation of HOCl within atherosclerotic plaques and γ-interferon stimulation of macrophages results in the synthesis and release of 7,8-dihydroneopterin [8,9]. This would suggest that a probable source of the plasma neopterin is activated macrophages within atherosclerotic plaques.

The exact function of macrophage 7,8-dihydroneopterin synthesis and release in response to γ-interferon stimulation is uncertain. 7,8-Dihydroneopterin is a redox active compound capable of acting as either a pro-oxidant or antioxidant depending on the chemical environment. We have previously shown that at low μM concentrations 7,8-dihydroneopterin is a potent antioxidant capable of protecting cells, cellular membranes [6], free proteins, cellular protein and protein thiols [10–12] from oxidant damage. Copper, peroxyl radical and cell mediated LDL oxidation is

Abbreviations: AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); EDTA, Ethylenediaminetetraacetic acid disodium salt; GSH, glutathione; HPLC, high performance liquid chromatography; LDL, Low density lipoprotein; oxLDL, oxidised low density lipoprotein; PS, phosphatidylserine; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate

* Corresponding author. Tel.: +64 3 364 2860; fax: +64 3 364 2024.
E-mail address: Steven.Gieseg@canterbury.ac.nz (S.P. Gieseg).

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also effectively inhibited by μM levels of 7,8-dihydroneopterin [13–15]. Much of this protection is due to the potent radical scavenging ability of 7,8-dihydroneopterin with peroxyl radicals, superoxide, hypochlorite and hydroxyl radicals [12,16–18]. These observations have led us and others to suggest that 7,8-dihydroneopterin may be synthesised to protect macrophages from the oxidants present in inflammatory sites such as atherosclerotic plaques [12,13]. At mM pterin concentration neopterin and 7,8-dihydroneopterin have been reported to promote radical generation and apoptosis in a range of cell types exposed to different forms of cellular stress [19–22].

One of the major oxidants present within atherosclerotic plaques is oxidised LDL (oxLDL) [23]. The uncontrolled uptake of oxLDL by macrophages results in the formation of lipid loaded foam cells. It is the build up of foam cells within the artery wall which characterises the atherosclerotic plaque [24]. The cytotoxicity of oxLDL appears to cause the death of macrophages and smooth muscle cells deep within the plaque, leading to the formation of a necrotic core region [23,25,26].

To provide some insight into the possible effect of 7,8-dihydroneopterin within plaques, we have examined the effect of this pterin in two different human monocyte-like cell lines exposed to oxLDL. The THP-1 and U937 cell lines have been extensively used to model aspects of atherosclerosis and are equally susceptible to oxLDL induced cell death. We have previously reported that oxLDL triggers caspase-3 activation and apoptotic cell death in THP-1 cells, while cell death in the U937 cells occurs independently of caspase-3 activity and is characterised by a massive loss of intracellular thiols [27].

2. Methods

All chemicals used were of AR grade or better and supplied by either the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited. Tissue culture media including the heat inactivated foetal calf serum was supplied by Invitrogene New Zealand Limited and plastic ware from Sarstedt Australia Pty Ltd. through Global Science and Technology Ltd. (New Zealand). Neopterin and 7,8-dihydroneopterin were obtained from Schirck's Laboratories, Switzerland. 2,2′-azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company Inc. Chelex-100 resin was supplied by Bio-Rad Laboratories (NZ). All solutions were prepared using ion-exchanged ultrafiltered water from a NANOpure ultrapure water system supplied by Barnstead/Thermolyne (Iowa, USA). Phosphate buffered saline (PBS) solution consisted of 150 mM sodium chloride and 10 mM sodium phosphate pH 7.4.

LDL was purified from EDTA treated plasma collected by venapuncture from healthy male and female donors following an overnight fast. After removal of cellular components by centrifugation, the plasma from multiple donors was pooled and frozen at −80 °C for up to 6 months. LDL was purified by a single 22 h ultracentrifugation of the pooled EDTA-plasma using a buoyant density four step discontinuous gradient in a Beckman SW41 rotor [28,29]. LDL concentration (total mass) was determined by enzymatic cholesterol determination using the "Chol MPR 2" kit supplied by Roche Chemicals (New Zealand), assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6% [29]. Purified LDL was desalted by dialysis against nitrogen-gassed chelex-treated phosphate buffered saline (pH 7.4). OxLDL was prepared by incubating LDL at a concentration of 4 mg/ml with 200 μM $CuSO_4$ solution for 24 h at 37 °C before stirring with chelex-100 resin for 2 h to remove copper ions. HPLC analysis[14] showed the oxidation had increased the LDL TBARS level from 0.2 to 1.9 nmol/mg LDL. The electrophoretic mobility of the oxLDL was 3.2-fold higher than the native LDL. Native and oxLDL were concentrated with Urifil-10 concentrators (Millipore, MA, USA) and sterilised through a 0.22-μm membrane filter (Pall Gelman Laboratory) before addition to the cells.

THP-1 and U937 cells were grown in RPMI 1640 with 2 mM glutamine, 5% heat-inactivated foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at a density of not more than 2×10^6/ml. During experiments, cells were incubated for up to 48 h in RPMI 1640 alone at 5×10^5 cells/ml with the described additives before washing in warm PBS to remove the media before analysis.

Cell viability was determined by trypan blue dye exclusion [30] and the cells' ability to reduce MTT (Sigma) [31] by using 10% $^{W}/_{V}$ sodium dodecyl sulphate (SDS, final concentration) to lyse the cells and solubilise the insoluble MTT-formazan salt.

Total cellular thiol content was determined by incubating the PBS washed cells in 10% $^{W}/_{V}$ SDS and 30 μM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, final concentration shown) for 30 min at room temperature and reading the absorbance at 412 nm [32].

Cellular glutathione (GSH) was measured in PBS washed cells by derivatisation with monobromobimane and analysis by gradient reverse phase high performance liquid chromatography (HPLC) with fluorescence detection [33]. The GSH concentration was calculated from comparison to peak area of known concentrations of glutathione solutions, standardised using DTNB.

7,8-Dihydroneopterin and neopterin concentrations in the media were measured by reverse phase HPLC with electrochemical and fluorescence detection as previously described [34].

Caspase-3-like activity was determined by measuring the rate of cleavage of the artificial substrate DEVD-AMC (Sigma) [35]. PBS washed cells (1×10^6) were lysed in 100 μL of caspase reagent buffer containing 100 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid),