ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 10, 2010

DOI: 10.1089/ars.2009.3065

FORUM ORIGINAL RESEARCH COMMUNICATION

Oxidant Production, oxLDL Uptake, and CD36 Levels in Human Monocyte–Derived Macrophages Are Downregulated by the Macrophage-Generated Antioxidant 7,8-Dihydroneopterin

Steven P. Gieseg, Zunika Amit, Ya-Ting Yang, Anastasia Shchepetkina, and Hanadi Katouah

Abstract

The severity of atheroma burden in patients strongly correlates to increasing levels of plasma neopterin, the oxidation product of 7,8-dihydroneopterin. Interferon-γ stimulation of macrophages causes the synthesis of 7,8-dihydroneopterin, a potent antioxidant that inhibits oxidative damage to cells, and the cytotoxicity of oxidized low-density lipoprotein (oxLDL) to monocyte-like U937 cells but not THP-1 cells. With human monocyte–derived macrophages (HMDMs), oxLDL triggered a large oxidative stress, causing the rapid loss of cellular glutathione, glyceradehyde-3-phosphate dehydrogenase (GAPDH) inhibition, and eventual loss of viability without caspase-3 activation. Inhibition of oxLDL cytotoxicity to HMDMs occurred at 7,8-dihydroneopterin concentrations $>100\,\mu M$. The oxLDL-mediated glutathione loss and GAPDH inactivation was inhibited by 7,8-dihydroneopterin. 7,8-Dihydroneopterin rapidly entered the HMDMs, suggesting that much of the protective effect was scavenging of intracellular oxidants generated in response to oxLDL. OxLDL uptake by HMDMs was reduced by 30% by 7,8-dihydroneopterin. Immunoblot analysis suggests that this decrease in oxLDL uptake was due to a significant downregulation in the levels of CD36. These results imply that 7,8-dihydroneopterin protects human macrophages both by scavenging oxidants generated in response to oxLDL and by decreasing CD36-mediated uptake of oxLDL. *Antioxid. Redox Signal*. 13, 1525–1534.

Introduction

Increased clinical use of plasma neopterin is seen as a marker of a patient's level of inflammation in conditions as diverse as cancer and tuberculosis infection. Neopterin is the oxidation product of 7,8-dihydroneopterin, a pterin synthesized by macrophages when stimulated by γ-interferon released by Th-1 cells (17, 52). The increase of plasma neopterin levels has been strongly correlated with increased atherosclerotic burden (1, 43, 47). Significant amounts of neopterin and 7,8-dihydroneopterin have been reported in the interior of an atherosclerotic plaque (13, 17), which represents localized sites of chronic inflammation (36). Although neopterin is a sensitive marker of inflammation, it is becoming increasingly apparent that 7,8-dihydroneopterin and neopterin have distinct biologic properties. They may have significant influence on the inflammatory process, especially in chronic inflammation, such as atherosclerosis.

In vitro studies have shown that 7,8-dihydroneopterin is a potent antioxidant that can protect a range of biomolecules and cells from various oxidants, including hydroxyl and peroxyl radicals (21, 24, 25, 31). Oxidized low-density lipoprotein (OxLDL) formation with copper, peroxyl, or cellular oxidants is effectively inhibited by scavenging of the chain carrying lipid-peroxyl radical within the LDL particle by 7,8-dihydroneopterin (13, 15, 23, 24). 7,8-Dihydroneopterin accelerates copper-mediated oxidation if added after the lag phase because of increased metal reduction (26).

7,8-Dihydroneopterin is effective in protecting the monocyte-like U937 cells from the cytotoxic effects of oxidized low-density lipoprotein (oxLDL) (5). Cell death due to oxLDL cytotoxicity is considered to be a major factor in the development of a necrotic core in advanced atherosclerotic plaques (28, 30). Our previous studies have shown 7,8-dihydroneopterin protects U937 cells from oxLDL by preventing the loss of intracellular glutathione and the initiation of necrosis (5).

Free Radical Biochemistry Laboratory, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.

Although oxLDL itself is not capable of directly generating oxidants, exposure to U937 cells causes a large intracellular oxidant flux, which can be scavenged or inhibited by 7,8-dihydroneopterin.

Surprisingly, 7,8-dihydroneopterin cannot protect the related monocyte-like THP-1 cells from oxLDL-induced cytotoxicity (5). In THP-1 cells, oxLDL triggers caspase activation and apoptosis without the loss of glutathione (4). This non-oxidative stress mechanism in THP-1 cells does not appear to be inhibited by 7,8-dihydroneopterin. OxLDL-induced cell death occurs through a number of mechanisms, depending on the type of cell under study and the level to which the LDL has oxidized (22). The proposed mechanisms include calpain activation through oxysterol-induced alterations in lipid rafts–associated calcium channels (6), dephosphorylation and inhibition of protein kinase B, leading to mitochondrial dysfunction through Bcl-2 and MAPK (9, 45), lysozyme destabilization (7, 34), and CD36-linked NADPH oxidase activation (33, 37).

Human macrophages derived from peripheral blood monocytes, usually described as human monocyte–derived macrophages (HMDMs) have been described undergoing both caspase and caspase-independent cell death (3, 22, 53). We have observed oxLDL causing HMDMs to undergo a caspase-independent cell death characterized by a rapid loss of glutathione, caspase inactivation, and the appearance of necrotic cell morphology (22). In this study, we examined whether 7,8-dihydroneopterin can inhibit oxLDL-induced death in HMDMs and whether the mechanism involves the reduction of intracellular oxidative stress, as observed with U937 cells.

Materials and Methods

All reagents and chemicals were AR grade or better and obtained from either the Sigma Chemical Company (St. Louis, MO) or BDH Chemicals New Zealand Limited. Solutions were prepared by using high-purity water generated from an NANOpure ultrapure water system from Barnstead/Thermolyne (Dubuque, IA). Cells were grown in Nunc plates supplied by In Vitro New Zealand Ltd., and the remaining plastic ware was supplied by Greiner Bio-one through Raylab New Zealand Ltd. Neopterin and 7,8-dihydroneopterin were obtained from Schirck's Laboratories, Jona, Switzerland. Phosphate-buffered saline (PBS) solution consisted of 150 m*M* sodium chloride and 10 m*M* sodium phosphate, pH 7.4.

LDL was purified by buoyant density-gradient ultracentrifugation by using a Beckman SW41 rotor from EDTA-treated plasma collected by venipuncture from healthy male and female donors after an overnight fast, as previously described (5, 10, 23). LDL concentration (total mass) was determined by enzymatic cholesterol assay with the Chol MPR 2 kit supplied by Roche Chemicals (Bern, Switzerland) (20). Heavily oxidized oxLDL with the properties previously described (5) was prepared by incubating LDL at a concentration of 3 mg/ml (600 μg/ml protein) with 400 μM $CuCl_2$ solution for 24 h at 37°C before stirring with chelex-100 resin for 2 h to remove copper ions. The oxLDL was concentrated by using a Vivapore membrane concentrator (Millipore, Billerica, MA) before filter sterilization through a 0.22-μm membrane filter and then stored at 4°C.

Human monocyte–derived macrophages (HMDMs) were prepared from whole blood donated by clinically healthy hemochromatosis patients at the NZ Blood Services (Christchurch) under ethics approval CTY/98/07/069 granted by the New Zealand Upper South B Ethics Committee. The monocytes were purified by centrifugation over Lymphoprep and differentiated into adherent macrophages in adherent 12-well plates (14). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated human serum plus 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was determined by MTT reduction (38). Cell protein concentration was determined by using the BCA Protein Determination kit supplied by Pierce, (Rockford, IL) with bovine serum albumin as the standard.

Cellular glutathione levels were measured by derivation with monobromobimane and reverse-phase C_{18} high-performance liquid chromatography (HPLC) analysis of the fluorescent derivative by using monobromobimane-derivatized GSH as a standard (11). Neopterin and 7,8-dihydroneopterin were measured with reverse-phase HPLC analysis of cell lysates after removal of proteins by precipitation with trichloroacetic acid (13, 21). GAPDH activity was measured in cell lysates by monitoring the rate of conversion of $NADP^+$ to NADPH in the presence of glyceraldehyde-3-phosphate over a 4-min period (49). OxLDL uptake by HMDMs was measured by using DiI-labelled oxLDL (12, 50). The DiI uptake was measured by lysing cells into isopropanol and measuring the native DiI fluorescence intensity. The cellular protein concentration was determined by analysis of cell pellets collected after centrifugation of the isopropanol. Intracellular superoxide release was detected with dihydroethidium (DHE) staining (8) of cells grown on sterile glass coverslips. The cells were incubated for 20 min with DHE before examination with fluorescence microscopy.

Caspase-3, CD36, and scavenger receptor-A (SCR-A) were detected by immunoblotting after SDS-PAGE electrophoresis cells were harvested by scraping in lysis buffer (40 m*M* HEPES, 50 m*M* NaCl, 1 m*M* EDTA, and 1 m*M* EGTA, plus one tablet of Roche Complete mini protease inhibitor per 10 ml). Immunoblots were probed with either anti-CD36 rabbit polyclonal (NB400-145, Novus Biologicals, Cambridge, UK), diluted 1:1,000, followed by peroxidase-conjugated goat polyclonal anti-rabbit IgG (NB730-H, Novus Biologicals) at 1:2,000 dilution; or anti-caspase-3 (E-8) (SC-7272, Santa Cruz Biotechnology, Santa Cruz, CA) followed by conjugated goat anti-mouse IgG (F_c) (31434; Pierce Biotechnology); or anti-SR-A (I-20) (SC-20441, Santa Cruz Biotechnology) followed by peroxidase-conjugated donkey anti-goat IgG (SC-2020, Santa Cruz Biotechnology). Lane loading was accessed by reblotting with anti-β-actin (A5316, Sigma-Aldrich Chemical, St. Louis, MO) followed by peroxidase-conjugated sheep anti-mouse IgG (RPN4401, Amersham Biosciences, Amersham, England). Immunoblots were visualised by using Supersignal West Dura chemiluminescence from Pierce. The image was recorded on a Syngene Chemigenius-2 bioimaging system by using Genesnap software (Global, Aotearoa, NZ).

Phosphatidylserine (PS) translocation to the cell surface as a marker of apoptosis was measured by staining cells grown on coverslips with annexin-V-FITC by using the Annexin V Apoptosis Kit form Santa Cruz Biotechnology and viewing by fluorescence microscope by using Zeiss AxioImager.M1 epi-fluorescent microscope.

The data were analyzed by using the Prism software package supplied by Graphpad Software Inc. (San Diego,

CA). Comparisons among treatments were performed by using one-way analysis of variance (ANOVA). All results are expressed as the mean ± SD of triplicate treatments. Results shown are from single experiments, representative of a minimum of three. Where appropriate, significance is indicated as (***)$p \leq 0.001$; (**)$p \leq 0.01$, or (*)$p \leq 0.05$.

Results

OxLDL induced cell death in the HMDMs only after 12 h of incubation (Fig. 1A). Up to the 12-h time point, the cell morphology appeared normal, but after this, increasing numbers of lysed cells were observed. The appearance of lysed cells corresponded with the loss of cell viability, as measured by the ability to reduce MTT to the purple formazan product. The oxLDL-induced loss of viability was significantly inhibited by 50 μM 7,8-dihydroneopterin added 10 min before the oxLDL treatment (Fig. 1B). One hundred micromolar 7,8-dihydroneopterin reduced the loss of viability to a mean value of 20%, with the majority of the cells displaying normal morphology.

To understand the nature of the 7,8-dihydroneopterin protection, the mechanism of the oxLDL-induced death in the HMDMs was further examined, as conflicting reports in the literature exist on the oxLDL-induced death mechanism (22). Western blot analysis constantly failed to show any sign of proteolytic activation of caspase-3 in the oxLDL-treated HMDMs. The 32-kDa band of procaspase-3 was clearly seen (Fig. 2A), but no active 17- or 20-kDa band was evident. This confirms our earlier investigations, in which no caspase enzyme activity was observed with oxLDL exposure (22). Phosphatidylserine externalization in the plasma membrane appeared to increase, as indicated by staining with annexin-V after 3 and 6 h after the treatment with oxLDL (Fig. 2B–E). This process was significantly slowed and reduced in the presence of 7,8-dihydroneopterin (Fig. 2F–H). Although the effector caspase-3 appears not to be activated by oxLDL exposure, phosphatidylserine translocation, another classic marker of apoptosis, occurs.

We previously observed the loss of caspase activity during oxLDL-induced death of U937 cells (4). This was also accompanied by a large loss of glutathione, suggesting inhibition of caspase-3 activity through oxidative loss of key thiols within the enzyme. With the HMDMs, we observed a similar and very rapid loss of glutathione (Fig. 3A). Within 6 h of adding the oxLDL, nearly 50% of the glutathione was lost, with the level dropping to 36% of the control by 24 h. Generally, no significant loss was observed in the absence of oxLDL, although a small loss was observed at 24 h in this experiment. As with previous studies using 7,8-dihydroneopterin on macrophage-like cells (5, 25), addition of 7,8-dihydroneopterin alone had no significant effect on the levels of glutathione, but in the presence of oxLDL, a dose-dependent protection of the glutathione levels was noted at the end of a 24-h incubation (Fig. 3B). The 200 μM 7,8-dihydroneopterin maintained the glutathione level at 68% of the control value.

Based on studies with monocyte-like U937 cells (4, 29) and endothelial cells (51), we suspect that oxLDL induced the HMDM cells to generate reactive oxidants that depleted the intracellular glutathione levels. Treatment of cells with the fluorescent dye DHE confirmed that oxLDL was inducing an oxidative stress over the first 6 h (Fig. 4A–C), which was reduced by the addition of 7,8-dihydroneopterin (Fig. 4D and E). This suggested that the 7,8-dihydroneopterin was either inhibiting the oxidant production or scavenging the oxidant directly.

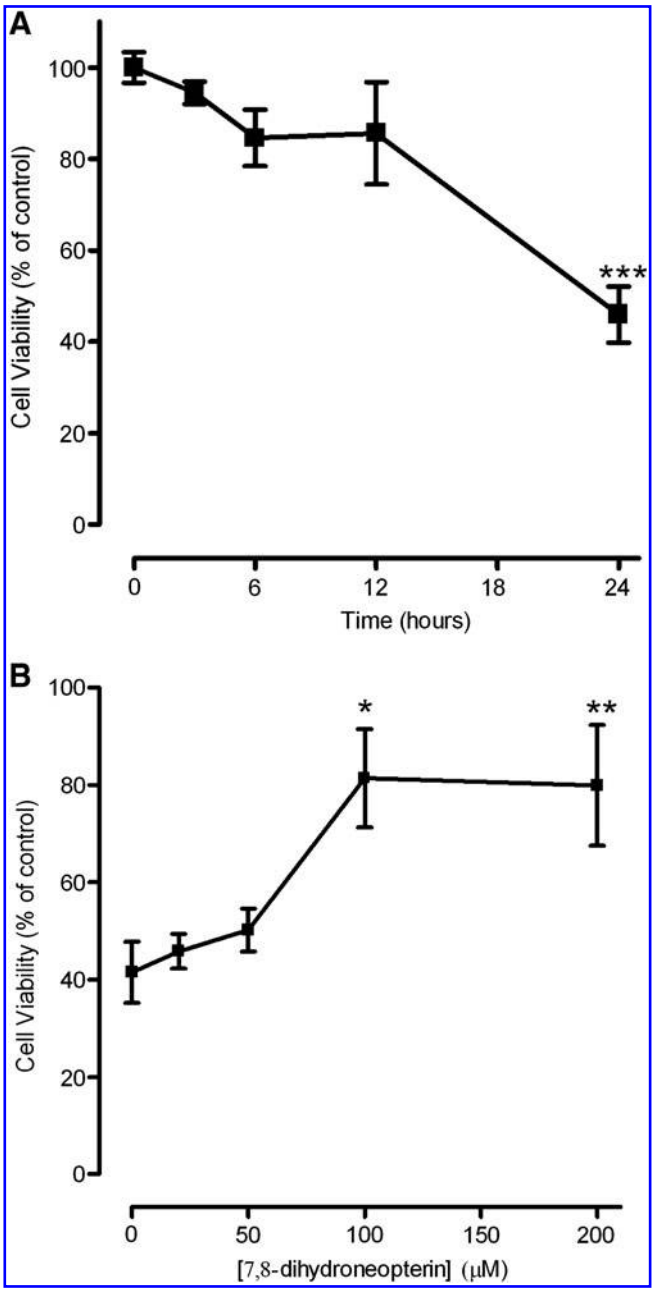

FIG. 1. OxLDL loss of HMDM cell viability is inhibited by 7,8-dihydroneopterin. (**A**) HMDMs were incubated with 1 mg/ml oxLDL in RPMI-1640 supplemented with 10% human serum. At indicated time points, the medium was removed, and the cell viability was determined by measuring MTT reduction. (**B**) Increasing concentrations of 7,8-dihydroneopterin inhibited oxLDL-induced loss of HMDM cell viability during a 24-h incubation with 1 mg/ml oxLDL. The 7,8-dihydroneopterin was added 10 min before the oxLDL. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Like the caspases, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has a critical thiol within its active site that, when oxidized, results in the loss of

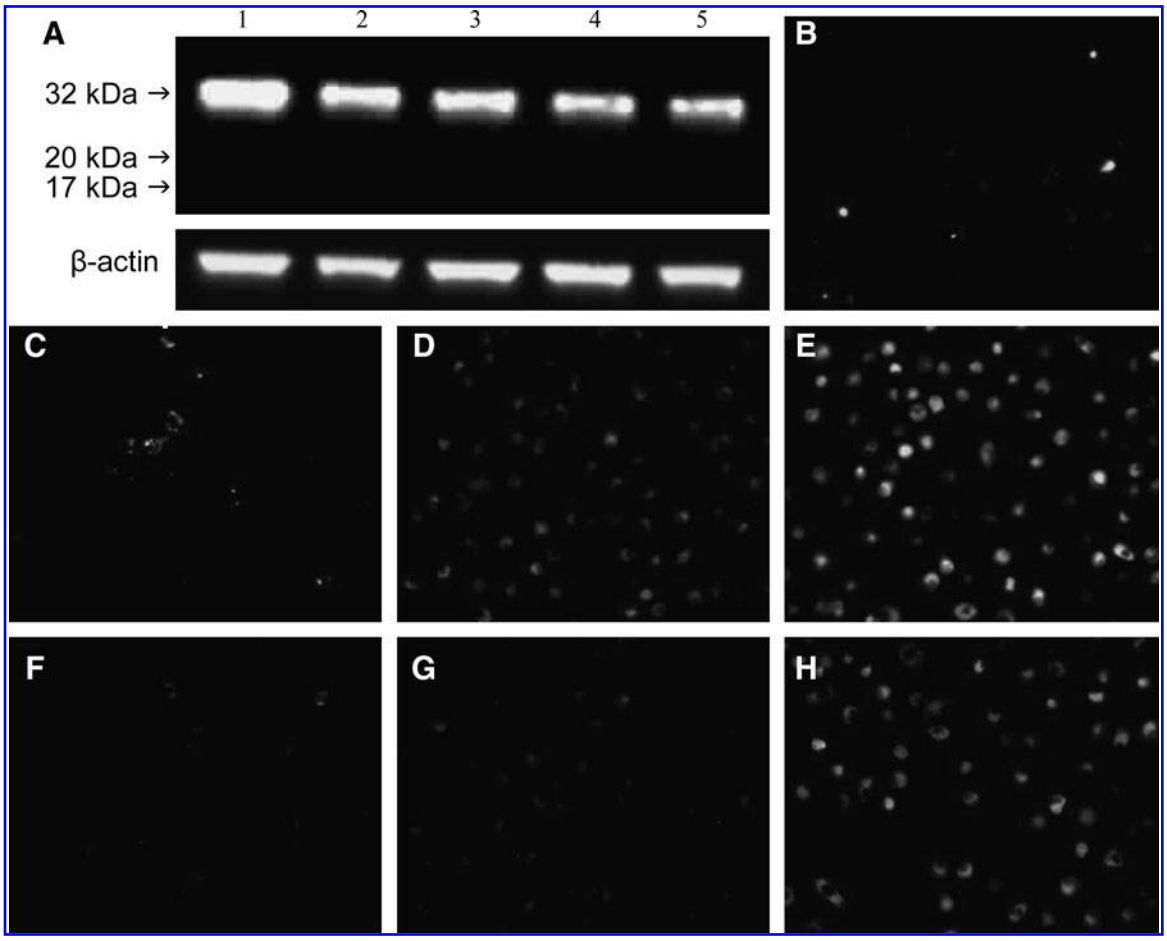

FIG. 2. OxLDL does not activate HMDM cell caspase-3 but causes phosphatidylserine translocation to the plasma membrane, which is inhibited by 7,8-dihydroneopterin. (**A**) HMDMs were incubated under various conditions before washing, and cell lysates were collected for immunoblot staining for caspase-3 activation and β-actin as a loading control. Lane 1: control cells at zero time; lane 2: with 1 mg/ml oxLDL for 12 h; lane 3: with 2 mg/ml oxLDL for 12 h; lane 4: with 1 mg/ml oxLDL for 24 h; lane 5: with 2 mg/ml oxLDL for 24 h. Cells were grown on coverslips before exposure to 1 mg/ml oxLDL for 0 h. (**B**) 3 h (**C, F**), 6 h (**D, G**), and 12 h (**E–H**) with (**F–H**) or without 200 μM 7,8-dihydroneopterin (**C–E**). Phosphatidylserine exposure on the cells surface was visualized by staining with annexin-V under fluorescence microscopy.

enzymatic activity. In HMDMs, oxLDL was observed to cause a rapid reduction of GAPDH activity, which paralleled the loss of glutathione (Fig. 5A). Within 6 h of oxLDL addition, the GAPDH activity had been reduced to 50% of the control. No loss in GAPDH activity was observed in the absence of oxLDL or in the presence of only 7,8-dihydroneopterin. At 50 μM 7,8-dihydroneopterin, a small but statistically significant protection of the GAPDH activity steadily increased with 7,8-dihydroneopterin concentration. 7,8-Dihydroneopterin at 200 μM completely prevented the oxLDL-induced loss of GAPDH activity (Fig. 5B).

The data strongly suggest that 7,8-dihydroneopterin may be scavenging oxidants generated by the cell in response to the oxLDL binding or internalization. Such a mode of activity would require 7,8-dihydroneopterin to enter the cells. This was examined by incubating HMDMs with 7,8-dihydroneopterin and analyzing cell lysates at various time points. A gradual loss of 7,8-dihydroneopterin was observed in the media, with the concentration decreasing by 50% over a 24-h period (Fig. 6). This loss is due to oxidation of the 7,8-dihydroneopterin to 7,8-dihydroxanthopterin and neopterin (13, 18). Intracellularly, the 7,8-dihydroneopterin level increased rapidly, with half the maximum concentration reached within 4 h and maximum at 12 h (Fig. 6). No significant loss of intracellular 7,8-dihydroneopterin was noted over the following 12-h period, even though the media concentration dropped by 25%. As the cells were not stimulated with γ-interferon, the intracellular generation was negligible.

Although intracellular oxidant scavenging by 7,8-dihydroneopterin appeared to explain the cellular protection from oxLDL, reduced uptake of oxLDL into the cells was also explored. By using DiI-labeled oxLDL, we measured oxLDL uptake by HMDMs and found that 7,8-dihydroneopterin reduced the uptake, but only by a third when compared with cells without 7,8-dihydroneopterin (Fig. 7A). The concentration of oxLDL used was below the cytotoxic concentration, thus removing the compounding factor of cell death and lysis from the analysis. A simple explanation for this decrease in oxLDL uptake is downregulation in the levels of the main scavenger receptors of oxLDL, scavenger receptor-A (SR-A)

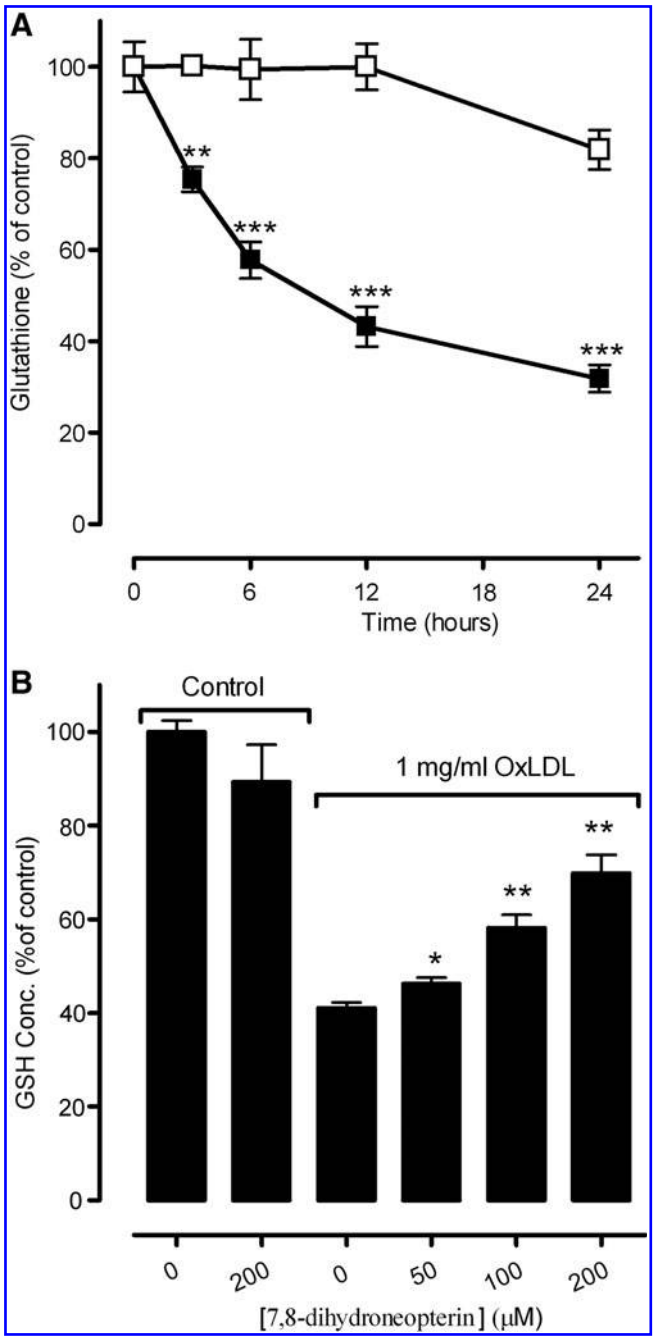

FIG. 3. OxLDL causes a rapid loss of intracellular glutathione, which is inhibited by 7,8-dihydroneopterin. (**A**) Cells in RPMI1640 supplemented with 10% human serum were incubated with (■) and without (□) oxLDL before cell GSH levels were measured with HPLC analysis at indicated time points. (**B**) HMDMs were incubated for 24 h in the indicated 7,8-dihydroneopterin concentrations, without (control) or with 1 mg/ml oxLDL, before the intracellular GSH concentration was determined by HPLC analysis. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

and CD36. Immunoblot analysis of HMDMs treated with 7,8-dihydroneopterin showed no significant change in SR-A levels (data not shown), but a significant concentration-dependent loss of the CD36 was observed with both the 83- and 100-kDa bands (Fig. 7B). The greatest loss appears with the 100-kDa plasma membrane form, in which 200 μM 7,8-dihydroneopterin almost completely inhibited the appearance of this isoform.

Discussion

OxLDL induces a large oxidative stress in the HMDMs, causing cellular death, similar to that with U937 cells (4). This study shows that the oxidant release, detected as DHE fluorescence, is associated with rapid loss of glutathione, inactivation of GAPDH, and possible loss of caspase activity. The oxidative stress develops rapidly with >25% of the glutathione and GAPDH activity lost within 3 h of oxLDL addition (Figs. 3 and 5). It is not until 12 h later that this intracellular damage appears as a significant loss of cell viability (Fig. 1). Also similar to the U937 cells (5) is that the presence of 7,8-dihydroneopterin effectively inhibits oxLDL-induced oxidant production and cell death in HMDMs, as shown by decrease in DHE staining, and maintenance of glutathione levels and GAPDH enzyme activity. The presence of 7,8-dihydroneopterin within the cells and the known radical-scavenging properties of 7,8-dihydroneopterin (19, 24, 41) strongly support an intracellular scavenging mechanism for the observed protection.

The heavily oxidized oxLDL used in this study is relatively inert chemically, because most of the LDL components have been oxidized and can no longer sustain any significant oxidant production (16, 51). Treatment of oxLDL with antioxidant before addition to cells does not reduce the oxLDL cytotoxicity (5, 51). It is, therefore, reasonable to assume that the oxLDL-induced intracellular oxidant production is a cellular response to oxLDL. In the case of HMDMs, we have shown that this response is large enough to be cytotoxic.

A number of mechanisms responsible for oxLDL-induced intracellular oxidant production have been reported. Mitochondrial dysfunction (3), NADPH-oxidase activation (33), and lipoxygenase activity (51) have all been demonstrated to occur in response to oxLDL. Our DHE data suggest that superoxide may be the primary reactive oxygen species generated within the cells, possibly due to NADPH-oxidase activation, as has been reported in U937 cells (40, 48). However, DHE is not sufficiently discriminative between superoxide and other ROS, so it is likely that a range of reactive oxygen species is generated.

A strong experimental link exists between CD36 binding of oxLDL and superoxide/oxidant generation. Cell experiments in which CD36 binding of oxLDL is inhibited by anti-CD36 antibodies, or in which the receptor is absent, still take up oxLDL, (likely through SR-A), yet have significantly reduced levels of reactive oxygen species (ROS) production, caspase activation, and cell death (51, 53). OxLDL binding to CD36 of HMDMs has been reported to generate large amounts of hydrogen peroxide (37), which would have originated as superoxide. 7,8-Dihydroneopterin is an effective scavenger of superoxide and also appears to be able to inhibit NADPH-oxidase (32). Both mechanisms may operate within the HMDMs. In addition to radical scavenging, 7,8-dihydroneopterin induced downregulation of the 100-kDa plasma membrane glycoform of CD36 on the HMDMs may also reduce the level of superoxide production. The superoxide generated is then scavenged by the 7,8-dihydroneopterin that has collected within the HMDM cells.

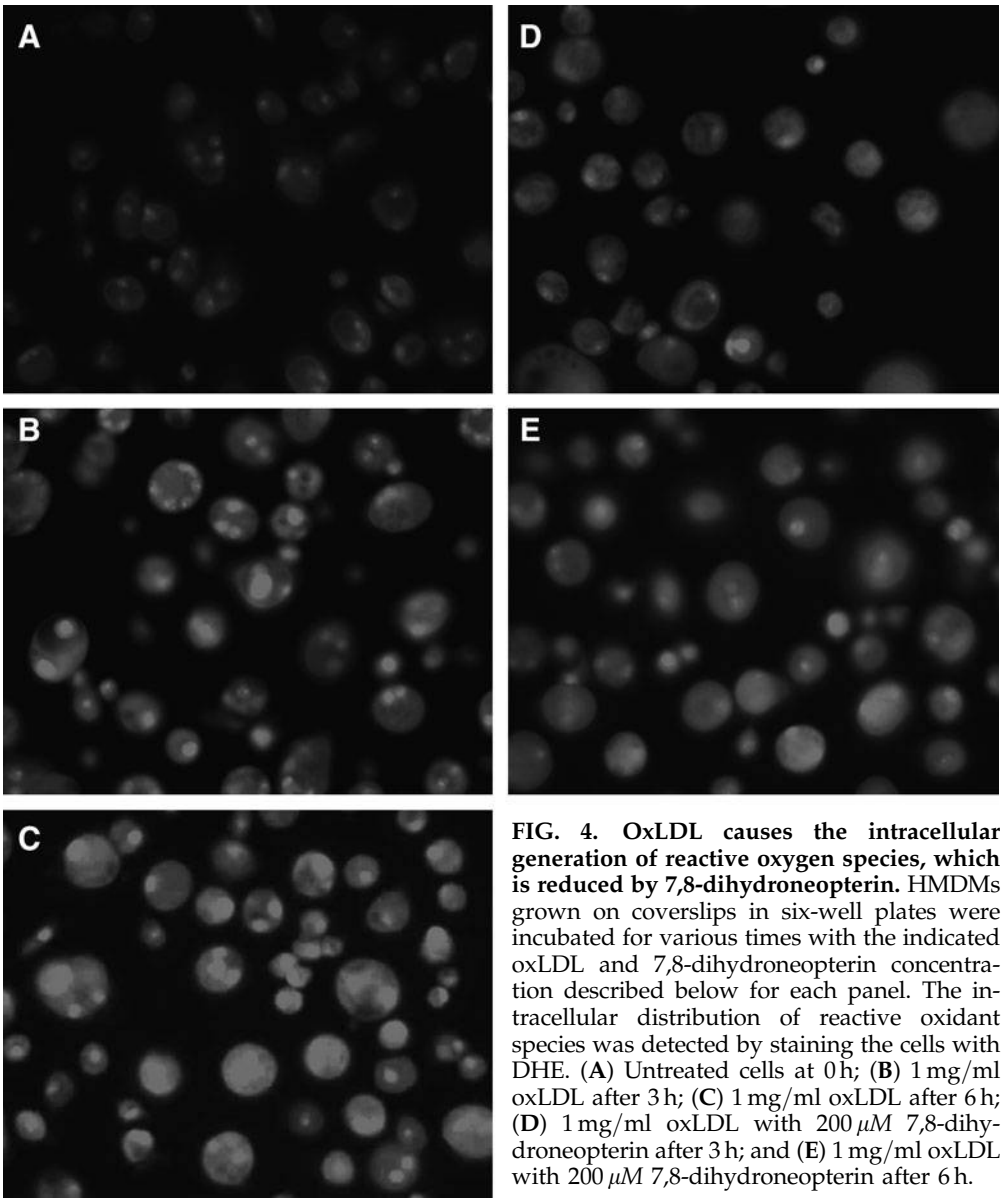

FIG. 4. OxLDL causes the intracellular generation of reactive oxygen species, which is reduced by 7,8-dihydroneopterin. HMDMs grown on coverslips in six-well plates were incubated for various times with the indicated oxLDL and 7,8-dihydroneopterin concentration described below for each panel. The intracellular distribution of reactive oxidant species was detected by staining the cells with DHE. (**A**) Untreated cells at 0 h; (**B**) 1 mg/ml oxLDL after 3 h; (**C**) 1 mg/ml oxLDL after 6 h; (**D**) 1 mg/ml oxLDL with 200 μM 7,8-dihydroneopterin after 3 h; and (**E**) 1 mg/ml oxLDL with 200 μM 7,8-dihydroneopterin after 6 h.

7,8-Dihydroneopterin only decreased DiI-oxLDL uptake by 30% over a 24-h period, suggesting that oxLDL was still entering the cell through another receptor(s), possibly SR-A. This is similar to the effect reported when CD36 was blocked by using an antibody, causing a decrease but not inhibition of oxLDL uptake (53). Increased SR-A activity has been reported to decrease oxLDL cytotoxicity to THP-1 cells, suggesting that SR-A is responsible for uptake and foam cell formation (35) but not for the toxic effects after oxLDL binding and internalization. This suggests that the CD36 downregulation in the HMDMs has an important contribution to the observed protection against oxLDL.

The combination of these arguments led us to advocate that with HMDMs and U937 cells, oxLDL triggers an excessive respiratory-burst response, which is effectively inhibited or quenched by 7,8-dihydroneopterin. This response is not seen with THP-1 cells (4) or cells treated with 7-ketocholesterol, in which changes in the key intracellular kinases and lipid raft–associated calcium channels results in cytochrome *c* release, caspase activation, and apoptosis (6). In the apoptotic mechanism, the oxidative stress is much reduced, and the glutathione levels are reasonably well maintained during the first 12 h of the response (4, 46). Monocyte-like THP-1 cells have significantly lower levels of CD36 expression on the plasma membrane compared with U937 cells (2). This may explain the much lower oxidative-stress levels observed in THP-1 cells exposed to oxLDL. The reason the mechanism triggered in THP-1 cells is not activated in our HMDMs treated with 7,8-dihydroneopterin is uncertain. The oxLDL preparations used in these experiments were rich in oxysterols and therefore should have triggered the caspase-mediated mechanism seen in THP-1 and other cell types. The mechanism we have proposed for 7,8-dihydroneopterin action should only have changed the HMDM death mechanism from necrosis to apoptosis by protecting the caspase activity. Ascorbate has been reported to do just that with HMDMs and, as a result, increased the strength of the apoptotic response to oxLDL (27). The exact mechanism by which oxLDL causes deactivation

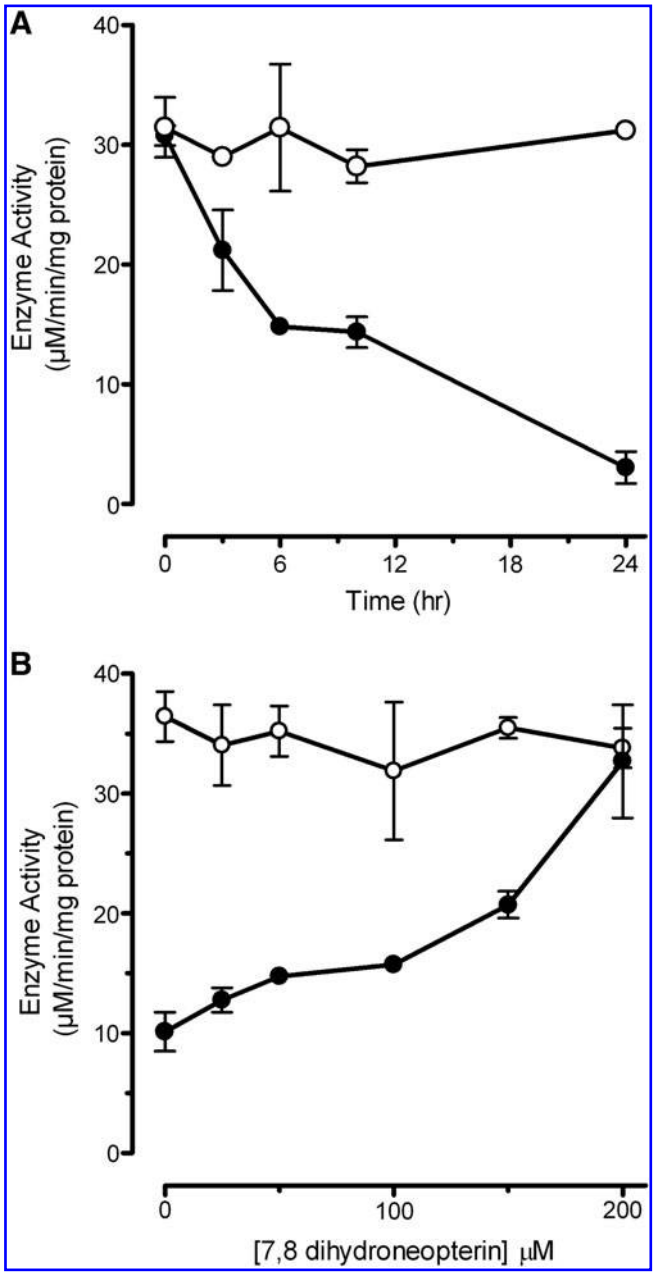

FIG. 5. OxLDL loss of macrophage GAPDH enzyme activity is inhibited by 7,8-dihydroneopterin. (**A**) Monocyte-derived macrophages in RPMI1640 supplemented with heat-inactivated human serum were incubated with 2 mg/ml oxLDL for ≤24 h (●), or 200 μM 7,8-dihydroneopterin was added to the medium before the oxLDL (○). At indicated time points, the cells were collected, and the GAPDH activity was measured. (**B**) Level of GAPDH protection from oxLDL is related to 7,8-dihydroneopterin concentration. Cells were incubated for 24 h with increasing concentrations of 7,8-dihydroneopterin with 2 mg/ml oxLDL (●) or without (○).

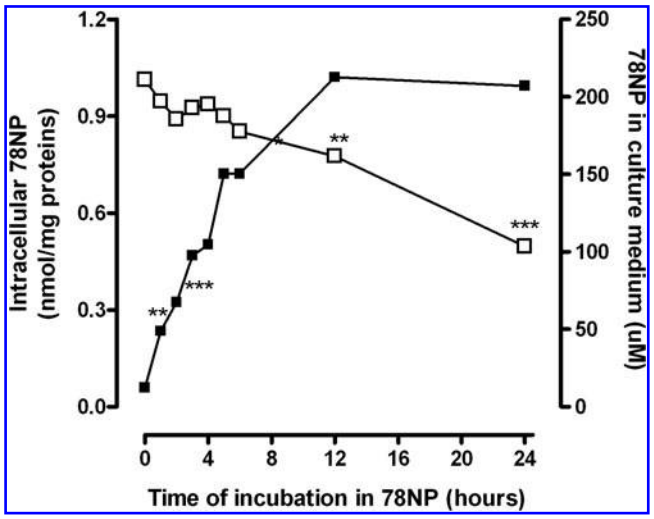

FIG. 6. 7,8-Dihydroneopterin is taken up by HMDMs. HMDMs were incubated with 200 μM 7,8-dihydroneopterin in RPMI-1640 with 10% human serum. At indicated time points, the cells were washed and lysed, and the intracellular 7,8-dihydroneopterin concentration (■) was determined with HPLC analysis. The incubation medium (□) was also analyzed by HPLC to measure the oxidative loss of 7,8-dihydroneopterin over time.

of AKT kinase with subsequent cJNK activation and apoptosis is uncertain (9, 45), and from our data, does not appear to occur in U937 or our HMDMs.

How 7,8-dihydroneopterin is downregulating the level of the CD36 protein in HMDMs also is uncertain. Studies on CD36 in THP-1 and U937 cells clearly show that the 100-kDa band is the active plasma membrane form, whereas the 83-kDa form is the less-glycosylated intracellular precursor form, possibly held within the Golgi (2). The 7,8-dihydroneopterin caused the complete disappearance of the 100-kDa form, whereas only a partial decrease in the intracellular 83-kDa form was observed (Fig. 7). The downregulation with 7,8-dihydroneopterin is very different from the inhibition of CD36 transcription induced by another antioxidant, α-tocopherol (44). α-Tocopherol has an antagonistic effect on oxLDL-induced CD36 expression by preventing oxLDL-induced protein kinase B phosphorylation action on the peroxisome proliferator–activated receptor-γ (PPAR-γ) (39). As 7,8-dihydroneopterin downregulation of CD36 still occurs in the absence of oxLDL, we suspect a different mechanism from that reported with α-tocopherol.

The loss of GAPDH activity within the first hours of oxLDL exposure would potentially exacerbate cellular stress. GAPDH inhibition would cause the glycolytic carbohydrate intermediates to be shunted into the pentose phosphate pathway, generating more NADPH (42). Although increased NADPH generation would normally increase the cells' ability to regenerate GSH (42), with HMDMs and U937 cells, it also provides more NADPH for the NADPH-oxidase. The loss of GSH in the HMDMs (Fig. 3) and U937 cells (4) shows that the increase in GSH regenerative capacity was not enough to protect the cells. As MTT reduction is dependent on NADPH reductases (38), this would also explain the high MTT-reducing potential of the HMDMs for the first 12 h, even though GAPDH and general cell metabolism was failing.

Within actual atherosclerotic plaques, total neopterin levels (neopterin plus 7,8-dihydroneopterin) have been measured in the low micromolar range (13), and this concentration varies along the length of the plaque (17), suggesting that a range of pathologies is occurring. These are advanced plaques in which the possible protective mechanisms of 7,8-dihydroneopterin on

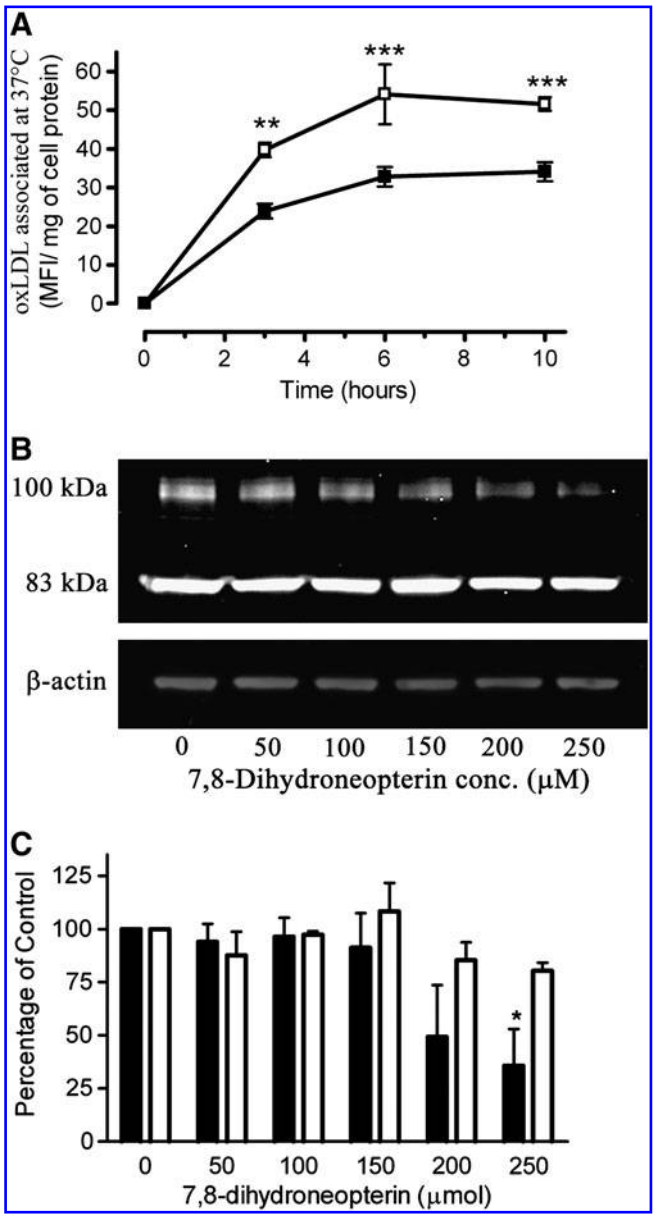

FIG. 7. 7,8-Dihydroneopterin treatment decreases DiI-oxLDL uptake and CD36 levels in HMDMs. (**A**) HMDMs were incubated in RPMI-1640 with 10% human serum and 0.2 mg/ml DiI-labeled oxLDL with (■) and without (□) 200 μM 7,8-dihydroneopterin for ≤ 10 h. Cells were washed with PBS and lysed with isopropanol, and the fluorescence intensity (FI) was measured. Cell protein was recovered from the isopropanol and measured as described in Methods. (**B**) HMDM cells were incubated in RPMI-1640 with 10% human serum containing 200 μM 7,8-dihydroneopterin. At indicated time points, the cells were washed, and the lysates were analyzed with immunoblot analysis for CD36 and β-actin as an internal loading control. (**C**) Graphic display of the CD36 signal of the Western blot in **B**, normalized to the control values with no 7,8-dihydroneopterin. The *black bars* are the 100-kDa isoform, and the *clear bars* are the 83-kDa isoform. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

macrophage cell death explored in this research, and the inhibition of oxLDL formation (14, 24), have been overwhelmed. In middle-aged and older subjects with low levels of vascular disease, the action of 7,8-dihydroneopterin may be an important process in slowing or preventing the effects of oxLDL formation.

Acknowledgments

The work was partly funded through a project grant from the National Heart Foundation of New Zealand. Zunika Amit was supported by a Ph.D. Scholarship from the Tunku Abdul Rahman Sarawak Scholarship Foundation and the Universiti Malaysia Sarawak. Anastasia Shchepetkina was supported by a University of Canterbury Postgraduate Scholarship, and Hanadi Katouah, through the Suadi Arabian Postgraduate Scholarship Scheme. We thank our healthy blood donors for our source of blood for lipoprotein preparation and Dr. Jinny Willis for carrying out the blood collection. We also thank the staff and blood donors of Christchurch Blood Service for the blood to prepare the monocytes. This article is dedicated to the memory of Alan M^{c}Curdy, who inspired a love of chemistry in so many students.

Author Disclosure Statement

No competing financial interests exist.

References

1. Adachi T, Naruko T, Itoh A, Komatsu R, Abe Y, Shirai N, Yamashita H, Ehara S, Nakagawa M, Kitabayashi C, Ikura Y, Ohsawa M, Yoshiyama M, Haze K, and Ueda M. Neopterin is associated with plaque inflammation and destabilisation in human coronary atherosclerotic lesions. *Heart* 93: 1537–1541, 2007.
2. Alessio M, Demonte L, Scirea A, Gruarin P, Tandon NN, and Sitia R. Synthesis, processing, and intracellular transport of CD36 during monocytic differentiation. *J Biol Chem* 271: 1770–1775, 1996.
3. Asmis R and Begley JG. Oxidized LDL promotes peroxide-mediated mitochondrial dysfunction and cell death in human macrophages: a caspase-3-independent pathway. *Circ Res* 92: E20–E29, 2003.
4. Baird SK, Hampton M, and Gieseg SP. Oxidised LDL triggers phosphatidylserine exposure in human monocyte cell lines by both caspase-dependent and independent mechanisms. *FEBS Lett* 578: 169–174, 2004.
5. Baird SK, Reid L, Hampton M, and Gieseg SP. OxLDL induced cell death is inhibited by the macrophage synthesised pterin, 7,8-dihydroneopterin, in U937 cells but not THP-1 cells. *Biochim Biophys Act* 1745: 361–369, 2005.
6. Berthier A, Lemaire-Ewing S, Prunet C, Monier S, Athias A, Bessede G, De Barros JPP, Laubriet A, Gambert P, Lizard G, and Neel D. Involvement of a calcium-dependent dephosphorylation of BAD associated with the localization of TRPC-1 within lipid rafts in 7-ketocholesterol-induced THP-1 cell apoptosis. *Cell Death Differ* 11: 897–905, 2004.
7. Brodeur MR, Brissette L, Falstrault L, Ouellet P, and Moreau R. Influence of oxidized low-density lipoproteins (LDL) on the viability of osteoblastic cells. *Free Radic Biol Med* 44: 506–517, 2008.
8. Burnaugh L, Sabeur K, and Ball BA. Generation of superoxide anion by equine spermatozoa as detected by dihydroethidium. *Theriogenology* 67: 580–589, 2007.

9. Choi YK, Kim YS, Choi IY, Kim SW, and Kim WK. 25-Hydroxycholesterol induces mitochondria-dependent apoptosis via activation of glycogen synthase kinase-3 beta in PC12 cells. *Free Radic Res* 42: 544–553, 2008.
10. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, and Cone JT. Single vertical spin density gradient centrifugation. *Methods Enzymol* 128: 181–209, 1986.
11. Cotgreave IA and Moldeus P. Methodologies for the application of monobromobimane to the simultaneous analysis of soluble and protein thiol components of biological systems. *J Biochem Biophys Methods* 13: 231–249, 1986.
12. Devaraj S, Hugou I, and Jialal I. Alpha-Tocopherol decreases CD36 expression in human monocyte-derived macrophages. *J Lipid Res* 42: 521–527, 2001.
13. Firth CA, Crone EM, Flavall EA, Roake J, and Gieseg SP. Macrophage mediated protein hydroperoxide formation and lipid oxidation in low density lipoprotein is inhibited by the inflammation marker 7,8 dihydroneopterin. *Biochim Biophys Acta* 1783: 1095–1101, 2008.
14. Firth CA, Yang Y, and Gieseg SP. Lipid oxidation predominates over protein hydroperoxide formation in human monocyte-derived macrophages exposed to aqueous peroxyl radicals. *Free Radic Res* 41: 839–848, 2007.
15. Gieseg SP and Cato S. Inhibition of THP-1 cell-mediated low-density lipoprotein oxidation by the macrophage-synthesised pterin, 7,8-dihydroneopterin. *Redox Rep* 8: 113–119, 2003.
16. Gieseg SP, Crone E, and Amit Z. Oxidised low density lipoprotein cytotoxicity and vascular disease. In *Endogenous Toxins: Diet, Genetics, Disease and Treatment*, edited by O'Brien PJ and Bruce WR. Weinheim, Germany: Wiley-VCH 2009, pp. 620–645.
17. Gieseg SP, Crone EM, Flavall EA, and Amit Z. Potential to inhibit growth of atherosclerotic plaque development through modulation of macrophage neopterin/7,8-dihydroneopterin synthesis. *Br J Pharmacol* 153: 627–635, 2008.
18. Gieseg SP, Cruz T, Glubb D, Muzghal D, and Whybrow J. 7,8 Dihydroneopterin can protect cells from free radical mediated damage. *Free Radic Biol Med* 25: 32, 1998.
19. Gieseg SP, Duggan S, Rait C, and Platt A. Protein and thiol oxidation in cells exposed to peroxyl radicals, is inhibited by the macrophage synthesised pterin 7,8-dihydroneopterin. *Biochim Biophys Acta* 1591: 139–145, 2002.
20. Gieseg SP and Esterbauer H. Low density lipoprotein is saturable by pro-oxidant copper. *FEBS Lett* 343: 188–194, 1994.
21. Gieseg SP, Glubb D, and Maghzal G. Protection of erythrocytes by the macrophage synthesized antioxidant 7,8 dihydroneopterin. *Free Radic Res* 34: 123–136, 2001.
22. Gieseg SP, Leake DS, Flavall EM, Amit Z, Reid L, and Yang Y. Macrophage antioxidant protection within atherosclerotic plaques. *Frontiers Biosci* 14: 1230–1246, 2009.
23. Gieseg SP, Pearson J, and Firth CA. Protein hydroperoxides are a major product of low density lipoprotein oxidation during copper, peroxyl radical and macrophage-mediated oxidation. *Free Radic Res* 37: 983–991, 2003.
24. Gieseg SP, Reibnegger G, Wachter H, and Esterbauer H. 7,8-Dihydroneopterin inhibits low density lipoprotein oxidation *in vitro*: evidence that this macrophage secreted pteridine is an antioxidant. *Free Radic Res* 23: 123–136, 1995.
25. Gieseg SP, Whybrow J, Glubb D, and Rait C. Protection of U937 cells from free radical damage by the macrophage synthesized antioxidant 7,8 dihydroneopterin. *Free Radic Res* 35: 311–318, 2001.
26. Greilberger J, Oettl K, Cvirn G, Reibnegger G, and Jurgens G. Modulation of LDL oxidation by 7,8-dihydroneopterin. *Free Radic Res* 38: 9–17, 2004.
27. Harris LK, Mann GE, Ruiz E, Mushtaq S, and Leake DS. Ascorbate does not protect macrophages against apoptosis induced by oxidised low density lipoprotein. *Arch Biochem Biophys* 455: 68–76, 2006.
28. Hutter R, Valdiviezo C, Sauter BV, Savontaus M, Chereshnev I, Carrick FE, Bauriedel G, Luderitz B, Fallon JT, Fuster V, and Badimon JJ. Caspase-3 and tissue factor expression in lipid-rich plaque macrophages - Evidence for apoptosis as link between inflammation and atherothrombosis. *Circulation* 109: 2001–2008, 2004.
29. Kappler M, Gerry AJ, Brown E, Reid L, Leake DS, and Gieseg SP. Aqueous peroxyl radical exposure to THP-1 cells causes glutathione loss followed by protein oxidation and cell death without increased caspase-3 activity. *Biochim Biophys Acta* 1773: 945–953, 2007.
30. Kockx MM, De Meyer GRY, Muhring J, Bult H, Bultinck J, and Herman AG. Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol fed rabbits. *Atherosclerosis* 120: 115–124, 1996.
31. Kojima S, Icho T, Kajiwara Y, and Kubota K. Neopterin as an endogenous antioxidant. *FEBS Lett* 304: 163–166, 1992.
32. Kojima S, Nomura T, Icho T, Kajiwara Y, Kitabatake K, and Kubota K. Inhibitory effects of neopterin on NADPH-dependent superoxide-generating oxidase of rat peritoneal macrophages. *FEBS Lett* 329: 125–128, 1993.
33. Leonarduzzi G, Vizio B, Sottero B, Verde V, Gamba P, Mascia C, Chiarpotto E, Poli G, and Biasi F. Early involvement of ROS overproduction in apoptosis induced by 7-ketocholesterol. *Antioxidants Redox Signal* 8: 375–380, 2006.
34. Li W, Yuan XM, and Brunk UT. OxLDL-induced macrophage cytotoxicity is mediated by lysosomal rupture and modified by intralysosomal redox-active iron. *Free Radic Res* 29: 389, 1998.
35. Liao HS, Kodama T, and Geng YJ. Expression of class A scavenger receptor inhibits apoptosis of macrophages triggered by oxidized low density lipoprotein and oxysterol. *Arterioscler Thromb Vasc Biol* 8: 1968–1975, 2000.
36. Libby P, Ridker PM, and Maseri A. Inflammation and atherosclerosis. *Circulation* 105: 1135–1143, 2002.
37. Maxeiner H, Husemann J, Thomas CA, Loike JD, El Khoury J, and Silverstein SC. Complementary roles for scavenger receptor a and CD36 of human monocyte-derived macrophages in adhesion to surfaces coated with oxidized low-density lipoproteins and in secretion of H_2O_2. *J Exp Med* 188: 2257–2265, 1998.
38. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
39. Munteanu A, Taddei M, Tamburini I, Bergamini E, Azzi A, and Zingg JM. Antagonistic effects of oxidized low density lipoprotein and alpha-tocopherol on CD36 scavenger receptor expression in monocytes: involvement of protein kinase B and peroxisome proliferator-activated receptor-gamma. *J Biol Chem* 281: 6489–6497, 2006.
40. Nguyen-Khoa T, Massy ZA, Witko-Sarsat V, Canteloup S, Kebede M, Lacour B, Drueke T, and Descamps-Latscha B. Oxidized low-density lipoprotein induces macrophage respiratory burst via its protein moiety: a novel pathway in atherogenesis? *Biochem Biophys Res Commun* 263: 804–809, 1999.
41. Oettl K, Dikalov S, Freisleben HJ, Mlekusch W, and Reibnegger G. Spin trapping study of antioxidant properties of neopterin and 7,8-dihydroneopterin. *Biochem Biophys Res Commmun* 234: 774–778, 1997.

42. Ralser M, Wamelink MM, Kowald A, Gerisch B, Heeren G, Struys EA, Klipp E, Jakobs C, Breitenbach M, Lehrach H, and Krobitsch S. Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *J Biol* 6: 10, 2007.
43. Ray KK, Morrow DA, Sabatine MS, Shui A, Rifai N, Cannon CP, and Braunwald E. Long-term prognostic value of neopterin a novel marker of monocyte activation in patients with acute coronary syndrome. *Circulation* 115: 3071–3078, 2007.
44. Ricciarelli R, Zingg JM, and Azzi A. Vitamin E reduces the uptake of oxidized LDL by inhibiting Cd36 scavenger receptor expression in cultured aortic smooth muscle cells. *Circulation* 102: 82–87, 2000.
45. Rusinol AE, Thewke D, Liu J, Freeman N, Panini SR, and Sinensky MS. AKT/Protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. *J Biol Chem* 279: 1392–1399, 2004.
46. Ryan L, O'Callaghan YC, and O'Brien NM. Involvement of calcium in 7 beta-hydroxycholesterol and cholesterol-5 beta, 6 beta-epoxide-induced apoptosis. *Int J Toxicol* 25: 35–39, 2006.
47. Schumacher M, Eder B, Tatzber F, Kaufmann P, Esterbauer H, and Klein W. Neopterin levels in patients with coronary artery disease. *Atherosclerosis* 94: 87–88, 1992.
48. Sellmayer A, Obermeier H, Danesch U, Aepfelbacher M, and Weber P. Arachidonic acid increases activation of NADPH oxidase in monocytic U937 cells by accelerating translocation of p47-phox and Co-stimulation of protein kinase C. *Cell Signal* 8: 397–402, 1996.
49. Steck TL and Kant JA. Preparation of impermeable ghosts and inside out vesicles from human erythrocyte membranes. *Methods Enzymol* 31: 172–180, 1974.
50. Stephan ZF and Yurachek EC. Rapid fluorometric assay of LDL receptor activity by Dil-labeled LDL. *J Lipid Res* 34: 325–330, 1993.
51. Sukhanov S, Higashi Y, Shai SY, Itabe H, Ono K, Parthasarathy S, and Delafontaine P. Novel effect of oxidized low-density lipoprotein: cellular ATP depletion via down-regulation of glyceraldehyde-3-phosphate dehydrogenase. *Circ Res* 99: 191–200, 2006.
52. Wachter H, Fuchs D, Hausen A, Reibnegger G, and Werner ER. Neopterin as marker for activation of cellular immunity: immunologic basis and clinical application. *Adv Clin Chem* 27: 81–141, 1989.
53. Wintergerst ES, Jelk J, Rahner C, and Asmis R. Apoptosis induced by oxidized low density lipoprotein in human monocyte-derived macrophages involves CD36 and activation of caspase-3. *Eur J Biochem* 267: 6050–6058, 2000.

Address correspondence to:
Dr. Steven P. Gieseg
School of Biological Sciences of Canterbury
Private Bag 4800
Christchurch
New Zealand

E-mail: steven.gieseg@canterbury.ac.nz

Date of first submission to ARS Central, December 20, 2009; date of acceptance, March 9, 2010.

Abbreviations Used

DHE = dihydroethidium
GAPDH = glyceradehyde-3-phosphate dehydrogenase
HMDMs = human monocyte–derived macrophages
oxLDL = oxidized low-density lipoprotein
PBS = phosphate-buffered saline
PPAR-γ = peroxisome proliferator–activated receptor-γ
PS = phosphatidylserine
SR-A = scavenger receptor-A

This article has been cited by:

1. Wim Martinet, Dorien M. Schrijvers, Guido R. Y. Meyer. 2012. Molecular and cellular mechanisms of macrophage survival in atherosclerosis. *Basic Research in Cardiology* **107**:6. . [CrossRef]
2. Ya-ting Tina Yang, Matthew Whiteman, Steven P. Gieseg. 2012. HOCl causes necrotic cell death in human monocyte derived macrophages through calcium dependent calpain activation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**:2, 420-429. [CrossRef]
3. Lucy D. Rutherford, Steven P. Gieseg. 2011. 7-Ketocholesterol is Not Cytotoxic to U937 Cells When Incorporated into Acetylated Low Density Lipoprotein. *Lipids* . [CrossRef]
4. Bridgette M. Cumming, Gregory M.F. Watson, J.P. Dean Goldring. 2011. Plasmodium falciparum: Effect of antimalarial drugs, malaria pigment (#-haematin) and Plasmodium falciparum lysate on monocyte GTP-cyclohydrolase 1 gene expression. *Experimental Parasitology* . [CrossRef]
5. Samir Samman . 2010. Antioxidants and Public Health. *Antioxidants & Redox Signaling* **13**:10, 1513-1515. [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]