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HYDROCHLOROTHIAZIDE ABOLISHES THE ANTI-ATHEROSCLEROTIC EFFECT OF QUINAPRIL

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SUMMARY

1. Antihypertensive treatment has been demonstrated to result in persistent reductions in morbidity and mortality due to stroke. However, the coronary risk attributable to hypertension has been only partially reversed. We hypothesized that diuretics could have unfavourable effects on atherosclerosis.

2. New Zealand rabbits were fed a 0.5% cholesterol-enriched diet for 12 weeks, followed by a 0.1% cholesterol diet for another 12 weeks. During the last 12 week period, 40 animals were randomly assigned to one of four groups: (i) group I was the control group; (ii) group II received hydrochlorothiazide (10 mg/day); (iii) group III received quinapril (30 mg/day); and (iv) group IV was treated with hydrochlorothiazide (10 mg/day) plus quinapril (30 mg/day).

3. The treatments did not affect either the lipid profile or serum electrolytes and oxidative stress. However, endothelium-dependent vasorelaxation in isolated aortic rings was significantly improved with quinapril (group III) treatment ($P < 0.001$ *vs* other groups). In addition, therapy with quinapril promoted a significant reduction in atherosclerosis (intima area, intima/media ratio and perimeter of vessel with plaque; $P < 0.05$ *vs* other groups), as well as in cholesterol content of the aorta ($P < 0.05$ *vs* groups II and IV).

4. In conclusion, hydrochlorothiazide did not modify atherosclerosis and, when added to quinapril treatment, impaired the anti-atherosclerotic effect seen with quinapril alone.

Key words: antihypertensive agents, atherosclerosis, endothelium, hypercholesterolaemia, rabbits.

INTRODUCTION

Previous studies have reported a uniform and persistent reduction in morbidity and mortality due to stroke that averages 40% with antihypertensive therapy, a reduction that fits the risk attributable to hypertension.[1,2] In contrast, the impact of blood pressure control on coronary artery disease reported in the past decade, mainly with diuretic therapy, was below its epidemiological importance.[3] More recently, two major clinical trials with angiotensin-converting enzyme (ACE) inhibitors have shown clear benefits of treatment to high-risk, normotensive individuals, as well as to those with hypertension, not only regarding stroke reduction, but also coronary events.[4,5]

Recent data suggest that the renin–angiotensin system may play an important role in the pathogenesis of atherosclerosis.[6–8] In fact, in animal models, treatment with ACE inhibitors induced a reduction in experimentally induced atherosclerosis and in some known mechanisms involved.[9,10]

The crucial role of the endothelium in cardiovascular homeostasis and protection against atherosclerosis has been firmly established in the past two decades,[11,12] but the impact of antihypertensives on these strategic cells has been poorly reported, especially regarding diuretics, recommended as first-line agents for blood pressure control.[13]

Therefore, the present study explores the hypothesis that diuretic therapy may have adverse effects on atherosclerosis.

METHODS

Experimental protocol

Three-month-old male New Zealand rabbits, weighing 2.8 ± 0.1 kg, were housed individually with free access to drinking water. All animals received proper care in compliance with the Ethics Committee for Research of the São Paulo Hospital/Federal University of São Paulo. Rabbits were fed a 0.5% cholesterol-enriched diet for 12 weeks, followed by a 0.1% cholesterol diet for another 12 weeks to achieve moderate hypercholesterolaemia during the period of active drug treatment. Animals were randomly assigned to one of four groups with matching of lipid profiles and bodyweight at the 12th week: (i) group I ($n = 10$) was the control group; (ii) group II ($n = 10$) was treated with hydrochlorothiazide (Clorana®; Sanofi-Synthelabo, Rio de Janeiro, Brazil; 10 mg/day); (iii) group III ($n = 10$) received quinapril (Accupril®; Pfizer, Cali, Colombia; 30 mg/day); and (iv) group IV ($n = 10$) was treated with hydrochlorothiazide (10 mg/day) plus quinapril (30 mg/day). All drugs were given orally, incorporated in 20 g of standard chow and placed on top of the atherogenic diet (approximately 100 g/day).

Measurements of lipids, electrolytes, tissue cholesterol and oxidative stress

Fasting blood for lipid profiles was obtained at 12 and 24 weeks. At this time, animals were anaesthetized with an intramuscular injection of 5 mg/kg xylazine (Rompun®; Bayer AG, Sao Paulo, Brazil;) and 35 mg/kg ketamine (Ketalar®; Parke-Davis, Buenos Aires, Argentina;). Lipids were measured by standard techniques using an enzymatic assay (Opera; Bayer, Leverkusen, Germany) and tissue cholesterol was examined as reported previously.[14] Serum sodium, potassium, calcium and magnesium were measured by colourimetric techniques (Advia 1650; Bayer, Tokyo, Japan). Plasma and tissue hydroperoxide concentrations were estimated by the

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ferrous oxidation–xylenol orange assay (FOX2),[15] and a technique based on thiobarbituric acid-reactive substances (TBARS).[16] These measurements were performed using a Spectronic Genesys 2 spectrophotometer (Spectronic UNICAM, Rochester, NY, USA) with readings at 560 nm and 535 nm, respectively.

Vascular responsiveness

The thoracic aorta was isolated and placed in a modified Krebs' solution of the following composition (in mmol/L): NaCl 122.0; KCl 5.9; $CaCl_2$ 1.2; $MgCl_2$ 1.2; $NaHCO_3$ 15.0; glucose 11.0. Ring preparations were made as reported previously.[14] Changes in isometric force under a resting tension of 2 g were monitored by a force-displacement transducer (Myograph F-60; Narco BioSystems, Houston, TX, USA). Rings were pre-incubated with 10^{-5} mol/L indomethacin for 1 h, contracted with 10^{-6} mol/L noradrenaline and the endothelium-dependent vasorelaxation tested with increasing doses of acetylcholine (10^{-9} to 10^{-4} mol/L). Endothelium-independent vasorelaxation was examined using 10^{-5} mol/L sodium nitroprusside.

Morphological study of the aorta

Specimens of the thoracic descending aorta and the proximal portion of the aortic arch from each animal were prepared as reported previously.[14] Macrophages were identified with a monoclonal antibody (anti-RAM-11; DAKO, Heidelberg, Germany). The labelled areas in the intima were delimited using CorelDraw 9 software (Microsoft Corporation, Redmond, WA, USA). Computer-assisted morphometric analysis was performed blindly with Image Tool for Windows software (University of Texas Health Science Center in San Antonio, San Antonio, TX, USA). Five sections, one of the upper and four of the descending aorta, were chosen for quantification.

Statistical analysis

Data are the mean±SEM. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls' multiple comparison test. When the data consisted of repeated observations at successive time points, a repeated-measures ANOVA was applied to determine differences between groups. Statistical significance was accepted at $P < 0.05$.

RESULTS

Lipid profile

Serum total cholesterol, low-density lipoprotein–cholesterol, high-density lipoprotein–cholesterol and triglycerides were significantly higher at 12 weeks compared with 24 weeks (Table 1). Treatment with hydrochlorothiazide and/or quinapril did not modify these lipid parameters.

Electrolytes

No differences were seen among groups regarding serum sodium, potassium, calcium or magnesium measured at the end of the experiment (Table 2).

Tissue cholesterol

Table 3 shows the values observed for the cholesterol content in the aorta, liver, kidney, spleen, brain and heart. Cholesterol content did

Table 1 Effect of treatment on serum lipid levels and bodyweight

Variable	*n*	Group I	Group II	Group III	Group IV
Total cholesterol (mg/dL)					
12 weeks	10	1310 ±122*	1286 ±79*	1163 ±105*	1450 ±117*
24 weeks	10	243 ±61	234 ±41	205 ±40	210 ±34
Low-density lipoprotein–cholesterol (mg/dL)					
12 weeks	10	1219 ±123*	1219 ±159*	1020 ±317*	1344 ±78*
24 weeks	10	215 ±58	178 ±27	168 ±36	186 ±33
High-density lipoprotein–cholesterol (mg/dL)					
12 weeks	10	36 ±4†	36 ±8†	40 ±5†	45 ±7†
24 weeks	10	18 ±3	16 ±2	19 ±3	18 ±2
Triglycerides (mg/dL)					
12 weeks	10	94 ±14†	110 ±19†	100 ±12†	138 ±28†
24 weeks	10	49 ±6	38 ±4	47 ±12	34 ±4
Weight (g)					
0 weeks	10	2.78 ±0.11‡	2.79 ±0.12‡	2.78 ±0.12‡	2.95 ±0.12‡
12 weeks	10	3.32 ±0.10	3.20 ±0.15	3.59 ±0.13	3.23 ±0.10
24 weeks	10	3.59 ±0.11	3.49 ±0.15	3.68 ±0.10	3.46 ±0.10

Data are the mean±SEM. †$P < 0.05$, *$P < 0.001$ compared with 24 weeks (Newman–Keuls' test); ‡$P < 0.05$ compared with 12 and 24 weeks (repeated-measures ANOVA).

Group I, control; group II, hydrochlorothiazide; group III, quinapril; gourp IV, hydrochlorothiazide + quinapril.

Table 2 Serum electrolytes at 24 weeks

Group	*n*	Sodium (mEq/L)	Potassium (mEq/L)	Magnesium (mg/dL)	Calcium (mg/dL)	Ionized calcium (mmol/L)
I	10	140 ±1	3.3 ±0.2	2.8 ±0.2	11.4 ±0.3	1.51 ±0.04
II	10	140 ±1	3.6 ±0.1	2.7 ±0.1	11.1 ±0.4	1.53 ±0.03
III	10	143 ±1	3.4 ±0.1	2.7 ±0.1	11.4 ±0.3	1.56 ±0.03
IV	10	138 ±2	3.2 ±0.2	2.4 ±0.1	11.2 ±0.4	1.59 ±0.02

Data are the mean±SEM.

Group I, control; group II, hydrochlorothiazide; group III, quinapril; gourp IV, hydrochlorothiazide + quinapril.

not differ in these tissues regardless of the type of treatment, except for in the aorta, where group III animals (receiving quinapril) had a lower cholesterol content than that observed in animals under diuretic therapy (groups II and IV).

Oxidative stress

No differences were observed in plasma or aortic concentrations of either hydroperoxide or TBARS (Table 4).

Morphology

Microscopic examination of atherosclerotic aortas revealed significant plaque development, which was attenuated by quinapril treatment. In fact, the intima area, intima/media ratio, plaque length and thickness were all significantly reduced following quinapril treatment. Moreover, in quinapril-treated rabbits, there was a 50% reduction in the intimal area occupied by macrophages, although this result did not reach statistical significance (Figs 1a–d,2).

Table 3 Tissue cholesterol at 24 weeks

Group	*n*	Cholesterol (mg/g tissue) Aorta	Liver	Kidney	Spleen	Brain	Heart
I	10	38.0 ±5.0	22.0 ±3.2	4.1 ±0.4	9.4 ±1.6	10.6 ±0.7	2.0 ±0.1
II	10	42.0 ±5.0	21.8 ±2.8	4.5 ±0.3	9.0 ±1.7	11.2 ±0.6	1.9 ±0.1
III	10	26.0 ±5.0*	21.4 ±2.9	4.4 ±0.2	6.9 ±1.3	10.7 ±0.9	1.8 ±0.1
IV	10	48.0 ±6.0	22.0 ±3.8	4.8 ±0.4	8.1 ±1.2	11.1 ±0.8	2.0 ±0.2

Data are the mean±SEM. *$P < 0.05$ compared with groups II and IV (Newman–Keuls' test).
Group I, control; group II, hydrochlorothiazide; group III, quinapril; gourp IV, hydrochlorothiazide + quinapril.

Table 4 Plasma and aorta concentrations of hydroperoxide and Thiobarbituric acid-reactive substances

Group	*n*	Hydroperoxides Plasma (μmol/L)	Aorta (μmol/100 g)	Thiobarbituric acid-reactive substances Plasma (nmol/mL)	Aorta (nmol/100 g)
I	10	1.00 ±0.30	2.52 ±0.41	1.27 ±0.24	1.23 ±0.33
II	10	1.33 ±0.48	2.54 ±0.57	1.32 ±0.23	1.86 ±0.51
III	10	1.16 ±0.40	1.88 ±0.17	1.59 ±0.19	1.26 ±0.43
IV	10	1.40 ±0.69	3.00 ±0.86	1.37 ±0.22	2.13 ±0.59

Group I, control; group II, hydrochlorothiazide; group III, quinapril; gourp IV, hydrochlorothiazide + quinapril.

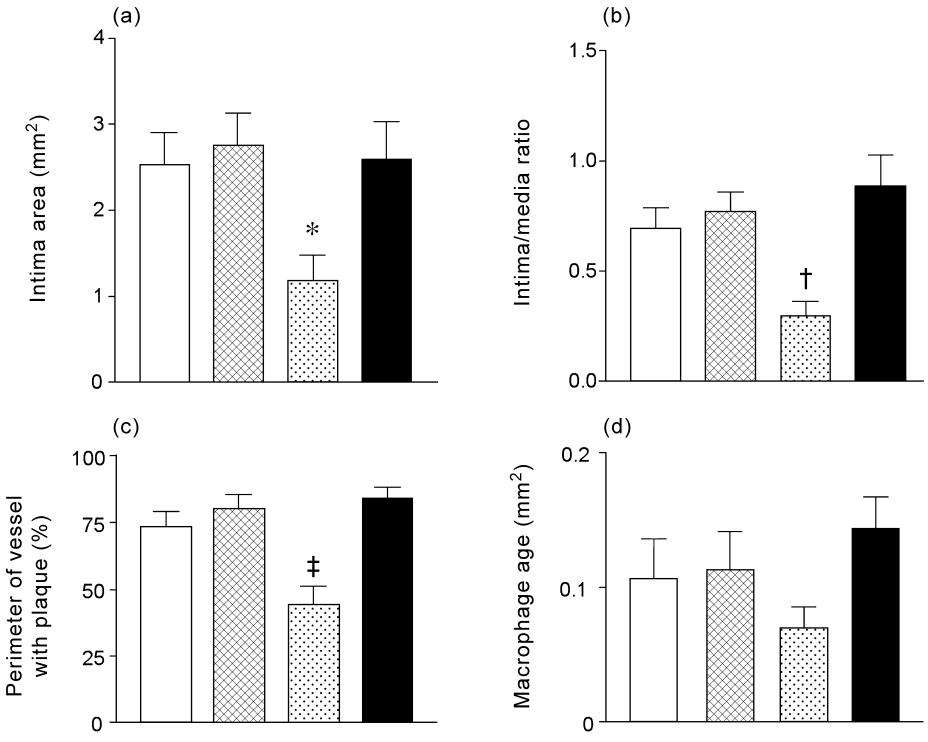

Fig. 1 Mean values for (a) intima area, (b) intima/media ratio, (c) perimeter and (d) macrophage area. Five specimens were chosen for measurements in each animal (one from the upper aorta and four from the descending aorta). (□), control (group I); (▨), hydrochlorothiazide (group II); (▩), quinapril (group III); (■), hydrochlorothiazide + quinapril (group IV). Data are the mean±SEM. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with groups I, II and IV (Newman–Keuls' test). For all morphometric analyses, specimens from 10 animals in each group were examined.

Vasoreactivity

The precontraction levels in response to noradrenaline were similar among groups (6.3 ± 0.4, 4.4 ± 0.3, 4.8 ± 0.5 and 5.1 ± 0.6 g for groups I–IV, respectively).

Concentration–response curves for the vasodilator effect of acetylcholine in aortic rings showed similar ED_{50} values among the four groups (6.11×10^{-8}, 3.24×10^{-8}, 3.46×10^{-8} and 3.31×10^{-8} mol/L for groups I–IV, respectively). However, Fig. 3 shows that dose–response curves to acetylcholine indicated a significant improvement in the vasorelaxant response in tissues from animals treated with quinapril (group III) compared with the control (group I) and hydrochlorothiazide groups (groups II and IV). Conversely, the dose–response curve to acetylcholine in the quinapril + diuretic-treated animals (group IV) was shifted to the right with the maximun response depressed.

Endothelial-independent vasorelaxation in response to sodium nitroprusside was not modified by drug treatment (data not shown).

DISCUSSION

Our results support the hypothesis that hydrochlorothiazide may have adverse effects on atherosclerosis. In fact, to our knowledge, the present study shows, for the first time, that diuretic therapy can abolish the beneficial effects of an ACE inhibitor with regard to endothelial function and atherosclerosis. Considering the widespread use of thiazide therapy for the control of blood pressure and the little benefit to coronary risk, at least with high doses, the present study sheds new lights on the importance of vascular homeostasis that is based not just on the efficacy of antihypertensive agents in reducing blood pressure.

Quinapril-treated animals showed consistent attenuation of atherosclerosis and improvement in endothelial function, a result that was expected based on the extensive literature and the results of recent clinical trials.[4–10] Conversely, hydrochlorothiazide treatment did not modify the development of atherosclerosis or endothelial function in this model. However, when added to quinapril, the benefits of the ACE inhibitor were lost. All these aspects were more striking considering that they were obtained while the same lipid and electrolyte profiles were maintained. In addition, no differences were observed among groups in bodyweight, which agrees with our observation that the intake of drug was comparable and uniform during the investigation. We have changed the amount of cholesterol in the diet during the active pharmacological treatment (final 12 weeks) to avoid excessive hypercholesterolaemia, which may affect endothelium function and could mask any potential benefit of drug treatment. The antiatherosclerotic effect of quinapril after 3 months treatment was of

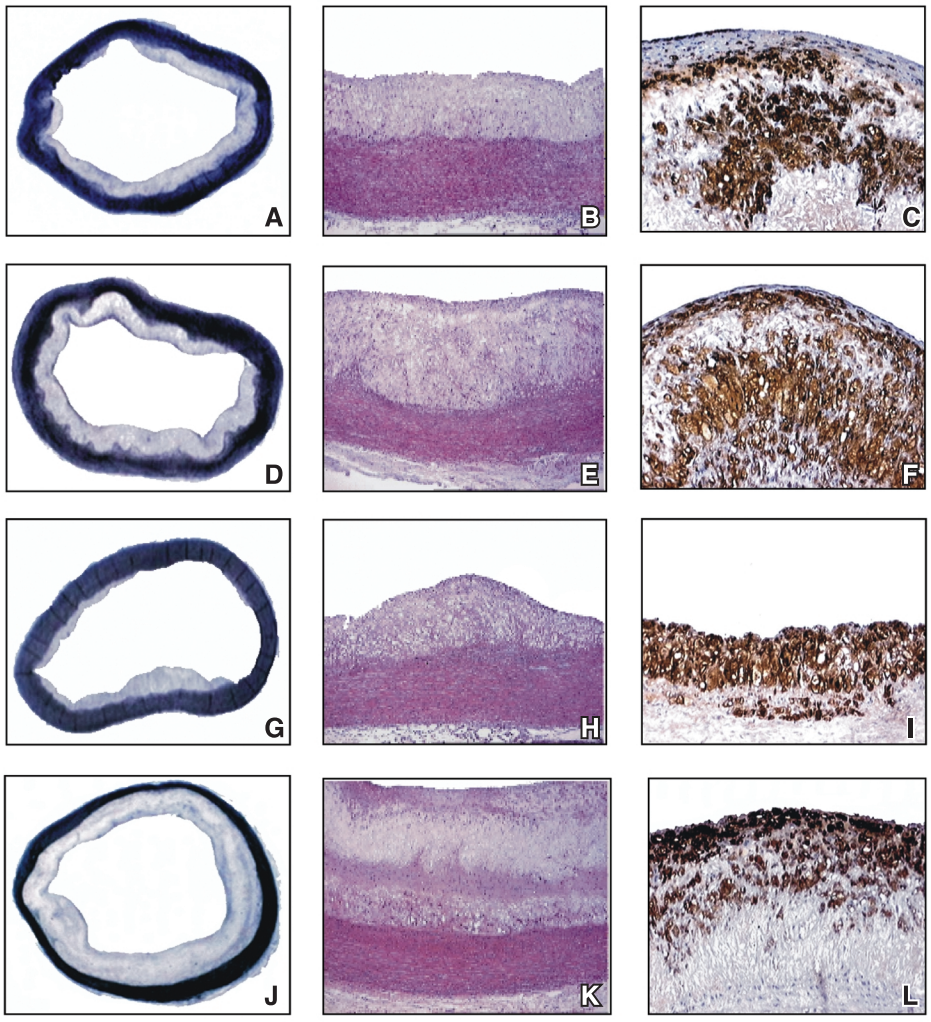

Fig. 2 Histological cross-sections of representative atherosclerotic aortas demonstrating marked reduction of intimal thickness following quinapril treatment. (a–c) Specimens from control animals (group I); (d–f) specimens from hydrochlorothiazide-treated animals (group II); (g–i) specimens from quinapril-treated animals (group III); (j–l) specimens from hydrochlorothiazide + quinapril-treated animals (group IV). (a,d,g,j) Verhoeff stain (original magnification ×6); (b,e,h,k) haematoxylin and eosin stain (original magnification ×40); (c,f,i,l) specimens treated with RAM-11 to identify macrophages (brown; original magnification ×100).

the same magnitude as that observed recently with pravastatin in a similar protocol developed by our group with regard to intima/media ratio, perimeter and maximal thickness of the atherosclerotic lesions.[14]

Previous studies have shown that patients with essential hypertension have reduced endothelium-dependent vasorelaxation[17,18] and some studies have suggested a decreased activity of endothelial nitric oxide (NO).[19–21] In the present study, hydrochlorothiazide treatment did not modify the endothelium-dependent vasodilatation in response to acetylcholine, whereas quinapril-treated animals (group III) showed significant improvement in this aspect. In addition, vasodilation to acetylcholine was more severely impaired (i.e. maximum response was depressed) in animals treated with hydrochlorothiazide plus quinapril.

Previous studies have shown that the production of NO by hypercholesterolaemic and atherosclerotic rabbit aorta is markedly enhanced rather than impaired.[22] However, hypercholesterolaemia increases superoxide anion production, which is associated with NO degradation due mainly to the formation of peroxynitrate.[22–24] In addition, a two- to fivefold increase in vascular ACE activity has been demonstrated in the atherosclerotic rabbit.[8] All components of the renin–angiotensin system have been found in cardiovascular structures.[25,26] More recently, an increase in the overall vascular responsiveness to angiotensin II was observed in atherosclerotic rabbits.[26] Therefore, the inhibition of ACE activity and, consequently, the reduction in angiotensin II formation may be of importance in the benefits seen in terms of endothelial function and atherosclerosis. Some protective mechanisms of ACE inhibition involves the reduction of superoxide anion concentrations in the intimal layer, decreases in endothelin transcription, leading to an improvement of endothelial function, and an attenuation of atherosclerosis. In our model, we did not measure ACE in the aorta, but overexpression of the renin–angiotensin system in vascular tissues following diuretic therapy could explain the lack of efficacy of quinapril. Furthermore, prevention of the degradation of endogenous kinins and other peptides by ACE inhibitors stimulates the production of endothelium-derived mediators, which include NO, prostacyclin and endothelium-derived hyperpolarizing factors (EDHF).[27] No differences were found in plasma concentrations of hydroperoxide or TBARS. However, examining these variables in the aorta, we found a trend for them to be higher with diuretic therapy. Moreover, the cholesterol content in the aorta was reduced following quinapril treatment compared with diuretic therapy.

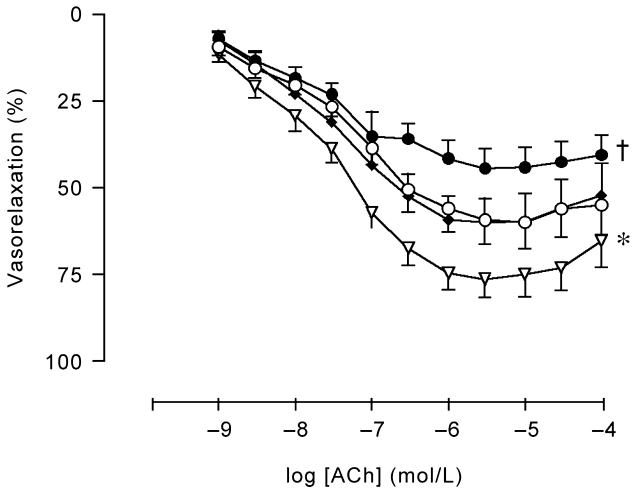

Fig. 3 Dose–response curves for the vasorelaxation induced by acetylcholine (ACh) in isolated aortic rings. Relaxations are expressed as a percentage of the initial constrictor response obtained to 10^{-5} mol/L noradrenaline. (○), control; (◆), hydrochlorothiazide; (▽), quinapril; (●), hydrochlorothiazide + quinapril. Data are the mean±SEM (n = 10 in each group). Vasorelaxation in quinapril-treated animals showed significant improvement compared with the other groups (*$P < 0.001$). Conversely, vasodilatation in the quinapril + hydrochlorothiazide-treated animals was significantly impaired compared with the other groups ($^{\dagger}P < 0.001$ versus other groups, ANOVA for repeated measurements followed by Newman-Keuls multiple comparison test).

Chronic administration of hydrochlorothiazide is associated with a reduction in serum magnesium and potassium via a mechanism that involves a reduction in intracellular potassium and magnesium, leading to membrane depolarization.[28] Furthermore, increased natriuresis has been reported following diuretic therapy in combination with ACE inhibitors.[29,30] Depletion of intracellular potassium and magnesium may affect important cellular responses, including the availability of calcium and cell membrane polarization. In the presence of atherosclerosis, the EDHF may have an important role in vasodilation and other endothelial functions, especially following ACE inhibitor treatment.[31] In addition, hydrochlorothiazide, as opposed to other diuretics, neither increases kallikrein gene expression nor has any anti-oxidant properties.[32,33] In fact, in the PROGRESS study,[5] the group that most benefited from ACE inhibitor (perindopril) treatment was the one in which the ACE inhibitor was combined with indapamide. However, this particular diuretic has no deleterious effect on glucose metabolism, serum lipid levels or renal function and also has anti-oxidant properties.[33,34]

In the recent ALLHAT study,[35] a larger trial in hypertensive patients, a low dose of chlorthalidone was better than the ACE inhibitor lisinopril in reducing some cardiovascular outcomes. However, 5 year systolic blood pressure was significantly higher in the lisinopril group compared with the chlorthalidone group. Furthermore, greater differences occurred in non-Caucasian patients (one-third of the entire study population) and these results are in accordance with many reports of poorer blood pressure responses with ACE inhibitor treatment in this population.[36,37]

We did not measure arterial blood pressure in the present study, but it is unlikely that a blood pressure reduction alone could explain our results on endothelial function as well as atherosclerosis because no benefit was observed in the quinapril + diuretic-treated animals. Combination of ACE inhibitors with low-dose diuretics is common in clinical practice and necessary for appropriate blood pressure control in many cases. However, as mentioned before, high doses of diuretics do not modify coronary outcomes in hypertensive patients. In the ALLHAT study, even a low dose of chlorthalidone was accompanied by an increase in fasting glucose levels. The precise role of the use of low-dose diuretics combined with ACE inhibitors in long-term cardiovascular outcomes for patients with appropriate blood pressure control, remains to be tested.

In conclusion, hydrochlorothiazide, considered as a first-line antihypertensive drug, showed a neutral effect on atherosclerosis in cholesterol-fed rabbits. However, in combination with the ACE inhibitor quinapril, hydrochlorothiazide abolished the benefit of quinapril drug on endothelial function and atherosclerosis. Our

results are in agreement with a new paradigm of antihypertensive therapy: the need of these agents to have beneficial effects on vascular homeostasis in addition to their ability to reduce blood pressure. The molecular mechanisms, including inhibition of the NO pathway, and how arterial blood pressure could affect these findings are important questions that remain to be answered.

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