Biochemical and Biophysical Research Communications 426 (2012) 106–111

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Degradation of heparan sulfate proteoglycans enhances oxidized-LDL-mediated autophagy and apoptosis in human endothelial cells

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ARTICLE INFO

Article history:
Received 8 August 2012
Available online 14 August 2012

Keywords:
Apoptosis
Atherosclerosis
Autophagy
HSPG
LOX-1

ABSTRACT

Background: Cell surface heparan sulfate proteoglycans (HSPG) play an important role in atherogenesis. We hypothesized that degradation of HSPG may increase the binding of atherogenic oxidized low density lipoprotein (ox-LDL) to endothelial cells, and result in extensive HSPG degradation as well as autophagy and apoptosis.

Methods: Primary human umbilical vein endothelial cells (HUVECs) were used to study the expression of lectin-like ox-LDL receptor-1 (LOX-1), HSPG, autophagy and apoptosis in response to ox-LDL and heparinase III (Hep III).

Results: As expected, ox-LDL treatment resulted in LOX-1 expression, ox-LDL uptake and reactive oxygen species (ROS) generation. Ox-LDL treatment also resulted in a modest degradation of HSPG and increase in autophagy (expression of LC3, beclin-1 and Atg5) and apoptosis (enhanced expression of caspases and Bax, and reduced expression of Bcl-2 and Bcl-xL). The effects of ox-LDL were blocked by pretreatment of cells with LOX-1 antibody or apocynin, an NADPH oxidase inhibitor. Hep III alone caused HSPG degradation and slightly, but significantly, increased ROS generation, and induced autophagy and caspase expression. However, autophagy and apoptosis induced by Hep III were not affected by apocynin or LOX-1 antibody. Importantly, Hep III pretreatment of cells significantly enhanced ox-LDL-induced HSPG degradation, LOX-1 expression, ox-LDL uptake and ROS generation as well as autophagy and apoptosis.

Conclusion: These data demonstrate that Hep III enhances the pro-atherosclerotic characteristics in HUVECs induced by ox-LDL.

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1. Introduction

Oxidized-low density lipoproteins (ox-LDL) accumulate in the arterial wall, and this accumulation results in early markers of atherosclerosis, such as generation of reactive oxygen species (ROS), cellular injury and expression of adhesion molecules [1,2]. Lectin-like ox-LDL scavenger receptor-1 (LOX-1) is one of the major receptors responsible for binding, internalizing and degrading ox-LDL [1]. Activation of LOX-1 has been known to be related to several pro-atherosclerotic characteristics, including endothelial cell (EC) activation, smooth muscle cell proliferation, and alterations in cell cycle signals [1,3,4].

Heparan sulfate proteoglycans (HSPG) are abundant cell surface receptors that interact with a variety of ligands through electrostatic interactions that are involved in several biological activities, such as regulation of basement membrane permeability; cell migration, adhesion and proliferation; receptor interaction, coagulation; and lipoprotein uptake [5,6].

Autophagy is an evolutionary conserved process involved in the degradation of long-lived proteins and excess number or dysfunctional organelles, which becomes manifest during tissue remodeling and starvation [7]. Although excessive autophagic activity leads to collapse of all cellular functions, moderately enhanced autophagy promotes cell survival. Apoptosis, on the other hand, represents programmed cell death meant to remove cells exposed to noxious stimuli [4,8]. The regulation of autophagy and apoptosis in response to ox-LDL is of interest in understanding endothelial cells biology in atherosclerotic regions wherein the concentrations of ox-LDL are high [9].

In the present study, we examined if ox-LDL induces HSPG degradation. Further, we studied autophagy and apoptosis responses to ox-LDL in ECs pretreated with heparinase III (Hep III), a known mediator of HSPG degradation [6].

Abbreviations: HSPG, heparan sulfate proteoglycans; Hep III, heparinase III; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low density lipoprotein; LOX-1, lectin-like ox-LDL receptor-1.

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0006-291X/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2012.08.044

2. Material and methods

2.1. Cell culture

Human primary umbilical vein endothelial cells (HUVECs) were obtained from ATCC (Manassas, VA), and maintained in vascular cell basal medium. High TBAR ox-LDL (90 nmol MDA/mg protein) was purchased from Biomedical Technologies Inc. (Stoughton, MA). Human LOX-1 antibody TS92 was a gift of Dr. T. Sawamura (Osaka, Japan). Heparinase III (Hep III) and ROS inhibitor (Apocynin, 1 mmol/l) were obtained from Sigma (St. Louis, MO).

2.2. Western blot

Primary and secondary antibodies were purchased from Abcam (San Francisco, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and Novus Biologicals (Littleton, CO). Details of Western blotting have been published elsewhere [4].

2.3. Dil-ox-LDL uptake

For visualization of specific ox-LDL uptake, triplicate cultures of HUVECs in 24-well format were incubated with Dil-ox-LDL 5 μg/ml for 2 h at 37 °C. After incubation, cells were gently washed with PBS three times and imaged using fluorescent microscopy.

2.4. MTT analysis of cell proliferation

Cell proliferation was assessed by MTT Cell Proliferation Assay (ATCC), as recently described [10].

2.5. Measurement of intracellular ROS

Intracellular ROS was measured with the use of the fluorescent signal dihydroethidium (DHE), a cell-permeable indicator for ROS generation, as recently described [10].

2.6. Analysis of apoptosis

Apoptosis was analyzed by Western blot and Polycaspase FLICA apoptosis detection kit (ImmunoChemistry Technologies, Bloomington, MN). Overall caspase activity was assessed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ), and the results were analyzed with the software WinMDI29.

2.7. Autophagy detection

Autophagy in HUVECs was detected by Western blot and Premo™ Autophagy Sensors (LC3B-FP) (Invitrogen, Grand Island, NY). In addition, we measured beclin-1 and Atg5 with fluorescence microscopy and flow cytometry.

2.8. Statistical analysis

Data from at least three independent experiments were used for statistical analysis. Results are shown as mean ± SD. Multiple means were compared using a one-way analysis of variance (ANOVA). Paired Student's *t*-test was used to assess significant differences. A P value <0.05 was considered significant.

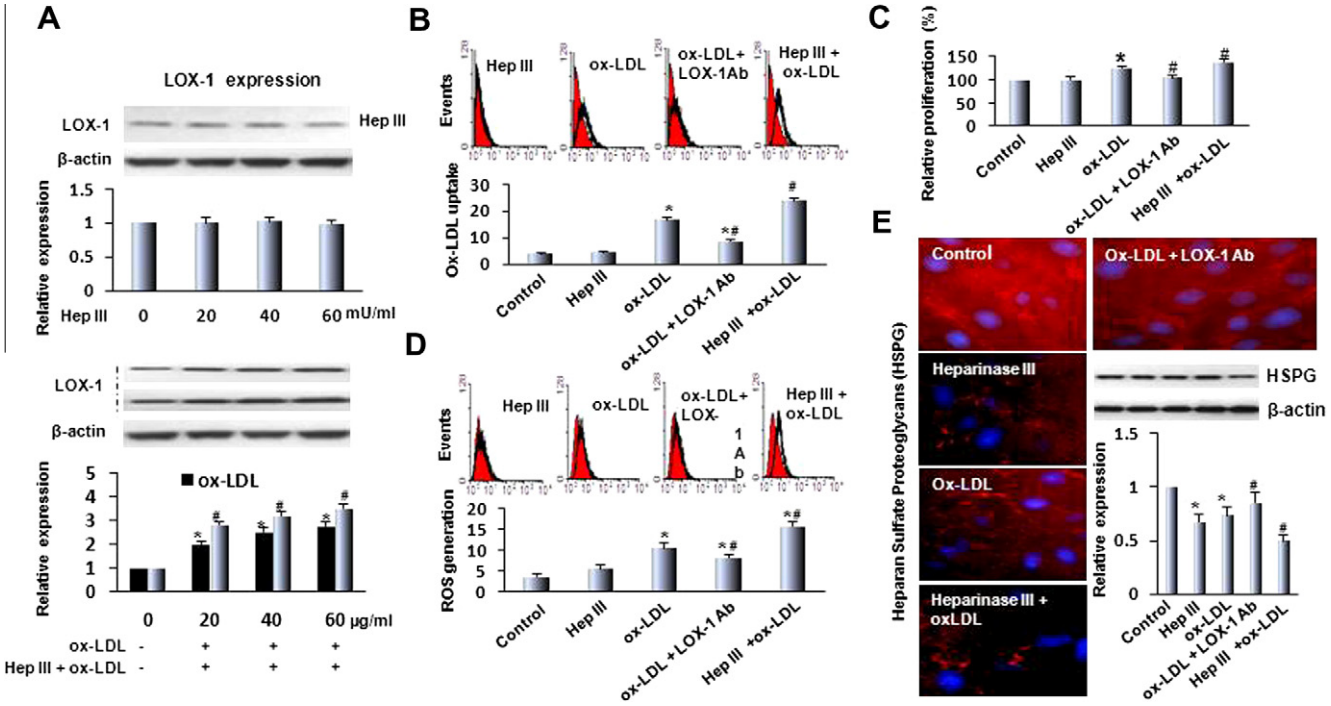

Fig. 1. Effects of ox-LDL and Hep III on LOX-1 expression, ROS generation and HSPG degradation in HUVECs. (A) Hep III had no effect on LOX-1 expression, while ox-LDL induced LOX-1 expression in a concentration-dependent manner. Hep III pretreatment enhanced ox-LDL-mediated LOX-1 expression. (B and C) Hep III had no effect on Dil-ox-LDL uptake or cell proliferation, while ox-LDL induced Dil-ox-LDL uptake or modest cell proliferation. However, Hep III pretreatment enhanced ox-LDL-mediated Dil-ox-LDL uptake and cell proliferation. LOX-1 antibody blocked most of ox-LDL uptake and inhibited cell proliferation in response to ox-LDL. (D) Hep III had no significant effect on ROS generation, but it enhanced ox-LDL-mediated ROS generation. Pretreatment of cells with LOX-1 antibody prevented ROS generation. (E) Immunofluorescence and Western blot showed that Hep III degraded HSPG; ox-LDL also caused modest HSPG degradation; and Hep III enhanced ox-LDL-mediated HSPG degradation. LOX-1 antibody protected ECs from ox-LDL-mediated HSPG degradation. Bar graphs represent data in mean ± SD, $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. ox-LDL.

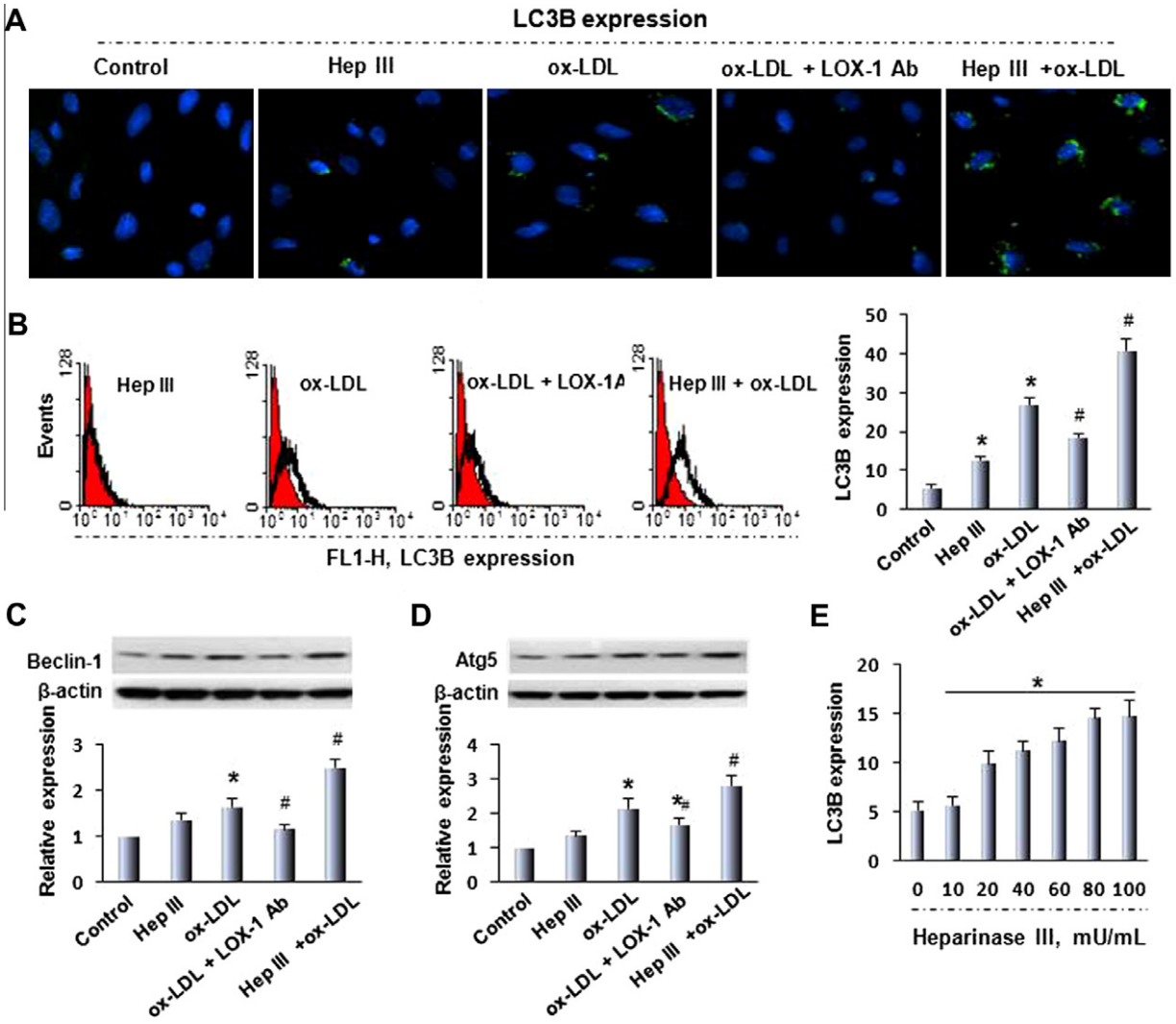

Fig. 2. Effect of Hep III and ox-LDL on markers of autophagy. (A–D) Hep III induced LC3B expression whereas ox-LDL induced all autophagy markers LC3B, beclin-1 and Atg5 expression. Pretreatment of cells with LOX-1 antibody prevented ox-LDL-mediated autophagy. Importantly, Hep III enhanced the effect of ox-LDL on autophagy markers. (E) Hep III induced LC3B expression in a concentration-dependent fashion. Bar graphs represent data in mean ± SD. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. ox-LDL.

3. Results

3.1. Effect of Hep III and ox-LDL on LOX-1 expression, ROS generation and HSPG damage

As shown in Fig. 1A, Hep III (up to 60 mU/ml for 24 h) treatment had no effect on LOX-1 expression, while ox-LDL induced LOX-1 expression in a concentration-dependent manner. Interestingly, Hep III pretreatment, modestly but significantly, enhanced the effect of each concentration of ox-LDL on LOX-1 expression ($P < 0.05$ vs. ox-LDL alone).

In subsequent experiments, we used Hep III 20 mU/ml and ox-LDL 20 μg/ml.

As shown in Fig. 1B and C, Hep III alone did not affect Dil-ox-LDL uptake or cell proliferation. Ox-LDL alone, as expected [11], increased Dil-ox-LDL uptake and induced cell proliferation. Both these effects of ox-LDL were blocked by treatment of cells with LOX-1 antibody (10 μg/ml). Importantly, pretreatment of cells with Hep III enhanced the effect of ox-LDL on Dil-ox-LDL uptake as well as cell proliferation ($P < 0.05$ vs. ox-LDL alone).

As an important secondary messenger, ROS play a key role in many cellular events including cell proliferation, apoptosis, hypertrophy and inflammation [11]. ROS are also key signaling molecules in the effects of LOX-1 [1,12]. As shown in Fig. 1D, ox-LDL, but not Hep III, induced ROS generation, and LOX-1 antibody blocked ROS generation in response to ox-LDL. Importantly, Hep III pretreatment of cells enhanced ox-LDL-mediated ROS generation ($P < 0.05$ vs. ox-LDL alone).

In keeping with the known effect of Hep III, there was evidence for degradation of surface HSPG in cells treated with Hep III (Fig. 1E). Ox-LDL also caused a modest, but significant, HSPG degradation which was blocked by LOX-1 antibody. Hep III pretreatment increased HSPG degradation by ox-LDL beyond the effect of ox-LDL alone. Western blot data confirmed these findings (Fig. 1E).

3.2. Effect of Hep III and ox-LDL on autophagy

As shown in Fig. 2A–D, Hep III had only a small effect on autophagy markers (LC3B and Atg5). Ox-LDL resulted in a significant increase in all autophagy markers (LC3B, beclin-1 and Atg5). LOX-1 antibody blocked autophagy in response to ox-LDL. Cells treated with Hep III and ox-LDL showed marked autophagy activity beyond that in response to ox-LDL alone ($P < 0.05$ vs. ox-LDL alone).

Since Hep III treatment of HUVECs induced some autophagy markers, we investigated if autophagy response to Hep III was

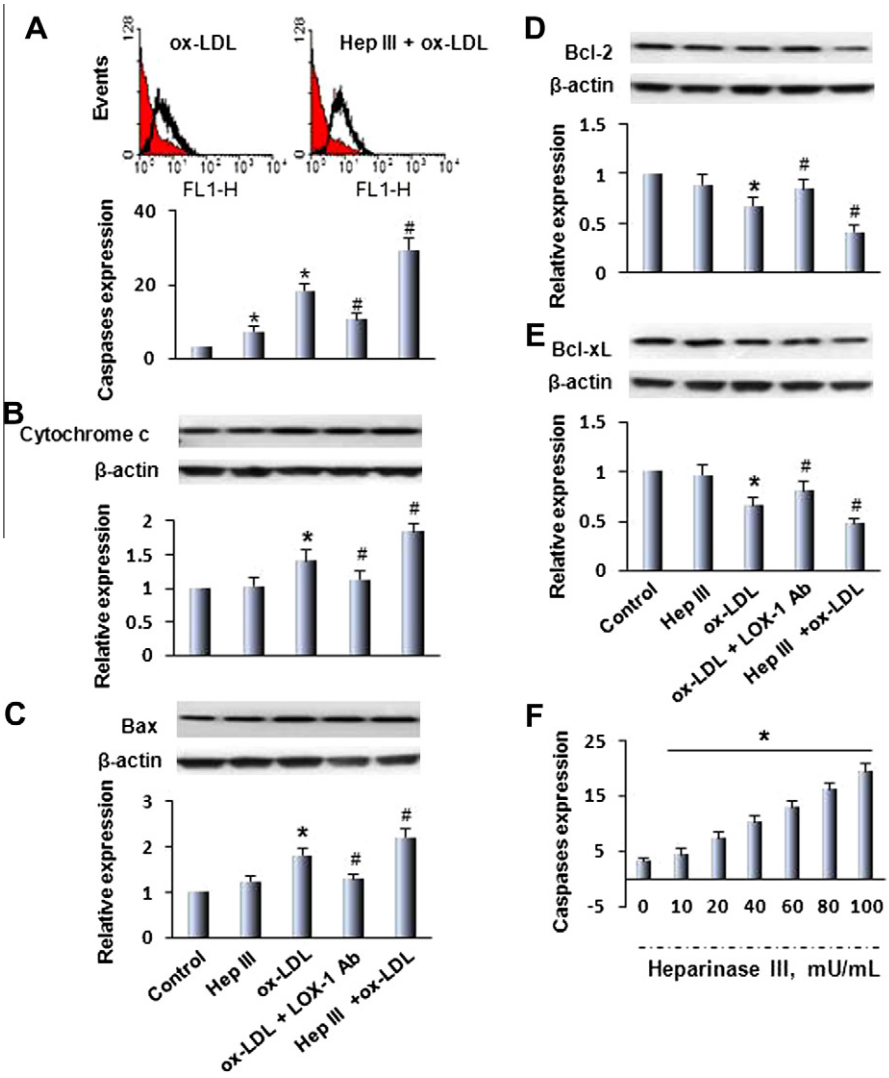

Fig. 3. Effect of Hep III and ox-LDL on markers of apoptosis. (A–E) Ox-LDL induced apoptosis measured as caspases, cytochrome c, Bax, Bcl2 and Bcl-xL expression. Pretreatment of cells with LOX-1 antibody prevented ox-LDL-mediated apoptosis. Hep III treatment of HUVECs increased caspases expression. Importantly, Hep III enhanced the effect of ox-LDL on apoptosis markers. (F) Hep III induced caspases expression in a concentration-dependent fashion. Bar graphs represent data in mean ± SD, $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. ox-LDL.

concentration-dependent. As evident from Fig. 2E, Hep III increased LC3B expression in a concentration-dependent fashion (Fig. 1E).

3.3. Effect of Hep III and ox-LDL on apoptosis

Ox-LDL induced apoptosis markers (caspase-3, cytochrome c and Bax, and reduced anti-apoptosis proteins Bcl-2 and Bcl-xL) (Fig. 3A–E). As expected, LOX-1 antibody markedly blunted the pro-apoptotic effects of ox-LDL.

Hep III induced the expression of caspases in a concentration-dependent manner (Fig. 3F) without a significant effect on cytochrome c, or the expression of Bax, Bcl-2 and Bcl-xL. Similar to autophagy activity data, pretreatment with Hep III significantly enhanced ox-LDL-mediated apoptosis ($P < 0.05$ vs. ox-LDL alone) (Fig. 3A–E).

3.4. Effects of apocynin on HSPG, autophagy and apoptosis in response to ox-LDL and Hep III

Ox-LDL mediated ROS play a key role in the development of apoptosis, and NADPH oxidase is a primary source of ROS in HUVECs [13]. We investigated if apocynin, a generalized NADPH oxidase inhibitor [13], would affect ox-LDL- or Hep III-mediated autophagy and apoptosis.

As shown in Fig. 4, apocynin significantly inhibited ox-LDL-mediated degradation of HSPG, and markers of autophagy (beclin-1 and Atg5) and apoptosis (altered expression of cytochrome c, Bax, Bcl-2 and Bcl-xL), suggesting that NADPH oxidase activation plays an important role in this process. In keeping with the data in Figs. 2 and 3, LOX-1 antibody pretreatment also reduced autophagy and apoptosis markers. In contrast, neither apocynin nor LOX-1 antibody modified Hep III-mediated changes in the expression of HSPG, or markers of autophagy and apoptosis.

4. Discussion

The purpose of this study was to test the hypothesis that a break in HSPG by Hep III may render ECs more susceptible to the effects of ox-LDL.

As expected, Hep III treatment resulted in degradation of HSPG. Interestingly, ox-LDL also caused a modest degradation of HSPG, hitherto an unknown effect of ox-LDL. More importantly, Hep III

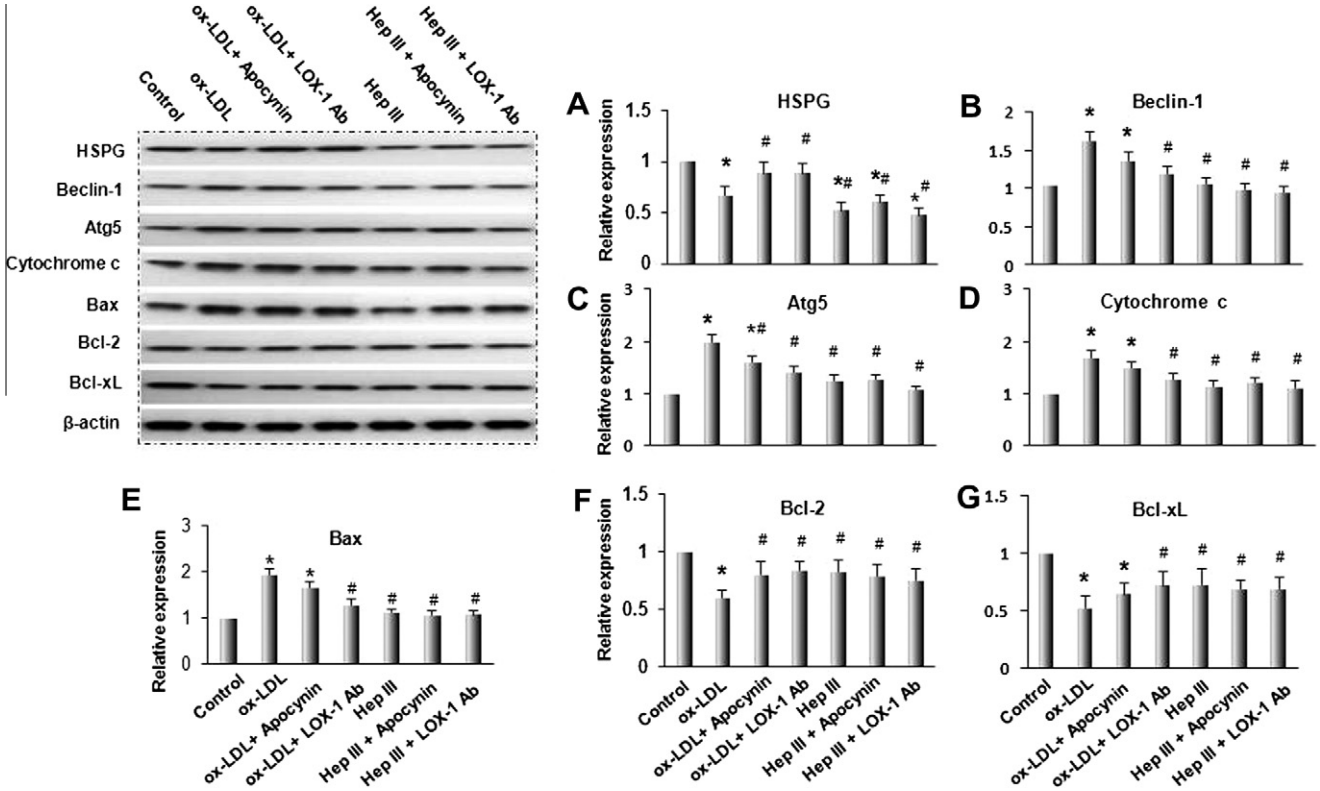

Fig. 4. Effects of apocynin and LOX-1 antibody on autophagy and apoptosis in response to ox-LDL and Hep III. (A–G) Both apocynin and LOX-1 antibody blocked HSPG degradation, as well as the markers of autophagy and apoptosis in response to ox-LDL, but had no effect on Hep III-mediated HSPG degradation, or the markers of autophagy and apoptosis autophagy. Bar graphs represent data in mean ± SD. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. ox-LDL.

pretreatment of ECs caused a significant increase in HSPG degradation beyond that caused by ox-LDL alone. These observations confirmed our major hypothesis.

This study confirms previous observations that ox-LDL induces ROS generation and development of apoptosis in a LOX-1 dependent fashion [1,4,14,15]. In keeping with this mechanistic basis, ox-LDL induced apoptosis was blocked by the NADPH oxidase inhibitor apocynin as well as LOX-1 antibody. While Hep III itself induced caspases expression, it also resulted in concomitant small increase in ROS generation. However, Hep III did not affect Dil-ox-LDL uptake or the expression of LOX-1. Therefore, as one would expect, LOX-1 antibody had no effect on caspase expression in response to Hep III. These observations collectively suggest that ox-LDL induces apoptosis in a ROS/LOX-1 dependent fashion, whereas Hep III induces caspases in a ROS/LOX-1 independent manner. Nonetheless and more importantly, we observed that Hep III significantly enhanced the pro-apoptotic effect of ox-LDL.

Autophagy is an evolutionary conserved process which becomes manifest during state of increased stress [16], and may be considered a cell survival mechanism [16,17]. We now show that HUVECs when exposed to ox-LDL develop autophagy. The results of three different indices of autophagy, LC3B, beclin-3 and Atg5, were similar and suggest that autophagy in HUVECs in response to ox-LDL reflects a stress response that allows HUVECs to survive during harsh conditions. Importantly, ox-LDL-induced autophagy was blocked by apocynin as well as LOX-1 antibody suggesting that the autophagic response in HUVECs is, like apoptosis, mediated via ROS-LOX-1 pathway. Notably, Hep III also elicited a small autophagic response, but similar to apoptotic response it was not modified by either apocynin or LOX-1 antibody. Thus, while the concomitant apoptosis and autophagy responses to Hep III and ox-LDL are qualitatively similar, the mechanisms of cell injury and survival are quite different. Nonetheless, when cells are pre-exposed to Hep III, apoptosis and autophagy responses to ox-LDL are exaggerated.

Our observations are novel and may have implications in the pathobiology of atherosclerosis and other conditions characterized by ROS generation and resultant EC injury/activation [17]. Ox-LDL is a major inducer of ROS, inflammation and injury to ECs. Degradation of proteoglycans by heparinase III or other mediators may enhance the deleterious effect of ox-LDL on EC organization. Autophagy response to ox-LDL is most likely a mechanism of cell survival during oxidant stress. The present observation of HSPG degradation by ox-LDL enhance our understanding of ox-LDL-mediated cellular injury.

Acknowledgment

This study was supported in part by funds from the Department of Veterans Affairs.

References

[1] J. Lu, S. Mitra, X. Wang, et al., Oxidative stress and lectin-like ox-LDL-receptor LOX-1 in atherogenesis and tumorigenesis, Antioxid Redox Signal 15 (2011) 2301–2333.
[2] D. Li, J.L. Mehta, Intracellular signaling of LOX-1 in endothelial cell apoptosis, Circ. Res. 104 (2009) 566–568.
[3] C.R. Kuhlmann, M. Schäfer, F. Li, et al., Modulation of endothelial Ca(2+)-activated K(+) channels by oxidized LDL and its contribution to endothelial proliferation, Cardiovasc. Res. 60 (2003) 626–634.
[4] J. Chen, J.L. Mehta, N. Haider, et al., Role of caspases in ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells, Circ. Res. 94 (2004) 370–376.
[5] U. Häcker, K. Nybakken, N. Perrimon, Heparan sulphate proteoglycans: the sweet side of development, Nat. Rev. Mol. Cell Biol. 6 (2005) 530–541.
[6] J.R. Bishop, M. Schuksz, J.D. Esko, Heparan sulphate proteoglycans fine-tune mammalian physiology, Nature 446 (2007) 1030–1037.

[7] B. Levine, N. Mizushima, H.W. Virgin, Autophagy in immunity and inflammation, Nature 469 (2001) 323–335.
[8] N. Ermak, B. Lacour, T.B. Drüeke, et al., Role of reactive oxygen species and Bax in oxidized low density lipoprotein-induced apoptosis of human monocytes, Atherosclerosis 200 (2008) 247–256.
[9] M.C. Maiuri, E. Zalckvar, A. Kimchi, et al., Self-eating and self-killing: crosstalk between autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 8 (2007) 741–752.
[10] X. Wang, J. Lu, M. Khaidakov, et al., Aspirin suppresses cardiac fibroblast proliferation and collagen formation through downregulation of angiotensin type 1 receptor transcription, Toxicol. Appl. Pharmacol. 259 (2012) 346–354.
[11] F. Jiang, Y. Zhang, G.J. Dusting, NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair, Pharmacol. Rev. 63 (2011) 218–242.
[12] I.V. Smirnova, T. Sawamura, M.S. Goligorsky, Upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in endothelial cells by nitric oxide deficiency, Am. J. Physiol. Renal. Physiol. 287 (2004) F25–F32.
[13] A. Taye, A.H. Saad, A.H. Kumar, et al., Effect of apocynin on NADPH oxidase-mediated oxidative stress-LOX-1-eNOS pathway in human endothelial cells exposed to high glucose, Eur. J. Pharmacol. 627 (2010) 42–48.
[14] Z. Ding, S. Liu, B. Yang, et al., Effect of oxidized low-density lipoprotein concentration polarization on human smooth muscle cells' proliferation, cycle, apoptosis and oxidized low-density lipoprotein uptake, J. R. Soc. Interface 9 (2012) 1233–1240.
[15] L. Cominacini, A.F. Pasini, U. Garbin, et al., Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species, J. Biol. Chem. 275 (2000) 12633–12638.
[16] J. Lee, S. Giordano, J. Zhang, Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling, Biochem. J. 441 (2012) 523–540.
[17] D.M. Schrijvers, G.R. De Meyer, W. Martinet, Autophagy in atherosclerosis: a potential drug target for plaque stabilization, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 2787–2891.