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Abstract—Angiotensin II (Ang II) is the major vasoactive component of the renin-angiotensin system. Several components of the renin-angiotensin system have been demonstrated in different tissues. Whereas the roles of tissue and renal renin-angiotensin system have been studied in detail, much less is known on whether the corpuscular elements of circulating blood contribute to Ang II production. Here we examined whether, in addition to vasculature, blood cells also contribute to the circulating Ang II levels. Mononuclear leukocytes were obtained from healthy subjects and were incubated. The resulting supernatant was chromatographed using different chromatographic methods. The vasoconstrictive effects of aliquots of the resulting fractions were tested. Each fraction with a vasoconstrictive effect was analyzed by mass spectrometry. In one fraction with a strong vasoconstrictive effect, Ang II was identified. Mononuclear lymphocytes produced Ang II in amounts sufficient to stimulate Ang II type 1 receptors. Moreover, in mononuclear leukocytes, renin as well as angiotensin-converting enzyme mRNA expression was detectable by RT-PCR. These findings demonstrate that mononuclear leukocytes are a source of Ang II. Ang II secretion by these cells may play a significant role in humoral vascular regulation. In conclusion, the isolation of Ang II in supernatants of mononuclear leukocytes adds a further physiological source of Ang II to the current view of angiotensin metabolism. The quantitative role of lymphocyte-derived Ang II secretion compared with the other sources of Ang II should be defined further, but the release found under the present conditions is at least sufficient to elicit vasoconstrictive effects. (***Hypertension*****. 2005;46:591-597.)**

Key Words: angiotensin II ■ leukocytes ■ vasoconstriction ■ chromatography ■ angiotensin II receptors

Whereas angiotensin II (Ang II) was first regarded merely as a potent vasoconstrictor, at present, its functions as a growth factor and as a cytokine are more and more recognized. The renin-angiotensin system (RAS) is known to mediate systemic Ang II production,[1,2] but Ang II is also produced locally in many tissues.[3–5] This local Ang II production, depending on tissue RAS, has recently attracted growing interest.[6–8]

In the classical RAS, circulating renal-derived renin produces Ang I by cleaving angiotensinogen. In the lungs, Ang I is converted to Ang II by the angiotensin-converting enzyme (ACE).[9–11] ACE is found in plasma as well as in most organs. Tissue ACE may play a role in the regulation of tissue perfusion.[12] Angiotensinogen and ACE, but not renin, have been found to be produced in all layers of the vessel wall.[13–15]

Therefore, local generation of Ang II is probably dependent on circulating renin. Of potential relevance to the pathophysiological role of RAS is the recent observation indicating that adipose tissue is a significant source of circulating angiotensinogen and hence possibly contributes to the regulation of blood pressure and sodium homeostasis.[16]

Whereas the roles of tissue and renal RAS have been studied in detail, much less is known on whether the corpuscular elements of circulating blood contribute to Ang II production. Therefore, we also examined whether cellular components of the circulating blood contribute to the circulating Ang II levels. The experiments showed that, indeed, a fraction of circulating mononuclear leukocytes is a source of Ang II in humans, especially if they are activated. These findings are more relevant because leukocyte activation and adhesion have been linked to vascular damage.[17]

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Methods

Chemicals

Details of the chemicals used in this study are available in the online supplement at http://www.hypertensionaha.org.

Mononuclear Leukocyte Isolation

Preparation of Mononuclear Leukocytes

The description of the mononuclear leukocytes preparation is available in the online supplement.

Incubation of Mononuclear Leukocytes

Mononuclear leukocytes were incubated without or with 10 mg L^{-1} lipopolysaccharide (LPS; LPS serotype 0111:B4 from *Escherichia coli*; Sigma-Aldrich) or 200 nmol/$L^{G\text{-}1}$ formyl-Met-Leu-phenylalanine (fMLP).[18] After incubation for 60 minutes, mononuclear leukocytes were centrifuged and the supernatant analyzed.

Isolation of CD8+ (T Lymphocytes) and CD19+ (B Lymphocytes)

The description of the isolation of CD8+ and CD19+ is available in the online supplement.

Analytical Techniques

The description of the chromatographic and mass-spectrometric procedures is available in the online supplement.

Measurement of Perfusion Pressure in Isolated Perfused Rat Kidney

The effects of aliquots (1/10) of the lyophilized fractions of the reversed-phase chromatography on vascular tone, aliquots (1/10) of supernatant of mononuclear leukocytes, and synthetic Ang II were evaluated in an isolated rat kidney perfused with a constant flow while perfusion pressure was monitored continuously. Details of the preparation are given previously[19] as well as in the online supplement.

Vasoconstrictor responses of the isolated perfused rat kidney were assessed at basal tone after an equilibration period of 30 minutes. Samples were dissolved in 200 μL of the perfusion solution described above. To characterize the receptor mediating the vasoconstrictive effect of the fraction, aliquots of fractions were also tested after the Ang II receptor antagonist saralasin (50 μmol/L^{-1}) was added to the perfusate, 30 minutes before challenge with the fractions to be tested.

Molecular Methodology

Preparation of RNA and RT-PCR

The preparation of RNA and the RT-PCR conditions are available in the online supplement.

Detection of Renin and ACE Activity of Mononuclear Leukocytes by MALDI Mass Spectrometry

To detect the renin and ACE activity of mononuclear leukocytes, a previously described method was used.[20] The description of the method is available in the online supplement. Briefly, the proteins potentially present in the lysate of mononuclear leukocytes were immobilized to bromine-cyan (CNBr)–activated Sepharose 6 macrobeads (MB; Amersham-Pharmacia Biotech). Next, 25-μL beads containing the immobilized proteins were transferred into a 500-μL reaction vial. A total of 10 μL of a suspension containing 5×10^{-4} mol/L^{-1} renin-substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser), 8×10^{-5} mol/L^{-1} Ang I, or 2×10^{-6} mol/L^{-1} angiotensinogen (from human plasma) was added. Moreover, to detect whether angiotensinogen was present in the cytosol of mononuclear leukocytes, 25-μL beads containing the immobilized proteins were incubated with the lysates of mononuclear leukocytes, prepared as described in the online supplement.

Each incubation experiment was performed in the absence and in the presence of an ACE inhibitor (Bachem; Ang I–converting enzyme [Cyanoac-Phe-Phe-OH]; 10^{-3} mol/L^{-1}) and a renin inhibitor (D-His-Pro-Phe-His-Leu-Psi-[CH_2NH]-Leu-Val-Tyr), which was used at a concentration achieving maximum renin inhibition. This inhibitor was used at a concentration achieving maximum renin inhibition (10^{-5} mol/L^{-1}).[21] From the reaction mixture, 0.5-μL aliquots were removed after 5 minutes, 2 hours, 4 hours, 12 hours, and 48 hours for analysis of the reaction products. The products of the enzymatic activity were analyzed by MALDI mass analysis using the conditions described above.

Verification of renin, ACE, angiotensinogen, Ang I, and Ang II content of the isotonic NaCl solution was used as washing step for isolation of mononuclear leukocytes.

To verify the efficiency of the washing procedure of the mononuclear leukocytes in removing plasma renin, ACE, and angiotensin, the proteins potentially present in the isotonic NaCl solution of the third washing step were also immobilized to activated CNBr–Sepharose 6 MB beads (Amersham-Pharmacia Biotech) using the method described in the online supplement. Next, these CNBr–Sepharose 6 MB beads were incubated with renin substrate (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) or Ang I in the absence or presence of ACE inhibitor and a renin inhibitor (D-His-Pro-Phe-His-Leu-Psi-[CH_2NH]-Leu-Val-Tyr) as described above. Moreover, the amount of Ang I and Ang II in the kept isotonic NaCl solution of the third washing step was quantified by chromatographic assays as described above.

Verification of Ang II Determination by MALDI Mass Spectrometry With Ang II Radioimmunoassay

Details of the verification of Ang II determination by MALDI mass spectrometry (MS) with the Ang II radioimmunoassay (RIA) are available in the online supplement.

Statistics

All data are presented as mean±SEM. Where error bars do not appear in the figure, errors are within the symbol size. Statistical analyses were performed with the Wilcoxon-Mann–Whitney test or with the Kruskal–Wallis test for multiple comparisons.

Results

After isolation of human mononuclear leukocytes from human blood by centrifugation, the cell viability was 98.1±0.4% (n=4). After incubation at 25°C for 60 minutes with or without stimuli, the supernatant was concentrated and fractionated by cation-exchange chromatography. A characteristic chromatogram is illustrated in Figure 1A. The fractions of each gradient step of the cation-exchange chromatography were then further chromatographed by reversed-phase chromatography. The reversed-phase chromatography, on the one hand, allows us to desalt the eluate of the cation exchanger and, on the other hand, to further fractionate the eluate.

The fractions obtained from each reversed-phase chromatography were tested for vasoactivity. In Figure 1A, the fraction yielding vasoactive on further fractionation is labeled by the arrow. The reversed-phase chromatography obtained with this fraction is shown in Figure 1B. The vasoconstrictive fraction as detected is indicated by the arrow.

The vasoconstrictive substance contained in the vasoactive fraction labeled in Figure 1B was identified by MS. In this fraction, a peptide with a molecular weight of m/z of 1046.5 was detected (Figure 2A). This peptide was fragmented to obtain sequence information that could be used for identification by searching in the Swissprot database (Figure 2B). Human Ang II was the highest-ranking match for the peptide at m/z 1046.5, with complete amino acid sequence coverage. This result was confirmed by comparison to a fragment ion

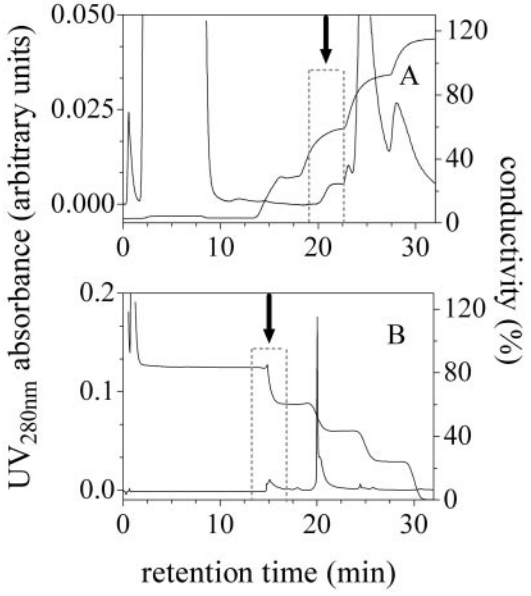

Figure 1. A, Cation-exchange chromatography of the supernatant of mononuclear leukocytes after 60-minute incubation. The vasoactive fraction is indicated by the arrow, the dotted line showing the extent of the fraction. B, Reversed-phase chromatography of the eluate of the cation-exchange chromatography. The fraction with a vasoconstrictive effect is indicated by the arrow, the dotted line showing the extent of the fraction.

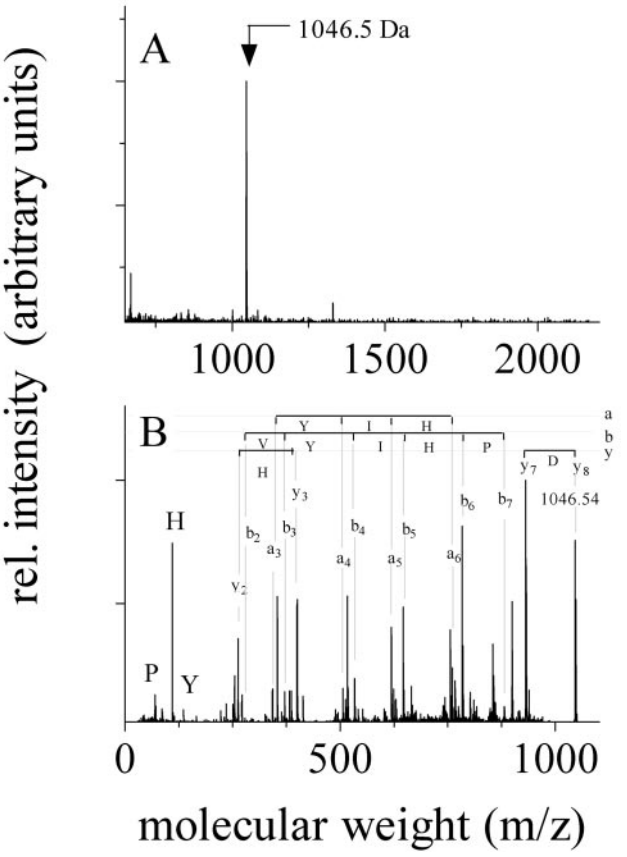

Figure 2. A, Identification of the substance with the vasoconstrictive effect labeled in Figure 1B by the arrow. MALDI-TOF mass spectra obtained from the vasoconstrictive fraction of the reversed-phase high-performance liquid chromatography separation. B, Fragment ion mass spectrum of the peptide at m/z 1046.5. In the top part of the panel, the amino acid sequences for the matched a, b, and y-ion series are indicated.

spectrum of purified human Ang II (Bachem). The mass signal of Ang I was not detected in Figure 2A because Ang I was not eluted in the same cation-exchange fraction as Ang II.

Figure 3A shows that, indeed, Ang II and the vasoactive fraction obtained from lymphocytes show very similar profiles of vascular effects. In 5 experiments, the effect of the vasoconstrictive fraction amounted to 74.1±8.4 mm Hg (Figure 3B). In the presence of saralasin, the vasoconstrictive effect decreased by 59.8±1.4%. A comparison to the concentration-response curve of Ang II (Figure 3C) shows that if the observed hemodynamic changes are attributable of Ang II, indeed, significant amounts of Ang II can be assumed to be present in this fraction.

From these findings, the question arose of which fraction of the mononuclear cells would be responsible for the production of Ang II. Therefore, the different fractions of mononuclear leukocytes were separated and were tested for expression of angiotensinogen, renin, and ACE. As shown in Figure 4, CD8+ (Figure 4A) and CD19+ (Figure 4B) expressed angiotensinogen–cDNA but also renin–cDNA and ACE–cDNA, thus being capable of producing Ang II without the need of being supplied with any of its precursors. Furthermore, in CD8+ and CD19+ lymphocytes, Ang II production was monitored separately. In both lymphocyte subsets, Ang II production was detectable by MS (Figure 4C and 4D). The identity of Ang II was confirmed by MS/MS time-of-flight/time-of-flight (TOF-TOF) analysis.

To also confirm the detection of lymphocytic angiotensinogen, renin, and ACE expression on a functional level, mass spectrometric Ang I and Ang II measurements were done under conditions chosen to prove the existence of these proteins. Immobilized lymphocytic proteins were incubated with lymphocytic cell lysate for 8 hours. Under these conditions, Ang II production was observed but was completely abolished by adding the renin inhibitor (data not shown). Ang I and Ang II production was also demonstrable when, instead of lymphocytic cell lysate, the synthetic renin substrate Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser was added (Figure 5A). Ang II production was abolished in the presence of a renin inhibitor (Figure 5A). Moreover, Ang II production was detectable after incubation with Ang I (Figure 5B). From these findings, the presence of renin in mononuclear leukocytes and the presence of the substrate of renin, angiotensinogen, can be concluded. After adding an ACE inhibitor (Figure 5B), Ang II production was blocked completely, additionally indicating the presence of ACE in the immobilized lymphocyte extract. Moreover, to exclude that Ang I might have been produced from the renin substrate by some other enzyme than renin, because the synthetic renin substrate may be cleaved unspecifically by several peptidases, purified angiotensinogen was added instead of the renin substrate. The experiments revealed that also after addition of angiotensinogen, Ang I and Ang II were produced (Figure 5C). Again, the Ang II production was abolished by renin inhibitor (Figure 5C). After addition of renin substrate,

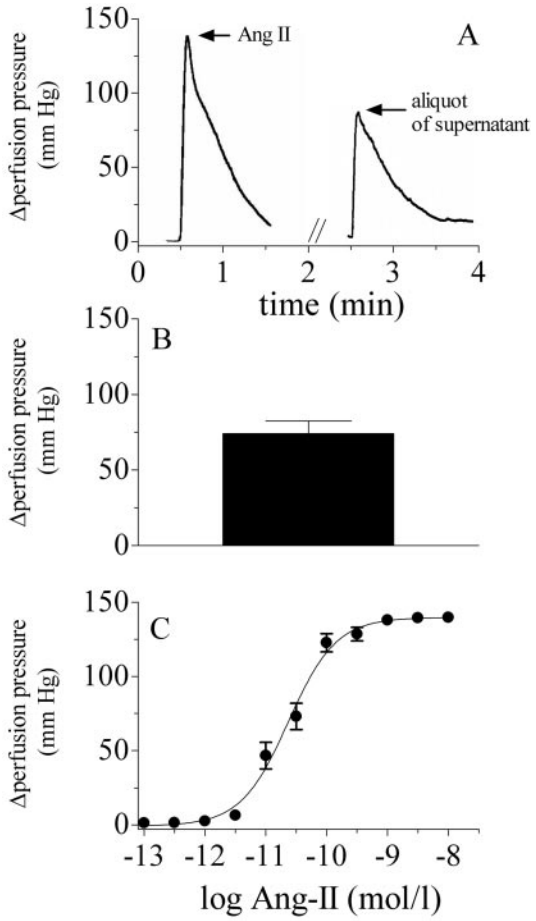

Figure 3. Effect of the supernatant of mononuclear leukocytes stimulated by 10 mg L^{-1} LPS on the perfusion pressure of the isolated perfused rat kidney. A, Original curve of the increase of the perfusion pressure of the isolated perfused rat kidney caused by synthetic Ang II (10^{-6} mol/L^{-1}) and an aliquot (100 μL) of the supernatant of mononuclear leukocytes. B, Quantification of the effect of an aliquot (100 μL) of the supernatant of mononuclear leukocytes on the perfusion pressure (n=5). C, Dose-response curve of synthetic Ang II on the perfusion pressure of the isolated perfused rat kidney.

Ang II concentrations in the supernatants exceeded those of Ang I, whereas after addition of angiotensinogen, Ang I was generated in higher concentrations than Ang II. These different patterns may be explained, assuming that renin has a higher affinity to angiotensinogen than to the renin substrate, so that Ang I is generated faster from angiotensinogen than from renin substrate. To ensure that mass spectrometry–derived data on Ang II concentrations are valid, we determined Ang II concentrations in supernatants from mononuclear leukocytes by mass spectrometry and a conventional RIA-based assay. Figure 5D shows that the mass-spectrometric method used in this study is in good accordance with the RIA-based method described by Hilgers et al.[22]

Next, we had to exclude that the detected renin and ACE activity was caused by contaminations from plasma renin and ACE. Therefore, the proteins of the isotonic NaCl solution of the third washing step of the isolation of mononuclear leukocytes were immobilized and incubated with renin substrate and Ang I. After an incubation time of 8 hours with renin substrate, no Ang II production was detected by the MALDI mass spectrometry assay (data not shown).

Next, the question arose whether mononuclear leukocytes do not only produce Ang II but also secrete Ang II into the circulation. The amount of Ang II in the supernatant was quantified (relative to internal standard). After a 120-minute incubation of mononuclear cells without stimulation, Ang II concentration in the supernatant was 0.17±0.05 (relative Ang II intensity [arbitrary units]). The addition of fMLP and LPS leads to a significant increase of Ang II concentration in the supernatant (fMLP versus LPS 0.37±0.06 versus 0.36±0.07 [relative Ang II intensity; arbitrary units]). From the quantitative relationship between MS signals and underlying Ang II concentrations, we estimated that after stimulation 9.9±6.6 fmol Ang II (10^6 lymphocytes)$^{-1}$ min^{-1} was secreted. The cell suspension used contained 5.08±0.98 10^3 mononuclear lymphocytes μL^{-1}, which is in the same order of magnitude as the mononuclear leukocyte count in human blood. For unstimulated cells, the same algorithm leads to an Ang II production rate of 4.7±1.7 fmol Ang II (10^6 lymphocytes)$^{-1}$ min^{-1}. Therefore, unstimulated lymphocytes should also contribute amounts of Ang II in the same order of magnitude as stimulated cells. To obtain a valid comparison between lymphocytic Ang II secretion rates and human Ang II plasma levels, the latter were also determined by mass spectrometry. In 6 healthy subjects, plasma Ang II concentrations of 1.80±0.80 10^{-10} mol/L^{-1} were found.

Discussion

The present findings demonstrate that human circulating mononuclear leukocytes are a source of Ang II, and that this secretion is sufficient to stimulate AT receptors. The mechanism whereby LPS stimulates Ang II release has been studied in different tissues.[23,24] These studies documented that LPS stimulates angiotensinogen mRNA expression and enhances angiotensinogen plasma concentrations. According to the present estimates, the amount of lymphocytes contained in 1 mL blood can produce ≈50 fmol Ang II per minute after stimulation and 20 fmol Ang II per minute without stimulation, thus increasing the concentration by 50 pmol/L^{-1} per minute after stimulation or 20 pmol/L^{-1} per minute without stimulation. In consideration of this secretion rate and given an EC_{50} in the nanomolar range,[25,26] lymphocytic Ang II production during ≈30 minutes would be sufficient to reach EC_{50}.

Obviously, from the rate of synthesis estimated in this study a steady-state plasma concentration, which is the net effect of synthesis and degradation, cannot be calculated without reliable data on degradation kinetics. Moreover, the degree of stimulation may be different between in vivo and in vitro conditions. Therefore, the conclusions that can be drawn from lymphocytic Ang II synthesis in vitro should be restricted to the point that the order of magnitude of lymphocytic Ang II production is sufficient to contribute to plasma Ang II.

Moreover, we demonstrated by a functional mass spectrometric assay that angiotensinogen, renin, and ACE are not only expressed in human lymphocytes but are also functionally relevant for lymphocytic Ang II production. Because after blocking each of these components, lymphocytic Ang II

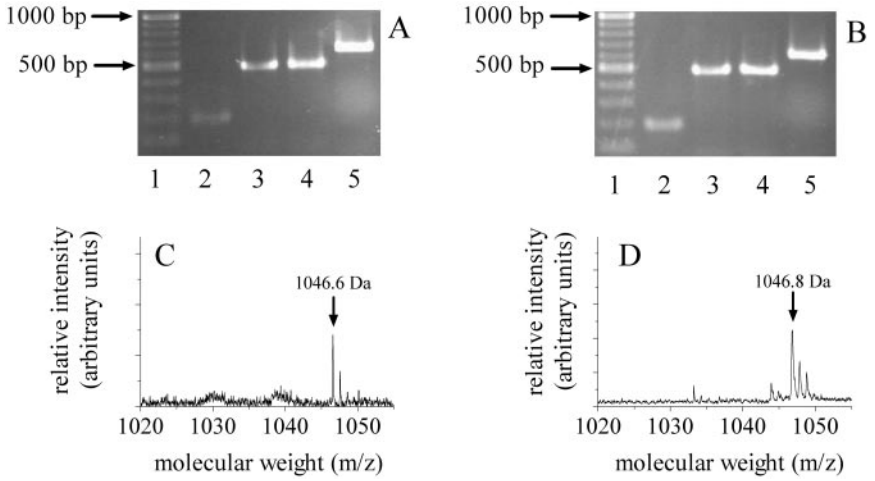

Figure 4. Representative gel electrophoresis of 4 experiments of products from RT-PCR using primer against ACE, renin, angiotensinogen, and β-actin cDNA. Lane 1, 100-bp marker; lane 2, renin; lane 3, ACE; lane 4, angiotensinogen; lane 5, β-actin from CD8+ cells (A) and CD19+ cells (B) . TOF-TOF mass spectra of the supernatants of CD8+ cells (C) and CD19+ cells (D). The molecular mass labeled in the spectra is conform to that of Ang II. The identity of Ang II was confirmed by MS/MS TOF-TOF analysis.

production was totally neutralized, other pathways of Ang II synthesis are not necessarily involved in these mononuclear leukocytes. From the present findings, it appears unlikely that mononuclear leukocytes merely take up Ang II by receptor internalization. The latter mechanism would lead to a continuous decrease of extracellular Ang II concentration, whereas in our experiments, extracellular Ang II increased continuously. We demonstrate that mononuclear lymphocytes produce and secrete Ang II, but we do not clarify whether these cells may also store Ang II.

There are several reports pointing out the possibility that in experimental animals and humans, Ang II might be produced by immune cells because the expression of several components of the RAS, such as ACE[27] and angiotensinogen,[28] has been demonstrated.

Several tissues have been shown to produce Ang II, including endothelial cells,[29] macrophages,[30] and adipocytes.[31] Moreover, angiotensin peptides are released by rat alveolar macrophages.[32] Local Ang II secretion was recognized as an important part of humoral vascular regulation. There have also been reports localizing Ang II in the cytoplasm of several types of leukocytes by immunocytochemistry, but secretion of Ang II by these cells, making them contributors to circulating Ang II levels, has not yet been demonstrated.[33] Furthermore, it has been shown that after adding angiotensinogen or Ang I to immune cells, Ang II production is increased,[34] indicating that all enzymes required

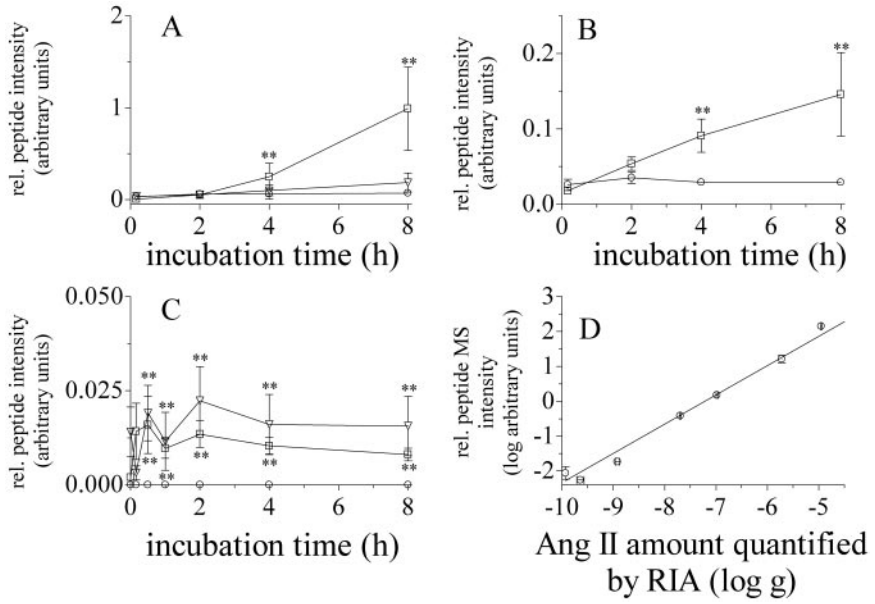

Figure 5. Detection of renin and ACE activity in a protein extract of mononuclear leukocytes by MALDI-MS. A, Incubation time–dependent generation by the protein extract of mononuclear leukocytes in the presence of renin substrate of the reaction products Ang I (▽) in the absence of a renin inhibitor, and of Ang II, either in the absence (□) or in the presence (○) of a renin inhibitor. B, Incubation time–dependent generation of the reaction product Ang II after the incubation of the protein extract of mononuclear leukocytes with Ang I in the absence (□) and in the presence (○) of an ACE inhibitor. C, Incubation time–dependent generation of the reaction products Ang I (▽) in the absence of a renin inhibitor and Ang II after the incubation of the protein extract of mononuclear leukocytes in the absence (□) and in the presence (○) of a renin inhibitor with purified angiotensinogen. Abscissa indicates incubation time; ordinate, ratios of the relative MALDI spectra signal intensities of the reaction product Ang II (n=5; *significance [calculated by Mann–Whitney test]) of relative peptide intensity in the absence or presence of the inhibitor used; $P<0.05$). D, Quantification of different Ang II concentrations by RIA vs MALDI mass spectrometry.

for Ang II production are present in these cells. However, it was not clear whether Ang II production in these cells was mainly mediated by renin and ACE, like in the classical pathway, or by other, renin- or ACE-independent enzymes.

What may be the specific role of lymphocyte-derived Ang II? We speculate that lymphocyte-derived Ang II has not only a direct vasoconstrictive effect but might also play a role in modulating immunologic processes in several ways. Using Agtr1a(−/−) mice, which lack Ang II type 1A (AT1A) receptors for Ang II, it was shown that Ang II triggers the proliferation of splenic lymphocytes.[35] These actions of the RAS to promote lymphocyte activation may contribute to inflammatory processes. Moreover, experimental mesangial proliferative glomerulonephritis developing in FcR-deficient mice surviving from lethal initial damage was prevented by an AT1 blocker.[36] Glomerular injury in FcR-deficient mice is associated with AT1 receptor–dependent CD4+ T-cell infiltration mediated by Ang II–activated renal mesangial cells showing chemotactic activity for T cells.[37] Furthermore, a role of the RAS has been demonstrated in interleukin-12 (IL-12) secretion by macrophages,[38] in hematopoietic processes in multiple cellular lineages including hematopoietic progenitor cells,[39,40] and in the regulation of transforming growth factor-b1 secretion by CD4+ cells.[41] Ang II is active as a cytokine stimulating IL secretion. Therefore, the Ang II production by lymphocytes may be regarded as part of an autocrine loop regulating lymphocytic immune response.

A yet unsolved issue regards the appropriate stimulus of lymphocytic Ang II secretion. Whereas in juxtaglomerular cells, the stimuli of renin secretion, which is the rate-limiting step of Ang II production from circulating angiotensinogen, are well known, the regulation of lymphocytic Ang II production has not been examined yet. Unlike juxtaglomerular cells, lymphocytes are not known to be sensors of whole body sodium chloride balance, thus being devoid of the afference felt to be most important in renin secretion. Partially analogous mechanisms may underlie renin secretion in lymphocytes and juxtaglomerular cells.[42]

In summary, circulating human CD8+ and CD19+ lymphocytes were shown to produce and to secrete Ang II. The findings thus add a further physiological source of Ang II to the current view of angiotensin metabolism. The physiological role of lymphocyte-derived Ang II secretion remains open. Nevertheless, the amounts of Ang II secreted by lymphocytes appear to be sufficient to stimulate AT receptors and to affect vascular tone.

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