Local formation of angiotensin peptides with paracrine activity by adipocytes

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A local paracrine angiotensin (ANG) system influences the insulin sensitivity and cell differentiation of adipose tissue. The limited view of a merely systemic renin-angiotensin-aldosterone-system with ANG II (1–8) as the main mediator of ANG-related effects may oversimplify the situation. The aim was to analyze the degradation of ANG by using capillary electrophoresis (CE) techniques. The supernatant of cultured 3T3-L1 adipocytes was used directly, and some data on degraded peptides were combined with a biological effect. The formation of several peptides such as ANG II (1–8), –III (2–8), –IV (3–8), and ANG (1–7) as degradation products is demonstrated; in addition low levels of ANG (3–7) are identified. The concentrations of the peptides ANG III (2–8) and ANG IV (3–8) (both are AT_4 receptor agonists) are modified in the vicinity of adipose tissue cells by amino-terminal degradation which resulted in ANG (3–8), –(4–8) and –(5–8). ANG IV (3–8) and ANG II (1–8) were biologically highly effective in inhibiting IRAP (insulin regulated aminopeptidase, part of the AT_4 receptor). It is observed that ANG (1–7) is the main degradation product derived from ANG I via ANG (1–9) and that ANG III (2–8) is one important regulated peptide for IRAP. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin; peptide degradation; IRAP assay

Introduction

Adipose tissue is not only a target for metabolic effects but also active in the secretion of angiotensinogen, leptin and adiponectin [1]. These hormones are of systemic importance by being involved in the metabolic homeostasis of blood glucose and lipid levels. The incidence of diabetes mellitus can be reduced by inhibitors of the angiotensin system, AT_1 receptor antagonist or ACE-inhibitors. In addition to this, systemic effect a local angiotensin (ANG) system influences glucose metabolism and preadipocyte differentiation [2] as part of the adipose tissue remodeling process [3]. This local system may be involved in metabolic syndrome (mainly diabetes and hypertension). Main compounds such as angiotensinogen, renin and ACE exist in adipocytes [4] although the expression of both renin-mRNA and renin-activity is low [5]: ACE inhibitors are rather without direct effect in fat cells mainly due to the extremely low renin activity. This low expression of renin could explain the differences in the effects of compounds acting on either the systemic or local ANG system. *In vivo* the reduction of body weight and body fat are clearly seen in ACE(−/−) mice [6] and systemically ANG II increases body weight and body fat [7] and induces adipocytes hyperplasia and hypertrophy [8,9] which can be reduced by an ACE inhibitor [7,8].

For a long time the local formation and degradation of ANG peptides was not in the focus with respect to adipocytes except with some authors [10–12]. Neprilysin (NEP), another ANG II degrading enzyme, was investigated in addition to ACE and has turned out to be important. In spite of the presence of NEP in human adipocytes its influence on one of its substrates (atrial natriuretic peptide and its effects) is low [13]. Local differences with respect to hormones and their local receptors expression exist when fat cells from varying body origins are investigated [14]. Thus ANG degradation products including those induced by NEP should be in the focus of further investigations.

From binding studies and peptide profile investigations it is far from clear which ANG receptors are involved and which of the locally produced peptides could be physiologically important. AT_1 receptors were investigated extensively in 3T3-L1 and human adipocytes, and AT_2 receptors were recently found in preadipocytes [15,16]. The first investigations were concentrating on the role of ANG II and AT_1 receptors [17]. The situation is complicated since the effects mediated by AT_1 and AT_2 receptors are contradictory and since AT_2 receptor stimulation causes antagonistic actions against AT_1 receptor signaling [18]. AT_2 receptors appear to be involved mainly in adipocytes differentiation and dysfunction using specific models [19]. But the presence and possible participation of active metabolites like ANG III, ANG IV (3–8), ANG (1–7) in paracrine feed back loops as described for other tissues were not considered [20]. From the CNS it is already known that many effects are not mediated by ANG II (1–8) itself but by degradation products like ANG III (2–8) and ANG IV (3–8) [21,22]. Both cross-reactivity of antibodies used in immunoassays and an insufficient separation of peaks by HPLC methods had been major obstacles for interpretation in the past. We applied a capillary electrophoresis (CE) method which in

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Abbreviations used: *ACE, angiotensin converting enzyme; ANG, angiotensin; brackets indicate the amino acid sequence of the peptide in respect to angiotensin I (e.g. 1–10); AT_1 receptor, angiotensin 1 receptor; AUC, area under the curve (indicative of summarized concentration); CE, capillary electrophoresis (we are using CZE = capillary zone electrophoresis); EDTA, ethylenaminetetraacetate; GLUT, glucose transporter; IRAP, insulin regulated aminopeptidase; Leu-NA, Leucyl-naphtylamine; NA, 2-amino-naphthalene.*

general is very well known for being highly efficient and highly sensitive for separation of peptides [23–25]. CE methods are often used routinely with complex biological matrices. CE's advantages over HPLC are clearly illustrated by its high separation efficiency and small necessary sample volume. The advantages of CE over HPLC and LC-ESI-MS are the simplicity of method and the higher resolution. The combination of CE with the high sensitivity and high selectivity offered by MS detection is very attractive [26,27].

Local concentrations of ANG and its degradation products may be assumed to be much higher than those concentrations in blood and, therefore, have different kinetics. The local metabolism of angiotensinogen, ANG I or ANG II by adipocytes needs detailed investigation in connection with their potential paracrine activity.

The enzyme IRAP (insulin regulated aminopeptidase) is known to possess a binding site for ANG IV (3–8) [28]. Besides others the peptide (agonist) ANG IV (3–8) inhibits the enzymatic activity of IRAP. IRAP as a aminopeptidase degrades especially *N*-terminal leucin- and cystein parts of peptides; substrates besides many others may be ANG III and ANG IV (3–8) [29]. The intracellular function of IRAP for signal transduction is not known yet [30].

Thus it was the aim of this study to examine the degradation products and pathways of ANG I (1–10) and the possible biological impact of the degradation product ANG IV (3–8) on IRAP by using a separation method better than earlier used HPLC.

Materials

3T3-L1 cells were obtained from ECACC (European Collection of Cell Cultures, Great Britain); cell culture supplements were from PAA Laboratories GmbH (Austria). ANG peptides were either obtained from Biotrend Chemicals GmbH (Germany) [ANG I (1–10), ANG III (2–8), ANG IV (3–8)] or Sigma-Aldrich GmbH (Germany)[(Sar1,Ile8)-ANG II (SARILE), ANG (1–7), Angiotensinogen 1–14] or Phoenix Pharmaceuticals, Inc (USA) [ANG (1–9), ANG II (4–8)] or Bachem Holding AG (Switzerland) [ANG (1–5), ANG (3–7), ANG (5–8), ANG II (1–8)].

Methods

Cell Culture

3T3-L1 cells were grown in Dulbecco's modified eagles's medium (25 mM glucose) supplemented with 10% NCS (normal calf serum) and 1% penicillin/streptomycin to confluence [31]. A quantity of 1.5×10^6 cells per ml was placed for the next passage as well as for starting incubations in 24–and 96 well plates. Differentiation was induced by changing to induction medium with 10% FCS (fetal calf serum), dexamethasone 0.25 μM, 3-isobutyl-methyl xanthine 0.5 mM and 1 μg/ml insulin) for 3 days, differentiation medium (standard medium plus 10% FCS, insulin 1 μg/ml) for 4 days and a DMEM-based standard medium for further 4–8 days. The ongoing differentiation was monitored by Oil red O staining. In brief, cells were washed twice with phosphate buffer solution (PBS) and fixed with neutral buffered formaline 4% (v/v) for 30 min. Lipid droplets were stained with Oil Red O solution (1.8 g/l) for 5 min., Nuclei were counterstained with Hematoxylin solution (7.5 g/l) for 1 min.

CE Assays

A Beckman P/ACE MDQ (Beckman Coulter, Munich, Germany) with UV-Detector (200 nm) was used with an untreated fused-silica capillary of 75 μm I.D/375 μm O.D, 60 cm effective length and 70 cm total length (BGB Analytik GmbH, Schloßböckelheim, Germany).

The electrophoresis buffer was a 100 mM phosphate buffer with pH 1.95 [32]. The samples (diluted with aqua bidest. 1:5) were injected hydrodynamically by vacuum (30 mbar, 10 s) out of the 96 well dishes. Separation was carried out by applying a voltage of 25 kV ($\approx$532 V/cm) with normal polarity mode (cathode at the outlet vial). The temperature of the capillary cartridge was maintained at 25 °C.

In preliminary experiments a borate buffer was used (boric acid 100 mM (618 mg), L-tartaric acid 3 mM (46 mg), $CuSO_4$ 1 mM (41.7 mg) in 100 ml H_2O, pH adjusted to 9.8 using 1 M NaOH). At this pH the neutral ANG peptides are deprotonated and the EOF (electroosmotic flow) is rather strong. The anionic peptides move against the EOF to the anode (method according to Lacher *et al*. [33]). This separation method using biuret reaction (Cu^{++}) did not yield sufficient results. For this reason we switched to a separation using a phosphate buffer, 100 mM) according to Lim and Sim [32] (H_3PO_4 100 mM (2.88 g of 85% solution), filled up to 250 ml, pH adjusted to 1.95). At this low pH the deprotonation of the silanol groups is low. There is apparently no EOF and all hybridionic peptides are cationic.

Preparation of Samples for CE Analysis

To analyze the enzymatic degradation activity of adipocytes, 3T3-L1 cells were grown and differentiated. The cell culture medium was replaced by incubation buffer (10 mM HEPES (2-(4-(2-hydroxy ethyl)-1-piperazinyl)ethanesulfonic acid) buffered saline), washed twice and 100 μM of the tested ANG peptide were added to a total volume of 300 μl while the cells remain bound to the surface. After 2 and 4 h incubation (37 °C) 50 μl supernatant was collected and frozen until being determined. For actual CE-analysis the frozen samples were thawed and 40 μl of each was diluted by adding distilled H_2O to a total volume of 200 μl in 96 well dishes. The HEPES peak was used as internal standard. This peak is dispersed because of electromigration or chemical interaction, but we used a precisely reproducible HEPES concentration, present in the peptides buffer. System stability testing was performed using a mixture of ANG I (1–10), ANG (1–9), ANG II (1–8), ANG IV (3–8), ANG (4–8) with final concentrations of 100 μg/ml each. By this method the peaks could be assigned to specific compounds.

For time-dependent formation of ANG peptides (Figure 2), capillary temperature was raised to 40 °C to shorten total run times from about 30 min to <10 min. Cells were grown in Nunclon 96 well dishes, washed twice and the degradation of the supernatant peptide solution was directly analyzed by repeated vacuum injection without prior dilution steps.

A representative electropherogram (Figure 1) shows the practicability of separation of a mixture of ten ANG peptides under normal incubation conditions (HEPES buffer).

IRAP Assay

For characterization of IRAP activity in 3T3-L1 adipocytes by a fluorescence assay the cleavage of 25 μM leucyl-naphthylamide (Leu-NA) to 2-amino-naphthaline (NA) was measured according to the group of Demaegt and Vanderheyden [34,35] in the presence or absence of peptidic ligands or EDTA. The calibration curve using NA as a fluorescence probe was linear over the concentration of 0–8 μM (whole range that was measured, $r^2 = 0.9984$ for eight experiments). The reaction was initiated by adding 50 μl
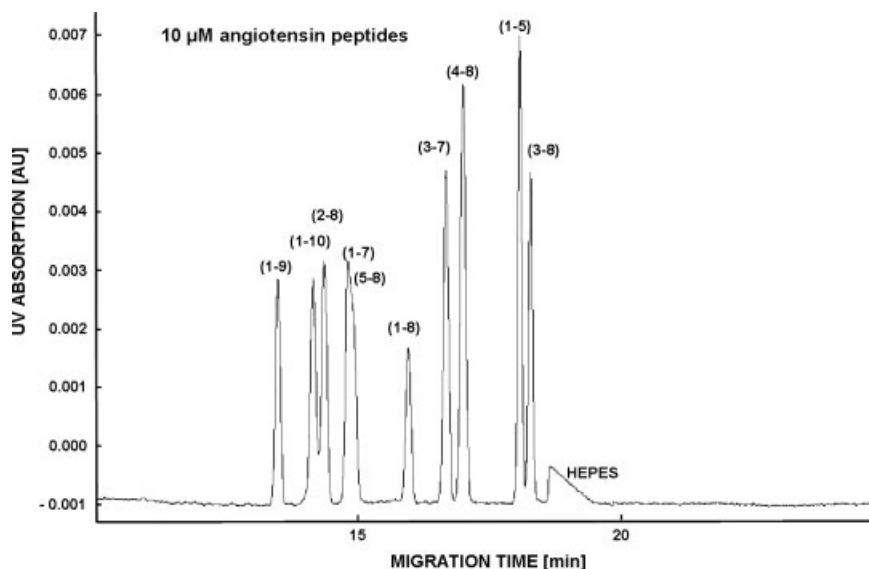
Figure 1. Electropherogram of a mixture of ten angiotensin peptides (final concentration 10 µM each). The sample was prepared by 1 : 10 dilution with double distilled water out of a stock solution (100 µM in HEPES buffered saline) in order to use the stacking effect. A 10 mM HEPES was used as an internal standard. A representative run out of four runs is shown.

adipocyte cell suspension to 100 µl iced Leu-NA and 50 µl ligand solution. The cell suspension was prepared immediately before the experiment by harvesting in a 75 cm^2 bottle flask using 15 ml ice cold HEPES buffered saline in the absence of EDTA and protease inhibitors. The amount of NA was determined after 10 min by measuring its fluorescence in black 96 well Nunclon dishes (Ex. 330 nm; Em. 390 nm) using a Fluostar galaxy plate reader (BMG Labtechnologies, Offenburg, Germany).

The used IRAP assay is not completely specific since substrates like the one used Leu-NA will react not only with IRAP, but also with arylamidases [36]. Arylamidase can be excluded by its sensitivity to puromycin. EDTA as a control compound complexes Zn^{++} and is specific when this enzyme is investigated alone; it may be thought not to be a specific inhibitor because other Zn-sensitive enzymes will react as well; one of these will be NEP (mentioned in the paper) which, however, does not use the substrate of the IRAP assay.

Statistics

Linear regression of curves for calibration of biological effects was done with SigmaPlot 8.0. Results are expressed as mean ± SEM. One-site models were used as model of choice unless statistical analysis (F-test) suggests a more complex model to be chosen. The two-site model was accepted if the P-value was less than 0.05.

Results

Establishment of a CE Separation Method

The half-lives of ANG III and ANG IV (3–8) are much lower than that of ANG II [37] indicating the existence of specific degrading enzymes for different peptides. The formation and degradation of ANG peptides locally should be complex since several enzymes such as chymases, aminopeptidases, carboxypeptidases and NEP could be involved simultaneously resulting in a wide variety of ANG peptides that are difficult to predict especially during AT-receptor blockade or ACE-inhibition. A CE method with better separation power than HPLC methods albeit with a comparable sensitivity was

established to analyze the most relevant peptides (Figure 1). We assume that the analysis of supernatants of whole cells is superior to incubation of cell lysates because the cellular compartmentalization of the enzymes remains intact and solely intracellularly located enzymes from broken cells cannot falsify the results.

Formation of ANG Peptides

Figure 2A shows the electropherograms of ANG I (1–10) and degraded peptides produced during a short time incubation with 3T3-L1 adipocytes. The curves of the indicated incubation times (0, 20, 50 and 70 min) are superimposed to show the development of degradation (similar presentation of data in further experiments). The peak of ANG (1–9) develops first which is followed (<50 min) by ANG (1–7). Electrodispersion of the saline sample leads to slight peak broadening.

Next a similar experiment is shown except for using a longer incubation period (2 and 4 h) (Figure 2B). There is a major development of ANG (1–9) and ANG (1–7) as already indicated by results shown in Figure 2A. After 4 h an additional peak for ANG II (1–8) is obvious. Unidentified peaks named X and Y were detected and may be assigned tentatively to ANG (2–9) and ANG (2–10) although this can only be proved after having synthesized these compounds. ANG (1–7) and ANG (5–8) coelute in this experimental setup. Considering the time-dependent formation of ANG (1–7) out of ANG (1–9) shown in Figure 2A and the results of comparable CE-experiments with a different separation technique (running at pH 9.8 in presence of copper ions; data not shown), the peak is assumed to be mainly ANG (1–7) with perhaps tiny amounts of ANG (5–8). Nevertheless this peak is named ANG (1–7)/(5–8) to avoid any misinterpretation. In the insert of Figure 2B the concentrations of both peptides (AUC of the peaks) are shown.

Since ANG (1–9) is one of the degradation products in the earlier experiment, an electropherogram of this added peptide and its degradation products was run next (Figure 3A). ANG (1–8) and ANG (1–7)/(5–8) were the major degradation products. An additional increase in ANG (1–7)/(5–8) after 4 h is observed.
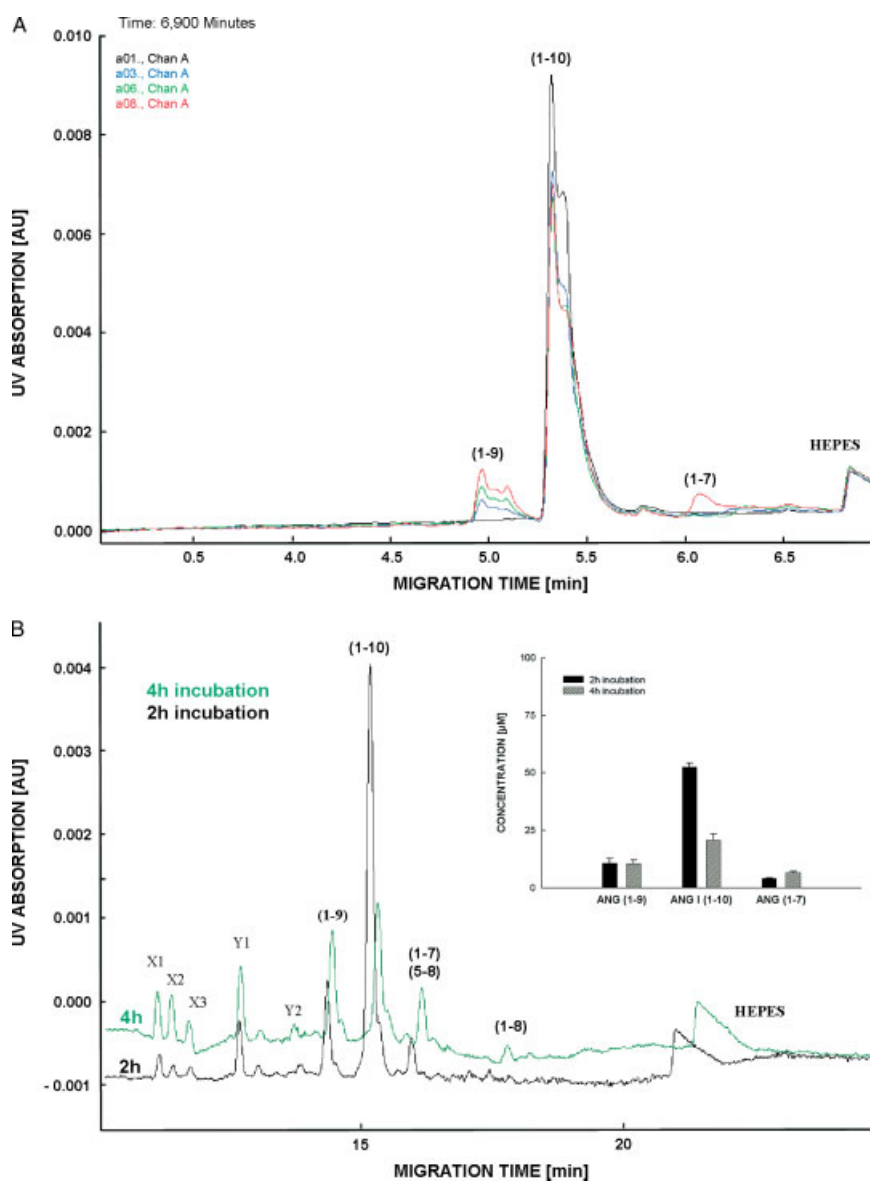
Figure 2. (A) Degradation of 100 µM ANG I (1–10) in the presence of 3T3-L1 adipocytes (within minutes). 3T3-L1 adipocytes were differentiated in 96 well dishes. After washing, the cells were incubated with 100 µM ANG I (1–10) at 37 °C. Using vacuum injection the cell supernatant was analyzed several times at 0, 20, 50 and 70 min by CE without prior dilution steps thereby carefully avoiding to destroy or pick up cells. The CE analysis was performed at 40 °C capillary temperature in order to reduce the analysis time (10 min). A representative run out of four runs is shown. (B) Degradation of 100 µM ANG I (1–10) in the presence of 3T3-L1 adipocytes (within hours). 3T3-L1 adipocytes were incubated in 24 well dishes with different angiotensin peptides at 37 °C in order to compare the peptide degradation as described. The supernatant was analyzed at 2 h (lower line) and 4 h (upper line) after the peptides had been diluted 1 : 4. Each experiment was repeated four times. Quantitative calculation is shown in the inlet (Mean + SEM); the electropherogram represents one of those experiments. A representative run out of four is shown. In the insert the AUCs (concentrations) of all four runs are summarized.

Unidentified peaks named X and Y were detected and may be assigned tentatively to ANG (2–9). In the insert of Figure 3A the concentrations of the peptides (AUC of the peaks) are shown.

Figure 3B shows the further breakdown of the degradation product (albeit bioactive) ANG II (1–8) now added under the same conditions as described before. The main metabolite is ANG (1–7)/(5–8), and ANG III (2–8) was reliably detected in each single experiment. ANG IV (3–8) and ANG (3–7) appear to be further metabolites but could not be quantified in a reproducible way. Unidentified peaks named X and Y were detected again. After 2 h roughly 30% of the added peptide ANG II (1–8) was degraded. The velocity of degradation is as fast as that of ANG (1–9) in the earlier experiment. The concentration of ANG III (2–8) was decreased

between 2 and 4 h of incubation time. In the insert of Figure 3B the concentrations of the peptides (AUC of the peaks) are shown.

The electropherogram of ANG (1–7) was run as well (Figure 3C), which was another degradation product shown in experiments of Figure 2. No relevant peptides could be detected except a small peak resembling ANG (3–7). This minor degradation is indicated in the insert. Unidentified peaks named X and Z were present.

In Figure 4A the electropherogram of added ANG III (2–8), an AT$_4$ receptor agonist, is shown. The degradation is rather quick: after 4 h ANG III (2–8) is no longer detected. ANG (3–8), ANG (4–8) and ANG (5–8) show up as products of amino-terminal degradation. In the insert of Figure 4A the low concentrations of the peptides (AUC of the peaks) are shown. Unidentified peaks
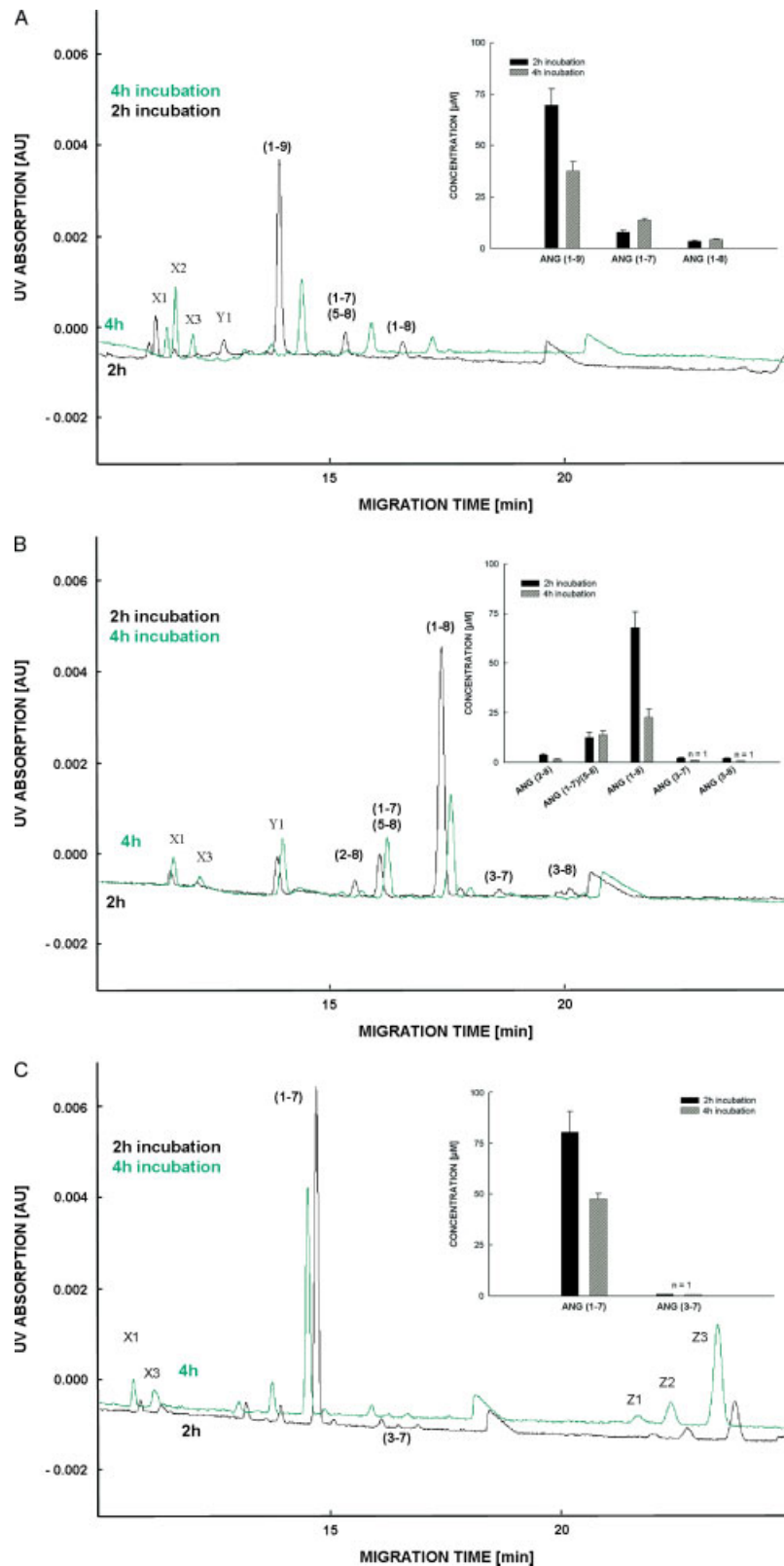
Figure 3. (A) Degradation of 100 µM ANG I (1–9) in the presence of 3T3-L1 adipocytes. The sampling and the analysis procedure were the same as described for Figure 2B. (B) Degradation of 100 µM ANG I (1–8) in the presence of 3T3-L1 adipocytes. The sampling and the analysis procedure were the same as described for Figure 2B (C) Degradation of 100 µM ANG (1–7) in the presence of 3T3-L1 adipocytes. The sampling and the analysis procedure were the same as described for Figure 2B.
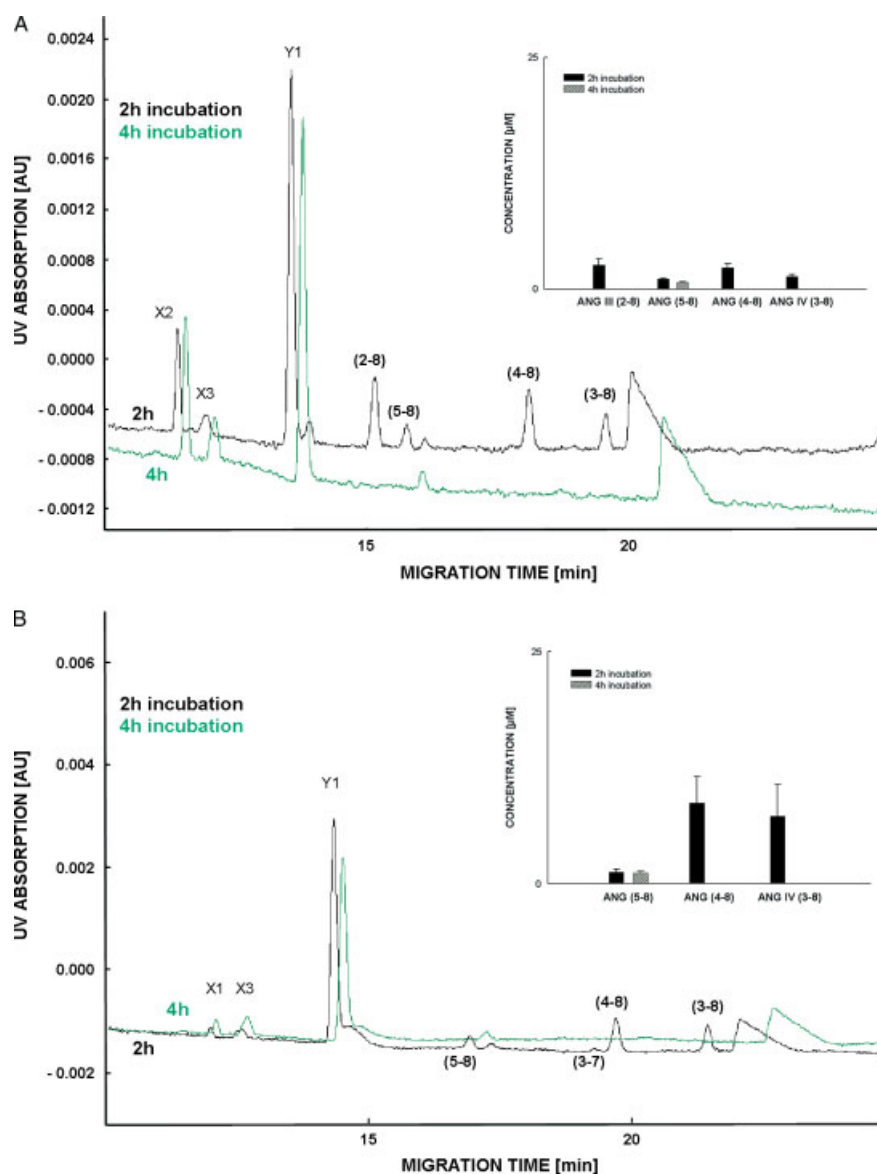
Figure 4. (A) Degradation of 100 μM ANG III (2–8) in the presence of 3T3-L1 adipocytes. The sampling and the analysis procedure were the same as described for Figure 2B. Please note the different scaling of the y-axis of the insert. (B) Degradation of 100 μM ANG IV (3–8) in the presence of 3T3-L1 adipocytes. The sampling and the analysis procedure were the same as described for Figure 2B. Please note the different scaling of the y-axis of the insert.

named X and Y were detected. In the insert of Figure 4A the concentrations of the peptides (AUC of the peaks) are shown.

As indicated in Figure 4B very similar metabolites were identified during incubation of ANG IV (3–8). ANG (4–8) and ANG (5–8) were identified as major metabolites of amino-terminal degradation processes. ANG (4–8) and ANG (3–8) completely vanished within 4 h (insert of Figure 4B). Unidentified peaks named X and Y were detected.

ANG Degradation Products Interacting with AT$_4$ Receptors

By using a fluorescence assay for the inhibition of a zinc-dependent IRAP activity it is shown that the precursor protein ANG II (1–8) has only low activity (range of 100 μM) in contrast to ANG IV (3–8) and ANG III (2–8), which were effective at high nanomolar concentrations (left panel of Figure 5A). Further amino-terminal loss of one amino acid leads to ANG (4–8) with decreased

activity. SARILE (Sar1; Ile8)-ANG II (1–8), Angiotensinogen (1–14), Zn – chelating EDTA (nonspecific inhibitor of IRAP-activity) and ANG (1–7) were not effective (Figure 5B).

The question was addressed whether interactions with one or two AT$_4$ receptor subtypes exist. Figure 6 again shows the effect of ANG III (2–8) on IRAP activity; however, in order to identify a second AT$_4$ binding site nonlinear regression was performed using both a one and two site model. The goodness of fit of a two-site model is slightly superior to the one-site model (correlation coefficient $r^2 = 0.96$ vs. 0.93). Statistical analysis by F-test shows that the more complex two-site model has to be accepted as the model of choice ($p = 0.037$). This is mainly due to the data points obtained at submicromolar concentrations. Thus ANG III (2–8) may interact with two different binding sites of IRAP (left panel of Figure 6).

In contrast the two-site model was not accepted when ANG IV (3–8) was used as the inhibiting ligand (right panel of Figure 6); in this case nonlinear regression with a one-site model is considered
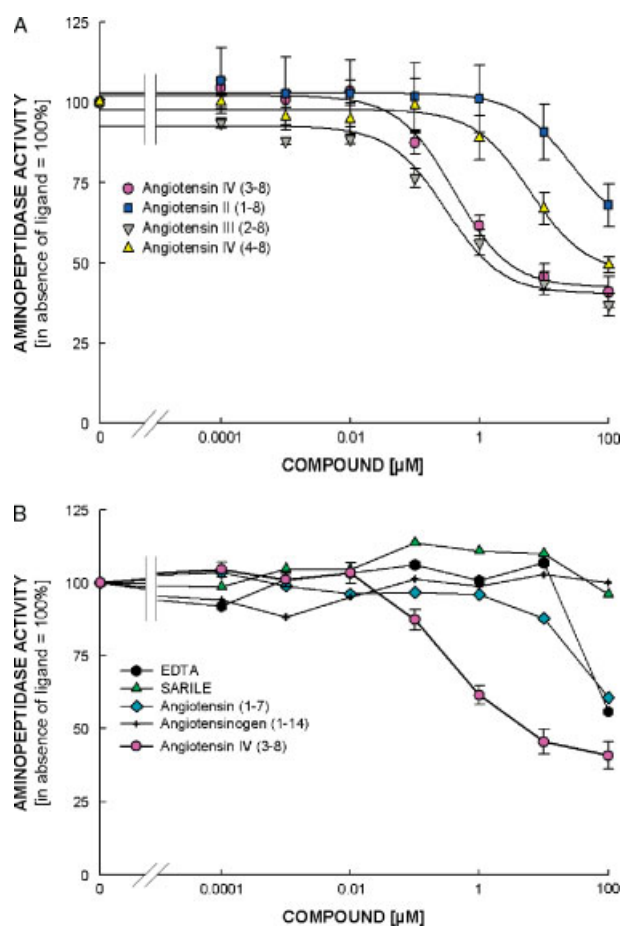
Figure 5. Inhibition of aminopeptidase activity in 3T3 – L1 adipocytes by angiotensin peptides. The compound Leu-NA was incubated with various concentrations of angiotensin peptides in presence of adipocytes for 10 min, and the resulting fluorescent product was measured. Mean ± SEM of 4–12 experiments run in eight replicates. The left panel (A) shows the inhibition by aminoterminal degradation products identified of ANG II (1–8), whereas the right panel (B) emphasizes the selectivity of this inhibition in comparison to other angiotensin peptides and derivatives and EDTA as nonselective inhibitors of IRAP-activity.

Figure 6. IRAP activity in presence of ANG III (2–8) and Nle-ANG IV. The compound Leu-NA was incubated with various concentrations of either ANG III (2–8) or Nle1-ANG IV (A) and (B) panel, respectively for 10 min, and the resulting fluorescent product was measured. Mean ± SEM of $n = 4$ experiments run in eight replicates. The figure shows the data of Figure 5A, supplemented by the modeled concentration-effect curves of a two-site and one-site model.

as the model of choice (compare left panel of Figure 5). The same holds for Nle1-ANG IV (3–8) as a more stable peptidic AT$_4$ agonist even though there is a tendency towards a biphasic effect (data not shown).

The IRAP data fit very well to AT$_4$ receptor binding data [unpublished].

Discussion

Degradation Products

The degradation was not that quick (Figure 2A) as expected so that it was sufficient to use 2 and 4 h during further experiments (Figures 2B to 4B). The data can be interpreted in a way that ANG (1–7) is produced from the intermediate ANG (1–9) (Figure 2B). ANG (1–7) and ANG (1–9) were produced more quickly than they were degraded (Figure 2B and Figure 3C). In contrast ANG II (1–8) appeared to have a short half life since it was not detected to a major degree (Figures 2B and 3A). This was corroborated by results from experiments shown in Figure 3B when ANG (1–8) was investigated directly. The metabolites ANG III (2–8) and ANG IV
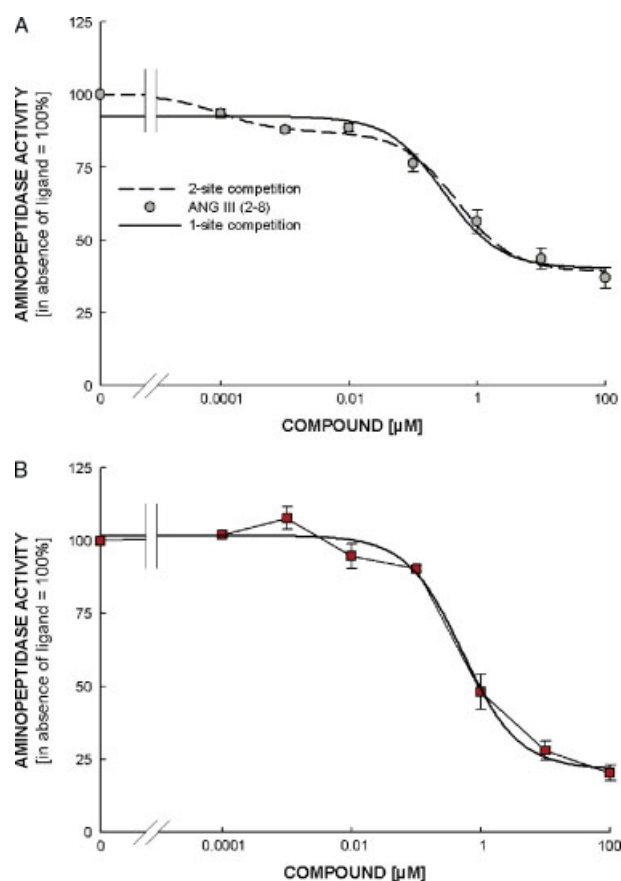
(3–8) were only transiently observed (Figure 3B) and appear to have a short half life. They may be important for AT$_4$ receptors and, therefore, included in biological investigation discussed below. ANG IV (3–8) is an active product of the aminoterminal degradation of ANG III (2–8). ANG III (2–8) and ANG IV (3–8) were degraded *in vivo* much more quickly than ANG II(1–8) [38,39]. The degradation of ANG IV (3–8) resembles that of ANG III (2–8) with respect to both the velocity of degradation and the types of peptides (Figure 4A and B). The quick kinetics may indicate an important role of this peptide and possibly important regulation for the adipocytes.

ANG (3–7) may be a metabolite of ANG (1–7) that was already expected from Figure 3B by using ANG II (1–8) and in Figure 3C by using ANG (1–7). As a bioactive peptide, ANG (1–7) is formed by ACE$_1$/ACE$_2$ – dependent pathways but also inactivated by ACE$_1$: the product is ANG (1–5). *In vivo* treatment with ACE inhibitors is associated with an increase in ANG (1–7) plasma levels. Our data corroborate the data of others [37] showing also a quick degradation for ANG IV (3–8) and for ANG II in a matter of seconds.

The inserts of Figures 3A to 5B summarize the degradation of all ANG peptides in a half-quantitative way. Most of the peptides are degraded rather slowly (25–50% within 2 h) whereas the AT$_4$ receptor agonistic peptides ANG III (2–8) and ANG IV (3–8) are
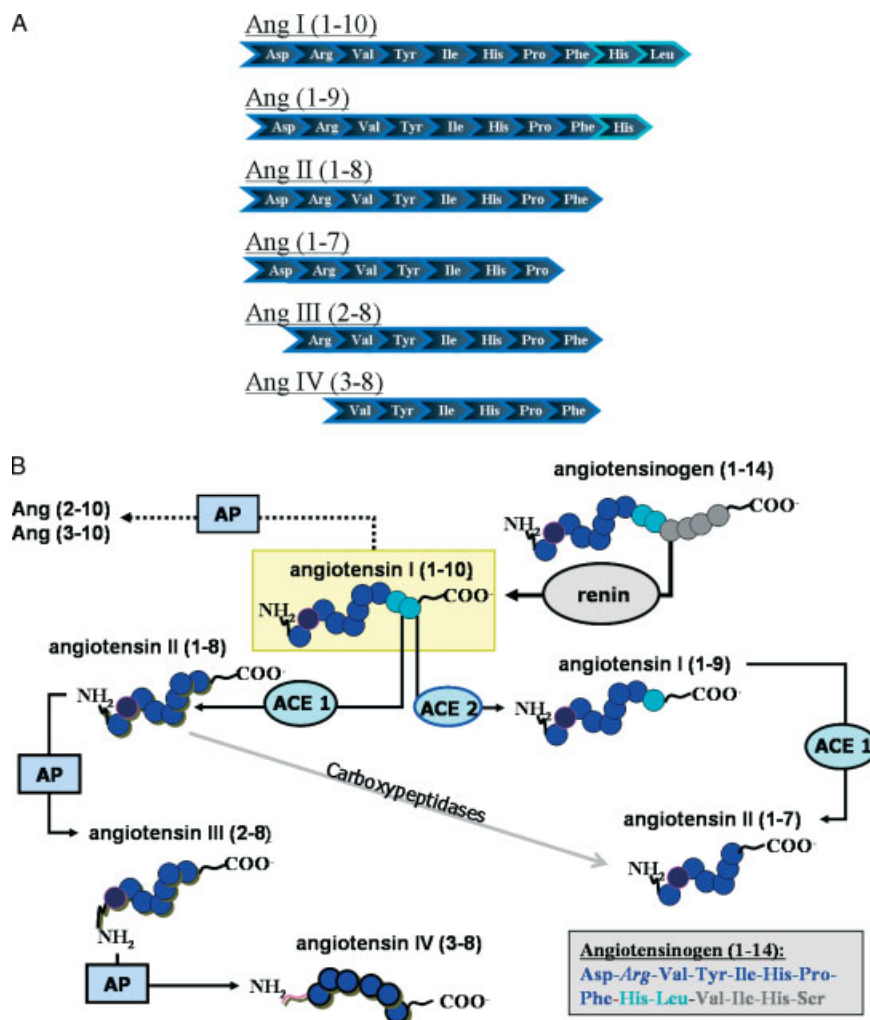
Figure 7. Chemical structure (A) and degradation pathway of angiotensin peptides (B).

degraded more quickly (>90% within 2 h) and are even rather completely eliminated within 4 h.

Unidentified Peaks

Some peaks in several figures were not identified. One peak in Figure 2B may be assigned to ANG (2–9). Since the peak Y2 of Figure 2B was no longer observed when ANG (1–9) had been added (see Figure 3A), this peak may be speculated to be ANG (2–10). Peaks Z1–3 (Figure 3C) were not investigated since they may be anionic degradation products. It cannot be ruled out that the results may be hampered by the possibility that degraded products were actively taken up by the 3T3-L1 cells and were not detected in the cell supernatant.

AT$_4$ Receptors and IRAP

Since degradation of ANG is controlled in the vicinity of adipocytes, an interaction of degradation products with AT$_4$ receptors may be of interest. The zinc-IRAP contains the AT$_4$ receptor which is a binding site at the luminal part of membrane bound IRAP. Its activity is completely inhibited by AT$_4$ receptor agonists. IRAP is involved in glucose uptake by GLUT4-transporters [40]. It is interesting to substantiate the biological role of various degraded

peptides for the IRAP/GLUT4 system. ANG III (2–8) and ANG (4–8) are already known to interact with AT$_4$ receptors [41,42]. In addition to this receptor interaction of ANG III (2–8) (Figure 5A) this peptide is also active on AT$_1$ – and AT$_2$ receptors and, therefore, a physiological peptide of considerable interest. The data show that ANG IV (3–8) and ANG (4–8) interact with IRAP (AT$_4$ receptor) but to a much lower degree than the other peptides. The very quick degradation of ANGIII and IV show their strict regulation. In addition ANG (3–7) was shown to be derived from ANG (1–7) at a low concentration which is known to have AT$_4$ agonistic effects [37].

ANG III and ANG IV (3–8) activate AT$_4$ receptors at the extracellular part of the membrane bound enzyme IRAP. The data strongly indicate that there may be more than one binding site for at least ANG III (2–8) (Figure 6).

For comparison the structures and degradation pathway of ANG peptides are summarized in Figure 7.

Conclusion

The different velocities of degradation of intermediate products may indicate an independent metabolism and specific roles of the compounds. ANG (1–7) is the main degradation product,

developing after the intermediate ANG (1–9) appeared. Both ANG III (2–8) and ANG IV (3–8) show a rapid turnover which indicates the relationship between finely regulated concentration and biological importance. This importance is verified in the IRAP assay. The findings of two different affinities, especially the AT_4 site with IC_{50} for ANG III (2–8) at subnanomolar concentrations, could gain further interest. Significant differences in IRAP inhibitory activity between metabolites were noted and correlated to a newly recognized fine control of AT_4 receptor activation at adipocytes by ANG peptides. Our data show for the first time adipocytes AT_4 receptor characteristics, which appear to be of major importance and support recent data on the ANG receptors [43].

Acknowledgements

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