Renin Inhibition by Aliskiren Prevents Atherosclerosis Progression

Comparison With Irbesartan, Atenolol, and Amlodipine

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Abstract—Hypertension is associated with increased risk of cardiovascular diseases. Antihypertensive treatment, particularly blockade of the renin-angiotensin system, contributes to prevent atherosclerosis-mediated cardiovascular events. Direct comparison of different antihypertensive treatments on atherosclerosis and particularly plaque stabilization is sparse. $ApoE^{-/-}$ mice with vulnerable (2-kidney, 1-clip renovascular hypertension model) or stable (1-kidney, 1-clip renovascular hypertension model) atherosclerotic plaques were used. Mice were treated with aliskiren (renin inhibitor), irbesartan (angiotensin-receptor blocker), atenolol (β-blocker), or amlodipine (calcium channel blocker). Atherosclerosis characteristics were assessed. Hemodynamic and hormonal parameters were measured. Aliskiren and irbesartan significantly prevented atherosclerosis progression in 2-kidney, 1-clip mice. Indeed, compared with untreated animals, plaques showed thinner fibrous cap ($P<0.05$); smaller lipid core ($P<0.05$); decreased media degeneration, layering, and macrophage content ($P<0.05$); and increased smooth muscle cell content ($P<0.05$). Interestingly, aliskiren significantly increased the smooth muscle cell compared with irbesartan. Despite similar blood pressure lowering, only partial plaque stabilization was attained by atenolol and amlodipine. Amlodipine increased plaque smooth muscle cell content ($P<0.05$), whereas atenolol decreased plaque inflammation ($P<0.05$). This divergent effect was also observed in 1-kidney, 1-clip mice. Normalizing blood pressure by irbesartan increased the plasma renin concentration (5932±1512 ng/mL per hour) more than normalizing it by aliskiren (16085±5628 ng/mL per hour). Specific renin-angiotensin system blockade prevents atherosclerosis progression. First, evidence is provided that direct renin inhibition mediates atherosclerotic plaque stabilization. In contrast, β-blocker and calcium channel blocker treatment only partially stabilize plaques differently influencing atherogenesis. Angiotensin II decisively mediates plaque vulnerability. The plasma renin concentration measurement by an indirect method did not confirm the excessive increase of plasma renin concentration reported in the literature during aliskiren compared with irbesartan or amlodipine treatment. **(*Hypertension*. 2008;51:1306-1311.)**

Key Words: hypertension ■ renin ■ atherosclerosis ■ angiotensin ■ vulnerable plaque

Atherosclerosis (ATS) is a complex chronic disease of multifactorial origin recognized to be a major health burden in modern society. A number of risk factors are strongly associated with the initiation and growth of atherosclerotic plaques. However, the mechanisms that cause a stable plaque to become vulnerable remain largely unknown. This is especially important because ATS proceeds clinically silently over time, as long as lesions remain stable. Conversion to an unstable or vulnerable phenotype renders plaques susceptible to rupture with dramatic consequences.[1,2] For these reasons, stabilizing the unstable plaques is a major goal in cardiovascular medicine. Hypertension is clearly associated with an increased risk of cardiovascular diseases and accelerated ATS. Therefore, antihypertensive treatments represent an essential pharmacological tool for the prevention of ATS-mediated cardiovascular events. Animal experiments and human studies directly or indirectly demonstrated that pharmacological blockade of the renin-angiotensin system (RAS) has beneficial effects on ATS.[3–6] A significant component of the cardiovascular protection observed in these studies appeared to be independent of blood pressure lowering, suggesting important and direct roles for the RAS within the arterial wall itself. Angiotensin (Ang) II, the effector hormone of the RAS (and eventually aldosterone), has been shown to exert several effects on various vessel wall cell components functioning as a growth, migration, prothrombotic, and proinflammatory factor.[7–10] Moreover, we have shown recently, in a mouse model of ATS, that Ang II

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induces the progression of ATS and mediates plaque vulnerability beyond its effect on blood pressure.[11] Therefore, the aim of this study was to evaluate the relative beneficial effect of various antihypertensive treatments on ATS progression, in particular, plaque vulnerability, using mouse models with differing stages of ATS.

Materials and Methods

Mouse Models of Stable and Vulnerable Atherosclerotic Plaques

ApoE$^{-/-}$ mice (C57BL/6J background, IFFA CREDO) were fed regular rodent chow and water ad libitum throughout the study. Both male and female mice were used. All of the experiments were approved by the local institutional animal committee. Two models of renovascular hypertension were generated, as described previously, in 14- to 16-week–old animals: the renin-dependent Ang II-mediated 2-kidney, 1-clip (2K1C; these mice develop vulnerable plaques, as reported previously),[11] and the renin-independent 1-kidney, 1-clip (1K1C; these mice develop stable plaques).[11] Briefly, mice were anesthetized by halothane inhalation (1% to 2% in oxygen), the left kidney was exposed, and the left renal artery was clipped to reduce renal perfusion. In the 1K1C model, other than the left renal artery clipping, right nephrectomy was performed.

Pharmacological Treatment

ApoE$^{-/-}$ 2K1C and 1K1C mice were treated during 3 weeks with a renin inhibitor (aliskiren, 50 mg/kg per day via SC minipumps, Novartis Switzerland), an Ang II type 1 (AT_1) receptor blocker (irbesartan, 100 mg/kg per day in drinking water, BMS), a β-blocker (atenolol, 120 mg/kg per day in drinking water, AstraZeneca), or a calcium channel blocker (amlodipine, 6 mg/kg per day in drinking water, Pfizer). In pilot experiments, the route of administration and optimal dose (similar blood pressure–lowering capacity) of these antihypertensive drugs were determined (data not shown). Treatment was started 1 week after clipping to allow plaque development in response to hypertension and RAS stimulation.

Blood Pressure, Heart Rate, Hormone, and Total Cholesterol Measurements

Four weeks after clipping, mean arterial blood pressure (MBP) and heart rate (HR) were measured as described previously.[11] Briefly, the left carotid artery was catheterized, and mice were allowed full recovery from anesthesia. The arterial line was connected to a pressure transducer, and 30 minutes thereafter, MBP and HR were recorded. Plasma renin concentration (PRC; active renin concentration) and plasma renin activity (PRA) were determined at the end of the 4-week clipping period as described previously using a modified microassay based on Ang I trapping by an antibody developed in our laboratory. Briefly, mouse PRC was measured by radioimmunoassay of Ang I generated from excess rat angiotensinogen (Ang-N) in the presence of high-affinity antibodies to Ang I (antibody-trapping technique)[12] (Figure S1A, available online at http://hyper.ahajournals.org). Rat Ang-N is preferentially cleaved by mouse renin, and it is conveniently available in the plasma of nephrectomized rats. The within- and between-assay precision coefficients of variation were 8% and 13%, respectively (n=12). The detection limit was at 10 ng of Ang I per milliliter per hour when 0.75 μL plasma was analyzed and PRC levels of saline containing plasma of nephrectomized rats (blank values) were the below detection limit. Normal PRC in nonclipped female mice carrying 1 renin gene (Ren-1) were 528 to 935 ng of Ang I per milliliter per hour and twice as high in male mice (n=26). Mouse PRA was measured by radioimmunoassay using high-affinity antibodies trapping generated Ang I from endogenous Ang-N (Figure S1B). The within- and between-assay precision coefficients of variation were 6% and 11%, respectively (n=10). The detection limit was 0.31 ng of Ang I per milliliter per hour when 25 μL of plasma were analyzed. The renin activity of water or trisalbumin buffer was below detection limit (blank values). Normal PRA in mice carrying 1 renin gene (Ren-1) were 5.8 to 9.0 ng of Ang I per milliliter per hour (n=26). Mouse Ang-N was measured by radioimmunoassay of generated Ang I after the incubation of plasma with an excess of mouse submaxillary renin and in the presence of angiotensinase inhibitors (Figure S1C). Excess submaxillary renin allows complete cleavage of endogenous Ang-N. The within- and between-assay precision coefficients of variation were 4% and 9%, respectively (n=8). The detection limit was 20 pmol/mL when 0.1 μL plasma was analyzed. No mouse Ang-N was detected when plasma of nephrectomized rats or mouse submaxillary extract containing angiotensinase inhibitors was analyzed (blank values). Normal Ang-N levels in mice carrying 1 renin gene (Ren-1) depended on the mouse strain and ranged between 170 and 837 pmol/mL (n=16 per group). Total cholesterol in the mouse plasma was measured using a commercially available kit (DiaSys Diagnostic Systems GmbH) following the manufacturer's instructions.

Evaluation of ATS Extension, Plaque Vulnerability, and Morphology

In euthanized mice perfused at physiological pressure, the thoracoabdominal aorta was dissected, fixed in formol, and en face stained with Oil-red-O.[11] Pictures of stained aortas were taken with a digital camera (Coolpix, Nikon), and the plaque area was quantified by computerized planimetry using the Qwin software (Leica Systems).[11] Analysis of plaque morphology and morphometry were carried out in 3-μm–thick serial histological sections.[11] (please see the online data supplement).

Immunostaining

For immunohistochemistry, sections were stained with a biotinylated mouse monoclonal IgG2a α-SM actin antibody[13] or with a rat monoclonal Mac-2 antibody (macrophage marker; Cedarlane; please see the online data supplement).

Statistical Analysis

Please see the online data supplement.

Results

Hemodynamic and hormonal results are summarized in the Table. Untreated and treated ApoE$^{-/-}$ 2K1C and 1K1C mice were analyzed. Untreated hypertensive ApoE$^{-/-}$ 2K1C and 1K1C animals served as controls. As expected, PRA and PRC were significantly above normal in the untreated ApoE$^{-/-}$ 2K1C mice (RAS-dependent hypertension model) as compared with normotensive untreated control mice (data not shown). Ang-N levels of untreated ApoE$^{-/-}$ 2K1C mice were at the lower end of the reference range. In contrast, PRA and PRC remained normal in the RAS-independent 1K1C model (volume overload hypertension), and the corresponding Ang-N levels were 50% higher than in 2K1C mice.

Antihypertensive Treatment and MBP, HR, Hormone, and Total Cholesterol Levels

Pharmacological treatment significantly lowered and actually normalized MBP as compared with untreated mice ($P<0.01$; Table). After treatment, MBP levels were similarly normalized in all of the animal groups; however, in 2K1C mice, atenolol treatment resulted in significantly higher normal MBP than irbesartan treatment ($P<0.05$; Table). In 1K1C mice, achieved MBP levels were normalized but tended to be higher than in similarly treated 2K1C animals (Table). HR was unchanged in all of the groups of mice except for the β-blocker–induced bradycardia ($P<0.01$; Table).

In both models of hypertension, aliskiren maximally decreased PRA and amlodipine maximally increased PRA,

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Table. Hemodynamic and Hormonal Parameters in Untreated and Treated 2K1C and 1K1C ApoE$^{-/-}$ Mice

	2K1C					1K1C				
Treatment	MBP, mm Hg	HR, bpm	Ang-N, pmol/mL	PRA, ng/mL per h	PRC, ng/mL per h	MBP, mm Hg	HR, bpm	Ang-N, pmol/mL	PRA, ng/mL per h	PRC, ng/mL per h
Untreated	144±2	660±9	224±22	14.9±1.8	3063±538	151±1	647±11	335±22	6.8±0.8	755±90
Aliskiren	106±3*	640±16	427±29*‡§	3.3±1*‡§	5932±1512	117±4*	663±10	423±289‡§	2.6±0.3‡§	3760±567
Irbesartan	103±3*†	643±18	62±29*†	16.5±2.6†§	16 085±5628*	111±6*	640±10	58±12*†	21.6±2.4*†	11 235±3001*‖
Atenolol	116±4*	528±14*‡§‖	341±97§	7.1±1.5*§	959±216‡	114±4*	511±14*‡§#	348±24	5.6±1§	542±93‡
Amlodipine	104±2*	641±35	138±35	25.4±2*	7552±2002‡	115±2*	610±46	213±70	24.5±2*	5834±2261

N=6 to 10 in each group.
*$P<0.05$ vs untreated.
†$P<0.05$ vs atenolol.
‡$P<0.05$ vs irbesartan.
§$P<0.05$ vs amlodipine.
‖$P<0.05$ vs aliskiren.

whereas PRC was comparably increased in these 2 treatment groups (Table). Atenolol, as a partial blocker of renin release, reduced PRA less strikingly than the direct renin inhibitor aliskiren, but it also tended to reduce PRC in both models of hypertension. In both 2K1C and 1K1C mice, irbesartan enhanced PRC to 3-fold higher levels than aliskiren despite a comparable antihypertensive effect of both drugs. During irbesartan treatment (and contrasting amlodipine treatment), the increase in PRA was less remarkable than the increase in PRC; particularly in the 2K1C mice, the 5-fold increase in PRC induced by irbesartan was not accompanied by an increase in PRA, possibly because of consumption of the endogenous renin substrate Ang-N (Table).

Total cholesterol levels were measured in all of the groups of mice. Results showed no significant difference among the untreated and treated mice (data not shown).

Assessment of ATS Extension and Atherosclerotic Plaque Vulnerability

Regardless of the type of antihypertensive drug used, normalization of blood pressure was accompanied by a significant and similar decrease in the ATS extension in all of the mice (decrease was not significant in irbesartan-treated 1K1C animals; Figure 1A and 1B). However, the morphology of plaques significantly differed among the various groups. Several signs of plaque vulnerability were assessed and quantified as detailed in the Method section (thickness of fibrous cap, surface of central lipid core, media degeneration, layering, SMC, and macrophage content). In untreated 2K1C mice, the fibrous cap was very thinned or absent in 100% of the analyzed animals. Pharmacological blockade of the RAS (renin inhibition and Ang II receptor blockade) and calcium channel blockade significantly prevented fibrous cap thinning ($P<0.05$ versus untreated or otherwise treated; n=10 to 13). On the contrary, no significant effect was observed after atenolol therapy. A large lipid core is linked to increased plaque vulnerability. In our mice, the total surface occupied by the central lipid/necrotic core exceeded 50% of the total plaque surface in only 8% and 18% of aliskirene- and irbesartan-treated mice ($P<0.05$ versus untreated and amlodipine or atenolol treated animals; n=10 to 13). Signs of media degeneration/atrophy and elastic lamina fragmentation were significantly less evident in aliskiren-treated mice than in untreated or otherwise treated mice ($P<0.05$; n=10 to 13). Mixed multiple layers of cells at different stages are suggested to be the consequence of previous clinically silent ruptures and after de novo plaque growth. This phenomenon was observed in lesions of 81% of untreated mice. Significant layering reduction was shown after antihypertensive treatment ($P<0.05$ versus untreated; n=10 to 13). Decreased SMCs and increased inflammatory cell plaque content are important markers of plaque vulnerability. In aliskiren- or irbesartan-treated animals, fibrous cap α-SMA content significantly increased after 3 weeks of treatment ($P<0.05$ compared with all of the other treatments; Figure 2A). This was especially true when the renin inhibitor was administered, suggesting a more stable phenotype. Along the same line, macrophage plaque content was significantly reduced

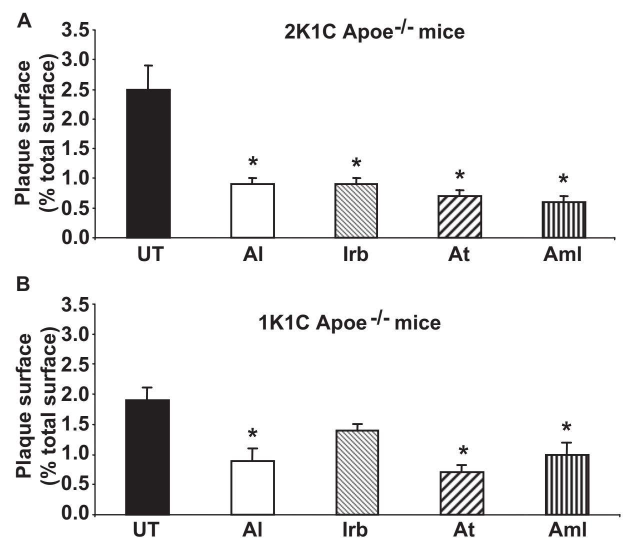

Figure 1. Extension of ATS measured by oil red–stained percentage of plaques over the total surface area of aorta. In 2K1C mice, pharmacological normalization of blood pressure reduced ATS by 60% to 80% (top). In 1K1C mice, pharmacological normalization of blood pressure reduced ATS by 25% to 60% (bottom). *$P<0.05$ vs untreated. UT indicates untreated; Al, aliskiren; Irb, irbesarta; At, atenolol; Aml, amlodipine; n=7 to 10 in each group.

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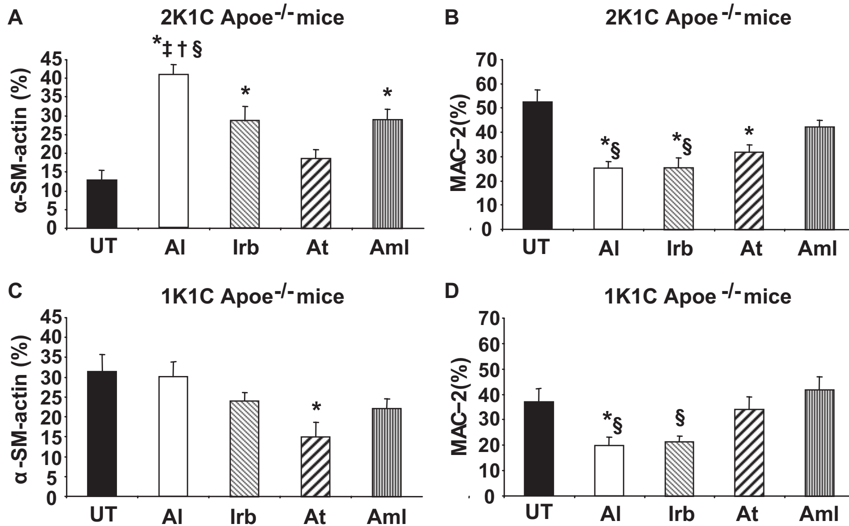

Figure 2. Plaque SMC and macrophage content assessed by α-SM actin (left) and MAC-2 (right). Vulnerability signs are enhanced in untreated 2K1C mice (top) as compared with 1K1C mice (bottom). Specific RAS blockade by aliskiren and irbesartan consistently reduces macrophage and increases SMC content. Atenolol and amlodipine have only partial or no effect on signs of plaque vulnerability (atenolol even decreases the α-SM actin area in 1K1C mice). *$P<0.05$ vs untreated; †$P<0.05$ vs atenolol; ‡$P<0.05$ vs irbesartan; §$P<0.05$ vs amlodipine; ‖$P<0.05$ vs aliskiren. UT indicates untreated; Al, aliskiren; Irb, irbesarta; At, atenolol; Aml, amlodipine; n=7 to 10 in each group.

after RAS blockade ($P<0.05$ compared with all of the other treatments; Figure 2A). Interestingly, compared with aliskiren and irbesartan, treatment with a β-blocker and a calcium channel blocker only partially affected ATS progression (Figure 2A and 2B), although a similar degree of blood pressure lowering was achieved (Table). In particular, amlodipine treatment in 2K1C mice positively affected SMC content within the fibrous cap (Figure 2A), but no significant consequence was seen in terms of inflammation (Figure 2B). In contrast, atenolol treatment was beneficial in inflammation reduction (Figure 2B), whereas no consequence on fibrous cap SMC content was observed (Figure 2A).

Plaque staging also different among the various groups of mice. Plaques were classified as early/intermediate (essentially composed of foam cells or with a small lipid core) and advanced (fibrofatty nodule or large necrotic/lipid core with multiple layers). Untreated 2K1C ApoE$^{-/-}$ mice developed mainly advanced lesions (82%), whereas only 23% and 25% of advanced plaques were found in the groups of mice treated with aliskiren or irbesartan, respectively ($P<0.05$ versus untreated; n=11). In contrast, 83% of plaques were in the advanced stage in the atenolol-treated group and 50% in the amlodipine administered mice (Figure 3A through 3E).

Treatment of 1K1C mice showed a less striking effect, because in these animals, plaques were already of a more stable phenotype (Figure 2C and 2D). However, RAS blockade proved efficient in further lowering the inflammatory cell content in these 1K1C mice (Figure 2D). On the contrary, amlodipine showed no effect, whereas atenolol treatment was associated with a decrease in SMC content (Figure 2C).

Discussion

Our results show that blood pressure normalization reduces ATS extension in hypertensive ApoE$^{-/-}$ mice. In 1K1C mice, irbesartan reduced the plaque surface slightly less than equally hypotensive comparator drugs (Figure 1B). One may speculate that exclusive blockade of the AT1 receptor and the subsequent increase in all of the plasma Ang peptides could contribute to plaque extension. Nevertheless, blood pressure normalization, per se, is not sufficient to prevent qualitative progression of ATS in hypertensive ApoE$^{-/-}$ mice with more vulnerable plaques. For this purpose, RAS blockade and, in particular, renin inhibition appear to be essential steps for plaque stabilization. These results are important because they show, for the first time in a mouse model of vulnerable atherosclerotic plaques, that ATS progression and plaque vulnerability can be efficaciously prevented by direct renin

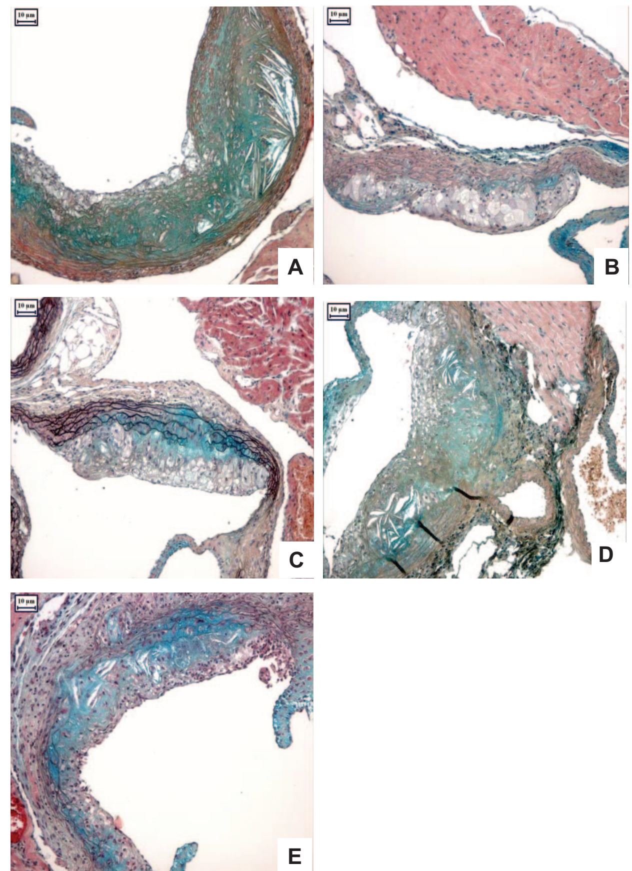

Figure 3. Morphology of atherosclerotic plaques of aortic sinus in 2K1C mice assessed by Movats staining. A, Untreated. B, Aliskirenn treated. C, Irbesartan treated. D, Atenolol treated. E, Amlodipine treated.

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inhibition. Moreover, our results emphasize the pivotal role of Ang II in atherogenesis. Indeed, treatment of 2K1C ApoE$^{-/-}$ mice with either a β-blocker or a calcium channel blocker failed to induce efficient plaque stabilization, although similar blood pressure lowering was achieved when compared with RAS blockers. Interestingly, atenolol and amlodipine showed dissimilar effects on ATS progression. Amlodipine significantly increased SMC content in the fibrous cap of 2K1C ApoE$^{-/-}$ mice, whereas atenolol significantly decreased plaque inflammation. This divergent effect was also observed in 1K1C mice. These observations suggest that, beyond a similar hemodynamic effect (blood pressure lowering), these 2 classes of drugs differently influence atherogenesis. In part, this may be explained by the fact that both atenolol and amlodipine have effects on the RAS. Atenolol is a partial inhibitor of renin secretion, as demonstrated by reduced PRA and PRC in our mice. Partial RAS blockade may be sufficient to reduce inflammatory cell content. On the contrary, amlodipine stimulates renin secretion (increased PRA and PRC in our mice), and this may partially counteract the beneficial effects of the drug on plaque composition.

From our data, it appears rather clearly that, beyond blood pressure control, blockade of the RAS is an essential step in plaque stabilization. This is demonstrated by the beneficial effect of both aliskiren and irbesartan. Mice treated with these compounds showed a more stable plaque phenotype: increased SMC content (at least in 2K1C mice) and decreased inflammation. Interestingly, aliskiren suppressed PRA in 2K1C mice from high initial levels to less than half of the PRA levels of untreated 1K1C mice. At the same time, plaques appeared more stable in the aliskiren-treated normotensive 2K1C mice than in the untreated hypertensive 1K1C mice (Figure 2). This stabilization of plaques was not found in amlodipine-treated, similarly normotensive 2K1C mice, where PRA was drastically increased. Atenolol in 2K1C mice reduced PRA levels to those found in untreated 1K1C mice, and plaque quality was comparable in both groups of mice (slightly less fibrous caps in atenolol-2K1C mice). Irbesartan, despite efficiently blocking the AT_1 receptors, did not outperform amlodipine concerning fibrous caps, but it did reduce inflammatory cells of the plaques. Renin inhibition appeared to increase SMC content more than AT_1 blockade (similar blood pressure–lowering effect). This suggests that this new class of drug may have a potential beneficial role in ATS stabilization and eventually reduces cardiovascular events. One concern raised from recently published trials in humans is the potential adverse effect of high circulating renin concentrations observed after treatment with a renin inhibitor. Indeed, aliskiren binds to the active site of renin, thus reducing its activity (low PRA) and, hence, Ang II production. Diminished Ang II concentrations stimulate, in turn, renin secretion (high PRC). The rationale of a potentially negative consequence of high renin concentration is the possibility that renin may bind to a renin receptor and trigger a series of yet unknown events.[14] However, AT_1 blockade also increases PRC (as well as PRA) via a similar mechanism. AT_1 blockers have been used successfully worldwide as a first-line therapy already for >15 years without any adverse effect linked to high PRC being reported. Authors have argued against renin inhibition, because in humans, PRC levels observed after renin blockade are higher than those observed after AT_1 blockade.[15] However, in our mice this was not the case. On the contrary, after AT_1 blockade, PRC was 3-fold higher than in mice treated with aliskiren. This apparent discrepancy was probably because of methodologic differences in the measurement of the renin concentration. In humans, a direct assay measuring active renin concentration is usually used. In this method, an antibody against the active site of renin is used.[16,17] Affinity of this antibody is higher than that of aliskiren. Therefore, in the in vitro assay, the antibody may displace the renin inhibitor and, thus, artificially increase the number of seemingly "active" renin molecules (PRC).[16,17] This is not the case for the PRC assay in mice, because an indirect method based on Ang I trapping by antibody is used.[12,18] Plasma aliskiren levels, which were in 100-fold excess of those required for comparable PRA suppression in humans, did not significantly reduce PRC (data not shown). Based on these considerations and on our present results, it appears that direct renin inhibitors should not cause more adverse events because of increased renin concentrations than AT_1 blockers.

Perspectives

Results reported here, if confirmed in clinical studies, which also warrant identical blood pressure–lowering effects, should have clinical implications in terms of cardiovascular event protection. In fact, for patients presenting with clinical or subclinical ATS, pharmacological blockade of the RAS may be an attractive therapy for the prevention of ATS progression. Under this aspect, patients with hypertension may also benefit more from RAS blockers than other antihypertensive drugs.

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