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REVIEW ARTICLE

Role of Heat Shock Protein 27 in Modulating Atherosclerotic Inflammation

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Abstract

Atherosclerosis is the primary cause of heart attacks, and while efforts to prevent its development or progression have historically focused largely on reducing cholesterol levels, there is now important proof-of-principle data that supports the role that inflammation plays in atherogenesis. Heat shock protein 27 (HSP27) is a novel biomarker of atherosclerosis that is also atheroprotective. Through a series of murine and in vitro experiments, an iterative narrative is emerging that demonstrates how HSP27 can act as an extracellular mediator that reduces plaque inflammation—either directly via transcriptional pathways, or indirectly via important effects on macrophage biology. While there is much more to learn about the biology of HSP27, we now review the strong foundation of knowledge that highlights the potential anti-inflammatory role of HSP27 as a novel therapeutic for not only atherosclerosis but potentially other inflammatory disorders.

Keywords Atherosclerosis · Inflammation · Heat shock protein 27

Introduction: the Role of Inflammation in Atherogenesis

Obstruction of blood flow to vital organs such as the heart muscle or brain is the primary cause of mortality in the developed world, with the burden of disease steadily growing in developing countries [1]. The common cause of such arterial obstructions is atherosclerosis, a disease process that begins early in life, and may remain clinically silent until a plaque ruptures, resulting in thrombus formation, ischemia, and end-organ damage. Although the focus of study for centuries, the factors that transform a non-obstructive plaque into a late-stage atherosclerotic lesion are incompletely understood [2]. Much attention has been centered on understanding and modifying the role of lipoproteins in plaque development, instability, and quiescence. Though progress has been made with the widespread use of statins, the current standard of care for reducing cholesterol levels, there remains a substantial residual risk in these patients—even with the use of newer therapies that target PCSK9, an important negative regulator of cholesterol metabolism that drive plasma low-density lipoprotein cholesterol down to levels previously thought to be unattainable [3, 4]. Indeed, the 5-year risk of recurrent myocardial infarction, stroke, or cardiovascular death is > 20% in statin-treated patients, and although some of this risk may be due to suboptimal treatment of low-density cholesterol levels, it is now becoming clear that residual inflammatory risk is also important [5–7].

In the context of atherosclerosis, what is inflammation? In any disease process or injury, the inflammatory process is designed to contain the insult, initiate appropriate wound healing responses, and restore homeostasis. However, persistent (or chronic) inflammation indicates that this process is incomplete or lacks the cues to resolve—thereby signaling the transformation from a physiological to a pathological response [8]. Indeed, many of the steps that promote the transformation of benign intimal thickening to a complex lipid-laden plaque with a necrotic core that is vulnerable to rupture or ulceration are due to unbridled inflammation [9, 10]. While the role of inflammation in experimental atherosclerosis

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models is very well studied—it is only recently that the clinical evidence for the role of inflammation in the pathogenesis of atherosclerotic coronary artery disease (CAD) has emerged. As arterial inflammation is challenging to directly study, even by non-invasive means [11], there is a heavy reliance on the circulating inflammatory biomarker C-reactive protein (CRP; measured using a high sensitivity assay or hsCRP) to prognosticate cardiovascular events [12].

CRP is downstream from the inflammatory cytokine IL-1β, which is activated by the NLRP3 (NOD-like receptor P3) inflammasome and then released into the circulation [13]. Perhaps the importance of CRP in cardiovascular prevention trials was initially highlighted by JUPITER (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin) that showed how a statin (rosuvastatin) reduced the incidence of cardiovascular events in asymptomatic subjects with normal low-density lipoprotein cholesterol (LDL-C) but elevated hsCRP—as the degree of protection was proportional to the diminution in hsCRP [14]. However, with the concomitant reduction in LDL-C in JUPITER, it was impossible to conclusively attribute the improvement in CV outcomes to the attenuation of inflammation. While it is clear that statins can optimally reduce LDL-C (defined as < 70 mg/dL) and hsCRP (defined as < 2 mg/L) in symptomatic CAD patients—typically, only the minority of patients reach these goals. For example, in *post hoc* analyses of two large trials of acute coronary syndrome patients treated with aggressive statin therapy, the relative proportion of patients with residual inflammatory risk is significant (e.g., 29–33%) [15, 16].

Hence, the need to address inflammation as a major pathophysiological contributor to CAD is large. This is where novel biological therapies are now entering the CAD therapeutic landscape. In particular, there is a renewed interest in the cytokine IL-1β, as it is produced by macrophages in human atherosclerotic lesions in response to the presence of cholesterol crystals and plays an integral role in "inflammageing" [17, 18]. Inflammageing is essentially chronic inflammation and is characterized by elevated levels of blood inflammatory markers such as hsCRP and is associated with the development of persistent inflammatory conditions (such as atherosclerosis) that arise due to genetic, lifestyle, and co-morbidity factors [19].

Given the importance of IL-1β in atherosclerosis, and the availability of a human monoclonal antibody that targets IL-1β (i.e., canakinumab, approved for use in patients with rheumatological disorders), the recently completed CANTOS trial (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) was designed to test the inflammatory theory for the pathogenesis of atherosclerosis [20]. In approximately ten thousand myocardial infarction patients prescribed optimal statin therapy, subjects were randomized to receive one of three doses of canakinumab administered subcutaneously every 3 months for approximately 4 years. Only patients with an elevated hsCRP level (> 2 mg/L) were enrolled—as this was considered a surrogate marker for arterial wall inflammation. Briefly, with the intermediate (150 mg) dose of canakinumab, there was a reduction in the primary clinical endpoint (a composite of nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death). Hence, this trial emphasizes the fact that inflammation is treatable and associated with improved cardiovascular outcomes. Moreover, it identified IL-1β as a therapeutic target for "atheroprotection." There were other intriguing results from this trial, including a reduction in cancer mortality with canakinumab and, unfortunately, a higher death rate from an infection that was statistically significant but relatively low (a 1-year annual risk of 1/750 patients). Finally, the cost of this drug is currently prohibitive (approximately $64,000 per year) [21]. Although some [22, 23] but not all [24, 25] recent pre-clinical and clinical trials support the anti-inflammation approach for the treatment of atherosclerosis, CANTOS stands out as an intriguing proof-of-concept trial that highlights the need for additional anti-inflammatory studies. To this point, we now review the accrued knowledge of the anti-inflammatory properties of a specific heat shock protein as an anti-inflammatory modulator of atherogenesis and how it is being developed as a potential clinical therapy.

Heat Shock Protein 27 in the Context of Atherosclerotic Inflammation

Briefly, heat shock proteins are highly conserved proteins grouped according to their molecular size. HSP27 (205 amino acids, also known as HSPB1) is part of the small HSP (sHSP) family of proteins. sHSPs are ATP-independent molecular chaperones that are transiently upregulated in various cell types subjected to stress stimuli. Similar to other (larger) HSPs, sHSPs recognize and bind unfolded or misfolded proteins, thus preventing their irreversible aggregation—which is important for a variety of protein-misfolding disorders (e.g., Parkinson's, Alzheimer's diseases, and the inflammatory/apoptotic nature of atherosclerosis). To varying degrees, HSP27 is expressed in human cells, including vascular smooth muscle and endothelial cells.

Approximately 15 years ago, three independent laboratories (including our own) almost simultaneously published their findings on the potential role of HSP27 in atherosclerosis [26–28]. While looking for clues to the paradox that women are relatively protected against CAD until menopause, we discovered HSP27 as an estrogen receptor beta (ER-β)–associated protein [27, 29]. Importantly, we examined the expression of HSP27 in human CAD and noted a progressive diminution of protein expression as the stage of atherosclerosis progressed (Fig. 1). These results have subsequently been confirmed by two additional laboratories using objective proteomic approaches [30, 31]. Moreover, we and others noted

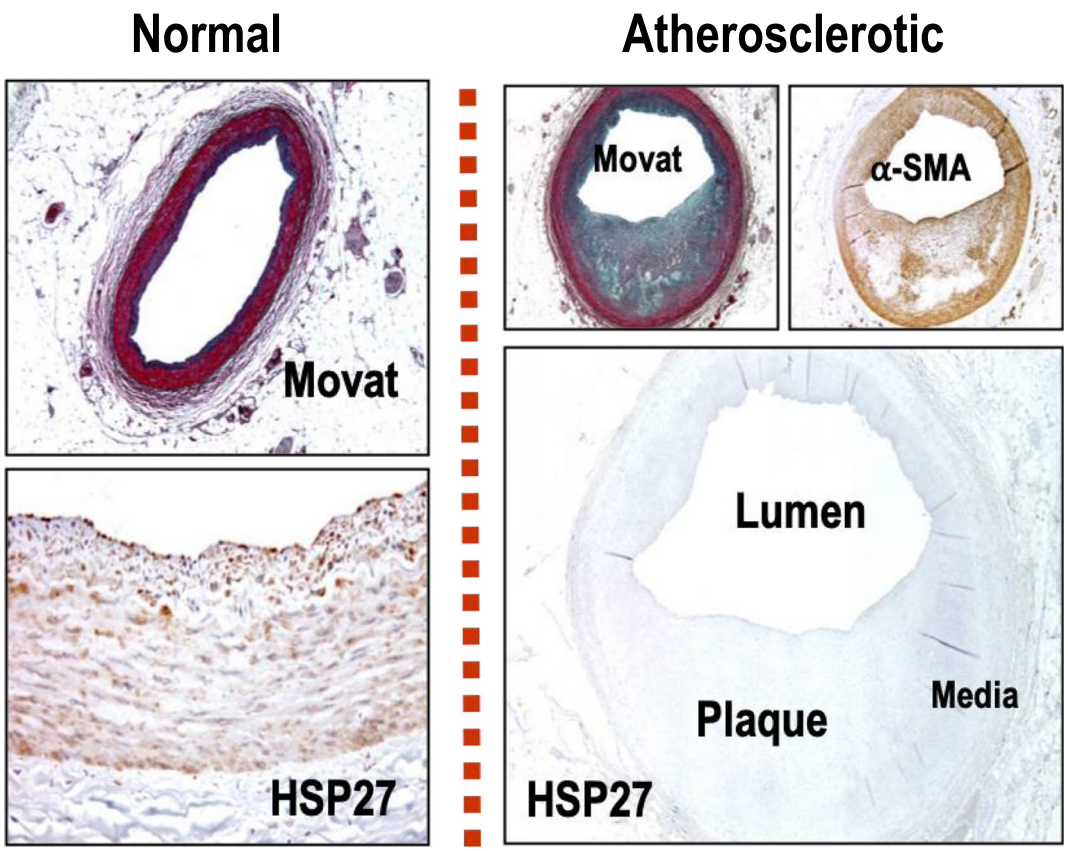


Fig. 1 Photomicrographs of human coronary artery cross-sections: Left: Cross-section of benign intimal thickening in a coronary artery of a young individual free of atherosclerosis (top: Movat pentachrome stain, magnification × 40; bottom: immunolabeled for HSP27, magnification × 400). Right: Advanced atheroma with necrotic, cholesterol-laden intimal core (Movat pentachrome stain and α-smooth muscle actin immunolabeling). HSP27 immunolabeling is essentially negative (below right; magnification × 100). All positive immunolabeling yielded a brown color reaction product, with a hematoxylin nuclear counterstain. Adapted from [27]

that blood HSP27 levels were lower in atherosclerosis patients compared with healthy subjects and showed that lower HSP27 levels were predictive of subsequent major clinical events (i.e., heart attack, stroke, cardiovascular death) over a 5-year period [26, 32, 33]. Hence, HSP27 blood levels have also emerged as a new biomarker for atherosclerosis that is predictive of adverse cardiovascular events. With these clinical insights regarding HSP27 expression, we then developed an experimental platform to study HSP27 in vascular biology.

Endogenous HSP27 Overexpression Attenuates Atherogenesis and Inflammation

To examine the functional significance of HSP27 in atherogenesis, we studied both the early and late phases of lesion development using a common murine model of inflammatory atherogenesis: the *Apoe*$^{-/-}$ mouse fed a high-fat diet (HFD). To determine if HSP27 would alter atherogenesis in this model, mice overexpressing human *HSP27* (*HSP27*$^{o/e}$) were crossbred with *Apoe*$^{-/-}$ mice [34]. After 4 weeks of the HFD, there was a 35% reduction in aortic atherosclerotic burden in female (but not male) Apoe$^{-/-}$ HSP27$^{o/e}$ vs. *Apoe*$^{-/-}$ control mice. While baseline HSP27 blood levels were virtually undetectable in all mice, after 4 weeks of the HFD, there was a > 10-fold increase in HSP27 blood levels in female *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice (and little change in the levels in male mice). Moreover, there was an inverse correlation between HSP27 blood levels and the extent of atherosclerotic burden. With regard to inflammation, rHSP27 treatment of macrophages in vitro was associated with a reduction in foam cell formation, as reflected by decreased uptake of acetylated LDL (acLDL). These macrophages released less IL-1β (pro-inflammatory) and more IL-10 (anti-inflammatory) into their cell culture media. Hence, HSP27 had an indirect effect on inflammatory cytokine generation, likely due to a reduction in the transformation of macrophages into foam cells.

Chronic overexpression of HSP27 is also atheroprotective in *Apoe*$^{-/-}$ mice as it facilitates favorable plaque remodeling [35]. In *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice of both sexes fed a HFD for 12 weeks, blood HSP27 levels rose by more than 16-fold from baseline. Of note, HSP27 blood levels were higher in the female mice—which is in keeping with our earlier in vitro and in vivo observations on the key role that estrogens play

in promoting the extracellular release of HSP27 [34, 36]. Presumably, the rise in HSP27 blood levels in the male mice was due to prolonged exposure to the metabolic stress of an atherogenic HFD. While the reductions in atherogenesis were significant but modest in the *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ compared with *Apoe*$^{-/-}$ counterpart mice (e.g., for both sexes, the reductions in aortic lesion area were 21–35% for *en face* and 24–30% for cross-sectional aortic sinus tissue sections), there were impressive changes in lesion morphology that are consistent with less inflamed plaques, including:

(a) Reduced aortic lesion cholesterol content, as reflected by the number of intimal cholesterol clefts, as well as the areas occupied by lipid and free cholesterol (Fig. 2),
(b) Reduced plaque macrophage content (Fig. 2),
(c) Increased lesion smooth muscle and collagen content (Fig. 2), and
(d) Increased lesion stiffness (as reflected in stress-strain curves generated during ex-vivo mechanical stretching experiments).

Taken together, these histological changes are consistent with plaque inflammation that has not gone unabated and may in fact be in the process of resolving [37]. Further study of the long-term effects of HSP27 on atherogenesis is warranted. However, to date, we do know that *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice fed a normal chow diet for 32 weeks show a persistent decrease (− 29%) in aortic lesion burden with a concomitant (−26%) reduction in total plasma cholesterol levels compared with *Apoe*$^{-/-}$ control mice [33]. Hence, it appears that HSP27 has a durable atheroprotective effect.

Exogenous HSP27 Is Atheroprotective and Attenuates Arterial Inflammation

As we demonstrated the secretion of HSP27 from macrophages in vitro, we sought to determine what cells were driving the increase in HSP27 blood levels in vivo by transplanting bone marrow from *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice into *Apoe*$^{-/-}$ littermates, which do not have the *HSPB1* gene encoding for HSP27 [33]. While serum HSP27 levels were undetectable in the control mice, transplantation of bone marrow from *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice resulted in marked elevation of HSP27 blood levels and reductions in aortic lesion burden (e.g., − 50% for *en face* and − 28% for aortic sinus cross-sections). Again, other important salutary effects were noted in the plaques of the mice receiving the *HSP27*$^{o/e}$ marrow (e.g., reduced cholesterol cleft and necrotic core areas). Hence, the data from this experiment suggest that HSP27 derived from blood-borne cells is sufficient to attenuate de novo atherosclerotic lesion formation and perhaps overshadows the functional importance of the artery wall (i.e., smooth muscle or endothelial cell) expression of HSP27.

Additionally, we synthesized recombinant HSP27 (rHSP27) to explore its potential therapeutic effects in de novo lesion formation [33]. Twice-daily subcutaneous injections of either rHSP27 (100 μg) or vehicle for 3 weeks increased HSP27 blood levels and reduced total aortic lesion area (31% and 40% for aortic *en face* and sinus analyses, respectively). Interestingly, total plasma cholesterol levels were 42% lower with rHSP27 treatment ($p < 0.001$) due to reductions in very-low-density lipoprotein and intermediate-IDL/low-density sub-fractions. The mechanisms involved in lowering plasma cholesterol levels are now an active area of research in the O'Brien laboratory and appear to be due to important transcriptional effects on key mediators of cholesterol metabolism (unpublished data).

HSP27 Stabilizes Existing Plaques and Lowers Inflammation

As clinical therapies are often initiated in patients with established disease after events such as myocardial infarction or stroke, we tested the ability of rHSP27 to modulate the progression and morphology of established atherosclerotic lesions. *Apoe*$^{-/-}$ mice were fed an atherogenic diet for 4 weeks to establish a "baseline" atherosclerotic state, then switched to a normal chow diet to simulate lipid management strategies such as lifestyle modifications and/or the prescribing of statin therapy [33]. After developing these baseline lesions, rHSP27 (100 μg) or vehicle was administered twice daily for 3 weeks. Total plasma cholesterol levels were similar in both groups at baseline and decreased after switching to the chow diet, yet were 27% lower with rHSP27 treatment at the time of euthanasia ($p = 0.004$). Again, aortic lesion area was modestly but significantly reduced with rHSP27 vs. control treatment—and comparable with that of the baseline atherosclerotic state—thereby implying that lesion progression was halted. Moreover, rHSP27 therapy was associated with the genesis of intimal plaques with less inflammation (e.g., fewer macrophages, less free cholesterol accumulation, and less area occupied by cholesterol clefts) and structurally more resilient morphologies (e.g., more abundant collagen and smooth muscle content). Whether all of the salutary HSP27 effects on lesion morphology are directly related to the effect of this protein on macrophage biology is unclear. Certainly, there are other possible cellular effects to consider—including the role of HSP27 in accelerating re-endothelialization of arterial lesions via the upregulation of vascular endothelial growth factor [38].

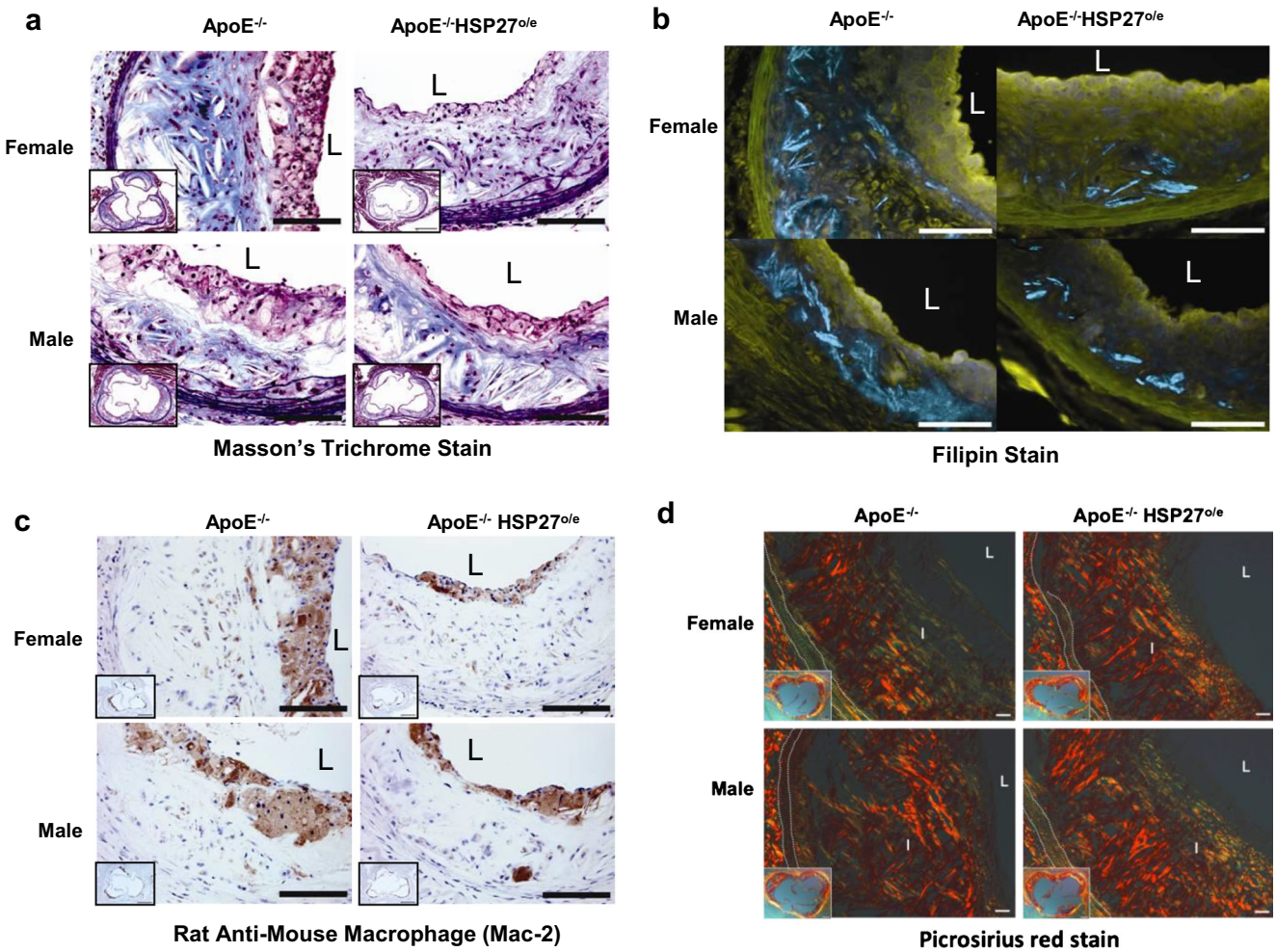


Fig. 2 Chronic HSP27 overexpression in *Apoe*$^{-/-}$ mice reduces aortic lesion cholesterol content. **a** Cholesterol cleft area is reduced within lesions from *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ vs. *Apoe*$^{-/-}$ mice as observed using the Masson's trichrome stain. **b** Attenuated arterial wall unesterified cholesterol content in *Apoe*$^{-/-}$ *HSP27*$^{o/e}$ mice as denoted by fluorescent blue Filipin staining. Scale bars = 100 μm for **a** and **b** and 500 μm for insert photo for **a**; L lumen. **c** Reduction in arterial wall foamy macrophage content and apoptosis with overexpression of HSP27. Macrophages (brown anti-Mac-2 immunolabeling reaction product) localized mainly in luminal layers of aortic sinus cross-sections. Scale bars = 100 μm and 500 μm for insert photo. **d** Increased intimal collagen content with HSP27 overexpression as demonstrated by polarized microscopy on picrosirius red-stained sections. Bright yellow/orange birefringence of collagen, due to co-aligned molecules of type I collagen. Scale bars = 20 μm and 200 μm for insert photo; L lumen, I intima; dotted lines delineate the media. Figure reproduced with permission from [35]

HSP27 Modulation of Inflammatory Signaling

The reductions in atherosclerotic lesion formation with either endogenous HSP27 overexpression or the administration of exogenous rHSP27 occurred in the presence of modest lowering of plasma cholesterol levels. Given that the pathogenesis of atherosclerosis is broadly considered to be due to the dual effects of hypercholesterolemia and inflammation, we sought to focus on how HSP27 may interact with the NF-kB master transcriptional regulatory pathway that plays a key role in modulating inflammation. Briefly, NF-κB transcription factors regulate a vast number and diversity of gene targets, including those involved in cell proliferation, apoptosis, the cell stress response, inflammation, and both innate and adaptive immune responses [39, 40]. Normally found in the cytoplasm as inactive dimers associated with IκB, NF-κB translocates to the nucleus once IκB is phosphorylated by the upstream IκB kinase complex (IKK), leading to the transactivation of numerous gene targets—including many atherosclerosis-related gene programs [39, 41].

Hence, to remove the potential confounding HSP27 lipid-lowering effect and the resultant indirect effects on inflammation, we performed the following in vitro experiments. Studying macrophages in tissue culture, we observed that rHSP27 activates the NF-κB pathway and alters downstream transcription, resulting in up-/downregulation of the key genes for pro- and anti-inflammatory cytokines such as IL-6, GM-CSF, TNF, or IL-10 [42]. Such a result is not unexpected. While NF-kB is often regarded to have a pro-inflammatory role, this may be an oversimplification. Indeed, while a "pan-

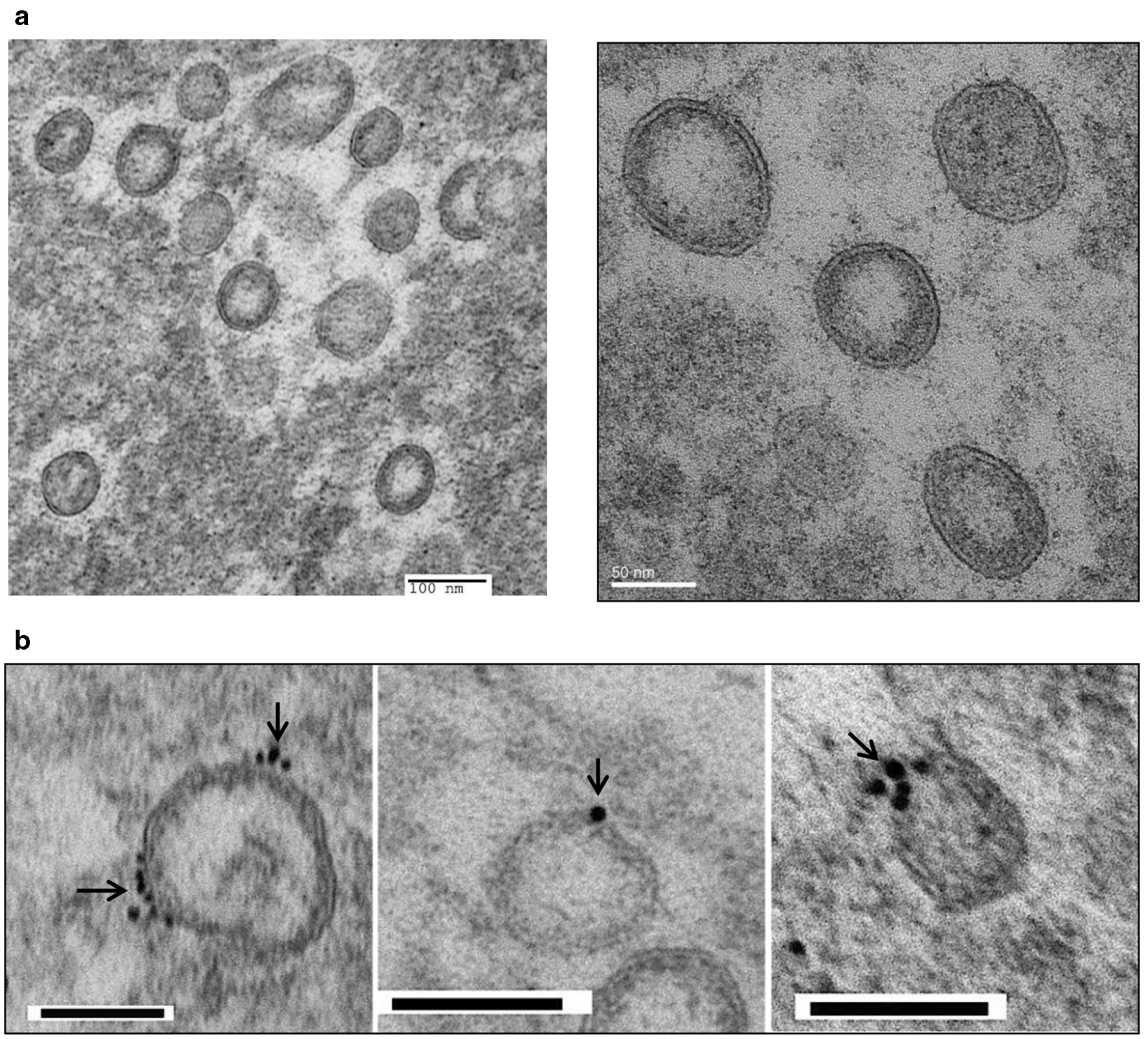


Fig. 3 Transmission electron microscopic images of exosomes harvested from THP-1 monocytes in vitro. **a** Left and right showing × 50,000 and × 100,000 magnification, with scale bars of 100 and 50 nm; respectively. **b** Exosomes immunolabeled with anti-human HSP27 primary mouse antibody followed by anti-mouse IgG secondary antibody conjugated to 5-nm gold particles. Arrows indicate HSP27 immuno-gold labeling (black dots) on the exosome surface. Scale bar = 100 nm. Figure reproduced with permission from [48]

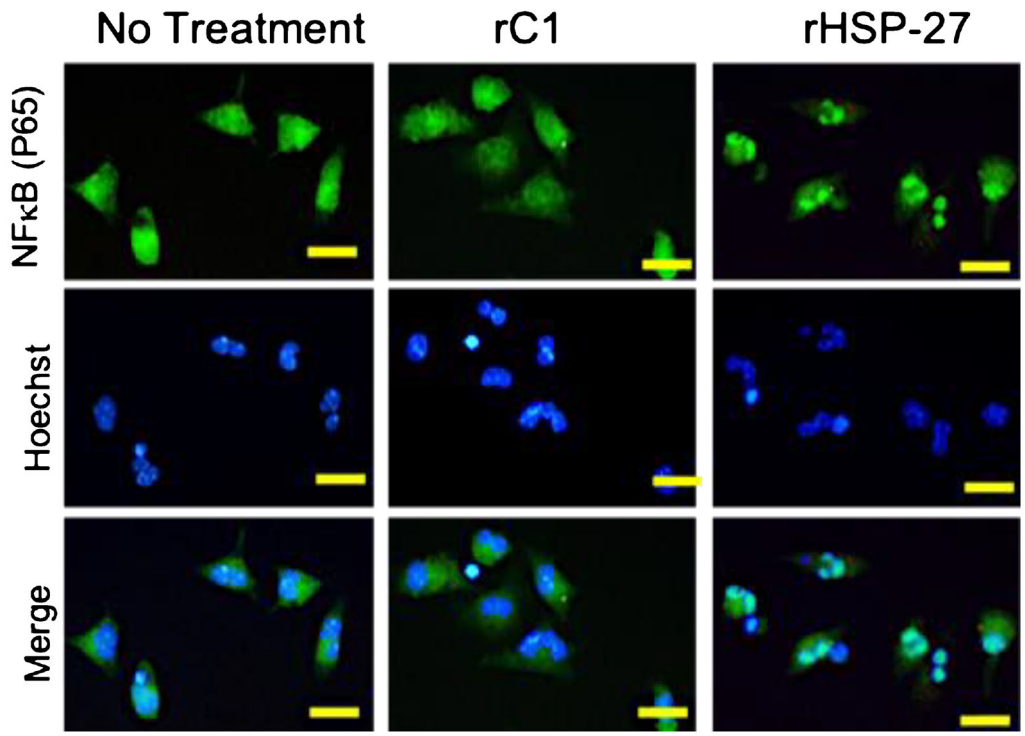
hematopoietic" knock-out of NF-kB reduces experimental atherogenesis, [43] knocking out macrophage [44] NF-kB actually worsens atherogenesis. As macrophages will of course produce anti-inflammatory factors such as IL-10 (in response to HSP27), activation of NF-kB is in some instances a critical determinant of inflammatory balance, yet may change over time, depending on the disease stage (e.g., early inflammatory stage of atherogenesis vs. later progressive plaque remodeling). Recently, it has been pointed out that perhaps, the macrophage functional response is determined by interactions between triggered transcription factors and depends on the microenvironment and interdependent signaling cascades [45]. Finally, it is important to note that many studies focus on the role of *intracellular* HSP27 on the NF-κB pathway and that we are only now beginning to better understand whether *extracellular* HSP27 has similar or different effects as an inter-cellular messenger.

Future Perspectives

While there has been much progress understanding the atheroprotective role of HSP27—including its important anti-inflammatory effects—several questions remain for future study:

(1) How is HSP27 released from cells?

Initially thought to be exclusively an intracellular protein that plays a major role in facilitating the refolding of denatured proteins, it is now clear that HSP27 exits viable cells to serve inter-cellular signaling functions [46]. Lacking a classical N-terminus signal sequence required for conventional protein secretory pathways, HSP27 is released into the extracellular space via unconventional pathways. For example, we previously showed that HSP27 is released



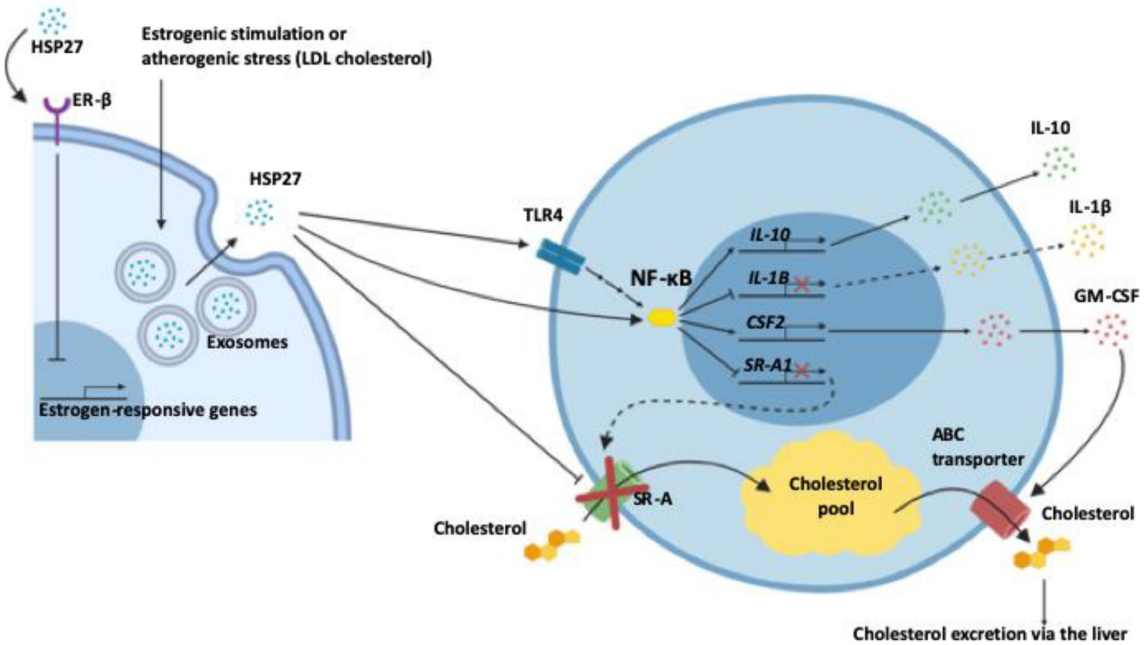


Fig. 4 Top panel showing peritoneal macrophages isolated from *Apoe*$^{-/-}$ mice and subjected to treatment with rHSP27, rC1 (negative control peptide), or no treatment. rHSP27 but not rC1 treatment for 30 min resulted in increased nuclear localization of the NF-κB p65 subunit (green fluorescence with blue Hoechst nuclear stain). Scale bar = 10 μm. Figure reproduced with permission from [52]. The bottom panel is a schematic overview of HSP27 cellular release and signaling. As an ER-β binding protein HSP27 acts as a co-repressor of estrogen signaling. In response to estrogenic stimulation or to atherogenic stress induced by elevated serum cholesterol levels, HSP27 is released as EVs (e.g., exosomes). The proportion of extracellular HSP27 that is exosomal vs. non-exosomal is currently unclear. HSP27 may communicate with other cells via pattern recognition receptors like toll-like receptor 4 (TLR4) on macrophages to alter transcription via the NF-κB pathway. Shown here are various examples of HSP27-induced upregulation (IL-10, GM-CSF) and downregulation (IL-1, SR-A1) of gene expression. HSP27 can also competitively bind SR-A to reduce uptake of acLDL. GM-CSF can up-regulate ABC transporters involved in reverse cholesterol transport

from macrophages treated with estrogens in vitro, especially estrogen receptor-β agonists [34, 36, 47]. This secretion to the extracellular space is not due to release from nonviable cells. Moreover, the pharmacological inhibitor of exosomal transport, dimethylamiloride, reduces HSP27 levels in the culture media. In contrast, inhibitors of the Golgi apparatus (brefeldin A), protein synthesis (cycloheximide), transcription (actinomycin D), or ABC transporters (glibenclamide) do not alter the secretion of HSP27 in response to estrogen receptor modulation. In addition, we recently demonstrated that HSP27 localizes to macrophage-derived extracellular vesicles (EVs) using 4 methods, including transmission electron microscopy with immuno-gold labeling (Fig. 3) [48]. When these EVs are treated (presumably coated) with excess HSP27, they activate NF-kB and release IL-10. Hence, these data suggest that HSP27 may be an important cargo protein for EVs that can participate in paracrine signaling processes that attenuate inflammation. Whether HSP27-laden EVs can be utilized as therapeutic agents—either endogenously upregulated or exogenously produced and administered—is currently being studied.

(2) Potential sex-specific mechanisms involved in HSP27-mediated atheroprotection

Linked to the discovery of HSP27 as EV cargo, is the fact that estrogens facilitate the release of EVs. There has been a long-standing link between HSP27 and estrogen—particularly estrogen receptors [49]. Indeed, this association appears to be sex-specific, as female mice or women tend to have higher HSP27 levels than men—particularly premenopausal women [33, 34, 50, 51]. Moreover, while HSP27 overexpression protected mice from atherosclerosis, particularly in female mice [34]—this process was abrogated by ovariectomy but rescued by administration of exogenous estrogens [36]. Taken together, these data indicate that secretion of HSP27 into the circulation is at least facilitated by estrogens—which of course has implications for postmenopausal women, who are faced with a steep increase in symptomatic CAD once they surpass menopause [29]. Currently, we are studying the effects of "surgical menopause" (i.e., removal of ovaries because of an inherited risk of cancer) as a model for understanding the changes in HSP27, cholesterol, and other inflammatory markers when ovarian function ceases [51].

Summary

HSP27 represents an interesting extracellular molecule that plays a key role in atheroprotection, reducing not only cholesterol levels but also indices of inflammation. However, more research is needed to better characterize the cellular mechanisms by which extracellular HSP27 signals at the cell membrane, alters transcriptional regulation pathways, and reduces inflammation because of direct and indirect effects on macrophage biology (Fig. 4).

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and are listed in all studies performed by the O'Brien laboratory and cited in this review manuscript.

All procedures performed in human studies performed by the O'Brien laboratory and cited in this review manuscript were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in any research studies from the O'Brien laboratory that are cited in this review manuscript.

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