Cardiac Angiotensin II Participates in Coronary Microvessel Inflammation of Unstable Angina and Strengthens the Immunomediated Component

Gian Gastone Neri Serneri, Maria Boddi, Pietro Amedeo Modesti, Morella Coppo, Ilaria Cecioni, Thomas Toscano, Maria Letizia Papa, Manuela Bandinelli, Gian Franco Lisi, Mario Chiavarelli

Abstract—Angiotensin (Ang) II is now recognized to be a mediator of a wide variety of inflammatory processes. This study investigated renin-angiotensin system (RAS) components and a number of inflammatory mediators in left ventricular biopsies from 2-vessel disease unstable angina (UA) (n=43) and stable angina (SA) (n=15) patients undergoing coronary bypass surgery. Biopsy samples from 6 patients undergoing valve replacement for mitral stenosis served as controls. UA patients were randomly assigned to angiotensin-converting enzyme (ACE)-inhibitor (ramipril), AT1 antagonist (valsartan), or placebo and treated during the 5 days preceding coronary bypass surgery, performed from 6 to 9 days after coronary angiography. During coronary angiography coronary blood flow was measured and samples were obtained from aorta and coronary sinus for determination of Ang I and Ang II gradients. The hearts of UA patients produced Ang II in a greater amount than in SA patients (P<0.01). UA biopsy samples showed numerous DR+ cells, identified as lymphocytes, macrophages, and endothelial cells. Reverse-transcriptase polymerase chain reaction showed overexpression of AGTN, ACE, and AT1-R genes, as well as upregulation of TNF-α, IL-6, IFN-γ, and iNOS genes (P<0.01), with no differences between nonischemic and potentially ischemic areas. AGTN, ACE, and cytokine genes were mainly localized on endothelial cells. Ramipril and valsartan markedly decreased the expression levels of TNF-α, IL-6, and iNOS, and, to a lesser extent, of IFN-γ genes, but did not affect the number of DR+ cells, with no significant difference between the 2 treatments. These results show that locally generated Ang II amplifies the immunomediated inflammatory process of coronary microvessels occurring in unstable angina. (Circ Res. 2004;94:1630-1637.)

Key words: angiotensin II ■ unstable angina ■ myocardial inflammation

Unstable angina (UA) classes IIB and IIIB of Braunwald classification are an acute manifestation thought to be caused by recent inflammatory activation within coronary atherosclerotic plaque, often resulting in the plaque erosion or rupture with consequent nonocclusive thrombus development. Unstable or vulnerable plaques are characterized by increased accumulation of inflammatory cells, particularly macrophages and T-lymphocytes, and by a large lipid core and a thin fibrous cap. A high number of inflammatory cells express DR molecules and IL-2 receptors, thus indicating that antigen presentation, as well as an immunological reaction, occurred. However, the concept that UA is only an intraplaque event is strongly restrictive because recent studies have shown that >1 coronary plaque is activated in acute coronary syndromes. Moreover, we showed that also coronary microvessels are involved in UA by an acute inflammatory process with cellular and molecular characteristics of an immunomediated reaction. The complex pathophysiology of UA is also suggested by the link that seems to exist between renin-angiotensin system (RAS) and atherosclerosis. Several immunohistochemical and biochemical studies have shown a marked accumulation of angiotensin-converting enzyme (ACE), angiotensin (Ang) II, angiotensin type 1 receptor (AT1-R), and the co-expression of Ang II and IL-6 in human atherosclerotic plaques of patients with acute coronary syndromes. In a recent study, we demonstrated that cardiac RAS is activated in UA, but not in patients with stable angina (SA), resulting in an increased Ang II formation. Experimental studies in transgenic rats have shown that an exaggerated Ang II cardiac production can induce coronary vasculopathy. These findings are potentially relevant for the pathophysiology of UA, because there is growing evidence that local RAS has important modulatory activity in the atherogenic process and is provided with significant proinflammatory actions.
Therefore, we planned this study to investigate whether locally generated Ang II induces myocardial inflammation and, if so, to also investigate the cellular and molecular features of this inflammatory reaction.

Materials and Methods
Please note that additional information is included in the online data supplement available at http://circres.ahajournals.org.

Study Population
We examined 43 patients with UA in classes IIB (n=18) and IIB (n=25) and 15 patients with SA in Canadian classes II (n=4) and III (n=11). Patients with UA and SA were considered for inclusion in the study if they had a 2-vessel disease, I of which had to be the left anterior descending (LAD) artery, and if they had been scheduled for a coronary artery bypass graft because the condition was unsuitable for percutaneous transluminal angioplasty. The distribution of coronary artery disease and baseline clinical characteristics of the study population are reported in Table I of the online data supplement (see http://circres.ahajournals.org). Diagnosis of UA and SA and risk evaluation of UA patients followed the American College of Cardiology/American Heart Association Guidelines.18,19 High-risk patients and patients with recent (ie, within the preceding 3 months) myocardial infarction were not accepted for the study. We considered eligible for the study only patients who had had the onset of UA <5 days before admission and who experienced at ≥2 episodes of angina at rest or 1 episode lasting >20 minutes, associated with transient ischemic ST segment changes and troponin T levels <0.1 (troponin T-negative). All UA patients received aspirin (100 mg/d) and nitrates. In addition to this therapy, 18 patients were treated with beta-blockers, 27 with heparin, and 13 received calcium-antagonists. Anginal episodes were recorded until to surgery. Patients with SA were using aspirin (100 mg/d) and nitrates. Patients with conduction disturbances or heart failure or patients treated with ACE inhibitors in the 2 weeks preceding the hospital admission were also excluded from the study, as were patients who at admission had myocardial ischemia associated with pulmonary edema, hypotension, or threatening arrhythmias. Eligible patients were enrolled in the study after coronary angiography that was performed from 48 to 96 hours after admission. We also assessed severity of coronary lesions and presence and grade of coronary collateral circulation. Patients who at admission had had recent infectious diseases, known or suspected neoplasia, and erythrocyte sedimentation rate >20 mm/h were not considered eligible for the study. UA patients were randomly assigned to ramipril (5 mg/d, n=14), valsartan (80 mg/d, n=14), or placebo (n=15) groups. The patients were treated during the 5 days preceding the scheduled coronary artery bypass graft, which was performed from 6 to 9 days after coronary angiography. None of the patient showed a decrease in systolic or diastolic arterial blood pressure ≥5 mm Hg during the 5 days of drug administration. The protocol of this study complies with the principles of the Helsinki declaration and was approved by the Ethical Committee of our Institution. All patients gave informed consent to participate and to have blood samples and biopsy specimens taken for the study.

Experimental Procedures
During coronary angiography, we measured coronary blood flow with the thermodilution technique (mean of 3 measurements)20 and drew blood samples from aorta and coronary sinus for the determination of cardiac oxygen extraction and for the measurement of aorta–coronary sinus Ang I and II gradients.14 Ten patients who underwent coronary angiography for atypical chest pain and did not show significant lesions served as the control group for the measurement of Ang I and II aorta–coronary sinus gradients. In UA patients, we performed a transmural biopsy (10×0.5 mm) through a biopsy needle (MN1416; diameter 2.1 mm; BIP Gembh) from the anterolateral wall of the left ventricle close to the apex in the distribution territory of the LAD artery (potentially ischemic area) immediately after sternotomy and before inducing cardioplegia. In the 12 UA patients with LAD and coronary right artery (CRA) disease, a second biopsy was performed in the free left ventricle wall in an area that was supplied by obtuse marginal branches and was normally perfused at 211Thallium scintigraphy (nonischemic area). The surgeon did not take the biopsy specimens if the area selected for biopsies appeared as a site of an old infarction. Cardiac specimens were also obtained from the anterolateral wall of the left ventricle of 6 patients with mitral stenosis who were undergoing surgical valve replacement (control hearts [CHs]). Handling of biopsy specimens for reverse-transcriptase polymerase chain reaction (RT-PCR), in situ hybridization, and immunohistochemical studies were performed as previously described21 (see http://www.circresahajournals.org).

Measurement of Cardiac Ang II Formation
Cardiac Ang I and II formation was measured by means of aorta–coronary sinus concentration gradient indexed by coronary blood flow, following the procedure detailed in an earlier article.14 Plasma concentrations of both angiotensins were determined by radioimmunoassay after C18 Sep-Pak cartridge extraction and high-performance liquid chromatography separation.14,21

Immunohistochemical Detection of Inflammatory Cells and Morphometric Analysis
The presence and identification of activated inflammatory cells in the myocardium were determined by immunohistochemical analysis using human monoclonal antibodies for major histocompatibility class II molecules (human leukocyte antigen [HLA]-DR), CD68 macrophages, CD3 T-lymphocytes, and CD31 and von Willebrand endothelial cells on serial sections. We used double staining for investigating colocalization of DR and ACE proteins with von Willebrand-positive cells using a commercial kit ( Vectastain, Elite, ABC Kit; Vector Laboratories). We tested the presence of neutrophils by immunostaining for elastase. The number of DR-positive (DR+) inflammatory (lymphocytes and macrophages) and endothelial cells was expressed as cells/mm².

RT-PCR Quantification of mRNAs for RAS Components, Chymase, Cytokines, MCP-1, and iNOS
RT-PCR was used to evaluate gene expression of renin, angiotensinogen (AGTN), ACE, AT1-R, AT2-R, chymase, inducible nitric oxide (iNOS), IL-1β, tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ, and monocyte chemotactic protein (MCP)-1. RT-PCR was performed as previously reported6 by means of specific primers, and gene levels were expressed as ratios to the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Characteristics of primers and operative conditions for RT-PCR are reported in the online section.

In Situ Hybridization Studies of mRNAs for AGTN, ACE, Cytokines and iNOS
The in situ hybridization procedure was performed with specific cDNA photobiotin-labeled probes6,14,21 on adjacent serial sections (see http://www.circresahajournals.org).

Immunohistochemical Analysis for Cytokine and iNOS Proteins
The endothelial source of cytokine proteins were assayed by double-staining immunohistochemical analysis using primary monoclonal antibodies against human TNF-α, IL-6, IFN-γ, and iNOS, and second primary monoclonal antibody against human von Willebrand according to the recommendations provided by the supplier ( Vectastain, Elite, ABC Kit; Vector Laboratories).

Statistical Analysis
Data are expressed as means±SD. Comparisons between groups were made with Student t test for paired and unpaired data. We used 1-way ANOVA followed by Tukey multiple-range comparison test,
as appropriate, to examine the differences among the 3 groups. Relationships between cardiac Ang II formation and severity of angiographic coronary lesions were tested by linear regression analysis. We used BMDP statistical software (BMDP Statistical Software Inc) for all calculations.

Results

Clinical Course
No patients had heart failure, myocardial infarction, or died before, during, or after the surgical procedure. Sixteen of 43 UA and 3 of 15 SA patients experienced anginal episodes during the interval between their enrollment and surgery; none of these episodes lasted >5 to 6 minutes. Only 3 patients experienced angina in the 2 days preceding surgery. After the randomization codex was opened, 2 of these patients turned out to be using placebo and the other 1 was using an ACE inhibitor. Collateral blood flow was angiographically demonstrable in 14 of 43 UA patients, and in 8 supplied the area of ventricular wall from which biopsy specimens were taken.

Cardiac Angiotensin Formation
Baseline concentrations of Ang I and II and PRA in peripheral venous blood of UA patients did not differ from values of SA patients or controls. By contrast, cardiac Ang II formation was higher (P<0.01) in UA than in SA patients or controls (6.9±4.8 pg/mL versus 0.7±0.6 pg/mL and −0.1±0.3 p/mL, respectively) (data are shown in Table III of the online data supplement available at http://circres.ahajournals.org.) No correlation was found between cardiac Ang II formation and severity of angiographic coronary lesions (r=0.23, NS).

Expression Levels of Cardiac RAS Components
RT-PCR revealed that AGTN, ACE, AT1-R, AT2-R, and chymase genes were expressed in all myocardial ventricular specimens from anginal patients who were using the placebo and from CH (Figure 1). mRNA for renin was not detected. The expression levels of AGTN, ACE, and AT1-R genes in UA biopsy samples were significantly higher (P<0.01 for all genes) than in those from SA or CH. There were not significant differences between nonischemic and potentially ischemic areas in the patients with LAD and CRA disease (Figure 1). AT2-R and chymase gene expression did not differ among anginal patients and controls (Figure 1).

Negative and positive controls for in situ hybridization studies showed that the signal was specific for mRNA and that the mRNA in the biopsy samples was intact. AGTN and ACE genes were expressed only in trace amounts in myocardial biopsy samples from SA and CH, whereas they were overexpressed in the biopsy samples from UA patients and were detected almost exclusively in microvessel endothelial cells (Figure 1).

Coronary Microvessel Inflammation and Ang II Blockade
Numerous cells that expressed HLA-DR molecules, were observed in UA biopsy samples, but were very rare in SA biopsy samples, and no DR+ cells were found in CH (Figure 2). The majority of DR+ cells was represented by endothelial cells because they were positively immunostained by both von Willebrand and CD31+ antibodies (Figure 2). The remaining DR+ cells were identified as macrophages and lymphocytes and were detected in the myocardial interstitium, but not in the lumen of microvessels. In SA hearts, lymphocytes and macrophages minimally contributed to DR positivity and their number was significantly lower than in UA hearts (Figure 2). In UA hearts, the number of DR+ cells did not differ between nonischemic and potentially ischemic areas (Figure 2). Double immunostaining showed that DR and ACE proteins colocalized on von Willebrand–positive cells (ie, endothelial cells) (Figure 3). No elastase-positive cell was detected.

RT-PCR revealed that expression of mRNAs for cytokines and iNOS was negligible or absent in CH. In SA biopsy
specimens, only TNF-α and iNOS genes were clearly expressed at a higher level than in CH (P<0.05 and P<0.01, respectively) (Figure 4). Conversely, in myocardial biopsy specimens from UA patients TNF-α, IL-6, IFN-γ, and iNOS genes were overexpressed from 1.6-fold for TNF-α (P<0.001) to 7.1-fold for IL-6 (P<0.001) when compared with SA hearts (P<0.001). IL-1β and MCP-1 genes were barely expressed in both SA and UA hearts (Figure 4). In patients with LAD and CRA disease, expression levels of mRNA for cytokines and iNOS did not differ between nonischemic and potentially ischemic areas (Figure 4).

In situ hybridization studies revealed that in SA biopsy specimens, signals for TNF-α and iNOS appeared only in trace amounts on endothelial cells, and signals for IFN-γ and IL-6 were undetectable. By contrast, in UA biopsy samples, genes for cytokines and iNOS were overexpressed and almost exclusively localized in the endothelium of microvessels (online Figure I, see http://www.circresahajournals.org). Cardiomyocytes did not express mRNAs either for cytokines or for iNOS (online Figure I). Double staining showed that immunoreactivity for TNF-α, IL-6, IFN-γ, and iNOS proteins colocalized with von Willebrand-positive cells (Figure 5). No signal was detected on cardiomyocytes (Figure 5). The cellular localization of the genes for the various cytokines and iNOS did not differ between nonischemic and potentially ischemic areas. We did not find any immunoreactivity for cytokine and iNOS proteins in CH, and only weak signals for TNF-α and iNOS proteins were found in SA biopsy specimens.

Ramipril or valsartan treatment significantly reduced the mRNA expression levels of cytokines and iNOS in comparison with biopsy specimens from UA patients using placebo without differences between the 2 treatments (Figure 6). The decrease in the expression levels of TNF-α and IL-6 was 91±7% and 89±5%, respectively, and that of iNOS was 78±5%, whereas IFN-γ gene expression decreased, 53±5%, significantly less than those of the other genes (P<0.01). Conversely, the blockade of Ang II activity did not significantly change either the total number of DR⁺ cells or the number of the endothelial and inflammatory cells (Figure 7). The effect of Ang II blockade slightly, but not significantly,
modified mRNA expression levels of RAS components and was similar in potentially ischemic and in nonischemic areas (data not shown).

Discussion

Cardiac RAS Activation and Myocardial Ischemia

The present results provide additional evidence\textsuperscript{14} that cardiac Ang II formation is increased in UA patients and suggest that locally generated Ang II plays a role in coronary microvessel inflammation. This statement is supported by the presence of inflammatory cells (T lymphocytes and macrophages) and endothelial cells expressing DR molecules, and by the up-regulation of mRNAs for iNOS and various inflammatory cytokines (TNF-\(\alpha\), IL-6, IFN-\(\gamma\)). The expression of HLA-DR molecules on the inflammatory and endothelial cells as well as the overexpression of IFN-\(\gamma\) genes provide evidence of an immunomediated reaction,\textsuperscript{22} thus confirming our previous study.\textsuperscript{8} The possibility that both cardiac RAS activation and coronary microvessel inflammation may be a reaction to myocardial necrosis,\textsuperscript{23} surgery,\textsuperscript{24} or ischemia should be considered. Myocardial necrosis or surgery may be reasonably excluded as causes of RAS activation and microvessel inflammation because the enrolled UA patients were troponin-negative, and myocardial biopsies were performed before extracorporeal circulation. The lack of differences in the cytokine levels and in the number and types of DR\textsuperscript{7} cells between the nonischemic and potentially ischemic areas in the UA patients with LAD and CRA disease without lesions of left circumflex (LCX) artery makes it highly unlikely that the RAS activation and coronary microvessel inflammation are consequence of myocardial ischemia. Acute myocardial ischemia can induce an inflammatory reaction according to the classic scheme of ischemia reperfusion injury. In this condition, neutrophils are principally involved, but the absence of elastase in our biopsy specimens is against this hypothesis. Likewise, various histological and molecular features differentiate the coronary microvascular inflammation found in UA patients from the late phase of ischemic preconditioning and hibernating myocardium. In the late phase of ischemic preconditioning, IL-1\(\beta\) and TNF-\(\alpha\) genes are coexpressed and iNOS is selectively upregulated in the myocytes,\textsuperscript{25,26} but not in small vessels and fibroblasts as, instead, we found. Hibernating myocardium is associated with degeneration of cardiomyocytes, loss of contractile material, fibrosis, and MCP-1 gene overexpression.\textsuperscript{27,28} By contrast, all these findings were not observed in UA coronary microvessel inflammation.

Ang II–Dependent Inflammation

ACE inhibitor (ramipril) or AT1-R antagonist (valsartan) treatment markedly reduced (from 70% to 90%) the expres-
Neri Serneri et al  Angiotensin in Unstable Angina Heart Inflammation 1635

Figure 6. A, Effect of ramipril and valsartan on expression of mRNAs for GAPDH, TNF-α, IL-6, IFN-γ, and iNOS in UA biopsy specimens. Representative experiments are shown. B, Bar graphs show densitometric quantification of RT-PCR products of cytokines and iNOS after Ang II blockade (* P<0.01 vs placebo). Bars show mean±SD of densitometric quantification obtained from specimens of 14 patients for each of the 2 groups.

Figure 7. Top, Effect of Ang II blockade on the presence of DR+ cells in UA specimens. The figures show representative data for each treatment (A, placebo; B, ramipril; C, valsartan). Immunostaining signal was revealed by avidin–biotin peroxidase system (magnification ×400). D, Bar graphs show the effect of Ang II blockade on the mean number±SD of all DR+ cells, T lymphocytes (CD3+), macrophages (CD68+), and endothelial cells (CD31+) in biopsy specimens from UA hearts.

Immunomodulated Inflammatory Component

The Ang II-dependent inflammation was associated with an immunomodulated inflammatory component demonstrated by the expression of HLA-DR molecules on T cells, macrophages, and endothelial cells, as well as by the overexpression of IFN-γ gene and protein, which reflects a recent immunological reaction.22 ACE inhibition and AT1-R blockade caused a marked decrease in the expression levels of TNF-α, IL-6, and iNOS genes, whereas they did not affect the number of inflammatory cells and decreased the expression levels of the IFN-γ gene significantly less than the other cytokines (−53±5% versus −84±9%, P<0.01). These data suggest a major effect of Ang II to stimulate expression of some, but not all, cytokines by endothelial cells rather than to influence activated (ie, DR+) inflammatory cells. However, these findings do not exclude the fact that Ang II can affect T-cell activity, because these cells are provided with AT1 receptors.35,36 And Ang II increases the number of IFN-γ-secreting cells37 and stimulates the production of IFN-γ and IL-2.30 The decrease in the IFN-γ gene expression after ramipril or valsartan administration was milder than that of differences between the 2 drugs in the cytokine reduction exclude a nonspecific effect. The incomplete reversion of cytokine and iNOS levels after Ang II blockade does not speak against the notion that Ang II plays a role in the microvessel inflammation. This partial effect may result from a variety of causes, including a nonoptimum dosage and duration of ramipril or valsartan administration, the impossibility to fully inhibit cardiac RAS, and the possible contribution of AT2 receptors in inflammatory signal transduction mechanism.29 Thus, the notable and ready attenuation of the expression levels of cytokine and iNOS genes corroborate the hypothesis that the increased cardiac Ang II generation plays a major role in the Ang II-associated inflammatory process. It is, however, to be emphasized that in the present study, ramipril or valsartan was used as a tool for investigating the contribution of Ang II to the UA microvessel inflammation and not for a clinical assessment of UA treatment for which large-scale clinical trials are required.

In this study, we did not investigate the mechanisms by which Ang II induces the expression of the inflammatory genes in UA myocardium. Specifically addressed studies are needed because Ang II can elicit cellular inflammatory responses through a variety of signal transduction mechanisms, such as nuclear factor (NF)-κB and activating protein (AP)-1.30 Moreover, Ang II may induce cellular responses through other molecular pathways, such as free radical generation, particularly through NAD(P)H complex,31,32 that mediates the activation of Akt/protein kinase B33 and JAK-STAT signaling.34 The inhibitory effect of ramipril and valsartan on the expression of TNF-α, IL-6, and iNOS genes indirectly suggests an Ang II-dependent NF-κB activation, because these genes and iNOS are controlled by this system. The lack of MCP-1 gene activation suggests that AP-1 and Rho were not activated because MCP-1 expression is mediated by these signal transduction systems.30,34 However, no certain conclusion can be drawn because the timing and the duration of MCP-1 activation are not well-defined.
the other cytokines and is consistent with the minor effect of Ang II on the immunomediated component than on the nonspecific inflammatory component.

The overexpression of IFN-γ gene on the microvascular endothelial cells reflects the immunological activation of the endothelium, which is followed by endothelial ACE activation, because there is evidence that IFN-γ induces the transcription of ACE gene and upregulates its enzymatic activity in the endothelial cells. It is noteworthy that the enhanced Ang II formation in UA patients is essentially caused by a selective increase in ACE activity. This mechanism appears to be different from that responsible for the enhanced Ang II generation in heart failure, which is mainly supported by an increased cardiac Ang I formation, related to the elevated plasma renin activity.

Microvessel Inflammation and Unstable Angina

The clinical significance of this study remains to be established. The locally increased Ang II formation does not seem to directly contribute to myocardial ischemia with its vasoconstrictor activity, because coronary vascular resistance was not augmented and Ang II appears to be generated downstream the resistance arterioles. However, it is worth stressing that we investigated our patients from 9 to 18 days after the onset of anginal attacks. Therefore, it cannot be ruled out that the inflammatory process of coronary microvessels, which is presumably more intense at the onset of angina, can hamper coronary flow at the capillary level, and so may contribute to myocardial ischemia. Only additional specifically addressed studies could clarify this issue. Likewise, the potential clinical meaning of the inhibition of Ang II activity remains to be investigated. The present study has a pathophysiological significance because it extends current knowledge of UA by demonstrating that the inflammatory process that supports UA is not confined into the culprit plaques, but is widespread in the coronary vascular bed. This fact challenges the concept of a single vulnerable plaque as the pathological basis of UA. Our results are consistent with recent angiographic, angioscopic, and intracoronary ultrasound investigations that, in patients with myocardial infarction or UA, have shown the simultaneous presence of numerous eroded and thrombosed plaques, diffused on the coronary arteries, even if only 1 plaque may be the culprit plaque. Our research, together with these studies, suggests that the pathomorphological substrate underlying the majority of acute coronary syndromes is not a fissuration or rupture of individual plaque, but rather an acute widespread inflammatory process of the coronary vascular bed, in the context of which 1 or more atherosclerotic plaques may easily break. Both the activated plaques and coronary microvessel inflammation show similar cellular and molecular characteristics, including the upregulation of local RAS, the high number of DR lymphocytes and macrophages, and the endothelial activation. This points to common mechanism(s) underlying both conditions. The high number of DR cells, the upregulation of IFN-γ gene and the overexpression of IL-2 receptor on plaque lymphocytes suggest that immunological factors may have a role in triggering UA.

Acknowledgments

The financial support of the Ministero dell’ Università e della Ricerca Scientifica e Tecnologica, Rome, Italy (Grant 9906108278) is gratefully acknowledged.

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*Circ Res.* 2004;94:1630-1637; originally published online May 6, 2004; doi: 10.1161/01.RES.0000130944.49657.b8

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Title of Manuscript:

Cardiac angiotensin II participates in coronary microvessel inflammation of unstable angina and strengthens the immunomediated component.

Online Supporting Data:

Expanded materials and methods section and supplementary results

Transmural left ventricle biopsies and tissue processing

Immediately after sternotomy and before inducing cardioplegia, transmural biopsies (10 x 0.5 mm) were taken through a biopsy needle (MN1416 diameter 2.1 mm; BIP Gembh). One of the biopsies was collected in sterile RNase-free tubes immediately frozen in liquid nitrogen in the operating theater and thereafter transferred to -80°C for storage. The other one was used for immunohistochemical studies. This sample was immediately immersed in buffered 10% formalin for 18 hours, dehydrated in an alcohol series (50%, 75%, 95% ethanol and absolute) and, then, in xylene and finally embedded in a paraffin block. The amount of tissue obtained was sufficient to allow quantification and localization of mRNAs and immunohistochemical studies of the corresponding proteins.

Immunohistochemistry

Paraffin blocks obtained from bioptic specimens were serially cut into sequential 3-5 µm sections by microtomy. Sections were deparaffinized with xylene (3 times for 5 min) and immediately treated with 3% hydrogen peroxide (10 min) to block endogenous peroxidase. After washing in phosphate buffered saline (PBS) (3 times for 5 min), non specific binding was blocked with normal blocking serum and the avidin-biotin blocking kit (Vector Laboratories) was applied. Sections were then incubated 30 min at room temperature with the primary antibody. All antibodies were diluted in PBS containing 10% normal blocking serum. After washing (3 times for 10 min) with PBS, the sections were incubated at room temperature for 30 min with biotinylated secondary antibody. After an extensive wash with PBS (3 times for 10 min), antibody binding was revealed with the avidin-biotin peroxidase complex technique (Vector Laboratories) and 3-amino-9-ethylcarbazole was used as the chromogenic substrate. Cell nuclei were counterstained with hematoxylin. Negative
controls were obtained by omitting the primary antibody. Each stained histological section was examined under a microscope (DMRB, Leica) connected to a computerized image-analysis system (Qwin, Leica). For double staining, sections were again incubated with a second primary antibody according to the procedure already described. The second primary antibody binding was revealed with the avidin-biotin peroxidase complex technique (Vector Laboratories) and 3,3′-diaminobenzidine (DAB) was used as the second chromogenic substrate. Each double stained section was again examined under the microscope.

**Immunohistochemical detection of inflammatory cells**
The presence and identification of activated inflammatory cells in the myocardium were determined by immunostaining for major histocompatibility class II antigen (HLA-DR) on sections adjacent to serial sections stained for macrophages, T lymphocytes and endothelial cells. Double staining was used to investigate the colocalization of both HLA-DR+ and angiotensin-converting enzyme (ACE) protein with von Willebrand positive endothelial cells. The presence of neutrophils was also investigated by immunostaining for elastase. The following primary antibodies were used: mouse monoclonal antibody anti human HLA-DR (Dako, 1:50 dilution), mouse monoclonal antibody anti macrophages CD68 (Dako, 1:50 dilution), mouse monoclonal antibody anti T lymphocytes CD3 (Santa Cruz Biotechnology, 1:30 dilution), mouse monoclonal antibody anti endothelial cells CD31 (Dako, 1:30 dilution) and von Willebrand (Sigma, 1:150 dilution), and mouse monoclonal antibody anti neutrophil elastase (Dako, 1:40 dilution). The entire stained biopsied sample was scanned using Adobe Photoshop software (Adobe Systems) and a Leaf Microlumina digital camera. The number of DR positive (DR+) inflammatory (lymphocytes and macrophages) and endothelial cells was expressed as cells/mm².

**Immunohistochemical analysis for cytokines and iNOS**
Cytokine proteins were assayed by immunohistochemical analysis according to the avidin-biotin peroxidase method. Double staining was used to investigate the colocalization of cytokine and inducible nitric oxide synthase (iNOS) proteins with von Willebrand positive endothelial cells. The following primary antibodies were used: mouse monoclonal antibodies against human interferon(IFN)-γ (Sigma, 1:50 dilution), interleukin(IL)-6 (RD System, 1:400 dilution), tumor necrosis factor(TNF)-α (Santa Cruz Biotechnology, 1:50 dilution) and iNOS (Transduction Laboratory, 1:50 dilution).
Reverse transcriptase-polymerase chain reaction (RT-PCR) quantification of angiotensinogen (AGTN), ACE, AT1 receptor (AT1-R), chymase, cytokine (TNF-α, IL-6, IFN-γ, IL-1β), monocyte chemoattractant protein (MCP)-1 and iNOS mRNA levels

Total mRNA was isolated from homogenized frozen samples using TRIzol reagent (BRL-Life Technologies), as outlined by the manufacturer, and reverse transcribed using oligo dT-20. Levels of mRNA for renin angiotensin system (RAS) components, chymase, cytokines, MCP-1 and iNOS were quantified by RT-PCR as previously described (1,2). To ensure that different amounts of RT-PCR products on myocardial biopsies were not due to different starting concentrations of mRNA, RT-PCR analysis was performed for the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA on serial 2-fold dilutions of cDNA for each sample. The last dilution giving a positive reaction for GAPDH was used to equalize the amount of cDNA used in each RT-PCR. The sequences of the primers used and RT-PCR conditions are showed in Table 2A and 2B. All primers were purchased from Pharmacia. The RT-PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide by UV light transillumination. Band density was analyzed using a computerized image densitometer (Qwin, Leica) and the expression level for each target gene was calculated as the ratio of the density of the target gene band versus that of the GAPDH band (densitometric ratio: mRNA for AGTN, ACE, AT1-R, TNF-α, IL-6, IFN-γ, IL-1β, MCP-1 and iNOS/mRNA for GAPDH).

The variability coefficients of the RT-PCR analysis was assayed by measuring band densities obtained for each target gene for 5 samples assayed five times in the same amplification and again in four different amplifications. The intra-assay and inter-assay variation coefficients were 4.1±1.2% and 4.8±1.4%, respectively.

Localization of AGTN, ACE, TNF-α, IL-6, IFN-γ and iNOS mRNAs in the myocardium by in situ hybridization

The in situ hybridization procedure was performed using complementary cDNA photobiotin labelled probes for GAPDH (pHcGAP, ATCC n.57090), AGTN (HFBDR96, ATCC n.82996), ACE (kindly donated by Prof Soubrier, INSERM, Hopital Saint-Louis, Paris, France), TNF-α (pE4, ATCC n.39894), IL-6 (PT7T3D, ATCC n.3127523), IFN-γ (p52, ATCC n.65948) and iNOS (ATCC n.1351820) purchased by Vector Laboratories as previously described in details (1,2). Localization of TNF-α, IL-6, IFN-γ, iNOS and ACE genes was performed on serial adjacent sections. The biotinylated probe was mixed in 60
µL hybridization buffer, applied to each section, and hybridizations were performed at 55°C overnight in humidified chamber. Washes included stringent wash in 1x SSC for 30 minutes and 0.1x SSC for 1 hour at 55°C. The streptavidin-biotinylated horseradish peroxidase complex in buffered sodium chloride was used as detection reagent and 3-amino-9-ethylcarbazole (AEC, Sigma) as chromogenic substrate was applied for 5 min at room temperature. The cell nuclei were counterstained with hematoxylin (Mayer's haemalum).

Negative controls were performed by testing the sections with hybridization mixture 1) without the probe, 2) after incubation with RNase A (0.05 mg/mL = 4.7 Kunitz units/mL) for 1 hour at 37°C, and 3) with application of inappropriate probe (plasmid vector pBR322). Positive controls were obtained for each sample using a cDNA probe for the constitutively expressed gene for GAPDH to ensure that mRNA in myocardial biopsies was intact. Each stained histological section was examined under a microscope (DMRB, Leica) connected to a computerized image-analysis system (Qwin, Leica) at x400 magnifications.
Legend of the online Figure.

In situ hybridization for TNF-α (A), IL-6 (B), IFN-γ (C), iNOS (D) and ACE (E) in adjacent serial sections of myocardial biopsies from UA patients. Positive mRNA signal was revealed by red-brown staining. In UA hearts mRNAs for cytokines, iNOS and ACE were expressed in interstitial and endothelial cells, but not in cardiomyocytes. Magnification x200.
References


Table 1. Characteristics of controls and anginal patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=10)</th>
<th>Stable angina (n=15)</th>
<th>Unstable angina (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/Females</td>
<td>7/3</td>
<td>13/2</td>
<td>32/11</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>53±11</td>
<td>64±7</td>
<td>59±12</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.2±6.9</td>
<td>78.5±6.9</td>
<td>77.4±7.8</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>6(60)</td>
<td>10(66)</td>
<td>31(72)</td>
</tr>
<tr>
<td>Cholesterolemia (mmol/liter)</td>
<td>4.2±0.9</td>
<td>5.4±0.7</td>
<td>5.6±0.7</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>1(10)</td>
<td>4(27)**</td>
<td>11(25)**</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>3(30)</td>
<td>7(47)*</td>
<td>20(46)*</td>
</tr>
<tr>
<td>Left ventricular mass (g/m$^2$)</td>
<td>113±14</td>
<td>112±13</td>
<td>113±12</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>61.3±5.4</td>
<td>53.6±5.2</td>
<td>54.6±6.1</td>
</tr>
<tr>
<td>Mean aortic pressure (mmHg)</td>
<td>96.2±11.5</td>
<td>99.2±11.4</td>
<td>100.7±10.3</td>
</tr>
<tr>
<td>Mean angiographic score$^#$</td>
<td>-</td>
<td>16.1±5.2</td>
<td>16.4±4.2</td>
</tr>
<tr>
<td>Coronary blood flow (ml/min)</td>
<td>85.1±8.6</td>
<td>81.5±4.6</td>
<td>83.4±12.9</td>
</tr>
<tr>
<td>CVR (mmHg/mL/min)</td>
<td>1.27±0.35</td>
<td>1.52±0.45</td>
<td>1.58±0.53</td>
</tr>
</tbody>
</table>

Localization of coronary lesions

<table>
<thead>
<tr>
<th>Localization of coronary lesions</th>
<th>-</th>
<th>9(60)</th>
<th>27(63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAD+CX (%)</td>
<td>-</td>
<td>4(27)</td>
<td>12(28)</td>
</tr>
<tr>
<td>LAD+CRA (%)</td>
<td>-</td>
<td>2(13)</td>
<td>4(9)</td>
</tr>
</tbody>
</table>

LVEF: left ventricular ejection fraction, CVR: coronary vascular resistance. Values are mean±SD or number (percentage).

*p<0.05, **p<0.01 vs controls.

$^#$ according to the American Heart Association.
Table 2A. Primer sequences and PCR conditions for RAS components

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Annealing temperature (°C)</th>
<th>Cycles (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (3)</td>
<td>Forward 5'-TGAAGGTCGGAGTCAACGGGATTGTG-3'   983pb  63  32</td>
<td>Reverse 5'-CATGTTGGGCCCATGAGGTCCACCAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGTN (3)</td>
<td>Forward 5'-CTGCAAGGATCTTTATGACCTG-3' 217bp  52  33</td>
<td>Reverse 5'-TACACAGCAAACAGGAATGGGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE (4)</td>
<td>Forward 5'-CCACATCAACCACAGAGCAAGATTC-3' 468bp  61  33</td>
<td>Reverse 5'-GGTGCTCTCGTACATAGACCTCCACGAGTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1-R (5)</td>
<td>Forward 5'-GATGATTGTCCCAAAGCTG-3' 255bp  51  33</td>
<td>Reverse 5'-TAGGTAATTGCCCAAAGGGCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2-R (5)</td>
<td>Forward 5'-AGTAAGCAGAATTCAAG-3' 293bp  58  33</td>
<td>Reverse 5'-AGTAAAGAATAGGAATTTGCAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHYMASE (6)</td>
<td>Forward 5'-GAAGGAGAAAGCCTGACCCTG-3' 323bp  60  35</td>
<td>Reverse 5'-CATCCGACGGCTCCATAGGATACGATC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENIN (7)</td>
<td>Forward 5'-AAATGAAGGGGTGTCTG-3' 376bp  62  35</td>
<td>Reverse 5'-AAGCCAATGCGTTGTTACGC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number within brackets indicates the respective reference.
Table 2B. Primer sequences and PCR conditions for cytokines and iNOS.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Annealing temperature (°C)</th>
<th>Cycles (n)</th>
</tr>
</thead>
</table>
| GAPDH (3) | Forward 5'-TGAAGGTCGGAGTCAACGGATTTG-3'  
Reverse 5'-CATGTGGGCCCATGAGGTCCACCAC-3' |
| 983pb | 63 | 32 |
| TNF-α (8) | Forward 5'-GCCTGTAGCCCATGTTGTAG-3'  
Reverse 5'-AATGATCCCAAAGTAGACCTGCCC-3' |
| 438bp | 57 | 30 |
| IL-6 (9) | Forward 5'-ATGAACCTCTTCTCCACAAGCGC-3'  
Reverse 5'-GAAGAGCCCTCAGGCTGACTG-3' |
| 628bp | 65 | 32 |
| IFN-γ (10) | Forward 5'-GGACCCATATGTAAGGAAGCGAG-3'  
Reverse 5'-TGTCCTCTCCTTTCCAATTCT-3' |
| 121bp | 55 | 35 |
| iNOS (8) | Forward 5'-CGGTGTCTGTATTTCTTACGAGCGAAG-3'  
Reverse 5'-GGTGTACTTTGTTAGAGAAGGAAGG-3' |
| 257bp | 62 | 35 |
| MCP-1 (11) | Forward 5'-TTGTGTGCTGCTGCTCATA-3'  
Reverse 5'-GGTTGTGTGCTGCTCATA-3' |
| 259bp | 55 | 35 |
| IL-1β (12) | Forward 5'-AGTACCTGAGCTCGGCAGTGAA-3'  
Reverse 5'-TACGATCCTGAACTGCAGCC-3' |
| 391bp | 55 | 35 |

Number within brackets indicates the respective reference.
Table 3. PRA and Ang I and II concentrations in peripheral venous blood and Ang I and II aorta-coronary sinus gradients (mean±SD).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=10)</th>
<th>Stable angina (n=15)</th>
<th>Unstable angina (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral venous blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRA (ng/ml/h)</td>
<td>0.8±0.2</td>
<td>0.8±0.3</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Ang I (pg/ml)</td>
<td>13.9±4.0</td>
<td>13.8±3.9</td>
<td>14.1±4.2</td>
</tr>
<tr>
<td>Ang II (pg/ml)</td>
<td>9.3±1.9</td>
<td>9.6±1.9</td>
<td>9.5±1.8</td>
</tr>
<tr>
<td><strong>Ang I (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td>13.4±3.7</td>
<td>13.8±4.1</td>
<td>13.6±3.8</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>11.8±2.7</td>
<td>12.1±2.6</td>
<td>20.5±4.3*</td>
</tr>
<tr>
<td>A-CS gradient</td>
<td>-1.6±1.2</td>
<td>-1.7±1.3</td>
<td>7.9±3.5*</td>
</tr>
<tr>
<td>Cardiac output#</td>
<td>-136.2±10.3</td>
<td>-144.7±59.8</td>
<td>672.3±174.2*</td>
</tr>
<tr>
<td><strong>Ang II (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td>9.1±1.9</td>
<td>9.4±1.8</td>
<td>9.3±1.9</td>
</tr>
<tr>
<td>Coronary sinus</td>
<td>8.3±1.2</td>
<td>8.6±1.5</td>
<td>16.2±5.1*</td>
</tr>
<tr>
<td>A-CS gradient</td>
<td>-0.1±0.3</td>
<td>0.7±0.6</td>
<td>6.9±4.8*</td>
</tr>
<tr>
<td>Cardiac output#</td>
<td>-8.5±2.6</td>
<td>-57.1±2.8</td>
<td>575.5±61.9</td>
</tr>
</tbody>
</table>

Values are mean±SD or number. A: aorta, CS: coronary sinus, CBF: coronary blood flow.

#cardiac output: A-CS gradient related to CBF (pg/min)

*p<0.01 vs controls and SA