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The kinetics of angiotensin-I metabolism in human carotid atheroma: An emerging role for angiotensin (1–7)

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ABSTRACT

Aim: Local levels of angiotensin peptides depend on their rates of production and degradation, which induce proatherogenic or atheroprotective effects. Here, we reveal the kinetics of Angiotensin-I metabolism in paired early and advanced atherosclerotic lesions.
Methods: Lesions were spiked with labeled Ang-I* and supernatants withdrawn after 0, 10, 20, 40 and 80 min. The concentration of produced Ang-II*, Ang-III*, Ang-IV* and Ang-(1–7)* peptides were measured using multiple reaction monitoring mass spectrometry coupled to ultra-performance liquid chromatography, normalized to tissue weight and initial [Ang-I*].
Results: Ang-(1–7)* was the major angiotensin peptide produced, showing increased levels in both tissue types, with 2–3 fold lower levels in advanced compared to early lesions. In contrast, Ang-II* was 2–3 fold higher in advanced compared to early lesions, showing a decrease between 0 and 40 min then an increase at 80 min in both tissue types. The levels of Ang-IV were stable in both tissue types across all time points. Finally, Ang-III was non-detectable in both lesions across all time points.
Conclusion: Our results suggest that progression of atherosclerosis depends on the increased levels of Ang-II along with the decreased levels of Ang-(1–7), which supports the use of Ang-(1–7) along with Angiotensin type-1 receptor (AT1R) blockers.

1. Introduction

Atherosclerosis remains the main cause of death and morbidity in the world, mainly in developed countries [1]. It develops over the years as a result of the combining effects of several intrinsic and extrinsic risk factors, including family history, hypertension, diabetes, metabolic syndrome, and local atherogenic processes in the arterial wall [1]. However, the burden of this disease has been decreasing during the last decade as a result of its better understanding and advances in its treatment [2]. Indeed, the identification and understanding of risk factors improve our ability to reach a better prevention and treatment of atherosclerosis [3]. An important system linking most of atherosclerotic risk factors and local atherogenic processes is the renin-angiotensin-aldosterone system (RAAS).

RAAS was originally defined as a circulating hormonal cascade that functions in the homeostatic control of arterial pressure, tissue perfusion, and extracellular volume [4]. In its classical view, RAAS includes the successive carboxylic cleavage of the "inactive" angiotensinogen (AGT) peptide by renin and angiotensin-converting enzyme (ACE) to yield the active peptide angiotensin II (Ang-II). The latter then binds to its specific membrane receptor, angiotensin type-I receptor (AT1R), in order to produce its cellular effects [5,6]. However, metabolism of angiotensinogen is more complicated than previously reported. During the last years, research is in favor of the new RAAS concept that includes multiple enzymatic pathways for the generation of different angiotensin peptides exerting their effects in a tissue- and condition-specific manner [7].

Studies have shown that RAAS is expressed in the arterial wall, leading to the generation of local angiotensin peptides that exert different, even opposite, effects [8]. However, this depends on the peptides produced, their levels and their receptors that are locally expressed. For example, Ang-II binds to Ang-II type 1 receptor (AT1R), leading to

Abbreviations: ACEi, angiotensin converting enzyme inhibitor; AGT, Angiotensinogen; Ang, angiotensin-; AT1R, angiotensin-II type 1 receptor; AT2R, angiotensin-II type 2 receptor; ARB, AT1R blocker; ATH, advanced atheroma; MasR, Mas1 receptor; MIT, macroscopically intact tissue; RAAS, renin-angiotensin-aldosterone system.

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proatherogenic effects such as vasoconstriction, endothelial dysfunction, inflammation, oxidative stress, fibrosis, vascular smooth muscle cell growth, proliferation and migration [9]. These effects are generally antagonized by Ang-II itself binding on Ang-II type 2 receptor (AT2R), but also by Ang-(1–7) acting on Mas1 receptor (MasR) [10]. In fact, each peptide is cleaved by multiple alternative enzymes and its level is defined by the rate of production and degradation. Therefore, identifying the kinetics by which each peptide is produced may help predict the outcome effects of RAAS in atheroma.

In this study, we reveal the kinetics of angiotensin peptides production from Ang-I using an ex vivo model of paired human atheroma plaque (ATH) and nearby macroscopically intact tissue (MIT). Our results show that different levels and kinetics of production for each peptide define its differential physiological significance.

2. Materials and methods

2.1. Samples collection and preparation

This study conforms to the principles outlined in the declaration of Helsinki [11]. All procedures were approved by the local ethical committee and patients gave informed consents prior to the use of their samples. A total of 7 carotid endarterectomy samples were collected from the Edouard Herriot hospital in Lyon (France). The carotid endarterectomy samples were collected in the operation room and dissected within 2 h after operation in two fragments: the atheroma plaque (ATH), corresponding to atherosclerotic lesion with Stary stage ≥4, with or without calcification and/or hemorrhage., and the macroscopically intact tissue (MIT) with Stary stage ≤2, with little, if any fatty streak accumulations [12].

2.2. Tissue treatment

The tissue treatment protocol was adapted from Bujak-Gizyka et al. [13]. A total of 10 mg of each tissue type was washed 3 times in phosphate buffer saline (PBS), then incubated in 1 ml Krebs Hensleit solution (KHS: $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, KCl 4.7 mM, NaCl 118 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM) for 5 min with shaking at room temperature and then 50 μl of supernatants were taken to measure background concentrations. Labeled Ang-I (Ang-I*, DRVYIH-P*-FHL, JPT Peptide Technologies, Germany) was next added to reach a final concentration of 1 μM and another 50 μl of the supernatants were retrieved to measure peptides concentrations at $t_0 = 0$ min. Following that, the mixture was incubated at 37 °C and 50 μl of supernatants were withdrawn after 10, 20, 40 and 80 min, respectively. Supernatants were then stored at −80 °C. After removing the remaining solution, the tissue was allowed to dry then weighed.

2.3. Liquid chromatography

The liquid chromatography system used was an Acquity UPLC® H-Class (Waters, Milford, USA). The system was equipped with a reversed phase column Acquity UPLC® BEH Shield RP18 (1.7 μm; 100 × 2.1 mm, Waters). The mobile phase consisted of varying proportions of acetonitrile and water with 0.1% formic acid. The elution gradient started with 5% acetonitrile for 0.1 min, followed by a linear gradient up to 55% acetonitrile during 5 min. The mobile phase remained at 95% acetonitrile for 2 min and then switched to 2%. The column was equilibrated with 5% acetonitrile for 5 min prior to the next injection. The flow rate was 0.4 ml/min and the injection volume was 5 μl. The column was heated at 40 °C to ensure a stable column temperature and a better repeatability between runs.

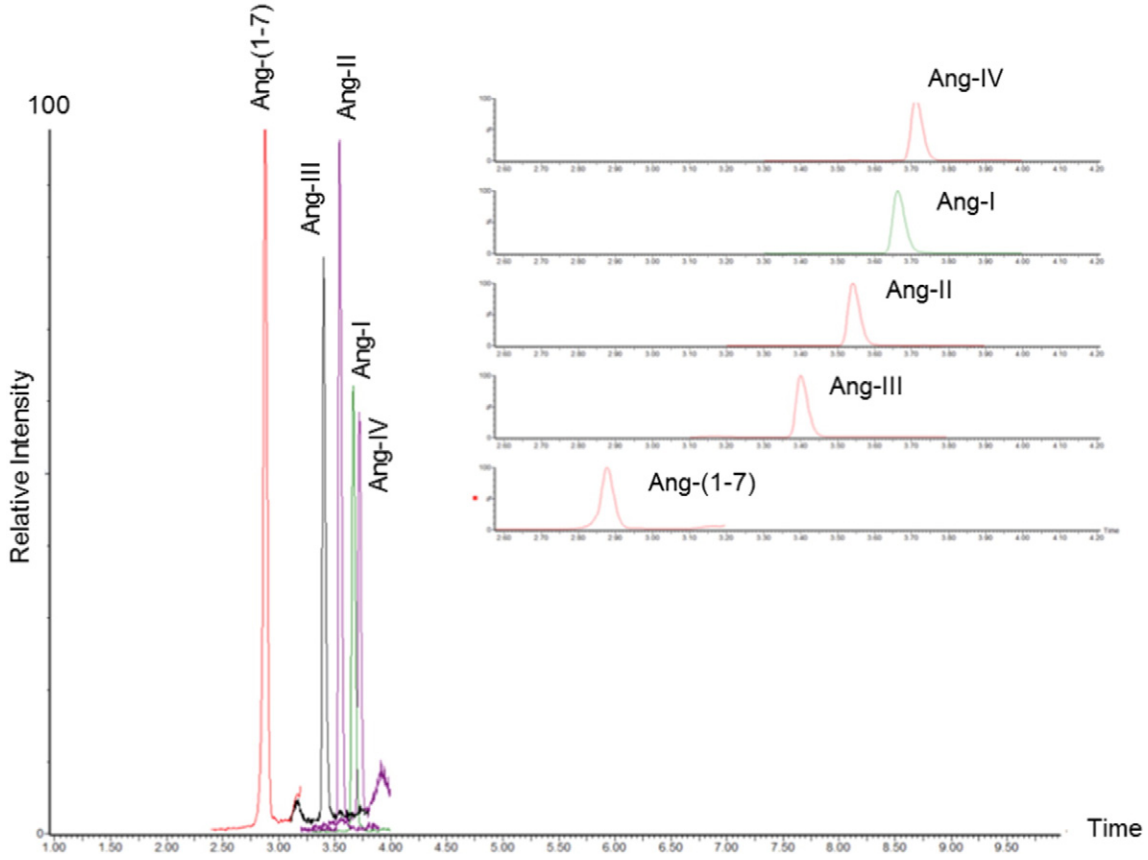

Fig. 1. Chromatograms of standards for labeled angiotensin peptides. Chromatographic separation by multiple reaction monitoring (MRM) mode (UPLC–MS/MS). Black-and-white, two-columns.

Table 1
Analytical parameters for specific detection by multiple reaction monitoring (MRM).

Peptide name	Transitions	Collision energy (eV)	Cone (V)
Angiotensin I*	652 > 269.3	22	40
	652 > 652	2	40
Angiotensin II*	526.8 > 469.4	15	40
	526.8 > 526.8	2	40
Angiotensin III*	469.4 > 335.4	15	40
	469.4 > 469.4	2	40
Angiotensin IV*	391.4 > 414.4	15	40
	391.4 > 391.4	2	40
Angiotensin 1–7*	453.3 > 647.6	16	40
	453.3 > 453.3	2	40

2.4. Mass spectrometry

The UPLC system was coupled to a triple quadrupole mass spectrometer Xexo TQS (Waters, Milford, USA). Compounds were quantified in multiple reaction monitoring mode (MRM), in positive electrospray ionization (ESI) and the following operating conditions were used: capillary voltage: 3.5 kV; source temperature: 150 °C; desolvation temperature: 650 °C; desolvation gas flow rate: 900 l/h; cone gas flow rate: 150 l/h; collision gas flow rate: 0.15 ml/min. The TQS data were analyzed using MassLynx and TargetLynx softwares (Waters, Milford, USA). Concentrations of angiotensin peptides were calculated using standard calibration linear regression curves constructed by plotting peak area versus angiotensin peptide concentration. Calibration curves were prepared using labeled peptides (purchased from JPT Peptide Technologies) for each of Ang-I (DRVYIH-P*-FHL), Ang-II (DRVYIH-P*-F), Ang-III (RVYIH-P*-F), Ang-IV (VYIH-P*-F) and Ang-(1–7) (DRVYIH-P*) at a concentration range of 0–500 ng/ml.

2.5. Statistics

Angiotensin peptides concentrations in each sample were normalized to tissue weight and initial Ang-I concentration. All values in the figures are expressed as the mean ± SEM (standard error of the mean). Concentrations of studied peptides were compared using binomial *t*-test with $p < 0.05$ considered significant.

3. Results

3.1. Angiotensin peptides separation and quantification

Stringent and rigorous selectivity, separation and symmetrical peaks of labeled angiotensin standards in U-HPLC separation were obtained using various proportions of acetonitrile and water with 0.1% formic acid (Fig. 1). Labeled standards of Ang-I, —II, —III, —IV and -(1–7) were first validated in MS and MS/MS mode with a high resolution mass spectrometer (Orbitrap, Supplementary Fig. 1). They were then analyzed by ESI-MS in positive mode using doubly charged molecular ions according to the MRM transition parameters listed in (Table 1). The developed LC-ESI-MS/MS method was validated for selectivity, precision, and stability. The calibration curve rank was used to determine angiotensin peptides in samples (Fig. 2). Incubation of Ang-I* resulted in the generation of significantly high amounts of Ang-II* after 40 min in advanced atherosclerotic lesion (ATH), in comparison to negligible amounts in nearby macroscopically intact tissue (MIT) (Fig. 2B). On the contrary, Ang-IV* was produced at relatively the same amounts in both tissues after 40 min of incubation (Fig. 2C). Finally, Ang-(1–7)* was produced in high amounts in both tissue types, but with lower amounts in MIT than in ATH (Fig. 2D).

3.2. Angiotensin peptides concentrations in MIT and ATH

Fresh MIT and ATH samples were incubated in labeled Ang-I* at 37 °C and supernatants were withdrawn for angiotensin peptides measurements in a time-course manner after 0, 10, 20, 40 and 80 min. Labeled Ang-I* and its downstream peptides were non-detectable in all supernatants taken for background measurements. In addition, Ang-III was non-detectable in both MIT and ATH across all time points (data not shown). Interestingly, Ang-(1–7) was the

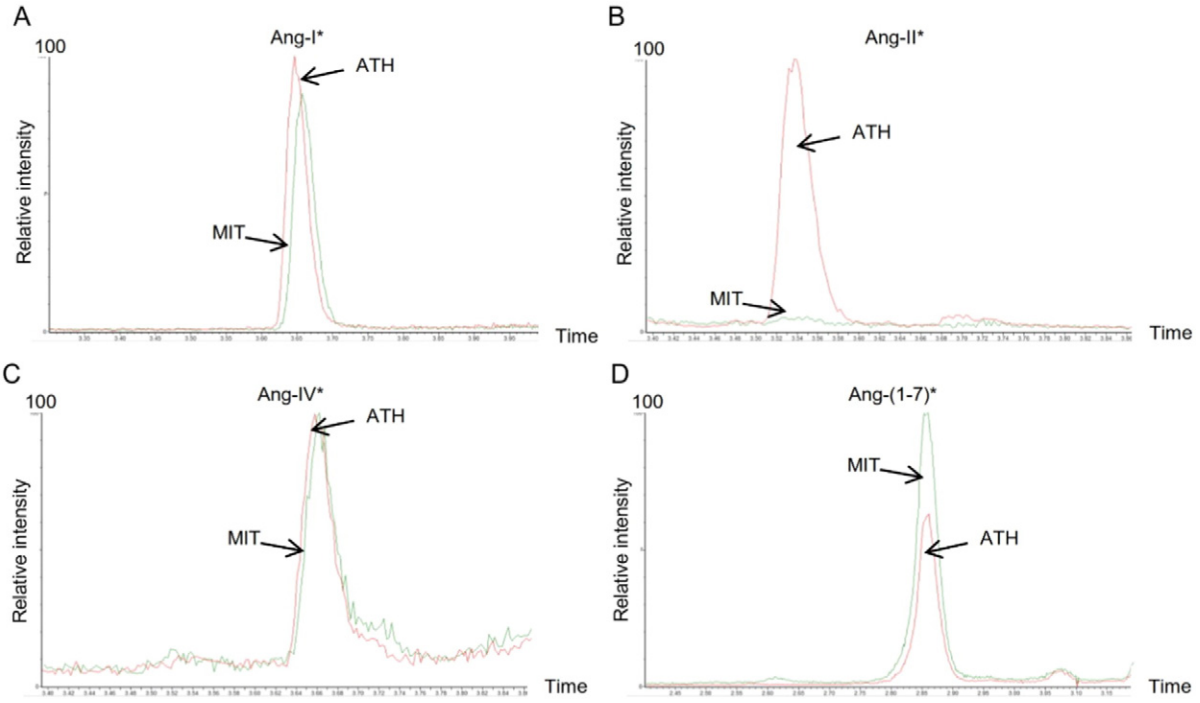

Fig. 2. Comparison of angiotensin peptides in MRM mode between macroscopically intact tissue (MIT) and atheroma (ATH). Chromatograms of (A) Ang-I*, (B) Ang-II*, (C) Ang-IV* and (D) Ang-(1–7)* measured in MIT (green) and ATH (red) after 40 min incubation with 1 μM of labeled Ang-I* ($n = 1$). Black-and-white, two-columns.

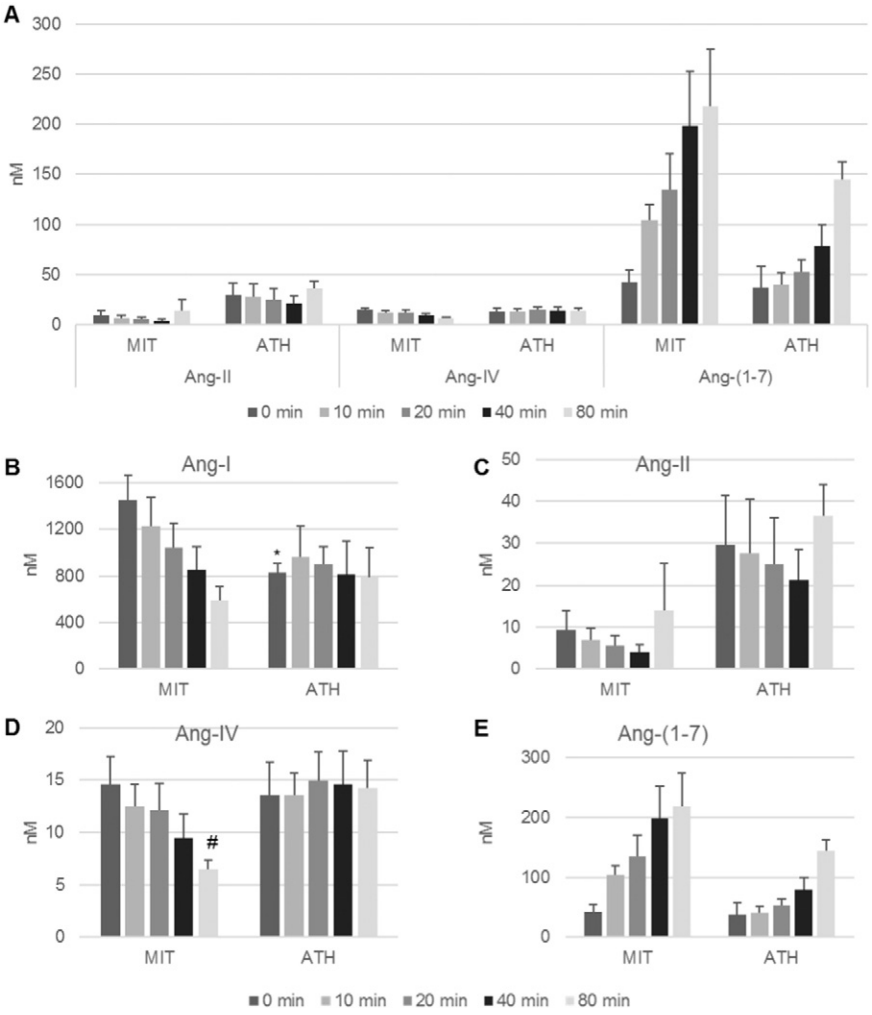

Fig. 3. Angiotensin peptides concentrations in macroscopically intact tissue (MIT) and atheroma (ATH). The mean concentration for each of the angiotensin peptides were calculated across samples (mean ± SEM) in MIT and ATH. Data are representatives of $n = 7$ samples. Black-and-white, two-columns.

major angiotensin peptide produced in both tissue types (Fig. 3A). The mean initial concentration of Ang-I* was 1451 ± 213 nM in MIT, which decreased to reach 589 ± 123 nM after 80 min (Fig. 3B). However, Ang-I* concentration remained relatively stable across all time points in ATH samples with a mean concentration of 858 ± 72 nM (Fig. 3B). On the other hand, the concentration of Ang-II was higher in ATH sample, in comparison to MIT, with respective mean concentrations of 8 ± 4 nM versus 28 ± 6 nM across all time points (Fig. 3C). On the contrary, Ang-IV was relatively stable across the different time points and in both tissue types with an average concentration of 13 ± 1 nM (Fig. 3D). Surprisingly, Ang-(1–7) showed 2-fold higher concentrations in MIT across all time points, compared to ATH, with a mean concentration of 140 ± 71 nM versus 71 ± 45 nM, respectively (Fig. 3E).

3.3. Angiotensin peptides kinetics in MIT and ATH

Since the initial Ang-I* concentration measurements were different among samples (Fig. 4A), and in order to calculate statistical significance, results were normalized to the initial Ang-I* concentration and to tissue weight (nM/intial [Ang-I] / tissue weight) in each sample. Interestingly, the kinetics of each peptide and its relative abundance in each tissue did not change after normalization (Fig. 5A). There was a significant decrease in the level of Ang-I in MIT, reaching 2-folds lower levels after 80 min of incubation, whereas it remained stable across all time points in ATH (Fig. 5B). Results showed that in both tissue types, the levels of Ang-II decreased between 0 and 40 min time points then increased at 80 min (Fig. 4C). The mean levels of Ang-II across samples were 2–3 times higher in ATH across all time points, in comparison to MIT ($p < 0.05$, Fig. 4C). On the contrary, and in an opposite manner to Ang-II, the levels of Ang-(1–7) increased with time in both tissue types, showing 2–3 times lower levels in ATH across all time points, in comparison to MIT (Fig. 4E). Moreover, the levels of Ang-IV kept relatively stable with no significant difference between ATH and MIT samples across all time points (Fig. 4D). Finally, Ang-III was non-detectable in all samples across all time points.

4. Discussion

RAAS regulates numerous molecular atherogenic mechanisms locally in the vascular wall, including endothelial dysfunction, inflammation, extracellular matrix remodeling, cellular growth, proliferation, differentiation, migration and apoptosis [14]. In fact, RAAS acts through two opposite arms leading to proatherogenic or atheroprotective effects. These effects depend on the types of the available angiotensin peptides and their levels, but also on the locally expressed receptors. However, the level of each angiotensin peptide is defined by its rate of production and degradation. In this study, we constructed a model of angiotensin peptides production in atheroma (Fig. 5), which allowed to reveal and for the first time the ex vivo kinetics of bioactive angiotensin peptides production in human carotid atheroma (ATH), in comparison to nearby vascular macroscopically intact tissue (MIT). Our results showed that

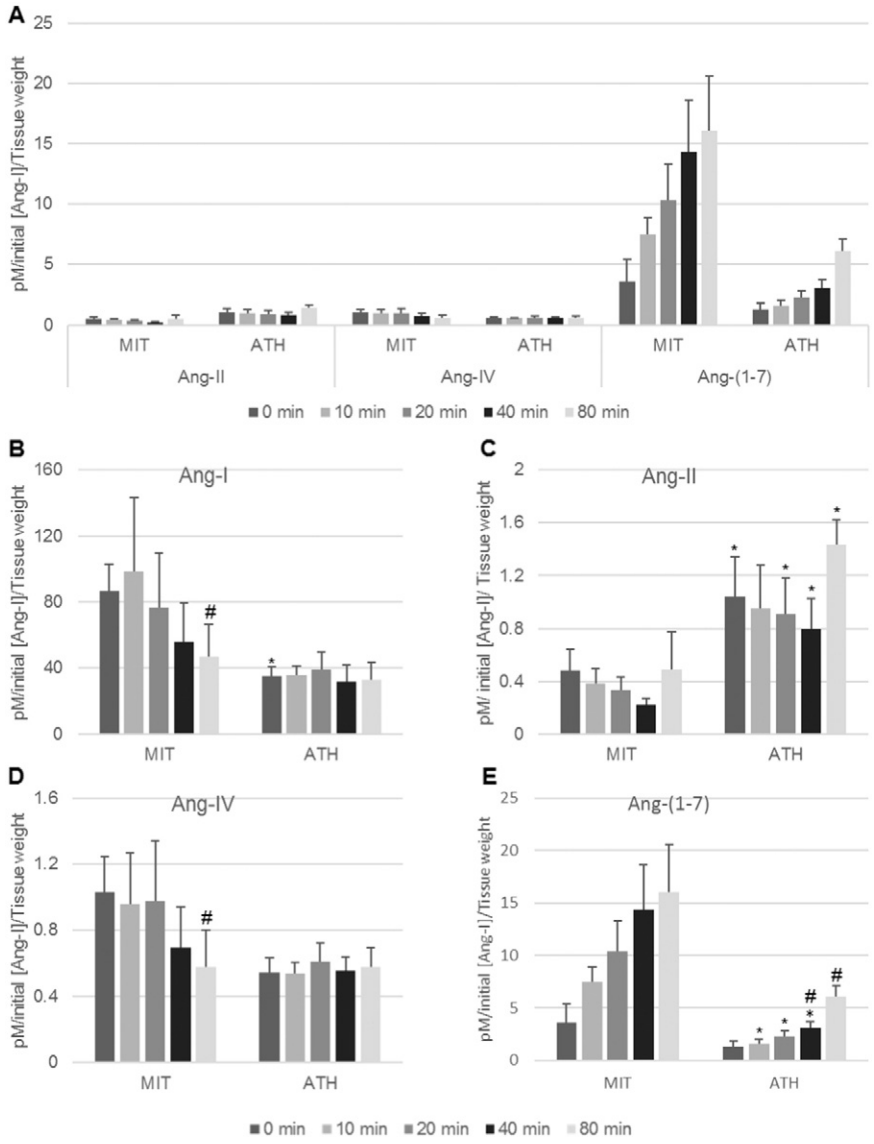

Fig. 4. Normalized angiotensin peptides concentrations in macroscopically intact tissue (MIT) and atheroma (ATH). The concentrations of angiotensin peptides were normalized to the initial angiotensin peptide concentration and tissue weight. The mean normalized value for each angiotensin peptide was calculated across samples (mean ± SEM) in MIT and ATH. Significance at $p < 0.05$: (*) between MIT and ATH; (#) against $t = 0$ min. Black-and-white, two-columns.

both tissue types possess similar kinetics, but different levels of angiotensin peptides production.

The levels of Ang-II were higher in ATH compared to MIT, which is in accordance with previous results showing an increased Ang-II production in atheroma [15,16]. The measured concentrations of Ang-II in MIT ($\text{Ang-II}_{\text{MIT}} = 8 \pm 4$) was significantly higher than the $IC50_{\text{Ang-II/AT2R}} = 0.52$ nM [17], but was equal to the $IC50_{\text{Ang-II/AT1R}} = 7.9$ nM [17], which suggests that the atheroprotective effects of Ang-II, through the AT2R, override its proatherogenic effects in MIT (Table 2, Fig. 5A). However, with a mean concentration of 28 ± 6 nM, Ang-II can induce proatherogenic effects in ATH by acting on the AT1R (Table 2, Fig. 5B) since the $IC50_{\text{Ang-II/AT1R}} = 7.9$ nM [17]. On the other hand, transcriptomic data in both human and mouse atheroma indicate that only AT1R is expressed in atheroma, whereas AT2R is not present (unpublished results), thus favoring proatherogenic effects by the Ang-II peptide.

Interestingly, our results confirm the local production of Ang-(1–7) and show that it is the major angiotensin peptide produced in atheroma. Indeed, both MIT and ATH samples demonstrated high and effective concentrations of Ang-(1–7), which may induce its effects mainly through MasR (Table 2, Fig. 5A and B). In fact, the mean Ang-(1–7) was well above the $IC50_{\text{Ang-(1–7)/MasR}} = 6.9$ nM [18] in both MIT and ATH, with mean concentrations across all time points equal to 140 ± 71 and 71 ± 45, respectively. Although no clear evidence is available about the protein expression of MasR in the vascular wall, studies investigating its effects indicate that MasR may be present and active in atherosclerosis [19–21]. In addition, most of the studies showing the atheroprotective effects of Ang-(1–7)/MasR were done using genetically altered models that either miss or over-express the MasR. Inversely, our results are in disagreement with other studies showing stable [22] or increased [23] Ang-(1–7) content in advanced atherosclerotic lesions of apoE knock out mice. Nevertheless, this could be attributed to several factors including the different model [24], arterial bed [25], lesion stability [26] and experimental procedure with respect to time points used in each study. The increase of Ang-(1–7) concentration with time implies that its rate of production is higher than its rate of degradation in both ATH and MIT. Indeed, the levels of Ang-(1–7) in both tissue types were found to be lower than its binding affinity (0.81 μM) to ACE, which cleaves Ang-(1–7) to Ang- (1–5) [27].

The absence of Ang-III supports transcriptomic data showing low levels of ENPEP and high levels of ANPEP (unpublished results). This suggests that Ang-IV is most probably synthesized from Ang-II, which

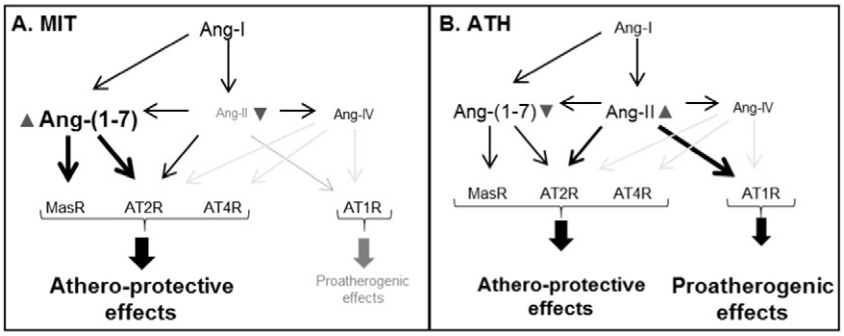

Fig. 5. Model of angiotensin peptides production in (A) advanced atherosclerotic lesions (ATH) and (B) nearby macroscopically intact tissue (MIT, early lesions). Font size and black gray scaling indicate the levels of peptides and effects produced. (▲) or (▼) corresponds to the increase or decrease in angiotensin peptides levels in ATH compared to MIT. Ang-, angiotensin; MasR, Mas1 receptor, AT1R, AT2R and AT4R correspond to angiotensin receptors type 1, type 2 and type 3, respectively. Black-and-white, two-columns.

may explain the decrease in Ang-II concentrations with time. In addition, and despite the high Ang-I* concentrations used, the levels of Ang-IV produced (13 ± 1 nM) seem to be biologically non-significant in both MIT and ATH, being lower than the IC50 of all receptors, including AT1R ($IC50_{Ang\text{-}IV/AT1R}$ = 10 μM), AT2R ($IC50_{Ang\text{-}IV/AT2R}$ = 48 nM) and AT4R ($IC50_{Ang\text{-}IV/AT2R}$ = 24 nM) [17,28].

The main RAAS-targeting molecules are ACE inhibitors (ACEi) and angiotensin type 1 receptor blockers (ARBs). Most studies showed that ACEi were superior over ARBs in the treatment of atherosclerosis by reducing ischemic events and mortality rate [29]. In fact, ACE enhanced atherosclerosis both by increasing proatherogenic effects through Ang-II production, and by decreasing atheroprotective effects through the cleavage of Ang-(1–7) [30]. In addition, the use of ARBs in combination with ACEi showed better effects than using either reagent alone [31]. However, a new clinical meta-analysis showed that combination therapy using ACEi and ARBs may lead to adverse effects on kidney function [32]. Our results showed an increase in Ang-II production, concomitant with a decrease in Ang-(1–7) levels, in advanced compared to early lesions. This raises the importance of using Ang-(1–7), as an alternative to ACEi, in combination with ARBs [33].

In this study, we used intact tissue which may reflect to a large extent the physiological metabolism of Ang-I in the vascular wall. However, in order to overcome substrate limitation in kinetics analysis, Ang-I was employed in excess which does not reflect physiological levels in atheroma. Thus, further studies should be performed in order to elucidate the physiological levels of angiotensin peptides in atheroma. In fact, angiotensin peptides need to bind to their corresponding receptors in order to perform their functions. Despite the fact that both AT1R [34] and AT2R [35,36] were shown to be expressed in the vessel wall, no relevant study has been performed to elucidate the physiological expression of MasR in the vasculature. Indeed, the expression of AT1R was shown to be several folds higher than AT2R in normal and atherosclerotic vessels, which indicate that proatherogenic effects of Ang-II, through AT1R, override its atheroprotective effects, through AT2R [37–39]. Thus, the determinant factor of Ang-II activity would be its level of production, which, as our study demonstrated, may only induce proatherogenic effects in advanced lesions. Nonetheless, further analysis of the expression of the angiotensin receptors, mainly MasR, should be performed using highly sensitive methods such as mass spectrometry.

Table 2
Affinity of angiotensin peptides to their receptors.

	Mean concentration (nM)		IC50 (nM)			
	MIT	ATH	AT1R [17]	AT2R [17]	AT4R [28]	MasR [18]
Ang-II	8 ± 4	28 ± 6	7.92	0.52	11×10^3	NA
Ang-III	NA	NA	21×10^{-8}	0.65	NA	NA
Ang-IV	11 ± 3	14 ± 1	1 × 104	48.6	24	NA
Ang-(1–7)	140 ± 71	71 ± 45	1 × 104	24.6	8×10^3	6.9

5. Conclusion

In conclusion, our results suggest that the progression of atherosclerosis depends on the increased levels of Ang-II along with the decreased levels of Ang-(1–7) in advanced compared to early atherosclerotic lesions (Fig. 5A and B), which support previous findings showing that the use of a combination treatment of Ang-(1–7) and losartan (ARB) induces stronger atheroprotective effects compared to the use of either molecule alone [33].

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Author Contributions Statement

AN and PM performed experiments. AN, PM, FK, RN and KZ analyzed data and wrote the manuscript. GB and KZ designed the study.

Conflict-of-interest disclosure

The authors declare that they have no conflict of interest.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.vph.2016.08.001.

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