Differential Effects of Angiotensin II on Cardiac Cell Proliferation and Intramyocardial Perivascular Fibrosis In Vivo

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Background—Growth effects of angiotensin II (Ang II) contribute to cardiac remodeling. Remodeling, in turn, may be influenced by proliferation of nonmyocytes. The aims of this study were to determine in vivo which cardiac cell types proliferate in response to Ang II, to evaluate whether proliferation is mediated by the Ang II AT₁ receptor, and to establish whether blood pressure affects cell proliferation by comparing proliferation in the normotensive right atrium and ventricle and pressure-overloaded left ventricle.

Methods and Results—Groups of 8 Wistar rats were implanted with miniosmotic pumps releasing 5-bromo-2′-deoxyuridine (BrdU) as a cell proliferation marker for 2 weeks. Two groups received Ang II infusions via a second minipump and drinking water. Two groups received vehicle. Cell proliferation was assessed as the percentage of nuclei that incorporated BrdU. Ang II increased proliferation within medial vascular smooth muscle cells (VSMCs) and in associated adventitial/interstitial fibroblasts of intramyocardial coronary arterioles but decreased proliferation of myoendothelial cells. Despite increased blood pressure, proliferation in atria and ventricles was similar. Aldosterone levels were not significantly elevated, suggesting direct proliferative effects of Ang II. Losartan reduced Ang II–induced VSMC and adventitial fibroblast proliferation but had no effect on myoendothelial cell proliferation.

Conclusions—These results indicate direct, differential effects of Ang II on proliferation of atrial and ventricular nonmyocytes. VSMC and fibroblast proliferation is AT₁ receptor–dependent, whereas myoendothelial cells are controlled by an AT₁-independent mechanism. The effects are independent of aldosterone and blood pressure and have important implications in renin-dependent hypertension and chronic cardiac failure when circulating Ang II is elevated. (Circulation. 1998;98:2765-2773.)

Key Words: angiotensin receptors cells

Stroke, myocardial infarction, and chronic heart failure occur with increased frequency in patients with essential hypertension.1,2 A number of underlying mechanisms are associated with hypertensive heart disease, including increased peripheral vascular resistance and alterations in the structure and muscle content of the heart and blood vessels.3-5 In the heart, complex changes occur, including the remodeling of intramyocardial coronary arterioles6 and accumulation of fibrillar collagen in the perivascular adventitia and interstitium of the myocardium.7-9 As a consequence of these changes, the myocardium becomes abnormally stiff and ventricular function is reduced. Recent evidence suggests that cardiac remodeling may be further complicated by growth interactions between nonproliferating myocytes and proliferating nonmyocyte cells.10 Because up to 60% of the myocardium is composed of nonmyocytes, such as fibroblasts, endothelial cells, smooth muscle cells, and macrophages, it is important to understand how proliferation of these cells is controlled.

Evidence from in vitro studies suggests that proliferation of endothelial cells, smooth muscle cells, and fibroblasts is controlled by a multitude of growth factors and cofactors.11-13 Fundamentally, studies have demonstrated the importance of the renin-angiotensin-aldosterone system in controlling tissue structure under pathophysiological conditions such as chronic heart failure, hypertension, and renal artery stenosis.14-17 For example, in the heart, both angiotensin II (Ang II) and aldosterone increase perivascular collagen deposition and interstitial fibrosis in vivo.9,14,18,19 Furthermore, Campbell et al17 reported temporal changes in adventitial and interstitial fibroblast proliferation in vivo and, using isolated proliferating cell nuclear antigen S-phase events as a marker for cell proliferation, suggested that Ang II and aldosterone control cell proliferation and, as a consequence, fibrosis by separate...
mechanisms. Campbell et al demonstrated direct proliferative effects of Ang II raised endogenously or exogenously on fibroblasts and indicated a direct action of circulating Ang II independent of aldosterone. To date, however, no studies have attempted to quantify the proliferative effects of Ang II after chronic infusion, to identify specifically which cells proliferate, or to address whether the Ang II AT1 receptors are involved in cardiac cell proliferation in vivo. These issues are the subject of the present investigation.

In vitro, Ang II promotes vascular smooth muscle cell (VSMC) and fibroblast proliferation, effects that are mediated via the AT1 receptor subtype. AT1 receptor–dependent inhibition of coronary endothelial cell growth by Ang II in vitro has also been described. Given the differential actions of Ang II as a growth promoter/inhibitor of a variety of cell types and that some of this information has been derived from in vitro cell culture experiments, in which conditions may not be physiological, it was important to determine the role of Ang II in vivo and to establish whether all proliferative effects of Ang II are AT1 receptor–dependent.

There has been considerable debate as to whether structural cardiac remodeling is due to direct effects of growth factors or whether ventricular hypertrophy develops as a consequence of hypertension per se and hemodynamic overload. Sun et al recently showed that perivascular fibrosis occurs in the normotensive atria and pulmonary artery and is independent of increased blood pressure. In the present study, we compare the effects of Ang II infusion on cell proliferation in the hemodynamically overloaded left ventricle with those in the normotensive right atrium and right ventricle, using a dose of Ang II that is known to cause a modest rise in blood pressure. Cell proliferation was assessed as the cumulative incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into nuclei during the synthetic phase of the cell cycle.

Methods

Animals

Adult male Wistar rats (n = 32; Harlan Olac, Bicester, UK) weighing 200 to 250 g were housed in a temperature- and light-controlled room (21°C ± 1°C; 12/12-hour light/dark cycle) and were given normal rat chow (Special Diets Services) and water ad libitum. Under pentobarbital sodium anesthesia, all rats were implanted with miniosmotic pumps (Alzet model 2002) that infused 1.25 mg/d BrdU (dissolved in a 1:1 vol/vol mixture of dimethyl sulfoxide and 0.154 mol/L NaCl) continuously. The dose of BrdU administered has been shown to have no effect on plasma corticosterone, aldosterone, adrenocorticotropic hormone, or plasma renin activity. Experiments were conducted under licensed approval by the Home Office, London, UK.

Ang II Infusion With and Without Losartan

The rats were divided into 4 groups. Two groups of rats were given a second minipump containing a subacute pressor dose of Ang II (6 mg/mL; 200 ng · kg⁻¹ · min⁻¹) as described by Griffin et al and were given drinking water with or without losartan (Merck Sharp & Dohme). A daily dose of losartan was based on an average fluid intake of 150 mL · kg⁻¹ · day⁻¹ to deliver 10 mg · kg⁻¹ · d⁻¹ (2.25 mg/d). Two other groups of rats were given vehicle instead of Ang II and received either tap water or losartan (2.25 mg/d) in drinking water. The rats were treated for 2 weeks to measure proliferation of cells that have a slow turnover, such as smooth muscle cells.

Blood Pressure

Blood pressure was measured by tail-cuff plethysmography. Rats were then killed by a blow to the back of the head followed by decapitation. Trunk blood was collected for measurement of plasma hormones. Hearts were cut longitudinally through the septum, fixed in formalin, and embedded in paraffin wax.

Immunocytochemistry

The technique for detecting BrdU in nuclei has been described previously. In brief, dewaxed 3-μm sections of heart were incubated overnight at 4°C with an anti-BrdU monoclonal antibody (Eurotech), then incubated with a rabbit anti-mouse alkaline phosphatase–conjugated IgG antibody for 1 hour at 22°C. In negative controls, anti-BrdU antibodies were replaced with an antibody of the same immunoglobulin class not directed against BrdU. BrdU was detected in cells with an alkaline phosphatase substrate and fuchsin red as a chromogen. To determine the presence of endothelial cells and smooth muscle cells, separate heart sections were treated with an antibody to rat endothelial cells (Griffonia simplicifolia lectin I [GSL I], Vector Laboratories) and an antibody specific for smooth muscle cell α-actin (Novo Castra Laboratories). Sections were counterstained with hematoxylin.

Double Immunolabeling

To determine whether proliferating cells lining myocytes were myoendothelial cells, BrdU was detected in randomly selected tissue sections with the anti-BrdU antibody described above and a standard avidin-biotin immunoperoxidase system (Dako). In the same tissue sections, cell membrane α-methyl-D-galactopyranosyl groups specific to endothelial cells were detected by an anti-GSL I antibody (Sigma). Myoendothelial cells lining myocytes in the right and left ventricles and right atrium were counted. Within the media of intramyocardial coronary arterioles, all BrdU-positive myoendothelial cells were counted in left and right ventricles and right atrium were counted. Within the media of transverse intramyocardial coronary arterioles, all BrdU-positive and -negative VSMCs were counted. On the outside of the same vessels, adventitial fibroblasts and fibroblasts that extended into the interstitial space were also counted with an automated image analysis system (Seescan Bioscience). Approximately 1500 myoendothelial cells were counted in left and right ventricles and in the right atrium by use of a camera lucida frame of fixed area and fixed magnification of ×40. Cells from 12 to 16 intramyocardial coronary arterioles were counted in 2 random sections from each heart. A BrdU index was calculated for each cell type as the percentage of the total number of cells counted in each heart or blood vessel section that was BrdU-positive.

Quantification

All tissues were analyzed in a blinded fashion in random sections. Myoendothelial cells lining myocytes in the right and left ventricles and right atrium were counted. Within the media of transverse intramyocardial coronary arterioles, all BrdU-positive and -negative VSMCs were counted. On the outside of the same vessels, adventitial fibroblasts and fibroblasts that extended into the interstitial space were also counted with an automated image analysis system (Seescan Bioscience). Approximately 1500 myoendothelial cells were counted in left and right ventricles and in the right atrium by use of a camera lucida frame of fixed area and fixed magnification of ×40. Cells from 12 to 16 intramyocardial coronary arterioles were counted in 2 random sections from each heart. A BrdU index was calculated for each cell type as the percentage of the total number of cells counted in each heart or blood vessel section that was BrdU-positive.

Statistical Analyses

Data collected from 2 heart sections from each animal were averaged. Values are expressed as mean ± SEM from 8 animals. Unpaired observations were assessed by Student's t test and 2-way ANOVA. A value of P < 0.05 was considered significant.
Results

General Pattern of Cell Proliferation
The antibody against BrdU showed a consistent intensity of staining of a range of cell types throughout the heart. BrdU-positive cells were distributed within the visceral pericardium, endocardium, and myocardium in atria and ventricles; within intracoronary arterioles; and in the interstitial space. In the myocardium, BrdU-positive cells lining myocyte fibers showed a pattern of staining similar to that of the endothelial cells identified with the anti–GSL I antibody (Figure 1A). Double labeling with anti-BrdU and anti–GSL I antibody confirmed that the largest proportion of BrdU-positive cells was myoendothelial (Figure 1B). However, there was no evidence of BrdU in nuclei of myocytes. In intramyocardial coronary arterioles, the endothelium, but not VSMCs or adventitial cells, was also consistently GSL I–positive and served as a positive internal control. Within the vascular media and adventitia, BrdU-positive cells were VSMCs and fibroblasts, respectively. SMCs were identified in the vascular media of blood vessels and occasionally in the visceral pericardium by positive staining with α–actin antibody (data not shown).

Intramyocardial Arteriolar Cell Proliferation
Figure 2 shows the effect of Ang II with and without losartan on cell proliferation in intramyocardial coronary arterioles. After 2 weeks’ treatment with Ang II, there were 3-fold increases in cell proliferation of VSMCs within the vascular media (P<0.01) and of fibroblasts (P<0.001) on the outer adventitia of intracoronary arterioles that extended from the blood vessels into the interstitial space (Figure 3). Losartan blocked the effects of Ang II in both VSMCs and fibroblasts (P<0.001). Compared with control tissues, treatment with losartan alone reduced proliferation of cells of the vascular media (P<0.01) and adventitial and interstitial fibroblasts (P<0.001).

Perivascular Collagen Deposition
Figure 4 shows the effects of Ang II with and without losartan on the degree of collagen deposition surrounding intramyocardial coronary arterioles. In many blood vessels after treatment with Ang II, perivascular collagen extended from the adventitia into the interstitium of the surrounding myocardium and protruded into viable myocardium. This effect was reduced but not abolished by losartan. In rats treated with losartan alone, adventitial collagen was less than in control blood vessels. Interstitial collagen was reduced by losartan.

Myoendothelial Cell Proliferation
Figures 5 and 6 illustrate the effect of Ang II with and without losartan on the proliferation of left and right ventricular and right atrial myoendothelial cells. In general, proliferation...
indices were greater within the left than the right ventricle of the heart, but these differences were not statistically significant. The degree of cell proliferation in the right atrium was also similar to that in the ventricles. Although losartan alone had no effect on myoendothelial cell proliferation, treatment with Ang II significantly decreased proliferation in both left and right ventricles and right atrium by >2-fold ($P<0.001$). Treatment with losartan failed to increase myoendothelial cell proliferation lowered by Ang II.

**Ang II AT$_1$ Receptor Distribution**

Figure 7A shows the presence of AT$_1$ receptors in an intramyocardial coronary arteriole. AT$_1$ receptors were present on endothelial cells, within medial smooth muscle cells, and on the outside of the vessels on adventitial fibroblasts. In blood vessels in which there was extensive perivascular fibrosis, AT$_1$ receptors were present in newly proliferating fibroblasts. Within the right atrium and ventricles, AT$_1$ receptors were present in fibroblasts and myoendothelial cells (Figure 7B).

**Plasma Hormone Levels**

The Table summarizes the effects of Ang II with and without losartan on plasma renin activity (PRA) and plasma aldosterone. Ang II suppressed PRA ($P<0.05$) and tended to increase plasma aldosterone (but not significantly). Losartan

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**Figure 3.** Proliferation of VSMCs and fibroblasts (red) in intracoronary arterioles of A, control rat; B, after treatment with losartan; C, after infusion of Ang II, proliferating fibroblasts protrude into surrounding myocardium; and D, combined treatment with Ang II+losartan. Sections are counterstained with hematoxylin; BrdU-negative nuclei are blue. Magnification ×240.
caused an increase in PRA ($P<0.05$) with or without a concomitant increase in plasma Ang II ($P<0.05$). Losartan alone or in combination with Ang II had no significant effect on plasma aldosterone.

**Blood Pressure**

After 2 weeks of treatment, Ang II increased systolic blood pressure (Ang II, $191\pm13$ versus control, $150\pm7$ mm Hg, $P<0.05$). Losartan blocked the effects of Ang II (losartan+Ang II, $135.5\pm8$ mm Hg, $P<0.05$). Without Ang II, losartan treatment lowered blood pressure compared with controls (losartan, $130\pm6$ mm Hg, $P<0.05$).

**Discussion**

The results from the present study show, in vivo, differential actions of Ang II as a growth promoter and growth inhibitor in the heart. Although Ang II stimulated ventricular and atrial...
VSMC and fibroblast proliferation, myoendothelial cell proliferation was reduced. There was no evidence of BrdU in cardiomyocytes, confirming that terminally differentiated myocytes do not divide, even after treatment with Ang II. Unlike the study by Malendowicz et al., who found that larger-dose bolus injections of BrdU increased adrenocortical hormone activity, the dose of BrdU administered in this study has been shown previously not to exhibit nonspecific effects on the activity of either the renin-angiotensin system or the pituitary adrenal axis.

Double immunolabeling with the anti–GSL I endothelial cell marker and anti-BrdU antibody confirmed that BrdU-positive cells lining myocytes were myoendothelial cells. Furthermore, whereas the positive effects of Ang II on proliferation of VSMCs and fibroblasts were blocked by AT₁ receptor antagonism, the inhibitory effect of Ang II on myoendothelial cell proliferation was unaffected by concomitant losartan. These results suggest that in vivo cell proliferation and inhibition are controlled either directly by different Ang II receptors or indirectly by different growth factors.

Several studies have shown that Ang II acts as a growth-promoting factor for VSMCs and fibroblasts in vitro and that this effect is mediated by the AT₁ receptor. We have confirmed these findings and have shown that losartan can reduce cell proliferation of medial VSMCs and adventitial fibroblasts of intramyocardial coronary arterioles and fibroblast proliferation within the interstitial space in vivo. Immunolabeling with an anti–AT₁ receptor antibody confirmed the presence of the AT₁ receptor on both fibroblasts and VSMCs in vivo. AT₁ receptors have been identified previously on fibroblasts and VSMCs. In terms of cardiovascular risk, the ability of losartan to reduce hyperplasia of VSMCs and

Figure 6. Myoendothelial cells lining myocyte fibers of ventricles of heart. BrdU-positive cells are red, BrdU-negative cells are stained with hematoxylin and appear blue. A, Control rat; B, losartan; C, Ang II; and D, Ang II+losartan. Note that there is no evidence of BrdU in nuclei of myocytes. Magnification ×384.
fibroblasts, thereby potentially limiting hypertrophy of the intramyocardial coronary arterioles, must be considered to be potentially of clinical benefit.

The present study provides evidence of a direct role for circulating Ang II as a growth factor for fibroblasts and VSMCs in vivo based on the fact that all of the chambers of the heart were affected similarly. Ang II increased cell proliferation within the left ventricle, which would be subjected to increased blood pressure and, as a consequence, left ventricular wall stress. However, similar positive effects on proliferation of cells within the normotensive right ventricle and right atrium throughout the treatment period suggest that the effects of Ang II are independent of blood pressure and promote the role of Ang II as a directly acting, circulating factor within the heart. In addition, the presence of Ang II AT1 receptors within the atria and ventricles in all of the cell types affected by Ang II further suggests that these actions are likely to be direct rather than secondary to other growth factors. The present results confirm findings by Sun et al, who demonstrated microscopic scarring, ACE binding at fibrous sites, and the presence of Ang II AT1 receptors in both pressure-independent left and right atria and increased adventitial collagen in stress-free pulmonary arteries in response to Ang II. Taken together, these results highlight the importance of Ang II as a circulating, proliferative factor that acts on cardiac cells and acts independently of arterial pressure.

Infusion of Ang II into rats caused an increase in collagen deposition surrounding intracoronary blood vessels and an extension of collagen into the myocardial interstitium. This was associated with a >2-fold increase in cell proliferation of adventitial and interstitial fibroblasts. It has been shown that reactive fibrosis is attributed to both Ang II and aldosterone and is associated with an increase in perivascular collagen production. In a definitive study, Campbell et al described temporal differences in cell proliferation in response to Ang II and aldosterone. In rat models in which Ang II was raised endogenously by unilateral renal ischemia or raised systemically by chronic Ang II infusion, clusters of proliferating cardiac fibroblasts were evident within the first week of treatment. In contrast, aldosterone had no effect on cell proliferation until week 4. In the present study, aldosterone levels were not significantly elevated, but there were large increases in cell proliferation after 2 weeks, suggesting that the proliferative effects are due to a direct effect of Ang II and not mediated by aldosterone. Indeed, concomitant treatment with losartan prevented proliferation caused by Ang II, further emphasizing the direct role of circulating Ang II and the potentially beneficial effects of losartan under pathophysiological conditions, such as renin-dependent hypertension and chronic cardiac failure, in which circulating Ang II is elevated. Furthermore, treatment with losartan alone decreased fibroblast proliferation and reduced perivascular collagen surrounding blood vessels, demonstrating that the

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**Figure 7.** A, Section of left ventricle showing distribution of AT1 receptors (brown) within endothelial, medial, and adventitial layers of an intramyocardial coronary arteriole. B, AT1 distribution within myocardium (brown) in myoendothelial cells and in fibroblasts. Nuclei are counterstained with hematoxylin. Magnification ×240.
AT1 receptor tonically regulates fibroblast proliferation in vivo. By inhibiting perivascular and interstitial fibrosis, losartan may help to reduce myocardial stiffness and improve cardiac function.

The role of Ang II as a growth factor/inhibitor for myoendothelial cells is an area of research that is less well investigated. However, in an elegant study, Stoll et al23 showed that in the presence of Ang II, treatment of cultured coronary endothelial cells with either an AT1, or an AT2 receptor antagonist reduced or increased cell proliferation, respectively. In the same study, the inhibitory actions of the AT2 receptor remarkably offset proliferation mediated by the AT1 receptor, suggesting that the AT2 subtype is the predominant receptor that preferentially inhibits the proliferation of myoendothelial cells. In the present study, myoendothelial cell proliferation was consistently decreased by Ang II, irrespective of the presence of AT1 receptors and even when the AT1 receptor was blocked, suggesting that the AT2 receptor is likely to be the predominant inhibitory receptor for these cells in vivo. Whether these effects are mediated directly by Ang II or indirectly by AT2 receptor–dependent activation of growth inhibitory factors remains to be elucidated. The evidence for a role of the AT2 receptor in the heart remains controversial. Radioligand binding studies by Sun et al26,30 failed to demonstrate the presence of AT2 receptors within atria and ventricles of the heart. However, a direct role for AT2-dependent inhibition of cell proliferation has been suggested for other tissues.43,44

Treatment with losartan alone tended to reduce myoendothelial cell proliferation slightly but not significantly, implying that the AT1 receptor does not promote proliferation of myoendothelial cells. In contrast, Stoll et al23 showed that endothelial cell proliferation is AT1-dependent. However, in the latter study, positive effects of Ang II were observed only in either quiescent cells when the AT2 receptor was blocked, leaving the AT1 receptor exposed, or cells of spontaneously hypertensive rats, which have different cell phenotypes and exhibit altered responses to Ang II.45,46 Moreover, treatment of quiescent cells with Ang II without receptor antagonists failed to stimulate or inhibit cell proliferation, suggesting that in vitro responses are dependent on cell status. By comparison, we have shown consistent inhibitory effects of Ang II regardless of the presence of AT1 receptors on these cells or AT2 receptor blockade, confirming that cells in vivo are receptive to Ang II without pharmacological manipulation and, importantly, that AT1 receptors do not control proliferation of myoendothelial cells in vivo.

If the principal effect of Ang II is to inhibit proliferation of myoendothelial cells, persistent inhibitory effects of Ang II would result in no new cell proliferation and eventual cell loss by apoptosis. Because ~10% of cells were BrdU-positive after a 2-week treatment period, this would imply that antiproliferation is partially counterbalanced by cell proliferation. It is possible that myoendothelial cell proliferation is mediated by growth factors other than Ang II and is therefore non–AT1 receptor–dependent or that factors that maintain cell turnover are indirectly regulated via AT2 receptors either within myoendothelial cells or on other target cells.

This study has shown that nonmyocyte proliferation is differentially regulated, such that some cells proliferate in response to Ang II but the growth of others is inhibited. Recently, Harada et al10 implicated nonmyocytes in the response of myocytes to Ang II. They showed that cultured rat cardiomyocytes failed to develop hypertrophy in response to Ang II unless they were cocultured with nonmyocyte matrix cells, such as endothelial cells, fibroblasts, and VSMCs. The regulation of proliferation and inhibition of nonmyocytes in vivo by Ang II suggests that Ang II may indirectly regulate myocyte hypertrophy.

In conclusion, Ang II differentially regulates cell proliferation in the myocardium. Ang II directly increases proliferation of medial VSMCs and adventitial and interstitial fibroblasts associated with intramyocardial blood vessels in atria and ventricles of the heart in vivo. These effects are mediated by the Ang II AT1 receptor and are independent of elevated aldosterone and arterial blood pressure. These observations suggest an important role for losartan in reducing hyperplasia of intramyocardial coronary arterioles. Inhibition of myoendothelial cell proliferation by Ang II is not AT1 receptor–dependent. These results have important implications not only in renin-dependent hypertension but also in chronic cardiac failure, when circulating Ang II levels are elevated.

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