THE ANGIOTENSIN GENERATING SYSTEM IN THE INSULINOMA CELL LINE INS-1

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Introduction

Evidence exists for a local angiotensin-generating system in the endocrine pancreas. Several components of a local RAS for example angiotensinogen, renin, ACE and AT_1-receptors could be found in islet cells. Autocrine and paracrine roles of a local angiotensin-generating system are discussed, for example islet blood flow, insulin secretion, cell proliferation or apoptosis. In both type 1 and 2 diabetes the pancreatic islet RAS is activated and increases inflammation, oxidative stress and free fatty acid levels. Chronic activation of a local RAS could contribute to beta-cell dysfunction[1].

Aim of study

Recent data showed that there is an angiotensin generating system in the endocrine pancreas, but it is not known which angiotensin peptides are enzymatically generated by the beta-cell line INS-1. Previous experiments were focused on activity of ANG II and the AT_1-receptor, although there are other active metabolites like ANG III, ANG 1-7 and ANG IV. The occurence and role of the angiotensin IV receptor (AT_4-rec.) which was identified as the transmembrane enzyme insulin regulated aminopeptidase (IRAP) is unknown[2].

AT_4-receptor / IRAP

Transfection

Transient transfections of IRAP-GFP pcDNA3 and GFP pcDNA3 plasmids into INS-1 cells were performed in Lab-Tek Chamberslides using Fugene®6 at a ratio of 1 µg plasmid DNA to 3 µl Fugene®6. Experiments started 48 h after transfection. For stable transfection INS-1 cells were selected with geneticin (200 µg/ml) over 20 days.

Western Blot

INS-1 and 3T3-L1 cells were lysed, treated with supersonic waves for 4 x 10 sec. and centrifuged by 2500 x g for 3 min. Supernatant was centrifuged again by 13.000 x g. Obtained supernatant was used for western blot. Proteins were separated by SDS-PAGE using a 12,5 % SDS-gel and transfered on a nitrocellulose membrane via semi dry blotting. The AT_4-receptor was detected by a specific primary antibody and a secondary HRP coupled antibody. The molecular weight of the AT_4-receptor was determined by standard protein markers.

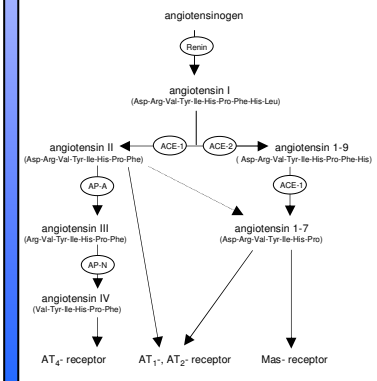
CLSM

We used a CLSM TCS SP2 AOBS® from Leica®. To visualise the GFP autofluorescence of transfected cells, the preloaded filter setup of the microscope was used (excitation wavelength 488 nm, detection wavelength 512 nm).

IRAP-ASSAY

IRAP activity was monitored by the rate of hydrolysis of L-leucyl β-naphthylamide to β-amino-naphthaline. 50 µl suspended INS-1 cells were incubated for 15 min at 20 °C in a black 96 well plate with 25 µM Leu-NA (100 µl) and 50µl ligand. Fluorescence intensity was measured with a Fluostar galaxy plate reader (ex.: 330 nm; em.: 390 nm).

Formation of angiotensin peptides



Angiotensin peptides

HPLC-MS

Angiotensin peptides were separated by HPLC and detected by LCQ® ion trap mass spectrometer.

Solid Phase:	Phenomenex Synergie 75x 2 mm, 4 µM, Hydro-RP 80
Flow:	0,5 ml/ min
Injektion:	20-30 µl
UV-Detection:	215 nm
Liquid Phase:	acetonitril : 20 mM ammuniumformiat buffer (2:98) containing 0,1 % HCOOH
	acetonitril : 20 mM ammuniumformiat buffer (85:15) containing 0,1 % HCOOH

Gradient:

t_{min}	A	B
0	100	0
30	70	30
35	100	0
40	100	0

ESI-Source:

Spray voltage (kV)	3
Sheat gas flow rate (arb)	80
Aux gas flow rate (arb)	0
Capillary voltage (V)	5
Tube lens offset (V)	10
Capillary temperature (°C)	200

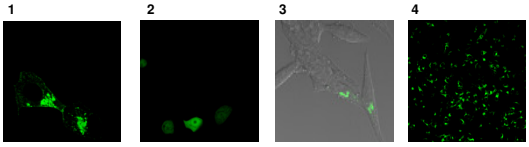
Determination of enzymatic angiotensin degradation

To analyse the enzymatic activity of insulinoma cells (Ins-1), cells were grown in 24 well plates. Cell cultur medium was replaced by KRH-buffer and spiked with angiotensin peptides. The degradation in presence of membrane bound enzymes was monitored at time point 2 and 4 hours.

RESULTS

CLSM

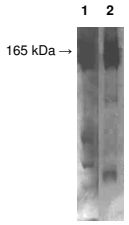
Fig. 1: Confocal images of transfected INS-1 cells



Confocal images displayed cross-section through transfected INS-1 cells with IRAP-GFP and GFP tagged plasmids. The IRAP/AT_4-rec. is located in a high amount in vesicles near the nucleus and a minor fraction at the plasma membrane **(1)**. In adipocytes IRAP is a companion of insulin-sensitve intracellular vesicles containing the glucose transporter GLUT4[3]. GFP tagged plasmid is unspecifically distributed in the cell **(2)**. Overlay of transmission and confocal image exhibit the cellulare localisation **(3)**. Stable transfection of INS-1 cells with IRAP-GFP **(4)**.

Western Blot

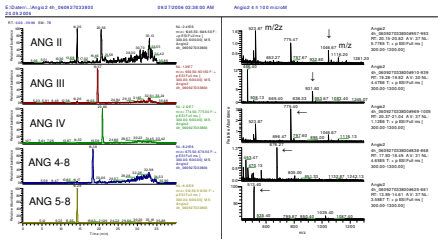
Fig. 2: Western blot of AT_4-receptor



The IRAP/ AT_4- rec. could be detected in 3T3-L1 cells **(1)** and in INS-1 cells **(2)**. Extrapolated molecularweight is shown by arrow.

HPLC-MS

Fig. 3: Sample HPLC-MS analysis of 100 µM ANG II (4 h)



We were able to separate and identify all known angiotensin peptides after incubation of ANG I or ANG II on INS-1 cells. ANG I was cleaved to the bioactive compounds ANG II in a low and ANG 1-7 in a high intensity. Several other N-terminal degradation products especially ANG 2-10, ANG 3-10, ANG 4-10 and ANG 5-10 could be found in high intensity. If products like ANG 2-9 or ANG 2-7 exhibit intrinsic activity is not known. ANG II was metabolised to ANG III. ANG III was cleaved to ANG IV and this was rapidly cleaved to ANG 4-8 and ANG 5-8. The precise degradation path and involved membraneous enzymes are not known.

IRAP-ASSAY

Inhibition of aminopeptidase activity by angiotensin IV
Fig. 4

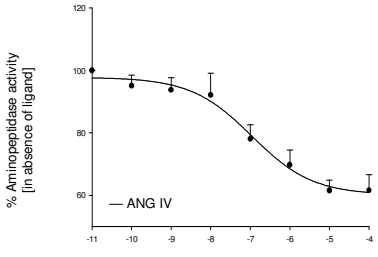


Fig. 4: The AT_4-ligand ANG IV inhibited the cleavage of the synthetic substrat Leu-β-NA by the aminopeptidase function of IRAP with an IC_{50} value of about 300 nM in INS-1 cells up to 60 % aminopeptidase activity. These findings conform with results from 3T3-L1 adipocytes[4]. Inhibition of IRAP by ANG IV may modify the processing of endogenic peptide hormones[5].

Inhibition of aminopeptidase activity by angiotensin peptides
Fig. 5

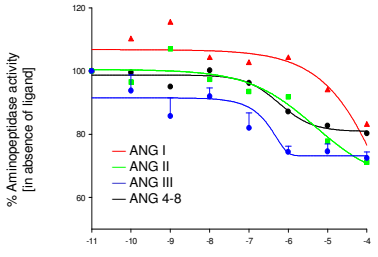


Fig. 5: ANG I (IC_{50} > 10 µM) and ANG II (IC_{50} > 1 µM) have only low affinity to the catalytic site of IRAP. ANG III (IC_{50} = 300 nM) has nearly the same affinity to the catalytic site as ANG IV but a lower efficacy (75 % aminopeptidase activity). Degradation product of ANG IV cleavage ANG (4-8) reduced aminopeptidase activity up to 80 % (IC_{50} = 800 nM).

Conclusion

- The beta-cell line INS-1 exhibits an autonomous angiotensin-generating system.
- A variety of degradation products indicate a simultanous N-terminal and C-terminal cleavage of ANG I by amino- and carboxypeptidases.
- The severe control of ANG III and ANG IV concentration as well as their control of IRAP activity indicate their importance for INS-1 cell function.
- AT_4-receptors / IRAP are expressed in vesicles of INS-1 cells near the nucleus, constantly trafficking to the plasma membrane in a small amount.
- The impact of AT_4-receptor ligands on INS-1 cell function is the aim of further research.

Literature

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Abbreviations

ACE	angiotensin converting enzyme	GFP	green fluorescence protein
ANG	angiotensin	HRP	horse radish peroxidase
AP	aminopeptidase	Leu-β-NA	L-leucyl β-naphthylamid
ESI	electro spray ionisation	IRAP	insulin regulated aminopeptidase
GLUT4	glucose transporter 4		