Journal of Molecular and Cellular Cardiology 46 (2009) 431–441

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Oxidized LDL affects smooth muscle cell growth through MAPK-mediated actions on nuclear protein import

Mirna N. Chahine [1], David P. Blackwood, Elena Dibrov, Melanie N. Richard, Grant N. Pierce *

Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre, Department of Physiology, Faculty of Medicine University of Manitoba, Winnipeg, Manitoba, Canada

ARTICLE INFO

Article history:
Received 2 September 2008
Received in revised form 2 October 2008
Accepted 6 October 2008
Available online 28 October 2008

Keywords:
Oxidized low-density lipoproteins
Nuclear protein import
Vascular smooth muscle cells
MAP kinase
Cell growth

ABSTRACT

Oxidized low density lipoprotein (oxLDL) plays an important role in the development of atherosclerosis partly through an action on cell proliferation and cell apoptosis. Nuclear protein import (NPI) is critical in regulating gene expression, transcription, and subsequently cell proliferation and apoptosis. The aim of this study was to determine if exposure of vascular smooth muscle cells (VSMC) to oxLDL affects cell growth by inducing alterations in NPI and nuclear pore density. VSMC were exposed for different times to oxLDL. Cells were then injected with a protein import substrate (Alexa488-BSA-NLS) to visually monitor nuclear transport with the confocal microscope. The effect of MAPK inhibitors (SB203580 and PD98059) was investigated and western immunoblottings were also performed. Shorter exposure times of VSMC to oxLDL, but not to native LDL, significantly increased NPI, nuclear pore expression (p62), PCNA expression, and cell number. These changes occurred through an ERK MAPK-dependent mechanism. However, longer exposures to oxLDL decreased NPI, nuclear pore expression, and increased apoptosis marker (cleaved PARP) expression through a p38 MAPK-dependent mechanism. We conclude that limited exposure to oxLDL may influence cell proliferation and apoptosis through an action on nucleocytoplasmic trafficking. The nucleus and NPI may represent a novel therapeutic target to control diseases like atherosclerosis that have changes in cell growth as a central feature.

1. Introduction

Atherosclerosis and its complications such as myocardial infarction, stroke, and peripheral vascular disease remain a major cause of death in industrialized countries [1,2]. Atherosclerosis is a complex process that involves, in early lesions, recruitment and proliferation of cells in the arterial wall [1]. In advanced lesions, a high rate of apoptotic vascular smooth muscle cells (VSMC) in atherosclerotic plaques may contribute to destabilize the fibrous cap and increase the risk of plaque rupture and thrombosis [3]. A large body of evidence has been gathered to support the hypothesis that oxidized low density lipoprotein (oxLDL) is potentially implicated in atherogenesis by inducing multiple functional modifications [2,4]. In vitro, oxLDL is capable of promoting various growth effects on cells [5], including the induction of transcription factors [6,7], stimulation of DNA synthesis, and cell proliferation [5,8]. Conversely, oxLDL is also known to induce VSMC apoptosis [9,10].

In light of these paradoxical findings, it is of interest to understand the mechanism through which oxLDL can induce both VSMC proliferation and apoptosis. Does this mechanism implicate the same pathway or does it occur via different ones? Many cell surface factors or intracellular sites in VSMC have been implicated in these effects including growth factor receptors that commonly involves the activation of mitogen-activated protein kinases (MAPKs) [11,12], protein tyrosine kinases, phosphatidylinositol 3-kinase [12], the lectinlike oxLDL receptor-1 [9] and Fas/FasL death pathway [13]. However, these cellular pathways represent only a portion of the downstream targets of oxLDL to induce vascular cell proliferation or apoptosis. It is important for example, to identify the targets of the kinases. In view of its central role in controlling cell growth, it is possible that the nucleus may be a target of this oxidative stimulus.

The nuclear envelope is a double-membrane barrier between the nucleus and the cytoplasm within the cell [14]. The nuclear pore complex (NPC) present on the surface of the nuclear membrane mediates the transport of key proteins such as transcription factors, kinases, DNA binding proteins, and polymerases into the nucleus to regulate gene expression and signal transduction and to induce cell differentiation, transformation, and proliferation [14]. Gene expression and transcription in any cell are clearly altered during a growth phase. Nuclear protein import (NPI) is also stimulated in proliferating cells in

Abbreviations: LDL, Low density lipoprotein; oxLDL, oxidized low density lipoprotein; naLDL, native low density lipoprotein; VSMC, vascular smooth muscle cells; NPI, nuclear protein import; NPC, nuclear pore complex; MAPKs, mitogen-activated protein kinases; PCNA, Proliferating cell nuclear antigen; PARP, poly ADP-ribose polymerase.

* Corresponding author. Tel.: +1 204 235 3206; fax: +1 204 235 0793.
E-mail address: gpierce@sbrc.ca (G.N. Pierce).

[1] Dr Chahine presented the data from this paper and received the Young Investigator Award at the Annual Meeting of the European Section of the International Society for Heart Research held in Athens in May 2008.

0022-2828/$ – see front matter.
doi:10.1016/j.yjmcc.2008.10.009

comparison to quiescent cells [15]. However, we know relatively little about the capacity of the NPC, one of the largest and most important channels in the cell, to change during the same diseases that have altered cellular growth as a critical component of the pathology. It is also important to understand if the same mechanisms are implicated in other diseases having cell growth as a central feature i.e. atherosclerosis.

Because cell growth is regulated by protein transport into the nucleus, it is hypothesized that the mechanism whereby oxLDL affects proliferation or apoptosis is through an action on NPI. These changes in NPI do not have to be large. It is obvious that complete blockade of nucleocytoplasmic trafficking will have dramatic effects on cell growth and viability. However, it is less clear what will happen if this transport process is modified in a more modest manner. It is also unknown if it can be modified at all by disease processes or factors associated with disease processes. The purposes of this study, therefore, were to: 1) determine if NPI and NPC protein density will change in response to relatively short and longer exposure times of VSMC in culture to native LDL (naLDL) and oxLDL and 2) examine specific molecules or pathways (i.e. MAPKs) that may participate in the regulation of NPI in these cells. Our work shows that limited exposure times of VSMC to oxLDL can affect cell proliferation and cell apoptosis through an action on NPI. Relatively short exposure times of VSMCs to oxLDL will increase NPI whereas longer exposure times will inhibit NPI. These effects correlated with changes in cell numbers and were specific to oxLDL. The effects appeared to be associated with changes in MAPK activity within the cell. Our study, therefore, provides novel insights into the nuclear mechanisms that may be responsible for the mitogenic actions of oxLDL during atherosclerosis.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin-streptomycin, and trypsin-EDTA were purchased from GIBCO-BRL. Cholesterol diet was purchased from Purina Test Diets (Richmond, USA). Transferrin, selenium, ascorbate, insulin, cholesterol oxidase, and cholesterol esterase were obtained from Sigma-Aldrich. The following antibodies were used: proliferating cell nuclear antigen PCNA, signalling antibodies p38, phospho-p38, p44/p42, phospho-p44/p42 (Cell Signalling, Beverly, U.S.A), nuclear pore protein antibodies mAb414 (Babco, Richmond, U.S.A), apoptotic marker antibody Poly ADP-ribose polymerase (PARP) (Zymed Laboratories/Invitrogen, Burlington, Canada) and loading control smooth muscle α-actin (Sigma, Saint Louis, U.S.A). Other reagents purchased included PD-98059 and SB-203580 (Calbiochem, San Diego, U.S.A) ALEXA488 BSA conjugate (BODIPY-FL conjugate-BSA) (Molecular Probes/Invitrogen, Burlington, Canada).

2.2. Vascular smooth muscle cells culture

Smooth muscle cells were isolated from the thoracic aorta of male New Zealand white rabbits by an explant technique, as described previously [16–18]. Briefly, aortic rings were incubated for several days in a culture media then transferred to a new plate for VSMC migration. The aortic tissue was then removed to allow the cells to grow to confluency. Cells were seeded on coverslips at a density of 40 thousand cells per well for microinjection purposes or directly into the wells at a density of 100 thousand cells per well for immunoblotting. Cells with no more than three passages were used. VSMC were left to attach to the membranes for 24 h and maintained in starvation (STV) media (DMEM, 5 μg/ml transferrin, 1 nM selenium, 200 μM ascorbate, 10 nM insulin, 2.5 μM sodium pyruvate, 1% fungizone) for 3 days to induce growth arrest [16–18].

2.3. Plasma lipoprotein isolation

LDL (density 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of male albino New Zealand White rabbits fed a 0.5% cholesterol-supplemented diet for a minimum of 8 weeks as referred previously [5,8,19]. Addition of EDTA (0.1 mmol/l) prevented oxidation of LDL throughout the isolation. The LDL fraction was dialyzed against 0.15 M NaCl and 0.1 mmol/l EDTA (pH 7.4), sterile filtered (0.2-μm pore size), and stored at 4 °C. The protein concentration of LDL was determined by Lowry's method, and cholesterol (free and esterified) was measured enzymatically as described [5,8,19].

2.4. Plasma lipoprotein oxidation

LDL was oxidized with a Fe-ADP free radical-generating system [5,8,19]. In a typical experiment, 1 mg/ml LDL was incubated at 37 °C for 3 h with freshly prepared 0.05 mM Fe and 0.5 mM ADP in sterile filtered 150 mM NaCl, pH 7.4. The same concentrations of ADP, in the absence of LDL, were added to control cells, and the same concentrations of ADP, in the presence of LDL, were added to native LDL treated cells [5,8,19].

2.5. VSMC treatments

Cells were incubated with 10, 25 or 50 μg cholesterol/ml LDL or oxLDL for various time points (3, 6, 12, 24, and 48 h) in DMEM (1% FBS, 1% Fungizone). Control cells were maintained in an identical medium without added LDL or oxLDL for the same period of time [5,8,19]. To demonstrate that the concentrations of oxLDL utilized in these experiments were not toxic, LDH release into the culture medium was assayed as an indicator of cell damage [5,8,19]. No significant differences were observed in levels of LDH release between oxLDL-treated cells and untreated controls over 3 h to 48 h for either 10, 25 or 50 μg/ml oxLDL (data not shown) [5,8]. Furthermore, we could detect no significant increases in the release of LDH after cells were incubated with any of the drugs, either alone or in combination with oxLDL.

2.6. Microinjection and measurement of nuclear protein import by confocal microscopy

At the end of the treatment, coverslips containing smooth muscle cells were placed in a Leyden dish, and 1 ml of prewarmed perfusate buffer (6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM dextrose, 6 mM HEPES, pH 7.4) was added. Temperature was maintained at 37 °C in a microperfusion chamber. The technique of microinjection consists of injecting an import substrate contained in a micropipette. A Flaming/Brown micropipette puller (Sutter Instruments, model p-97) was used to fashion micropipette from thin walled glass capillary tubes (1.0 mm, 3 in.). Approximately 10 μl of fluorescent substrate (ALEXA-BSA-NLS) in nuclear import buffer was added to the micropipette using a 1 ml-syringe. An ALEXA-BSA NLS fluorescent substrate was prepared in our lab by conjugating BODIPY-BSA to a SV40 large T antigen NLS (CGGGPKKKRKVED) [18]. Using a MS314 micromanipulator, the pipette was inserted into the cell cytoplasm in close proximity to the nucleus. Cells were injected four times using a PV830 Pneumatic PicoPump (World Precision Instruments, Sarasota, Florida, USA) and the pipette was slowly removed. Images of the microinjected cells were acquired on a MRC 600 CLSM confocal imaging system equipped with a Nikon Diaphot 300 microscope [16]. Images were taken of pre- and post-injection cells followed by set time points to observe the rate of nuclear import (represented as a ratio nucleus/cytoplasm) for each cell over time.

The signal strength in the nucleus is proportional to the amount of substrate present in the nucleus. There is no movement of the fluorescent marker into the cell nucleus when it does not contain an