Synergistic effect of angiotensin-converting enzyme (ACE) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibition on inflammatory markers in atherosclerotic rabbits

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ABSTRACT

In the present study, we compared the effect of atorvastatin (1 mg·kg⁻¹·day⁻¹) and quinapril (0.5 mg·kg⁻¹·day⁻¹) alone or in combination on inflammatory markers, endothelial function, intimal thickening and fibrinolytic balance in rabbits fed with either a control diet or a diet containing 1 % (v/v) cholesterol for 12 weeks. Atorvastatin alone or in combination partially prevented the increase in cholesterol plasma levels observed in rabbits fed with the cholesterol-rich diet, but did not modify blood pressure levels. Quinapril administration did not alter any of these parameters in any group. Hypercholesterolaemia increased plasma levels of interleukin-1β, interleukin-6, interferon-γ and C-reactive protein, reduced acetylcholine-induced relaxation and produced intimal thickening. Likewise, atherosclerotic rabbits had reduced plasma tissue-type plasminogen activator activity and D-dimer levels and an increase in plasminogen-activator inhibitor-1 activity. Both drugs enhanced acetylcholine-induced relaxation, reduced intimal thickening and improved fibrinolytic balance in atherosclerotic rabbits in a similar manner. Their combination did not induce additive effects on these parameters. However, only the combination of both drugs was able to prevent the increase in inflammatory markers induced by hypercholesterolaemia. In summary, these data suggest that quinapril and atorvastatin had comparable beneficial effects on the alterations of vascular function and structure as well as fibrinolytic balance in atherosclerotic rabbits. In addition, the combination of atorvastatin and quinapril exerts a synergistic effect on inflammatory markers, which individual treatment, at the doses used, was not able to modify.

Key words: atherosclerosis, angiotensin-converting-enzyme inhibitor, endothelial function, fibrinolytic balance, inflammation, statin.

Abbreviations: ACE, angiotensin-converting enzyme; CRP, C-reactive protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; oxLDL, oxidized low-density lipoprotein; NO, nitric oxide; PAI-1, plasminogen-activator inhibitor-1; t-PA, tissue-type plasminogen activator.

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INTRODUCTION

Hypercholesterolaemia produces numerous functional and structural alterations in the vascular wall and leads to the development of atherosclerosis [1–5], which is the major cause of morbidity and mortality in western populations. Endothelial dysfunction is a common consequence of hypercholesterolaemia and has usually been characterized by an impairment of endothelium-dependent relaxations [2–5]. However, endothelial dysfunction not only involves changes in the endothelial regulation of vascular tone, but also changes in vascular smooth muscle cell growth, leucocyte adhesion, platelet function and fibrinolytic activity [4–6]. Moreover, these alterations of endothelial function seem to contribute in a crucial manner to the development and progression of atherosclerosis and its complications [6,7].

Atherosclerosis not only represents a relentless accumulation of lipids within the artery wall, but it also involves a complex series of events that affect numerous cells, including macrophages and T lymphocytes. In fact, it has been proposed that atherosclerosis is a chronic inflammatory disease [8,9]. Local inflammatory cells, as well as smooth muscle and endothelial cells, can release cytokines in response to different stimuli, including angiotensin II, reactive oxygen species and oxidized low-density lipoprotein (oxLDL) [9–11]. Circulating cytokines not only participate in the development and complications of atherosclerosis [9,11], but are also considered to be independent markers for cardiovascular events [12,13]. Furthermore, C-reactive protein (CRP), a sensitive marker of ongoing inflammation, which is stimulated in atherosclerosis, shows a prognostic value for future risk of cardiovascular events in apparently healthy individuals and coronary artery disease patients [14,15].

Antihypertensive and hypolipidaemic drugs, such as angiotensin-converting enzyme (ACE) inhibitors and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, exert beneficial effects on both the development and complications of atherosclerosis [3,16–19]. However, there is not enough data comparing, in an integrated manner, the effects of statins and ACE inhibitors on the vascular and functional alterations associated with early phases of atherosclerosis. Moreover, it is not well known whether or not these drugs can exert additive or synergistic effects on atherosclerosis, as has been reported previously regarding blood pressure control [20]. Therefore we have tested the hypothesis that ACE inhibitors and statins could exert additive or synergistic effects on vascular alterations associated with atherosclerosis. To this end, we have compared the effects of treatment with quinapril and atorvastatin alone or in combination on endothelium-dependent relaxation, intimal thickening, fibrinolytic balance and systemic inflammatory markers in atherosclerotic rabbits.

METHODS

Sixty-four male New Zealand rabbits (2320 ± 36 g; Granja Cunicular San Bernardo, Navarra, Spain) were maintained under controlled light and temperature conditions and fed either a normal rabbit chow or a diet containing 1 % (v/v) cholesterole (UAR, Panlab, Spain) for 12 weeks with free access to tap water. Rabbis from each diet group were treated with atorvastatin (1 mg·kg−1·day−1) and quinapril (0.5 mg·kg−1·day−1) alone or in combination and were given in the food for the same period at the same doses. In order to observe the possible synergistic or additive effects of both drugs, the doses of atorvastatin and quinapril were chosen in preliminary experiments on the basis of the criteria of either a partial inhibition of ACE and no changes in blood pressure or a slight reduction in cholesterol levels. At the end of the experiment, systolic arterial pressure was measured directly in the medial ear artery in awake rabbits through a catheter connected to a pressure transducer (model P23XL; Spectromed, Oxnard, CA, U.S.A.). All experimental procedures were approved by the Animal Care and Use Committee of Universidad Complutense, according to the guidelines for ethical care of experimental animals of the European Community.

At the end of the experiment, blood samples were collected through a catheter inserted in the ear artery of awake rabbits in order to measure cholesterol, fibrinolytic balance and inflammatory markers. Plasma cholesterol levels were measured using colorimetric reactions employing commercial kits (Roche Diagnostics, Zurich, Switzerland). Plasma tissue-type plasminogen activator (t-PA) and plasminogen-activator inhibitor-1 (PAI-1) activities were evaluated using an immunooactivity assay using commercial kits (Biopool International, Ventura, CA, U.S.A.). D-dimers were measured by a semi-quantitative method with a highly specific monoclonal antibody to D-dimers (Dade International Inc., Miami, FL, U.S.A.). Plasma interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-α and interferon-γ (IFN-γ) were measured with quantitative sandwich enzyme immunoassay employing commercial kits for rats (R&D Systems, Minneapolis, MN, U.S.A.). CRP plasma levels were measured with a highly sensitive latex-based turbidimetric immunoassay on a Hitachi analyser for human samples (Sigma Chemical Co., St. Louis, MO, U.S.A.). Antibodies to rabbit cytokines are rare and are not commercially available. Therefore we have explored whether or not it is possible to use commercial kits for rats or human samples for measuring rabbit plasma levels. Our data indicate that they are specific enough to detect changes in rabbit samples. Moreover, cytokine similarities between rat and rabbits were explored by BLAST (National Center for Biotechnology Information), being 77 % for IL-1β, 56 % for IL-6 and 78 % between rabbit and human for CRP.
Finally, in order to eliminate the possibility that changes in inflammatory markers were consequence of a poor physical condition due to high cholesterol levels, liver function was evaluated by measuring plasma activities of both glutamate pyruvate transaminase and glutamate oxaloacetate transaminase. No differences were detected between both groups in glutamate pyruvate transaminase and glutamate oxaloacetate transaminase plasma activities (44 ± 6.3 compared with 50.2 ± 10 international units/l; and 20.3 ± 6 compared with 27 ± 5.7 international units/l respectively).

Descending thoracic aorta was excised from pentobarbital-anaesthetized animals and processed as described previously [3,5]. The vasorelaxing response to either an endothelium-dependent (acetylcholine; 10\(^{-5}\)-10\(^{-6}\) mol/l) or -independent (sodium nitroprusside; 10\(^{-10}\)-10\(^{-6}\) mol/l) vasodilator was studied in aortic rings from normolipidaemic and dyslipidaemic rabbits precontracted with a submaximal dose of phenylephrine (10\(^{-6}\) mol/l). In order to evaluate the degree of ACE inhibition, the response to angiotensin I (10\(^{-5}\) mol/l) was also studied.

Aortic segments were fixed in 15% formaldehyde/PBS, processed and cut in sections (3–4 µm). Lesion area was determined by tracing in digitalized segmented-coloured sections stained with Masson trichrome using a QWIN Leica image analyser (Leica Imaging Systems Ltd, Cambridge, U.K.) as described previously [3,5,21]. In order to determine the luminal area or the vessel area, the cross-sectional area enclosed by the internal or external elastic lamina respectively, was corrected to a circle by applying the form factor (π/4 a\(^2\)) to the measurement of the lamina, where \(l\) is the length of the lamina. This method was used to avoid miscalculation of vessel and luminal areas, since aortic segments might be deformed during preparation.

Products for morphological analysis were purchased from Merck (Darmstadt, Germany). Drugs for vascular reactivity were obtained from Sigma Chemical Co. Concentrations are expressed as final molar concentration in the organ chamber.

### Calculations and statistical analysis
Relaxation responses are expressed as a percentage of the reduction of tension in the phenylephrine-precontracted state. Contractile response was expressed as a percentage of the reference constrictor response to 120 mmol/l KCl. Results are expressed as means ± S.E.M. from eight rabbits, unless otherwise specified. Dose–response curves were compared by multivariate ANOVA for repeated measures using the SPSS program (SPSS Inc., Chicago, IL, U.S.A.). All other data were analysed using a one-way ANOVA, followed by a Bonferroni test if differences were noted. The null hypothesis was rejected when the \(P\) value was less than 0.05.

### RESULTS
As expected, rabbits fed a cholesterol-rich diet presented higher cholesterol plasma levels than animals fed a control diet (Table 1). Administration of atorvastatin, but not quinapril \((P < 0.05)\), reduced this increase without modifying plasma cholesterol concentration in control animals (Table 1). A combination of both drugs did not alter cholesterol plasma levels further in any group. Neither the diet nor the drugs were able to modify systolic arterial pressure among any group (Table 1). No differences in body weight were observed between control and atherosclerotic rabbits (3321 ± 63 g compared with 3209 ± 142 g). None of the treatments were able to modify this parameter (results not shown).

The vasorelaxing response to acetylcholine was reduced in rings from rabbits with atherosclerosis compared with aortic rings from control animals (Figure 1). Both atorvastatin and quinapril administration partially prevented this reduction in atherosclerotic animals. Combined treatment with atorvastatin and quinapril did not have any additional effects to those already observed with the drugs alone (Figure 1, right-hand panel). Atorvastatin alone or in combination with quinapril increased the response to acetylcholine in aortic rings from control animals (Figure 1, left-hand panel). By

<table>
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<th>Control diet</th>
<th>Atherosclerotic diet</th>
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<tr>
<td></td>
<td>(V)</td>
<td>(A)</td>
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<tr>
<td>Cholesterol (mM)</td>
<td>0.92 ± 0.05</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>120 ± 3.7</td>
<td>124 ± 8.4</td>
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\(V\), animals in the absence of atorvastatin or quinapril; \(A\), atorvastatin (1 mg · kg\(^{-1}\) · day\(^{-1}\)); \(Q\), quinapril (0.5 mg · kg\(^{-1}\) · day\(^{-1}\)); \(A + Q\), combination of atorvastatin and quinapril. Values are means ± S.E.M. of eight rabbits. *\(P < 0.05\) compared with untreated control rabbits; #\(P < 0.05\) compared with untreated atherosclerotic rabbits.
Figure 1 Vasorelaxation induced by acetylcholine \((10^{-9}–10^{-5}\ \text{mol/l})\) in aortic rings precontracted with a submaximal dose of phenylephrine \((10^{-6}\ \text{mol/l})\) from rabbits fed either a diet containing 1% cholesterol (Atherosclerotic) or a control diet (Control) and treated in the absence or presence of atorvastatin, quinapril or a combination of both.

V, Animals in the absence of atorvastatin or quinapril; A, atorvastatin \((1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1})\); Q, quinapril \((0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1})\); A+Q, combination of atorvastatin and quinapril. Values are means ± S.E.M. of six to eight rabbits. *P < 0.05 compared with untreated rabbits.

Table 2 Lumen, area of vessel, media and lesion areas in rabbits fed either a diet containing 1% cholesterol (Atherosclerotic) or a control diet (Control) in the absence or presence of atorvastatin, quinapril or both.

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<th>Control diet</th>
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<tr>
<td></td>
<td>V</td>
<td>A</td>
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<tr>
<td>Lumen area (mm²)</td>
<td>8.9 ± 0.6</td>
<td>8.8 ± 0.5</td>
</tr>
<tr>
<td>Area of vessel (mm²)</td>
<td>10.8 ± 0.6</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>Media area (mm²)</td>
<td>1.93 ± 0.11</td>
<td>1.96 ± 0.13</td>
</tr>
<tr>
<td>Lesion area (mm²)</td>
<td>—</td>
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<tr>
<td></td>
<td>6.5 ± 0.5*</td>
<td>8.4 ± 0.3#</td>
</tr>
<tr>
<td></td>
<td>9.5 ± 0.9</td>
<td>10.1 ± 1.03</td>
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<tr>
<td></td>
<td>1.87 ± 0.13</td>
<td>1.80 ± 0.10</td>
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<tr>
<td></td>
<td>—</td>
<td>0.93 ± 0.14*</td>
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<td>—</td>
<td>0.27 ± 0.06#</td>
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Both atorvastatin and quinapril treatment reduced the size of the lesion (Table 2 and Figure 2) and, consequently, the degree of stenosis \((4.1 ± 0.5\% \text{ and } 7.4 ± 1.6\% \text{ respectively})\) that produced an increase in lumen vessel area (Table 2 and Figure 2). A correlation was found between maximal acetylcholine-induced relaxation and the extent of the remaining lesion area in both atorvastatin- \((r = -0.723; P < 0.05)\) and quinapril-treated animals \((r = -0.763; P < 0.05)\). A combination of both drugs did not have additional effects on these parameters (Table 2). No differences were observed among any of the groups with respect to media and vessel cross-sectional areas (Table 2).

As shown in Figure 3, t-PA plasma activity and D-dimer plasma levels were reduced in atherosclerotic rabbits compared with control animals. By contrast,
PAI-1 activity was higher in atherosclerotic rabbits than in control animals. Both atorvastatin and quinapril treatment was able to prevent these changes in animals with atherosclerosis without provoking any modifications in control rabbits. A correlation was found between maximal acetylcholine-induced relaxation and t-PA and PAI-1 activities in atorvastatin- \((r = 0.692\) and \(r = -0.676\) respectively; both \(P<0.05\)) and quinapril-treated animals \((r = 0.651\) and \(r = -0.689\) respectively; both \(P<0.05\)). A combination of atorvastatin and quinapril did not have any additional effects on any of these parameters.

Plasma levels of IL-6, IL-1\(\beta\), IFN-\(\gamma\) and CRP were higher in atherosclerotic rabbits compared with control animals (Figure 4). The administration of either quinapril or atorvastatin alone was not able to modify these parameters in any of the groups. By contrast, the combination of both drugs prevented this increase in atherosclerotic rabbits, but did not affect them in normal rabbits (Figure 4). Neither diet nor drugs were able to modify plasma levels of tumour necrosis factor-\(\alpha\), which were too low to be quantified.

**DISCUSSION**

The present study shows that atorvastatin and quinapril, at the doses used, exerted a synergistic effect on inflammatory markers in atherosclerotic rabbits. By contrast, no synergism was observed in vascular function, atherosclerotic lesion or fibrinolytic balance induced by a high-cholesterol diet, all of which were prevented in a similar manner by the administration of either drug alone. These beneficial effects occurred with a slight reduction in cholesterol plasma levels in the case of atorvastatin, and in absence of blood pressure modifications in quinapril-treated rabbits.

As reported previously [3,17,22,23], treatment with statins prevented the reduction in endothelium-dependent relaxation observed in atherosclerotic rabbits. This beneficial effect on endothelial function could be initially explained by atorvastatin’s cholesterol-lowering action, which could affect not only plasma levels, but also vascular wall lipid content. We have reported previously [3] that atorvastatin decreased both extracellular and intracellular lipid deposits in intima and media layers. However, other mechanisms could be proposed to explain the effect of atorvastatin on endothelial function, since it enhanced acetylcholine-induced relaxation in control animals without modifying cholesterol levels, a fact reported previously [3,24]. An increase in nitric oxide (NO) availability could be hypothesized, because statins are able to up-regulate endothelial NO synthase expression and also prevent its down-regulation induced by oxLDL [25,26]. Furthermore, since an antioxidant action of statins has been suggested [27], a diminution of NO degradation by superoxide anions may also underlie the enhancement in NO availability. Moreover, taking into consideration that the maximal acetylcholine-induced relaxation inversely correlated with the extent of the lesion in atorvastatin-treated rabbits, the reduction
in intimal thickening lesion may also have accounted for the observed improvement in acetylcholine-induced relaxation. Inhibition of several mechanisms involved in the formation of atherosclerotic lesion, including monocyte infiltration, smooth muscle cell migration and proliferation, activation of oxLDL receptors and cell foam formation, could account for the reduction in intimal thickening induced by atorvastatin [16,17,27]. Similar to that observed with atorvastatin, quinapril ameliorated endothelial dysfunction and reduced intimal thickening in atherosclerotic rabbits. These effects were observed in the absence of changes in both arterial pressure and plasma cholesterol levels. These results are in agreement with previous studies [18,19,21,23], which showed that ACE inhibitors and AT1 receptor antagonists exerted beneficial effects on atherosclerosis development and supports further the proatherogenic role of angiotensin II. Since ACE inhibitors and statins share vascular effects, including NO enhancement, antioxidant activity, down-regulation of oxLDL receptors and inhibition of foam cell formation [27], all of these effects could account for the actions of quinapril on vascular function and structure.

Administration of either quinapril or atorvastatin was able to similarly improve the fibrinolytic balance in atherosclerotic rabbits. Comparable results have been reported previously [18,28,29]. This effect was due to the prevention of both the decrease in t-PA and the increase in PAI-1. A dual mechanism may be involved in the beneficial effect induced by quinapril, including an increase in t-PA levels via kinins enhancement [30] and a reduction in PAI-1 levels, which can be activated by angiotensin II [31,32]. Although the observed effect of atorvastatin on fibrinolytic balance could be a consequence of a cholesterol reduction, cellular mechanisms beyond...
its hypolipidaemic effect may also be involved. Indeed, statins are able to reduce PAI-1 levels and increase t-PA levels in cultured endothelial cells [33,34]. In addition, some of the mechanisms already mentioned for the amelioration of endothelial function, such as NO enhancement, may also have contributed to the normalization of fibrinolytic balance, since NO participates in the regulation of t-PA [35], and t-PA and PAI-1 activities correlated with maximal acetylcholine-induced relaxation in these animals.

The present data show no additional effects of a combination of atorvastatin and quinapril on endothelial function and vascular structure or on fibrinolytic balance. Similarly, in a previous study in patients with coronary atherosclerosis [36], the combination of enalapril and simvastatin did not improve further the increase in coronary artery diameter induced by the lone administration of the statin. The explanation for the lack of additive effects of atorvastatin and quinapril seems to be due to possible maximal effects on endothelial function, reduction of atherosclerotic lesion and fibrinolytic balance reached with the actual doses used. This supports further the concept that both types of drugs are very effective in the correction of the above-mentioned atherosclerotic alterations, even when the doses used do not reach maximal effects on their primary actions (plasma cholesterol level reduction or ACE inhibition).

In contrast with the above-mentioned results, the increase in plasma levels of cytokines (IL-6, IL-1β and IFN-γ) and CRP was prevented by the combined administration of atorvastatin and quinapril, but not by the individual treatments, suggesting a synergistic effect on inflammatory markers. This synergism does not appear to be a consequence of a further decrease in cholesterol or blood pressure levels, because no differences in these parameters were observed among the animals treated with these drugs alone or in combination. Similarly, Lauten et al. [37] have reported recently in patients with coronary heart disease treated with atorvastatin that the concomitant treatment with either quinapril or an AT1 receptor antagonist further reduced plasma IL-6 levels. The present results could be interpreted as a contradiction with previous studies showing anti-inflammatory actions of both statins and ACE inhibitors [15,38–41]; however, it could be proposed that the lack of effect of the individual treatments seems to be due to the actual doses used in the study. It seems that higher doses of both drugs are necessary to reduce inflammatory markers, indicating that quinapril and atorvastatin are less effective in reducing inflammatory markers than they are on endothelial function, atherosclerotic lesion and fibrinolysis. Consequently, dose-related differential effects of atorvastatin and quinapril on vascular alterations associated with atherosclerosis may be proposed. Supporting this idea are results in patients with cardiovascular disease showing that an intensive lipid-lowering regime is accompanied by a greater CRP decrease than a less intensive one [42,43].

Finally, it should be mentioned that, although the kits used to measure inflammatory markers had been designed for species other than rabbits and this could represent a limiting factor for the interpretation of the results, we consider that the specificity of these kits was good enough (see the Methods section) for the detection of differences in inflammatory markers in the present experimental model.

In summary, the results of the present study show that atorvastatin and quinapril exerted a synergistic effect on vascular inflammation at a dose for which the individual administration of each drug produced no effect. In addition, both drugs induced a protective role in the vascular wall by preventing, in a comparable manner, alterations in endothelial function, intimal thickening and fibrinolytic balance in atherosclerotic animals in the presence of high cholesterol levels. These beneficial effects seem to be only partially dependent on cholesterol lowering, totally independent of blood pressure levels, and could be mediated by common mechanisms shared by both drugs.

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