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Up-regulated Circulating T-cell Angiotensin Converting Enzyme Gene Expression and Activity in Acute Coronary Syndromes

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Abstract

Background: T-cells are endowed with a functional cell-based renin angiotensin system (RAS), related with and independent by circulating RAS that can autonomously synthesize angiotensin (Ang) II. Angiotensing converting enzyme (ACE) is the key step for the regulation of the synthesis of Ang II by RAS and T-cell ACE gene expression was reported to be up-regulated in hypertensives with low grade inflammation. Ang II and T-cells play a major role in the systemic inflammation that occurs in unstable angina patients, but whether T-cell based RAS is directly involved is unknown.

This study was aimed at measuring ACE gene expression and enzymatic activity in the cell pellet and in the supernatant of human cultured circulating T-cells obtained from control subjects, hypertensive or unstable angina patients. C reactive protein (CRP) levels as a marker of systemic inflammation were also investigated.

Methods: mRNA for ACE gene expression was obtained in T cells isolated from peripheral blood and quantified by real time transcriptase–polymerase chain reaction (PCR); mRNAs for INF-gamma was semi-quantified versus the housekeeping gene glyceraldeyde-3-phosphate dehydrogenase (GAPDH) by reverse transcriptase (RT) PCR. ACE activity in cell pellet and in the culture medium (supernatant) was measured by the high performance liquid chromatography (HPLC) assay of a synthetic substrate. Plasma renin activity (PRA) and Ang II levels were measured by radioimmunoassay and high sensitive C Reactive Protein (hsCRP) by a commercial kit.

Results: In hypertensive and more markedly in anginal patients increased mRNA levels for ACE, augmented cell-based ACE enzymatic activity and Ang II levels were measured in cultured circulating T-cells when compared with data obtained in T cells from controls ($p<0.05$ for alls). The addition of Ang II to the T-cell pellet further increase ACE activity and Ang II synthesis. In anginal patients who showed the highest ACE gene expression and enzymatic activity and hsCRP values, Ang II stimulation of T-cells induced an almost complete release of ACE in the supernatant.

Conclusions: In anginal patients with hsCRP levels >3 mg/dl a marked up-regulation of circulating T-cell-based ACE gene expression and activity did occur in T-cell culture, further amplified by Ang II. According to our *in vitro* findings, *in vivo* activated T-cells could autonomously increase local *de novo* Ang II synthesis in tissues where they migrate and play a role in coronary plaque rupture and microvessel damage of unstable angina.

Keywords: Angiotensin-converting enzyme; Angiotensin II; T-cell; Unstable angina; Hypertension

Introduction

Growing evidence supported a tight relationship between Ang II and inflammation, because Ang II up-regulates the inflammatory response and inflammatory T-cells are fully equipped with RAS components and deliver new synthesized Ang II to sites of inflammation, independently of the activation of systemic and tissue-based RAS [1-3]. Human circulating T-cells but not B-cells are endowed with autonomous cell-based RAS [4].

Ang II participates in the recruitment of T-cells *via* the direct activation of inflammatory cells or *via* the up-regulation of the synthesis of adhesion molecules and/or chemokines by resident cells [2,3].

The ACE gene expression and enzymatic activity have a key-role in the increase and decrease in Ang II synthesis associated to systemic, local or cell-based RAS activation or inhibition [5]. An increased expression of ACE gene and activity was reported in circulating T-cells in hypertensive patients with low grade inflammation [6]; augmented levels of tissue ACE strictly related to the amount of infiltrated inflammatory T-cells were shown in coronary plaques and microvessels of left ventricular tissues of unstable angina patients [7,8], but the direct involvement of T-cells based RAS in unstable angina is still a matter of debate. In acute coronary syndromes (ACS) an imbalance of T-cell homeostasis does occur and a higher systemic frequency of activated T-cells compared with those with stable angina was reported; T-cell repertoire perturbation plays a pivotal role in coronary instability [9-12]; however, at the best of our knowledge whether and how an up-regulation of circulating T-cell based ACE gene expression and activity could participate to ACS inflammation was not studied.

The present study was aimed at investigating the pattern of T-cell based ACE gene expression and enzymatic activity (assayed in pellet and in supernatant) in cultured circulating T-cells obtained by ACS patients with high (>3 mg/dl) sensible protein C reactive (hsCRP) values. The same data were also obtained in T-cell from control subjects with normal hsCRP values and from hypertensives with low grade inflammation (hsCRP>2 mg/dl).

Methods

Subjects investigated

T-lymphocytes were obtained from 11 (6 males, 5 females, 57-69 years) patients with unstable angina (UA) in class IIIB of Braunwald's classification admitted to our Coronary Care Unit and from 15 (9 males, 6 females, 45-67 years) subjects with World Health Organization stage II essential hypertension who attended to our out-patients' clinic. Ten (6 males, 4 females, 39-56 years) normotensive subjects were enrolled in the study as control group comparable for age and sex to anginal patients and hypertensive group.

Diagnosis of UA patients was performed according to the 2015 European Society of Cardiology Guidelines for the management of ACS in patients presenting without persistent ST elevation [13].

Only patients with recent onset (7 days before admission) of angina who had at least 2 episodes of angina at rest or 1 episode lasting more than 20 minutes during the preceding 24 hours associated with transient ischemic ST-segment changes and high sensitivity troponin T levels <0.1 ng/mL (troponin T–negative) were included in the study. UA patients received nitrates.

Exclusion criteria were secondary forms of arterial hypertension, ischemic heart disease/acute coronary syndrome (only for hypertensive and controls) or stroke in the previous 6 months, impaired glucose tolerance or diabetes mellitus, renal failure (creatinine clearance <60 ml/min), presence of a major illness such as cancer, liver disease, chronic and acute inflammatory disease or infectious disease in the previous 3 months. No subject was on treatment with ACE-inhibitors, AT1 receptor antagonists, diuretics, statin or anti-inflammatory drugs. Main demographic and clinical characteristics of enrolled patients are shown in table 1.

Characteristics	Controls (n=10)	Unstable angina patients (n=11)	Hypertensive patients (n=15)	ANOVA
Males/Females	6/4	6/5	9/6	
Age (years)	46 ± 9	63 ± 6	56 ± 11	p<0.01
Smokers (%)	2 (20)	8 (67)	10 (66)	p<0.01
Hypertension (%)	0	7 (46)	15 (100)	p<0.01
Fasting glucose (mg/dl)	84 ± 9	89 ± 10	96 ± 5	ns
Total cholesterol (mg/dl)	188 ± 24	191 ± 45	193 ± 40	ns
HDL- cholesterol (mg/dl)	52 ± 11	54 ± 16	46 ± 17	ns
Creatinine (mg/dl)	0.79 ± 0.09	0.89 ± 0.15	0.89 ± 0.27	ns
LVM (g/m2)	106.3 ± 17.3	106.5 ± 18.0	107.0 ± 22.4	ns
LVEF (%)	60.4 ± 5.2	54.9 ± 5.1	53.6 ± 4.5	ns
LVM: Left Ventricular Mass; LVEF: Left Ventricular Ejection Fraction				

Table 1: Clinical characteristics of controls, unstable angina and hypertensive patients.

According to hsCRP values at enrollment, only hypertensive patients with low grade inflammation (hsCRP>2 mg/l) had been included; assay of hsCRP levels and other general chemical tests were done as a part of a routine medical check-up.

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 their written informed consent to participate and to have their data analyzed for the study.

Experimental procedures

T-lymphocyte isolation: Fifty milliliters of peripheral venous blood were drawn from the antecubital vein, collected in a sterile flask containing 3.8% sodium citrate (9/1, vol/vol) and T-lymphocytes isolation was carried out according to our previous report [4,6] (Figure 1). Magnetic beads conjugated with antihuman CD 3 (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) were used for positive selection of T-lymphocytes. In each incubation experiment, mRNA expression of ACE, INF-γ and ACE activity and Ang II concentrations were studied in cell pellet. ACE activity was also investigated in the supernatants, since ACE can be shed by T-cells [6]. To study the effect of stimulation by Ang II on ACE expression and activity and Ang II concentrations, T-lymphocytes were cultured in humidified atmosphere with 5% for 6 or 18 or 24 h with and without 0.1 pmol/l Ang II addition to the culture medium.

Real-Time PCR for the assay of ACE gene expression and RT-PCR analysis for the assay of INF-gamma gene expression in T-cells from controls, hypertensive and anginal patients

Isolation of total RNA and real time PCR for ACE gene expression was performed as detailed in our previous paper [6]. Relative ACE

gene expression was calculated as $2^{-\Delta Ct}$ ($\Delta C_t = C_t$ of the target gene minus C_t of beta-actin). mRNAs for INF-gamma were semi-quantified versus the housekeeping gene GAPDH by RT-PCR (Applied Biosystems) using specific primers.

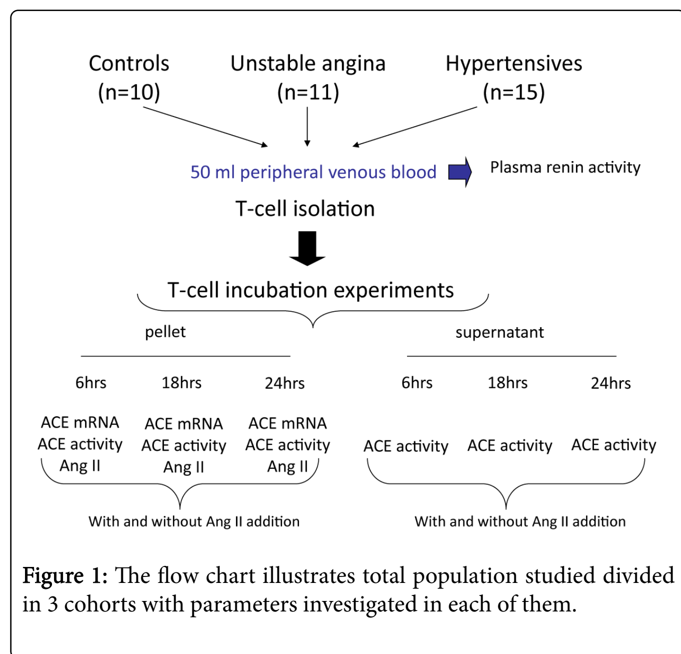

Figure 1: The flow chart illustrates total population studied divided in 3 cohorts with parameters investigated in each of them.

Assay of ACE activity in the T-cell pellet and in the supernatant of cultured T-cells from controls, hypertensive and anginal patients

T-cell ACE activity was measured by the quantification of hyppuric acid (HA), produced during the enzymatic reaction of ACE on a synthetic substrate hyppuril-hystidyl-leucine (HHL) and assayed by HPLC analysis using a spectrophotometric detector at 228 nm absorbance [6].

Measurement of plasma renin activity (PRA), Ang II and hsCRP

PRA measurement was performed with a commercial kit (Sorin Biomedica). Ang II levels were determined by radioimmunoassay after C18 Sep-Pak cartridge extraction and HPLC separation [6]. hsCRP was measured by a commercial kit (Beckman, Brea, CA) with the lowest detection point of 0.1 mg/dl [6].

Statistical analysis

Data have been stored in a dedicated data-base and analyzed by IBM-SPSS 20 for Windows® statistical software (IBM-SPSS Inc., USA). Variables were reported as mean ± SD. Within-groups comparisons (data at baseline vs. 24 h; baseline vs. 24 h plus Ang II and 24 h vs. 24 h plus angiotensin II) were made by paired Student t-test analysis. In order to analyze differences in ACE gene expression and enzymatic activity throughout the time-course among the three groups, an analysis of variance (ANOVA) for repeated measures was used. Statistical significance was taken as $p<0.05$ for all calculations.

Results

ACE gene expression and ACE activity without Ang II stimulation in T-cells from control subjects, ACS and hypertensive patients

Figure 2 shows the pattern of ACE gene expression in T-cells cultured without Ang II stimulation in controls and in patients: in control subjects T-cell ACE gene expression was detectable at baseline and did not significantly change at 18 and 24 hours of cell-incubation. T-cells from ACS patients showed significantly higher ($p<0.001$) ACE mRNA levels than those expressed by T-cells from controls at baseline and throughout cell-incubation. Hypertensive patients had higher ($p<0.01$) T-cells ACE mRNA levels than those observed in T-cells from controls, but lower ($p<0.01$) than those by T-cells from ACS patients. No changes in ACE gene expression were observed during the incubation time.

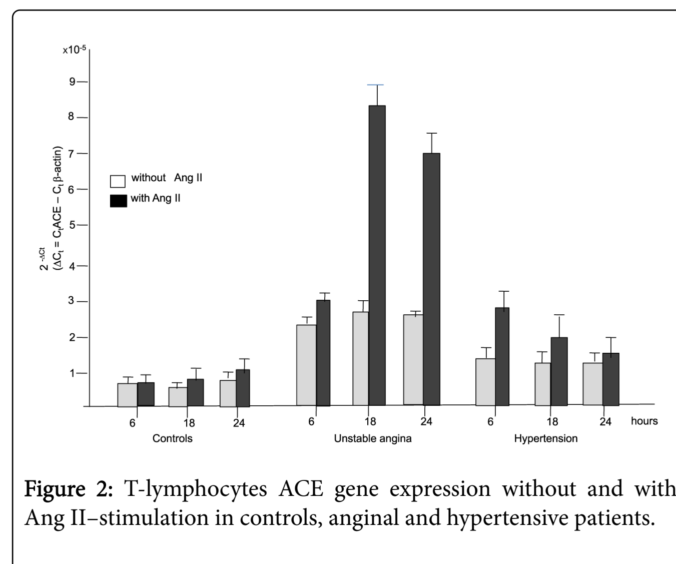

Figure 2: T-lymphocytes ACE gene expression without and with Ang II–stimulation in controls, anginal and hypertensive patients.

Table 2 shows the different pattern of ACE activity assayed in the pellet and in the supernatant of T-cells cultured without Ang II stimulation: in cultured T-cell from control subjects, ACE activity was detectable in the pellet at baseline and at 24 hours. In the supernatant T-cell ACE activity from controls was no detectable at baseline and was very low at 24 hours of incubation. In anginal patients T-cell ACE activity was significantly higher ($p<0.001$) than that assayed in controls both in the pellet and in the supernatant; specifically, at 24 hours T-cell ACE activity did not change in the pellet, but significantly increased in the supernatant ($p<0.001$ vs controls). In hypertensive patients T-cell ACE activity in the pellet and in the supernatant was significantly ($p<0.001$) higher than those assayed in controls at baseline and at 24 hours and not significantly different when compared with ACS levels; in the supernatant ACE activity was significantly ($p<0.001$) lower than those observed in ACS patients at 24 hours (Table 2).

ACE gene expression and ACE activity in cultured T-cell from control subjects, anginal and hypertensive patients after Ang II stimulation

In control subjects the addition of 0.1 pmol/l Ang II to the T-cell culture medium caused a significant ($p<0.01$ vs no Ang II stimulus) increase in T-cell ACE mRNA level at the 24 hours of incubation

(Figure 2). In T-cells from controls Ang II stimulus caused a significant increase (p<0.05) in T-cell pellet ACE activity at 24 hour and was associated with the spontaneous release of about the 20% of total T-cell ACE activity into the supernatant (Table 2).

ACE activity ($\mu U/10^6$ cells)	Baseline	24 hrs	24 hrs + Ang II
Control subjects			
Pellet	6.1 ± 2.1	6.5 ± 1.9	9.9 ± 1.6*
Supernatant	n.d.	0.6 ± 0.4$^\#$	2.2 ± 0.4$^\#$
Unstable angina			
Pellet	26.7 ± 8.9	28.1 ± 10.2	18.5 ± 3.8$^{*\#}$
Supernatant	n.d.	22.7 ± 9.5$^\#$	47.1 ± 10.4$^\$$
Hypertensives			
Pellet	20.30 ± 2.9	19.9 ± 1.65	17.2 ± 1.82
Supernatant	n.d.	1.22 ± 0.95	11.73 ± 1.05$^\$$
n.d.: non detectable			
within each group studied; *24 h + Ang II vs baseline p<0.01; $^\#$24 hrs vs. 24 h + Ang II p<0.01; $^\$$24 hrs vs 24 hrs + Ang II p<0.001			
at ANOVA unstable angina and hypertensives p<0.001 vs controls for pellet and supernatant ACE activity; unstable angina vs hypertensives p<0.001 for supernatant ACE activity			

Table 2: ACE activity in pellet and supernatant and Ang II pellet concentration of T-lymphocytes from controls, unstable angina and hypertensive patients without or with the addition of 10^{-13} mol/l Ang II in cell culture medium.

Also in T-cells from anginal patients the addition of Ang II to the cell medium significantly (p<0.001) increased T-cell ACE mRNA levels; the rate of increase was higher than that observed in T-cell from control subjects and the highest peak value for ACE gene expression was reached at 18 hours. In T-cell from hypertensive patients the Ang II stimulus caused a significant (p<0.01 vs no Ang II addition) early (peak at 6 hours) increase in T-cell ACE gene expression that was respectively significantly higher (p<0.001) and lower (p<0.001) than that shown by T-cells from controls and from anginal patients respectively (Figure 2).

In T-cells from anginal and hypertensive patients the addition of Ang II to the cell medium was not associated with an increase in the ACE activity of the T-cell pellet, but with a significant increase in the ACE activity in the supernatant that reached about the 64% of the pellet ACE activity in cultured T-cells from hypertensives and about the 260% in cultured T-cells from ACS patients.

Effect of Ang II–stimulation on T-cell INF-gamma gene expression

In controls and in patients, T-cell INF-gamma gene expression was detectable under baseline condition and did not significantly change at 18 and 24 hours of cell- Conversely, the addition of Ang II to the T-cell culture was associated with a significant increase in T-cell INF-gamma mRNA levels, that peaked at the 24th hour without differences between values observed in T-cells from controls, anginal and hypertensive patients (data not shown).

Ang II concentration in the pellet of cultured T-cells from controls, unstable angina and hypertensive patients

In the T-cell pellet from control subjects Ang II concentration was 176.5+17.9 fmol/mg proteins at baseline and did not significantly change after 24 hours of incubation; Ang II levels significantly increased after the addition of Ang II to the culture medium (Table 3). In T-cell pellet from anginal patients Ang II concentrations was significantly higher (p<0.01) than that assayed in controls both without and with Ang II stimulation (Table 3).

Ang II concentration in T-cell pellet from hypertensives was always significantly (p<0.01) higher than those assayed in controls (Table 3) and differed from ACS Ang II concentrations at baseline and at 24 hours with Ang II stimulation.

Ang II (fmol/mg proteins)	Baseline	24 hrs	24 hrs + Ang II
Control subjects			
24 hrs vs baseline	176.5 ± 17.9	180.2 ± 13.4$^{*\#}$	257.2 ± 18.1*
	p=0.520		
Unstable angina			
24 hrs vs baseline	408.0 ± 37.2	373.1 ± 39.6$^\#$	476.4 ± 30.7*
	p=0.570		
Hypertensives			
24 hrs vs baseline	274.9 ± 31.2	321.6 ± 34.8	378.9 ± 30.7*
	p=0.672		
within each group studied; *24 h + Ang II vs baseline p<0.01; $^\#$ 24 h vs. 24 h + Ang II p<0.01;			
at ANOVA unstable angina vs controls p<0.001, hypertensives vs controls p<0.01; unstable angina vs hypertensives p<0.01.			

Table 3: Ang II concentration in pellet of T-lymphocytes from controls, unstable angina and hypertensive patients without or with the addition of 10^{-13} mol/l Ang II in cell culture medium.

Plasma renin activity in controls, angina and hypertensive patients

In the control subjects, PRA in venous blood was 10.5+1.5 pmol. $L^{-1}.min^{-1}$ (range, 4.5 to 15.2 pmol. $L^{-1}.min^{-1}$). PRA levels in anginal and hypertensive patients were 10.1+1.2 pmol. $L^{-1}.min^{-1}$ (range, 4.8 to 14.7 pmol. $L^{-1}.min^{-1}$) and 11.2+0.9 pmol. $L^{-1}.min^{-1}$(range, 4.0 to 16.4 pmol. $L^{-1}.min^{-1}$) respectively, without significant differences between the three groups studied.

Discussion

Our data show that in patients with unstable angina (ACS) circulating T-cell based ACE mRNA expression and enzymatic activity were markedly increased when compared to levels found in T-cell from controls and hypertensive patients and resulted in a *de novo* enhanced cell-based Ang II production. Only in cultured T-cell from anginal patients ACE activity was detectable not only in the pellet but also in the supernatant under baseline conditions; remarkably, the addition of Ang II stimulus to these cultured T-cells caused the almost complete

release of ACE activity in the medium. This pattern of T-cell ACE genetic expression and enzymatic activity was peculiar of enrolled ACS patients with hsCRP >3 mg/dl and was markedly different not only from that observed in T-cell from controls but also from hypertensives with low grade inflammation (hsCRP >2 mg/dl). The normal PRA values found in ACS patients showed that the activation of T-cell RAS was selective and occurred in the absence of systemic RAS involvement. According to our data in ACS patients activated T-cell not only contribute to the acute systemic inflammatory reaction of unstable angina as cells of adaptive immunity [9,10], but also through the autonomous up-regulation of T-cell based RAS [5,6]. The increase in the synthesis of Ang II sustained by activated T-cells could have clinical relevance when it occurs into coronary plaques or microvessels where circulating T-cells are known to migrate [14,15]. No systemic effect can be hypothesized because of the low quote of Ang II synthesize by T-cells when compared to those produced by tissue-based or systemic RAS and because of the very short half-life of Ang II [16,17].

The enhanced *de novo* Ang II production by T-cells could start a dangerous positive loop in inflamed tissues and further amplify the activation of the immune response [18]. Remarkably, our *in vitro* findings of the marked release of ACE enzymatic activity in the supernatant by cultured T-cell from anginal patients, *in vivo* could translate in the local activation by *de novo* T-cell synthesized Ang II of other cells endowed with AT1-R such as endothelial and monocyte cells inside coronary plaques or microvessels [19,20]. Data available in literature reported that under Ang II stimulus AT-1 receptors of T-cells are upregulated 10 times and that T-cells exposed to Ang II have an increased ability to secrete TNF-alfa and INF-gamma as also confirmed in our *in vitro* experiments. In agreement with our *in vitro* findings, in myocardium of unstable angina patients an increased immunostaining for INF-gamma and TNF-alfa was shown next to inflammatory cells and was proved to be dumped in patients under pharmacological ACE inhibition [7,16]. Moreover, T-cells are involved in the process of endothelial dysfunction that contributes to the plaque destabilization in unstable angina and several studies have shown that T-cells infiltrate into atherosclerotic plaques and co-localize with ACE positivity at immunostaining [21-25]. According to our findings the instability of a plaque so to be a culprit lesion could be mediated by an increased *de novo* local Ang II production by activated circulating T-cells migrated inside plaques [17,18,26].

Finally, the local increased Ang II production by circulating cultured T-cells shown in our ACS patients could *in vivo* induce an oxidative burst in the endothelium, smooth cells , macrophages and T-cells of ischemic myocardium. Indeed, experimental studies reported that Ang II stimulus was shown to induce a marked increase in the ability of T-cells to produce reactive oxygen species, which in turn amplify the immune response [27,28].

Limits

Since different T-cell populations are known to operate with opposite roles on the regulation of the immune system, we are not able to specify whether T-cell ACE and Ang II upregulation was linked to a specific T-cell subpopulation. In experimental models, 8 weeks of exogenous Ang II treatment accelerated the development of atherosclerosis and prompted a switch from a stable-to-unstable plaque that was associated with a change in $CD4^+$ T lymphocyte activity; valsartan inhibited the effect of Ang II and significantly increased the frequency of Th2 and Treg regulatory cells [29]. In different atherosclerotic-prone models the proatherogenic properties of Th1 were shown and evidence from clinical investigations was collected that the upregulation of Th1 immune response was overwhelming in patients with ACS [30] and that an Ang II-due imbalance between Th1 and Th2 response might play a role in plaque rupture [29,30]. As a whole these findings strongly suggest that the activation of T-cell RAS found in angina patients could play a role in the dysregulation in T-cell populations underlying ACS. Dedicated studies are needed to investigate this issue.

Conclusions

In ACS patients with a systemic inflammatory reaction marked by high hs-CRP levels, a selective activation of circulating cultured T-cell based RAS did occur with enhanced ACE gene expression and activity that resulted in an increased *de novo* Ang II synthesis. Our *in vitro* findings strongly support that *in vivo*, once migrated into inflamed tissues, activated T-cells from ACS patients can autonomously increase the local concentration of ACE and Ang II and amplify the inflammatory reaction underlying coronary plaque activation and microvessel damage.

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