Cardiovascular Pathology 22 (2013) 42–48

Contents lists available at SciVerse ScienceDirect

Cardiovascular Pathology

Original Article

# Expression of angiotensin-converting enzyme 2 and its end product angiotensin 1-7 is increased in diabetic atheroma: implications for inflammation and neovascularization

K-Raman Purushothaman [a], Prakash Krishnan [a], Meerarani Purushothaman [a], Jose Wiley [a], Carlos L. Alviar [a], Fernando J. Ruiz [a], Yelena Zubatov [a], Annapoorna S. Kini [a], Samin K. Sharma [a], Valentin Fuster [a,b], Pedro R. Moreno [a,*]

[a] The Zena and Michael A. Wiener Cardiovascular Institute, Mount Sinai School of Medicine, New York, NY, USA
[b] Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain

ARTICLE INFO

*Article history:*
Received 25 November 2011
Received in revised form 27 March 2012
Accepted 21 May 2012

*Keywords:*
ACE2
Diabetes mellitus
Inflammation
Neovascularization
Peripheral vascular disease

ABSTRACT

*Aims:* The angiotensin-converting enzyme 2 (ACE2) and its end product angiotensin 1-7 (Ang1-7) are key counterregulatory proteins to offset the deleterious effects of angiotensin II. ACE2 is decreased in diabetic kidney disease but overexpressed in metabolically active atheroma. We tested the hypothesis that ACE2 is increased in diabetic peripheral atheroma, concomitantly with Ang1-7, angiotensin II receptor 1 (AT1R), proinflammatory cytokines, macrophage infiltration, and plaque neovascularization.
*Methods and Results:* Peripheral atherectomy plaques collected from 12 diabetic (DM) and 12 non-DM patients were immunostained for ACE2, Ang1-7, AT1R, and proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). Macrophage infiltration and neovascularization were counted using double-label immunochemistry with CD68/CD3 and CD34, respectively. Quantification was performed blindly by randomly counting positively stained cells in 20 high-power fields using previously validated methods. Tissue content of ACE2, Ang1-7, and AT1R was increased in DM when compared to non-DM ($P<.0001$). IL-6 and TNF-α were also increased in DM when compared to non-DM ($P<.0001$), as well as macrophage infiltration score and neovessel counting ($P<.0001$).
*Conclusion:* Expression of ACE2 and its end product Ang1-7 is increased in DM atheroma, along with overexpression of AT1R, IL6, TNF-α, macrophage infiltration, and neovascularization. These results suggest that the renin–angiotensin system counterregulatory pathway may be preserved in metabolically active atheroma, offering potential targets for future therapies in diabetic atherosclerosis.


## 1. Background

Angiotensin-converting enzyme 2 (ACE2) is now considered a key modulator of the renin–angiotensin system (RAS) in cardiovascular and renal disease [1]. This recently discovered homologue of ACE [2] is responsible for tissue degradation of angiotensin II (Ang-II) and the generation of angiotensin 1-7 (Ang 1-7), a vasodilatory and anti-inflammatory protein that counteracts the actions of Ang-II [3]. The proatherogenic effects of Ang-II are mediated by its specific Ang-II receptor type 1 (AT1R) [4]. This interaction between Ang-II and AT1R has been linked to atherosclerosis formation by means of endothelial dysfunction, increased inflammation, macrophage (MØ) infiltration, and adventitial neovascularization [5,6]. In renal disease, glomerular and tubular ACE2 expression is reduced in patients with type II diabetes [7]. While genetic deletion of ACE2 significantly accelerates atherosclerosis in ApoE KO mice [8], overexpression of ACE2 remarkably ameliorated glomerular injury and improved glycemic control in diabetic mice [9].

Diabetes mellitus (DM) is characterized by metabolically active atheroma, with increased inflammation and neovascularization [10,11]. Although poorly studied in human atherosclerosis, recent studies documented increased ACE2 in metabolically active atheroma, including vulnerable plaques [12]. Nevertheless, the role of ACE2 in diabetes atheroma has not been studied. This study tested the hypothesis that ACE2 is overexpressed in diabetic peripheral atheroma, which in turn leads to an increased production of Ang1-7. To evaluate possible mediators involved, we simultaneously quantified the expression of the AT1R along with proinflammatory cytokine expression, MØ infiltration, and plaque neovascularization.

The study was supported by intramural funding from Zena and Michael A Weiner Cardiovascular Institute, Mount Sinai School of Medicine, New York, NY, USA.
Disclosures: The authors have nothing to disclose for this manuscript.
* Corresponding author. Tel.: +1 212 241 3681; fax: +1 212 534 2845.
*E-mail address:* pedro.moreno@msnyuhealth.org (P.R. Moreno).

1054-8807/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
doi:10.1016/j.carpath.2012.05.004

## 2. Methods

### 2.1. Tissue collection

Peripheral atheroma specimens were obtained from 24 consecutive symptomatic patients admitted at the Catheterization Laboratory in Mount Sinai Medical Center for elective atherectomy using a Silverhawk atherotome (FoxHollow Technologies, Redwood City, CA, USA). Samples were processed into formalin-fixed, paraffin-embedded tissue sections for histological analysis. Hematoxylin–eosin staining was performed for the morphological evaluation of the atherosclerotic lesions. The study protocol was approved by the Mount Sinai Medical Center Institution Review Board, and informed consent before the procedure was obtained from every patient. Also, this study conforms with the Declaration of Helsinki. Demographic and clinical data were obtained for each patient. Data collection, tissue processing, staining, and histological analysis were performed in a blinded fashion by an investigator who was not aware of the clinical characteristics or diabetic status of the patients.

### 2.2. Immunochemistry and quantification of ACE2, Ang1-7, and AT1R

Immunochemistry was performed using specific, non-cross-reacting rabbit polyclonal antibodies against ACE2 (1:400 dilution; Abcam, CA, USA), Ang1-7 (1:200 dilution; Phoenix Pharmaceutical Corp, CA, USA), and AT1R (1:100 dilution; Abbiotec, CA, USA). Positive and negative controls using human renal tissue were included. Sections were examined under an Olympus BX 50 light microscope (Olympus America, Center Valley, PA, USA), and the expression of ACE2, Ang1-7, and AT1R was quantified in 20 random high-power fields (HPFs) using percentage of positive cells [endothelium, smooth muscle cells (SMCs), and MØs] stained per HPF. Using a modified version of a previously published methodology [13], a semiquantitative scale was used as follows: 0=absent; 1=1%–25%; 2=26%–50%; 3=51%–75%; and 4=76%–100%.

### 2.3. Immunochemistry and quantification of IL-6 and TNF-α expression

Polyclonal rabbit antibodies for IL-6 (Abbiotec, CA, USA) at 1:200 dilution and TNF-α, (Abbiotec, CA, USA) at 1:100 dilution were used with Avidin Biotin Complex (Vector Lab, CA, USA). The protein expression of these cytokines were detected by developing with 3'3'-diaminobenzidine (Sigma, MO, USA) chromogen. Positive and negative controls were also used by staining human tonsillar tissue.

### 2.4. Immunochemistry quantification of MØ infiltration and neovascularization

MØs and T-lymphocytes were identified using mouse monoclonal CD68/CD3 (Dako, CA, USA) in red chromogen, adapting an alkaline phosphatase method, and using CD34 antibody (Dako, CA, USA) in blue chromogen to stain the neovessels in blue color, as previously reported [13]. Using the high-power objective (40×) of the Olympus BX-50 planimetry microscopy, inflammatory cells were manually scored in 20 HPFs in the tunica intima and tunica media and reported as grade 0=<5 cells/HPF; grade 1=6–25 cells/HPF, and grade 2=>26 cells/HPF and enumerated to compute the mean inflammatory score per plaque as previously published [13]. Similarly, microvessels were counted manually in 20 HPFs in the tunica intima and tunica media and enumerated by absolute number to compute the mean total microvessel content per plaque.

### 2.5. Immunofluorescence quantification of cellular colocalization for MØs and SMCs with ACE2, Ang1-7, and AT1R

To identify the cell type (MØs and SMCs) that expresses ACE2, ATR1, and Ang1-7, immunofluorescence staining and confocal microscopy were used. Primary antibodies against the following antigens were adopted: rabbit polyclonal CD68 for MØ (M0814-DAKO, CA, USA; 1:100 dilution) and α-smooth muscle actin (αSMA) (FITC-F3777-Sigma Aldrich, MO, USA; 1:500 dilution) were used along with rabbit polyclonal ACE2 (ab65863-Abcam, MA, USA; 1:500 dilution), Ang1-7 (H-002-24-Phoenix Pharmaceuticals, CA, USA; 1:200 dilution), and AT1R (H-002-24-Phoenix pharmaceuticals, CA, USA; 1:100 dilution). Additionally, controls were included in parallel by substituting the primary antibody for IgG or specific IgG isotypes from the same species and at the same final concentration as the primary antibody. Secondary antibodies donkey anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 (A-21202 and A-21207, respectively; Invitrogen, NY, USA) were used at 1:500 dilution. Mounting medium containing DAPI (H-1200-Vector Lab, CA, USA) was then applied. Quantification of MØs and SMCs expressing ACE2, Ang1-7, and AT1R was performed in a blinded fashion. Three 20× magnification fields, randomly selected by the confocal Leica microscope, were quantified. Images were acquired using Mount Sinai's Shared Resource Facility-Leica TCS SP5 DMI, inverted confocal laser scanning microscope and analyzed using Leica LAS AF lite software system.

### 2.6. Statistical analysis

Continuous data are presented as mean and standard error of the mean. For two-group comparisons, Gaussian distribution samples were compared by the two-tailed Student's $t$ test, preceded by the Levene $F$ test for equality of variances. Non-Gaussian distribution samples were compared by the Mann–Whitney nonparametric test. For multiple comparisons, one-way analysis of variance was used. Discrete variables were compared with the $\chi^2$ test. ACE2 expression, AT1R expression, inflammation score, neovessel content, as well as IL-6 and TNF-α scores were compared between DM and non-DM peripheral plaques. SPSS 16.0 software (SPSS Inc., IBM Company, Chicago, IL, USA) was used for the analysis. Probability values of $P<.05$ were considered significant.

**Table 1**
Demographic characteristics from diabetic and nondiabetic groups

| Demographics | Diabetic patients ($n$,%) | Nondiabetic patients ($n$,%) | $P$ value |
|---|---|---|---|
| Age (years) | 71±10 | 76±9 | NS |
| Female | 5 (41.7%) | 5 (41.7%) | NS |
| Hypertension | 12 (100%) | 12 (100%) | NS |
| Hyperlipidemia | 11 (91.7%) | 9 (75%) | NS |
| Smoking | 8 (66.7%) | 7 (58.3%) | NS |
| Family history of CAD | 6 (50%) | 5 (41.7%) | NS |
| Presence of CAD | 5 (41.7%) | 4 (33.3%) | NS |
| Use of ACEi or ARB | 8 (66.7%) | 8 (66.7%) | NS |
| Fontaine classification for PAD | I=0%<br>IIa=0%<br>IIb=58%<br>III=42%<br>IV=0% | I=0%<br>IIa=0%<br>IIb=67%<br>III=33%<br>IV=0% | NS |
| Total cholesterol | 161±40.4 | 154±32 | NS |
| LDL | 92.9±35.5 | 84.16±25.51 | NS |
| HDL | 43.2±6.9 | 45.8±12.5 | NS |
| HbA1C | 7.6±1.8 | 5.42±0.51 | $P$=.005 |

CAD: coronary artery disease; ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; LDL: low-density lipoprotein; HDL: high-density lipoprotein; NS: not significant.