Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis

Julio Madrigal-Matute, Oscar López-Franco, Luis Miguel Blanco-Colio, Begoña Muñoz-García, Priscila Ramos-Mozo, Luis Ortega, Jesus Egido, and Jose Luis Martín-Ventura

1Vascular Research Lab, IIS, Fundación Jiménez Díaz, Autónoma University, Av. Reyes Católicos 2, 28040 Madrid, Spain; and 2Department of Pathology, Hospital Clínico San Carlos, Madrid, Spain

Received 22 September 2009; revised 5 February 2010; accepted 8 February 2010; online publish-ahead-of-print 12 February 2010

Time for primary review: 34 days

Aims
Heat shock protein 90 (HSP90) is a ubiquitous chaperone involved in the folding, activation, and assembly of many proteins. HSP90 inhibitors [17-allylamino-17-demethoxygeldamycin (17-AAG)/17-dimethyl aminothylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG)] bind to and inactivate HSP90, increasing the heat shock response and suppressing different signalling pathways. We aim to investigate the effect of HSP90 inhibitors in the modulation of inflammatory responses during atherogenesis.

Methods and results
In human atherosclerotic plaques, HSP90 immunostaining was increased in inflammatory regions and in plaques characterized by lower cap thickness. In cultured human macrophages and vascular smooth muscle cells, treatment with either 17-AAG or 17-DMAG increased HSP70 expression and reduced transcription factor [signal transducers and activators of transcription (STAT) and nuclear factor-κB (NF-κB)] activation and chemokine expression induced by proinflammatory cytokines. In vivo, hyperlipidaemic ApoE−/− mice were randomized to 17-DMAG (2 mg/kg every 2 days, n = 11) or vehicle injected (n = 9) during 10 weeks. Atherosclerotic plaques of mice treated with 17-DMAG displayed increased HSP70 expression and diminished NF-κB and STAT activation, along with decreased lesion, lipid, and macrophage content, compared with vehicle-injected mice. In addition, treatment with 17-DMAG significantly reduced monocyte chemoattractant protein-1 levels, both in plaques and in plasma.

Conclusion
HSP90 expression is associated with features of plaque instability in advanced human lesions. HSP90 inhibitors reduce inflammatory responses in atherosclerosis, suggesting that HSP90 could be a novel therapeutic target in atherosclerosis.

Keywords
Atherosclerosis • Inflammation • Heat shock proteins

1. Introduction
Atherothrombosis is the leading cause of mortality in the Western world. The underlying pathological process is a thickening of the arterial wall. However, it has been increasingly realized that lesions responsible for acute events may not necessarily be critically obstructive. Attenuation of the fibrous cap is a main determinant of plaque stability since the cap confers resistance to rupture due to its composition of collagen and other extracellular matrix (ECM) proteins, synthesized by vascular cells. Inflammatory cells are able to release different proteases, which lead to degradation of ECM proteins and promote plaque instability and rupture. Interestingly, the number of infiltrating cells and the expression of inflammatory mediators are increased in rupture-prone regions of culprit human atherosclerotic plaques.

Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone that is involved in the folding, activation, and assembly of many proteins, including key mediators of signal transduction and transcriptional regulation. It functions as part of a multichaperone complex via association with cochaperones (e.g. HSP70) and several client proteins [e.g. IκB kinase (IKK) and signal transducers and activators of transcription (STAT)]. Blocking the ATP-binding site of HSP90 by inhibitors such as ansamycins ultimately results in both up-regulation of HSP expression (specially HSP70) and degradation of some client proteins.
proteins via an ubiquitin–proteasome-dependent pathway. Among the different client proteins modified by HSP90 inhibitors, STAT and nuclear factor-kB (NF-kB) signalling pathways play a critical role in mediating inflammatory and immune responses. Janus kinase (JAK)/STAT is an important signalling pathway that functions downstream cytokine receptors. NF-kB activation requires phosphorylation of I kB by IKK and IkB degradation by the proteasome, allowing NF-kB to enter the nucleus. Both signalling pathways are activated by proatherogenic cytokines and control the expression of different proinflammatory genes, such as monocyte chemotactic protein-1 (MCP-1) or regulated upon activation, normal T cell expressed and secreted (RANTES). In this respect, it has been demonstrated that both clinical and experimental therapies that decrease inflammation in atherothrombosis may have beneficial effects by preventing the progression of this disease.

Among other experimental therapies, it has been recently observed that modulation of both NF-kB and STAT decreases inflammatory processes in the ApoE<sup>−/−</sup> model of atherosclerosis.

Although the main therapeutic application of HSP90 inhibitors is related to the field of cancer, it has been demonstrated that these drugs are also able to block the activity of certain proinflammatory mediators in different cell types. Moreover, the HSP90 inhibitor 17-allylamino-17-demethoxygeldamycin (17-AAG) is able to attenuate inflammation in several diseases. At present, there are no data regarding the effect of HSP90 inhibitors in cardiovascular diseases. In the present study, we hypothesized that HSP90 inhibitors may attenuate inflammatory processes associated with atherosclerosis. First, we performed an observational study to address the expression of HSP90 in human atherosclerotic plaques and its potential association with features of plaque instability. Furthermore, we analysed the intracellular mechanisms modified by HSP90 inhibitors, both in vitro (vascular cells) and in vivo (ApoE<sup>−/−</sup> experimental model).

2. Methods

2.1 Patients

Sixty consecutive patients undergoing carotid endarterectomy in our institutions were included in the study (69 ± 8 years, 26% women, 82% hypertensive, and 34% diabetics). Atherosclerotic plaques (starry stages V and VI) were fixed with paraformaldehyde and embedded in paraffin. The local committees on human research at Fundacion Jimenez Diaz-Autonoma University approved the study, which was performed in accordance with the principles outlined in the Declaration of Helsinki, and all participants gave written informed consent.

2.2 Cell culture

Human vascular smooth muscle cells (VSMC) were purchased from ATCC (CRL-1999) and maintained in HAM’s F12 (BioWhittaker) supplemented with 10% FBS (BioWhittaker), 2 mM l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). Cells were used between passages 3 and 7. Human THP-1 monocytic cell line was purchased from ATCC (CRL-1593) and cultured with RPMI 1640 (BioWhittaker) supplemented with 10% decomplemented FBS, 2 mM l-glutamine and antibiotics. THP-1 cells were differentiated to macrophages by incubation with 10<sup>−7</sup> M PMA for 48 h. For experiments, cells were pre-incubated with 0% FBS during 24 h.

2.3 Reagents

HSP90 inhibitors [17-AAG or 17-dimethyl aminothylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG), Biomol] were diluted in saline at 10 mmol/L and stored at −20 °C. A cytokine cocktail was composed of 100 U/mL of human interleukin 6 (IL6, PeproTech) and 1000 U/mL of human interferon gamma (INFγ, PeproTech).

2.4 RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen). One microgram of RNA was used to perform the reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems). Real-time polymerase chain reactions (PCRs) were performed on ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer’s protocol using the ΔΔC<sub>t</sub> method as described. Pre-developed primers and probe assays were obtained for human 18S, HSP70, HSP90, MCP-1, and IL6 from Applied Biosystems. Expression levels are given as a ratio to housekeeping gene 18S and data are expressed as fold vs. control values.

2.5 Western blot

Equal amount of total protein was separated on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Subsequently, membranes were blocked and incubated with mouse monoclonal anti-HSP70 (SPA-810, Stressgen), goat polyclonal anti-pSTAT3 (#9131, Cell Signaling), or mouse monoclonal anti-a-tubulin (T-5168, Sigma-Aldrich). Proteins were visualized by ECL Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer’s instructions.

2.6 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) for NF-kB-binding activity was performed with nuclear protein extracts from cells as described. The specificity of the assay was tested with a 100-fold excess of unlabelled NF-kB consensus oligonucleotide added to the <sup>32</sup>P-labelled probe-binding reaction.

2.7 Experimental atherosclerosis

Male ApoE<sup>−/−</sup> mice (12 weeks of age; Jackson Laboratory) were fed on a Western diet during 11 weeks. After 1 week at feeding, mice were randomized into two groups: DMAG treatment (n = 11) and control (n = 9). The treated group was ip injected with 2 mg/kg of 17-DMAG diluted in saline every 2 days during 10 weeks. Controls consisted on ApoE<sup>−/−</sup> mice injected with vehicle (saline). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by Fundación Jimenez Diaz Ethics Review Board.

2.8 Lipid determinations and enzyme-linked immunosorbent assay

Cholesterol and triglycerides were tested in serum samples from mice at fasting by a commercially available kit (TR13421 and TR0100, Sigma). Soluble MCP-1 levels were measured in the supernatants of cells after different experimental conditions and in mice serum with a commercially available enzyme-linked immunosorbent assay (ELISA; DCP00, R&D systems) following the manufacturer’s instructions.

2.9 Immunohistochemistry

Paraffin-embedded human carotid atherosclerotic plaques were cross-sectioned into 4 µm-thick pieces, dewaxed, and rehydrated. Mouse monoclonal anti-HSP90 (SC-13119, Santa Cruz Biotechnology) and anti-HSP70 (SPA-810, Stressgen) antibodies were applied. Negative controls using the corresponding IgG were included for checking non-specific staining. Cap thickness was evaluated at three different points in the histology sections.
by a pathologist blind to the clinical details. Thin caps were considered when the mean of these three measurements was <165 μm, as described previously. Data of computer-assisted morphometric analysis were expressed as percentage of positive staining/mm² as described.

Anaesthetized mice were saline perfused. Liver was paraffin embedded for histology. Aortic samples were frozen in OCT and serial 6 μm sections were stained with Oil red O/haematoxylin. In the root of each animal, maximal lesion size and lipid content were quantified by computerized morphometry and were averaged. In addition, to analyse the amount of lesion along the entire arch, lesion and lipid content were individually quantified as the sum of all measurements covering a total of 3000 μm. Data are presented as the mean of individual animal measurements in each group.

Activated NF-κB was detected by Southwestern histochemistry with digoxigenin-labelled probes, using competition and mutant probe as specificity controls. Rat monoclonal anti-monocytes/macrophages (clone MCA519, Serotec), goat polyclonal anti-MCP-1 (SC-1785, Santa Cruz Biotechnology), goat polyclonal anti-pSTAT3 (SC-7993, Santa Cruz Biotechnology), rabbit polyclonal anti-HSP90 (ab19021, Abcam), rabbit polyclonal anti-HSP70 (ab31010, Abcam), and rabbit polyclonal anti-RANTES (AB2109P, Millipore) antibodies were applied. In all cases, secondary antibodies and ABCComplex/HRP were added and sections were stained with 3,3'-diaminobenzidine and mounted in Pertex. Quantification was performed by a pathologist blind to the experimental groups analysed. Immunohistochemistry data are expressed as percentage of positive staining/μm² as described.

For colocalization studies in human plaques with VSMC and macrophages, double immunohistochemistry/immunofluorescence for HSP90 was carried out in serial sections along with α-actin (monoclonal CLONE 1A4, DAKO) and CD68 (macrophages) in serial tissue sections showed that both plaques with macrophages (MOMA-2, DAKO) and CD68 (macrophages) were stained with 3,3'-diaminobenzidine and mounted in Pertex. Quantification was performed by a pathologist blind to the experimental groups analysed. Immunohistochemistry data are expressed as percentage of positive staining/μm² as described.

2.10 Statistical analysis
Statistics were performed using GraphPAD InStat (GraphPAD Software). In vitro experiments were performed at least three times. Results are expressed as mean ± SEM and were analysed by the Mann–Whitney non-parametric, Wilcoxon paired or Student’s t-test when appropriate (two-tailed, significant differences at P < 0.05).

3. Results
3.1 HSP90 and HSP70 expression in culprit human carotid atherosclerotic plaques
We studied the expression and distribution of HSP90 and HSP70 in advanced human atherosclerotic plaques: the inflammatory region of the shoulder, characterized by a high macrophage accumulation, and the fibrous region, with increased VSMC and collagen content. Quantification of HSP90 and HSP70 immunostaining in 60 human atherosclerotic plaques showed an increased expression of HSP90 and HSP70 in the shoulder region in relation to the fibrous area (9.2 ± 1.1 vs. 6.6 ± 0.7% positive staining/μm², P < 0.05, and 5 ± 0.6 vs. 3.8 ± 0.5% positive staining/μm², P = n.s., respectively, Figure 1A and B). Interestingly, when atherosclerotic plaques were classified according to the cap thickness, we observed that plaques with thin caps (<165 μm, as described by Virmani and coworkers) displayed higher total HSP90 but lower HSP70 levels than those plaques with caps >165 μm (19.3 ± 2.5 vs. 12.7 ± 1.5% positive staining/μm², and 7.2 ± 1.5 vs. 10.7 ± 2% positive staining/μm², respectively, P < 0.05 for both, Figure 1C).

Moreover, immunostaining for HSP90 and α-actin (VSMC) and CD68 (macrophages) in serial tissue sections showed that both macrophages and VSMC present in human plaques are able to express HSP90 (see Supplementary material online, Figure S1). These results suggest that whereas HSP90 could be a marker of instability, HSP70 is associated with features of stability, in advanced human atherosclerotic plaques.

3.2 HSP90 inhibitors modulate HSP70 levels and inflammatory signalling pathways in vascular cells
Treatment of human cells with either 17-AAG or 17-DMAG dose-dependently increased mRNA and protein expression of HSP70 in VSMC (data not shown) and macrophages (Figure 2A and B). In contrast, HSP90 expression was not significantly modified by drug treatment at the doses tested (Figure 2A and B). Furthermore, the effect of

Figure 1 HSP90 and HSP70 immunostaining in human carotid atherosclerotic plaques. (A) Expression of HSP90 in the inflammatory and fibrous regions of the plaques. Atheroma is localized by an asterisk. Magnification ×100 (detail ×200). (B) Quantification of HSP90- and HSP70-immunostained area in different regions of carotid atherosclerotic plaques (n = 60, *P < 0.05). (C) Quantification of total HSP90 and HSP70 immunostaining in plaques with cap thickness <165 or >165 μm.

Downloaded from https://academic.oup.com/cardiovascres/article-abstract/86/2/330/372726 by guest on 08 January 2018
HSP90 inhibitors on client proteins was analysed. Stimulation of VSMC with a cytokine cocktail (IL6 + IFNγ) induced STAT3 phosphorylation at 30 min and this effect was abolished by HSP90 inhibitor pre-treatment (Figure 2C). Similar results were obtained in macrophages (data not shown). In order to determine whether HSP90 inhibitors could interfere with NF-κB signalling pathway in our experimental conditions, IκBα and DNA-binding activity were studied by western blot and EMSA, respectively. As observed in Figure 2D, the diminution of IκBα levels induced by cytokines was prevented by 17-AAG/17-DMAG pre-treatment. Accordingly, pre-incubation of cells with 17-AAG or 17-DMAG decreased cytokine-induced NF-κB activation (Figure 2E).

### 3.3 HSP90 inhibitors decrease cytokine levels in vascular cells

Monocyte recruitment to the vascular lesion is mainly mediated by MCP-1. Since this chemokine is transcriptionally regulated by NF-κB, we studied the effect of HSP90 inhibitors on MCP-1 levels. Incubation of VSMC and macrophages with cytokines for 3 h increased MCP-1 mRNA expression and 17-AAG/17-DMAG dose-dependently prevented this effect (Figure 3A). Similarly, HSP90 inhibitors decrease IL6 expression (% of reduction vs. stimulus: 17-AAG, 39 ± 9%; 17-DMAG, 69 ± 5% in VSMC, and 17-AAG, 36 ± 5%; 17-DMAG, 90 ± 15% in macrophages, P < 0.05 for all). In addition, we observed a decrease in MCP-1 levels in conditioned media of cells pre-treated with HSP90 inhibitors (Figure 3B).

### 3.4 17-DMAG decreases inflammatory cell infiltration in experimental atherosclerosis

To test the relevance of our in vitro results, in vivo studies were performed in hyperlipidaemic ApoE−/− mice. For that purpose, 17-DMAG was used due to its higher anti-inflammatory effects observed in vitro. The dose of the drug was chosen based on previous papers.21 Mice received 0.2 mL ip administration of 2 mg/kg 17-DMAG three times a week on alternate days, since after 48 h, these drugs are cleared from blood.21 Systemic effects of 17-DMAG treatment were evaluated in liver from ApoE−/− mice. Hepatic morphology was normal, without signs of inflammation, necrosis, or hepatocyte alterations (data not shown). Furthermore, 17-DMAG did not modify serum levels of cholesterol and triglycerides (1224 ± 392 vs. 1304 ± 496 mg/dL and 96 ± 15 vs. 81 ± 25 mg/dL, respectively, P > 0.05 vs. control group).
studied the effect of 17-DMAG on HSP70 expression and on the activation of its client proteins STAT3 and NF-κB. We observed that 17-DMAG increased HSP70 expression compared with vehicle-injected mice (6.8 ± 0.8 vs. 4.7 ± 0.5%, P < 0.05, Figure 5). In contrast, HSP90 levels were not modified by 17-DMAG treatment (2.9 ± 0.6 vs. 3.3 ± 0.9%, P > 0.05, data not shown). In addition, untreated mice displayed a higher staining for pSTAT3 than 17-DMAG treated mice (6.1 ± 1.1 vs. 2.8 ± 0.7%, P < 0.05, Figure 5). Moreover, untreated mice showed stronger nuclear staining for NF-κB compared with 17-DMAG-treated mice (Figure 5). Nuclear staining was not observed with unlabelled consensus competition. The percentage of NF-κB-positive cells decreased in aortas from 17-DMAG-treated mice compared with vehicle-injected mice (1.9 ± 0.4 vs. 3.6 ± 0.6%, P < 0.05, Figure 5).

Finally, HSP70 and pSTAT3 expression, as well as NF-κB activation, colocalized with VSMC and macrophages in mice plaques (see Supplementary material online, Figure S2).

### 3.6 17-DMAG decreases chemokine levels in ApoE−/− mice

To finally test the potential anti-inflammatory effect of 17-DMAG, chemokine expression was determined in atherosclerotic plaques of ApoE−/− mice. As observed in Figure 6, MCP-1 immunostaining was decreased in 17-DMAG-treated mice compared with vehicle-injected mice (3 ± 0.7 vs. 5.5 ± 0.3, P < 0.05). Both VSMC and macrophages expressed MCP-1 in mice plaques (see Supplementary material online, Figure S2). Similarly, 17-DMAG treatment decreased RANTES expression (4.4 ± 0.4 vs. 6.1 ± 0.7%, P < 0.05, data not shown). In agreement, 17-DMAG diminished MCP-1 serum levels (35 ± 4 vs. 62 ± 5 ng/mL, P < 0.05, Figure 6C).

### 4. Discussion

It is well established that the breakdown of atherosclerotic plaques occurs more frequently in thin cap atherosclerotic plaques and where there is a great amount of inflammatory cells. Studies on coronary arteries of patients suffering myocardial infarction demonstrated that the rupture of atheroma usually takes place in the shoulder region,22 an area characterized by a high inflammatory content, NF-κB activation, and MCP-1 expression.4,23 In this study, we observed strong HSP90 immunostaining in advanced human atherosclerotic plaques, preferentially in the shoulder region. Moreover, HSP90 levels were higher in those plaques where the fibrous cap was thinner, suggesting that HSP90 plays an important role in the instability of advanced human atherosclerotic plaques. It has been very recently reported that HSP90 is overexpressed both in plaque and serum from patients with atherosclerosis, potentially contributing to plaque instability by inducing an immune response.24 Our data extend these recent findings and suggest HSP90 as a possible therapeutic target in atherosclerosis.

Although the inhibitors of HSP90 are of therapeutic interest primarily in cancer,25 evidence is emerging for the potential beneficial role of HSP90 inhibitors in the treatment of other inflammatory diseases, such as rheumatoid arthritis,26 endotoxin-induced uveitis and murine sepsis.14,15 Whereas initial inflammatory response could help to prevent lipid accumulation inside the atherosclerotic plaques, a chronic inflammatory response is associated with plaque progression. For that reason, strategies that prevent this pathological

---

**Figure 4** Effect of 17-DMAG on lesion size and macrophage content in ApoE−/− mice. (A) Representative images of Oil-red/haematoxylin staining in aortic lesions of control and 17-DMAG treated mice (magnification ×25). (B) Quantification of the lesion and lipid content in the entire arch of ApoE−/− mice (n = 20, P < 0.05 vs. control). (C) Representative images of macrophages (MØ) in control and 17-DMAG-treated mice (magnification ×100). (D) Quantification of MOMA levels in the ApoE−/− experimental model (n = 20, P < 0.05 vs. control).

17-DMAG reduced the maximal aortic lesion size in the root (357 ± 24 vs. 432 ± 33 × 105 μm², P = 0.08) and the lipid content (116 ± 17 vs. 158 ± 31 × 105 μm², P = 0.07) compared with vehicle-injected mice, although it did not reach statistical significance. However, when we analysed the total amount of lesion along the entire arch (3000 μm) and their lipid content, we observed that 17-DMAG treated animals showed a significant reduction in both parameters (2376 ± 667 vs. 2973 ± 735 × 103 μm² and 605 ± 187 vs. 1028 ± 315 × 103 μm², respectively, P < 0.05 for both, Figure 4A and B). Interestingly, treatment with 17-DMAG significantly reduced the macrophage content of lesions (5.4 ± 0.6 vs. 8.6 ± 1%, P < 0.05, Figure 4C and D).

### 3.5 17-DMAG decreases inflammatory signalling pathways in lesions of ApoE−/− mice

To understand the potential mechanisms involved in the diminution of macrophage infiltration observed in 17-DMAG treated mice, we...
response would be of potential benefit. In this respect, the beneficial effect of HSP90 inhibitors in inflammatory diseases could be due to their double activity; degradation of client proteins involved in different inflammatory signalling pathways and up-regulation of anti-inflammatory HSP expression (especially HSP70). We have observed that HSP90 inhibitors could diminish the expression and activation of inflammatory mediators both in vitro and in vivo. However, further studies are needed to clarify whether the reduced inflammatory response observed with HSP90 inhibitors is due to HSP70 up-regulation, inhibition of HSP90 activity, or both.

Among the different client proteins of HSP90 involved in inflammatory diseases, STAT and NF-κB are the most representative examples. JAK/STAT is an important signalling pathway that functions downstream cytokine receptors and regulates the initiation/progression of atherosclerosis and the remodelling in response to injury.7,27 JAK/STAT activation has been previously found in cultured vascular cells under inflammatory conditions and in atherosclerotic lesions.10,28,29 Fatty streak formation was reduced in STAT3 conditional knockout mice when compared with their wild-type littermates.28 In contrast, STAT3 up-regulation by antisense oligodeoxynucleotides therapy targeting its negative regulator (suppressors of cytokine signalling 3) increases inflammatory responses in the ApoE<sup>−/−</sup> model of atherosclerosis.10 These results are in agreement with the present paper since we have observed that prevention of STAT3 activation by HSP90 inhibitors is able to decrease inflammatory responses in vascular cells and atherosclerotic plaques.

NF-κB signalling plays a critical role in mediating inflammatory and immune responses. Activation of NF-κB requires phosphorylation of IκB by IKK and degradation by the proteasome, allowing NF-κB to enter the nucleus to transcriptionally regulate the expression of different proinflammatory genes (e.g. MCP-1). Since IKK exists in complexes with HSP90, disruption of these complexes by HSP90 inhibitors blocks IKK function and, consequently, NF-κB activation. We have observed that NF-κB activation induced by proinflammatory cytokines is modulated by HSP90 inhibitors in vascular cells. In addition, it has been previously observed that NF-κB inhibition decreased inflammatory processes in the ApoE<sup>−/−</sup> model of atherosclerosis.9,30 Other approaches, such as NF-κB decoy oligodeoxynucleotides have also been successfully applied in animal models of vascular disease.31 In this respect, we have observed that HSP90 inhibitors decreased NF-κB activation, MCP-1 levels, and inflammatory cell infiltration in atherosclerotic plaques. These results are in agreement with previous papers in which HSP90 inhibitors attenuate NF-κB in other inflammatory diseases.14,15

**Figure 5** Effect of 17-DMAG on HSP70 and client proteins in vascular lesions of ApoE<sup>−/−</sup> mice. (A) Representative images of HSP70, pSTAT3 levels, and NF-κB activation in control and 17-DMAG-injected mice (magnification ×100). (B) Quantification of HSP70 (white), pSTAT3 (grey), and NF-κB activation (black) positive staining in aortic samples of ApoE<sup>−/−</sup> mice (n = 20, *P < 0.05 vs. control). Negative controls were performed with IgG isotype (for HSP70 and pSTAT3) or unlabelled consensus oligonucleotide (for NF-κB).

**Figure 6** Effect of 17-DMAG on chemokine levels in ApoE<sup>−/−</sup> mice. (A) Representative images of MCP-1 in control and 17-DMAG-injected mice (Magnification ×100). (B) Quantification of MCP-1 immunostaining in the ApoE<sup>−/−</sup> experimental model (n = 20, *P < 0.05 vs. control). (C) Quantification of MCP-1 serum levels in ApoE<sup>−/−</sup> mice (n = 20, *P < 0.05 vs. control).
Heat shock proteins are involved in protecting the tissue cells from a variety of insults. Although the mechanism of this protection has largely been thought to be due to their chaperone functions, it is known that some HSP, such as HSP70, displays anti-inflammatory properties in different diseases. In this respect, HSP70 inhibits leukocyte adhesion and recruitment. Moreover, mice overexpressing HSP70 showed decreased number of activated macrophages and inhibition of NF-κB in a model of brain inflammation. In a model of colitis, Tanaka et al. found that up-regulation of HSP70 in transgenic mice inhibited the expression of several cytokines, including IL-6. In this respect, it has been observed the beneficial effect of HSP70 induction by 17-AAG treatment in experimental autoimmune encephalomyelitis by suppressing glial inflammatory responses. Our results suggest that HSP70 induction by 17-AAG/DMA could be an additional mechanism to decrease inflammation in vascular cells and atherosclerotic plaques.

The clinical significance of our results is related to the emerging evidence pointing out a role for HSP as potential therapeutic targets in different diseases. In this respect, induction of HSP70 and degradation of client proteins by HSP90 inhibitors are able to decrease inflammation in different experimental models. However, since HSP90 inhibition affects several pathways at the same time, other pathological processes such as oxidative stress, neoangiogenesis, or apoptosis could also be affected by these drugs and would require further studies to clarify the protective role of HSP90 inhibitors in human diseases. In this respect, the dosing and toxicity of 17-AAG gained from Phase I clinical trials in cancer patients should facilitate the evaluation of HSP90 inhibitors in non-neoplastic disorders, such as cardiovascular diseases. For this purpose, 17-DMAG is a more potent and water soluble derivative than 17-AAG, which can be administered orally, thus possibly making it a more feasible long-term therapeutic agent. Our results demonstrate that HSP90 inhibitors reduce inflammatory responses in atherosclerosis, suggesting that HSP90 could be a novel therapeutic target in atherosclerosis.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
We want to thank Yolanda Vicente Lago for technical assistance, Carmen Gomez-Guerrero for helpful comments and Natalia Escribano for characterization of human plaques.

Conflict of interest: none declared.

Funding
This work was supported by the Spanish Ministerio de Ciencia y Tecnología (SAF2007/63648), Fundacion Ramon Areces, CAM (S2006/GEN-0247), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035), and European Network (HEALTH F2-2008-200647).

References
2. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coro-

5. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsph70-based chaperone machinery. Exp Biol Med (Maywood) 2003;228:
6. Zhang H, Burrows F. Targeting multiple signal transduction pathways through inhibi-

8. Blanco-Colio LM, Tufiñ, J, Martín-Ventura JL, Egido J. Antinflammatory and immuno-

11. Malhotra V, Stanley TP, Pittet JF, Welsh WJ, Wong HR. Geldanamycin inhibits NF-κB activation and interleukin-8 gene expression in cultured human respira-

12. Wax S, Piecyk M, Plantim B, Anderson P. Geldanamycin inhibits the production of inflammatory cytokines in activated macrophages by reducing the stability and trans-


16. Moreno JA, Muñoz-García B, Martín-Ventura JL, Madrigal-Matute J, Orbe J, Páramo JA et al. The CD163-expressing macrophages recognize and internalize TWEAK. Poten-

18. Wasserman BA, Wityk RJ, Trout HH 3rd, Virmani R. Response to Letter by Karapa-


21. Sauvalle EA, Tomaszewski JE, Iy P. Clinical development of 17-allylamino-17-

28. Gharaei NM, Alva JA, Mouilleseaux KP, Lai C, Yeung MV et al. Role of the JAK/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholi-


