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Mouse vs. Human ACE2: Novel Insights into Pharmacological Differences and Implications for the Renin-Angiotensin-Aldosterone-System

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List of contents

List of figures

List of abbreviations

ACE	Angiotensin-Converting Enzyme
ACE2	Angiotensin-Converting Enzyme 2
Ang	Angiotensin
ARB	Angiotensin II type 1 receptor blockers
AT_1	Angiotensin receptor $Type_1$
AT_2	Angiotensin receptor $Type_2$
BisTris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methan
CHO	Chinese Hamster Ovarian
CPA	Carboxypeptidase A
DEAE	Diethylaminoethyl
DIZE	Diminazene Aceturate
DMSO	Dimethyl Sulfoxide
E.C.	final concentration
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ER	Endoplasmatic reticulum
ERK2	extracellular-signal-related-kinase-2
EU	Endotoxin Unit
HIC	Hydrophobic Interaction Chromatography
HPLC	High Performance Liquid Chromatography
IC50	half-maximal inhibitory concentration
IP_3	Inositol triphosphate
iP	isoelectic point
LAL	Limulus Amoebocyte Lysate
LC	Liquid Chromatography
MS	Mass Spectroscopy
MW	Molecular Weight
NEP	Neprilysin/Neutrale Endopeptidase
NHS	N-hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis
PLC	Phopholipase C
PKC	Proteinkinase C
RP	Reversed Phase
RT	Room Temperature
SDS	Sodium dodecyl sulfate
SEC	Size-Exclusion-Chromatography
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
V	Volt
XNT	(1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one)

Zusammenfassung

Die Bedeutung des Renin-Angiotensin-Aldosteron-Systems (RAAS), einer Peptidhormon-Enzym-Kaskade, für die Regulation des Salz- und Wasserhaushalts sowie des Blutdrucks ist seit jeher bekannt. Mit verschiedenen Medikamenten wird therapeutisch in das Renin-Angiotensin-Aldosteron-System eingegriffen um Bluthochdruck und weitere Herz-Kreislauf-Erkrankungen zu behandeln. Einer der vielversprechendsten neuen Ansätze wurde im Jahr 2000 mit der membrangebundenen Zinkmetalloprotease ACE2 entdeckt, einem Enzym, welches das RAAS in Balance hält. Diverse Krankheitsbilder wie Herz- und Nierenversagen, Diabetes sowie Venen- und Lungenkrankheiten konnten mit unzureichenden endogenen Expressionslevels von ACE2 in Verbindung gebracht werden. Die komplementäre Verabreichung in Form von rekombinantem humanen ACE2 stellt somit eine neuartige und vielversprechende Therapie dar, welche es in unterschiedlichen Studien-Settings, unter anderem in Mäusen, zu untersuchen gilt. Da rekombinantes humanes ACE2 aber vom Immunsystem der Maus als fremd erkannt und eliminiert wird, braucht es rekombinantes murines ACE2, um Langzeitstudien mit ACE2 in Mäusen durchzuführen, speziell wenn therapeutische Effekte in chronischen Krankheitsmodellen untersucht werden.

Im Zuge der Masterarbeit wurden mehr als 600 mg rmACE2 in CHO-Zellen exprimiert und über verschiedene Chromatographie-Schritte gereinigt. Das Produkt wurde hinsichtlich Reinheit, Molekulargewicht, Modularität, isoelektrischen Punkts, Glykosylierung, hydrodynamischen Radius und Sekundärstruktur beschrieben und mit rekombinantem humanen ACE2 verglichen. Weiters wurden die katalytischen Aktivitäten beider ACE2-Homologe und deren Inhibierungssensitivität gegenüber DX600, *in vitro* als auch *ex vivo* in humanem Plasma mittels neuartiger LC-MS/MS basierender Methode untersucht. Rekombinantes murines und humanes ACE2 unterscheiden sich hinsichtlich ihrer Tertiärstruktur (hydrodynamischer Radius, Glykosylierung) sowie auch hinsichtlich der katalytischen Konversion von Angiotensin 1-10 zu Angiotensin 1-9, was auf eine Unterrepräsentanz der alternativen RAS-Achse in Mäusen hindeutet. Weiters konnte eine 100-fach schwächere Inhibierungssensitivität des murinen ACE2 gegenüber DX600 verglichen mit humanen ACE2 nachgewiesen werden. Diese Spezies-spezifische Aktivität und Inhibierung von ACE2 wurde erstmals im Zuge dieser Arbeit nachgewisen. Sie hat direkte Folgen für weitere ACE2 Studien in Nagern und sollten auch Anlass für eine stärkere Berücksichtigung von Spezies-Unterschieden in Tier-Studien anderer biochemischer Pathways geben.

Abstract

The importance of the enzyme peptide-cascade Renin-Angiotensin-System (RAS) or Renin-Angiotensin-Aldosteron-System (RAAS) for salt-retention, fluid-balance and blood pressure is well-known and target of different drugs to treat hypertension and cardiovascular diseases. In 2000, one of the most promising new approaches was discovered and published: ACE2, a membrane-bound zincmetallo-protease with rebalancing effects on RAS. Altered or decreased expression levels of ACE2 are associated with vascular and cardiac disorders, diabetes, lung damages and kidney injuries. The additional application of recombinant human ACE2 to complement insufficient endogen ACE2 levels can be a novel clinical treatment for various diseases which has to be investigated with further trials, such as mice-studies. But due to the immunogenic response in mice against human ACE2, the murine homologue of the enzyme is necessary to investigate long-term ACE2 applications in different study-settings in mice.

In the course of the Master Thesis, more than 600 mg of recombinant murine ACE2 were expressed in CHO-cells under serum-free conditions and purified by several chromatography steps. The product was characterized concerning size, purity, modularity, isoelectric point, glycosylation, hydrodynamic radius and secondary structure and compared to recombinant human ACE2. Furthermore, the catalytic activity and sensitivity towards the commercially available ACE2-inhibitor DX600 were investigated *in vitro* as well as *ex vivo* in human blood plasma using a novel LC-MS/MS based method. More than previously expected, the ternary structure (hydrodynamic radius, glycosylation) differs between human and murine ACE2 and also the catalytic conversion of Angiotensin 1-10 to Angiotensin 1-9 which may point to a under representation of the alternative RAS-axis in mice. Furthermore, rmACE2 was found to be 100 fold less sensitive towards DX600 than human ACE2. The species-specific activity and inhibition of ACE2 concern directly further ACE2 studies in piglets and rodents and shall give rise to major considerations of species-differences in animal-studies as well as for investigations in other biochemical pathways.

1. Introduction

The Renin-Angiotensin-System (RAS) or Renin-Angiotensin-Aldosterone-System (RAAS) is a system of peptide hormones and enzymes, which regulates blood pressure, salt retention and fluid balance[1]. The name RAS refers only to the peptides and simplifies the system. RAAS also includes the role of Aldosterone, a steroid hormone which affects sodium-retaining and high levels are associated with pathophysiological effects[2]. However, an overactive or misdirected RAAS is associated with several diseases like hypertension[3], heart failure[4], kidney failure[5], and diabetes[6]. Although the importance of the RAAS is well known for a long time, the far-reaching implications of the single components of the systems are not fully understood yet.

The RAAS is a common target of therapeutic treatments. Captopril, established in 1981, was the first therapeutic drug to treat hypertension by inhibiting ACE and rebalancing RAS[7]. In the nineties, a second generation of ACE-inhibitors was established, such as Lisinopril or Ramipril, but due to different side-effects of ACE-inhibitors[8] new strategies and drugs have been developed. For example, Aliskiren, a direct renin-inhibitor[9], or Losartan, an inhibitor of the AT_1-R of RAAS[10] which effects vasoconstriction and proliferation.

Recombinant human Angiotensin-Converting Enzyme 2 is a novel clinical treatment for various diseases associated with an imbalance of RAAS. ACE2 was discovered in 2000[11] and the functional importance of the zinc-metalloprotease has become unequivocal. Altered or decreased expression levels of ACE2 are associated with vascular or cardiac disorders, like endothelial dysfunction or atherosclerosis[12]. Furthermore, ACE2 also plays a critical role in kidney or respiratory disease and diabetic renal injury[13] [14]. Treatment with recombinant human ACE2 to complement insufficient activity of endogen ACE2 might be a novel therapy against various diseases. The clinical use of rhACE2 against Acute Lung Injury (ALI) is currently under investigation. But rhACE2 might also be used for treatment of other diseases why further pre-clinical studies are necessary.

Piglets have been established as animal model to investigate the effects of novel drugs, gene knockouts or other therapeutic approaches *in vivo.* Studies with piglets are associated with less effort than studies with animals which are closer to human, like apes. But due to the larger evolutionary distance to men, it is essential to know if the analyzed physiological condition is similar and comparable to humans. Also rhACE2 was investigated in additional, different therapeutic mice settings to get more information about ACE2 and awareness about the functionality of the enzyme and its role in several ailments. Different settings require a long-time application of recombinant human ACE2 but shortly after application, a humoral immune-response against the exogenous

human protein is built up which neutralize its enzymatic activity. For this reason, larger amounts of recombinant murine ACE2 are necessary for further studies.

In the course of this Master Thesis, **recombinant murine Angiotensin-Converting-Enzyme 2** should be expressed in CHO-cells under serum-free conditions in a chemical defined medium and be purified using serial chromatography steps. The final product shall be characterized concerning purity, size, glycosylation, substrate specificity/turn-over number, isoelectric and melting point, endotoxin level and secondary structure. Furthermore, the association respective dissociation constant of two different antibodies towards rmACE2 should be determined.

Despite the fact that the sequence identity of recombinant murine and human ACE2 is only 83 %[15], it has been supposed that the enzymes have a similar catalytic activity and the same sensitivity towards ACE2 inhibitors. In this course, the implications of a treatment with recombinant murine and human ACE2 for the RAS of human plasma will be shown and compared using a novel and highly sensitive LC-MS/MS based method. Furthermore, differences between rmACE2 and rhACE2 in terms of substrate specificity and sensitivity towards the ACE2-inhibitor DX600 will be demonstrated and highlighted *ex vivo*.

These experiments should pave the way for further studies in mice and compare pharmacological characteristics of mouse and human ACE2.

2. Background

2.1.Renin-Angiotensin-Aldosterone-System

The Renin-Angiotensin-Aldosterone-System is a complex network of different enzymes and peptides. Thereby the amount, formation and degradation of the peptides are important for the signaling, which regulates blood pressure, salt retention and fluid balance[1]. Formed peptides bind to different receptors and trigger varying answers of the cells to environment (See Figure 1).

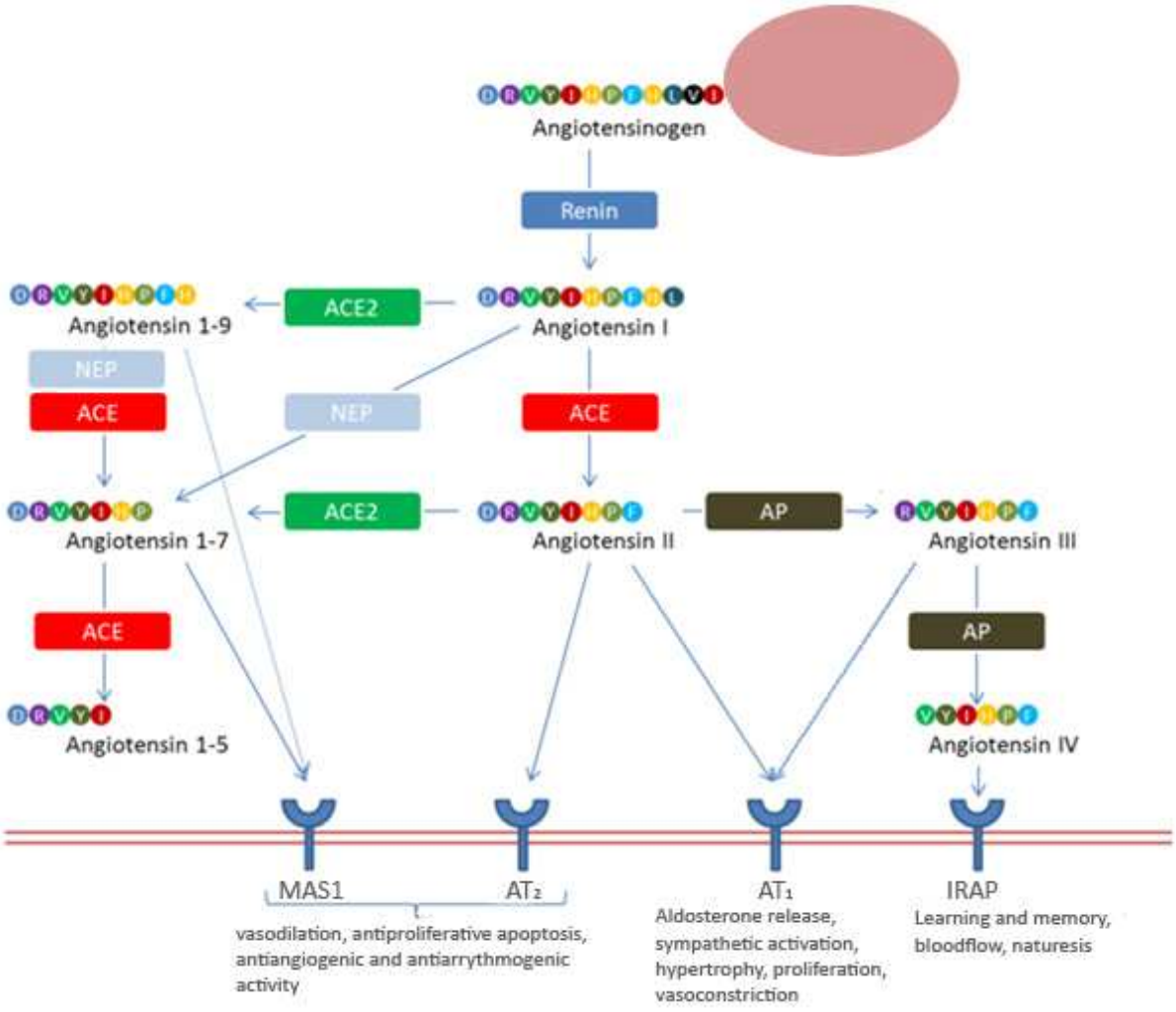

Figure 1 Schematic figure of the Renin-Angiotensin-Aldosterone-System. The system is a complex network between enzymes and peptides. The colored cycles represent peptides, where the letter in the middle is the short-letter-code of the amino acid the peptide is build of. The enzymes of the system are represented as boxes and the membrane-bound-signaling-receptors can be seen at the bottom of the figure. The binding consequences and signaling between specific peptides and receptors, shown by arrows, stand below the receptors. The specific binding between Angiotensin 1-9 and MAS1 is under investigation and not certain yet, so the arrow is dashed. To keep the figure simple, not all enzymes, which are also able to cleave the peptides, are shown. ACE: Angiotensin-Converting-Enzyme, ACE2: Angiotensin-Converting-Enzyme 2, NEP: Neprilysin/Neutrale Endopeptidase, AT_1: Angiotensin II Type 1 receptor, AT_2: Angiotensin II Type 2 receptor, IRAP: Insulin-Regulated-Aminopeptidase

The whole RAAS is started by and depends on its key-regulator: Renin. In juxtaglomerular cells of the kidney, Renin is synthesized and stored in vesicles. Its release is influenced by sympathetic nerves, tubular mechanisms, renal autacoids, and hormones to increase salt retention and blood volume[16]. Renin is an aspartyl protease and cleaves with high specify the tail of the C-terminus of Angiotensinogen to form the decapeptid Angiotensin 1-10, also known as Ang I. The major source of systemic circulating Angiotensinogen is the liver[17]. The further conversion of Ang 1-10 depends on the ACE/ACE2 ratio. ACE is part of the classical RAS-axis, ACE2 an activator of the alternative RAS-axis.

2.1.1. ACE, Ang 1-8 and Angiotensin II receptors – classical RAS-axis

Angiotensin-Converting-Enzyme, called ACE, is a zincmetalloprotease dicarboxypeptidase. In its membrane bound form, the enzyme is transmembrane anchored near to its carboxyterminal extremity. Caused by post-translational cleavage of the anchor it exists also in secreted form. The soluble enzyme consists of two large peptidic domains, designated the N- and C- domains, with high homology and both bearings a zinc-binding-domain and therefore a putative active site[18]. Testicular ACE contains only one active site on the C-domain[19]. Circulating and tissue ACE levels are associated with an insertion/deletion polymorphism of a 287-basepair *alu* repeat sequence in Intron 16 of the ACE-gen[20]. ACE is able to degrade bradykinin, a powerful vasodilator, and further it removes C-terminal histidyl-leucine from Ang 1-10 forming the physiological active oktapeptide Angiotensin 1-8, called AngII [21] [22].Furthermore, even Angiotensin 1-9, a nonapeptide and part of the alternative RAS-axis, formed by ACE2 and Carboxypeptidase A (CPA) is cleaved by ACE resulting in Angiotensin 1-7[23], which is also cleaved by ACE, but the physiological relevance of the product, Angiotensin 1-5, is not known yet and under investigation.

Angiotensin 1-8, called Ang II, is involved in the regulation of blood pressure and stimulates the release of the adrenal cortical hormone Aldosterone, which is a major regulator of potassium and sodium[24] levels and a potent vasoconstrictor, too. Increased levels of Ang 1-8 are associated with life threatening pathologic conditions including hypertension, progression of atherosclerosis and kidney diseases, heart failure and also tumor progression[25] [26]. The relevance of Ang 1-8 are mediated by the binding to two different receptors: AT_1-R and AT_2-R[27].

AT_1-R (Angiotensin receptor type I) is a G_q protein, membrane integrated by seven transmembrane glycoproteins and activates phopholipase C (PLC). As a result, PLC hydrolyzes Phosphatidylinositol 4-5 –biphosphate and the formed diacyl glycerol is an activator of protein kinase C. Further, the second cleavage product is IP_3 (Inositol triphosphate), which is responsible for the release of Ca^{2+}, a second messenger. The result is the activation of several serine/threonine and tyrosine kinases[28]. Summarized, the activity of AT_1-R leads to vasoconstriction and increased vasopressin secretion, proliferation and sympathetic activation[29].

AT_2-R (Angiotensin receptor type II) is also a G-coupled protein receptor and highly expressed during fetal development. In adult tissues, the receptor is less abundant and dramatically down regulated. Interestingly, the receptor is re-expressed under conditions of ischaemic or traumatic injury (target organ damage) in many tissues. Thus, under normal physiological circumstances, this receptor can hardly be detected in many organs or cellular systems, including those of cardiovascular relevance[30]. Its signaling pathways include serine and tyrosine phosphatases too, but also phospholipase A_2, nitric oxide and cyclic guanosine monophophate. The sequence similarity to its counterpart AT_1 is 30 %[27]. For a long time, the functionality of the receptor was unclear and contradictory due to the fact that Ang 1-8 binds both, AT_1-R and AT_2-R and no selective AT_2-R -antagonist was accessible. But it was assumed that the receptor counteracts several of the pathways of AT_1-R and cross-talks negatively with several receptor tyrosine kinases to inhibit cell growth[31]. The receptor also interacts with MUTS (Mitochondrial tumor suppressor 1), which consequences in inhibition of ERK2 (extracellular-signal-related-kinase-2)-activity, a proliferation-factor[31 32]. Recent studies with a novel orally active, non-peptidergic and highly selective AT_2-R agonist, compound 21, support an anti-inflammatory role for AT_2-receptors[33]. Chronic or intermittent inflammation is considered being a substantial mechanism in the progression of cardiovascular disease. Inflammatory responses to acute events such as myocardial infarction or stroke have been increasingly recognized as aggravating pathological factors[34]. Recent studies reported that even the disruption of the receptor promotes longevity in mice[35].

2.1.2. ACE2, Ang 1-7 and MAS receptor – alternative RAS-axis

Angiotensin-Converting-Enzyme 2, ACE2, was first described by two independent groups. Both groups identified an ACE homologue, which they called ACE2[36] or Captopril-insensitive Carboxypeptidase[11]. ACE2 is a zinc-metalloprotease and functions as a carboxymonopeptidase with a substrate preference for hydrolysis between proline and a C-terminal basic or hydrophobic residue. ACE2 is a type I membrane-anchored protein and present in many cardiovascular-relevant organs, such as heart, kidneys, blood vessels and lungs [25]. ACE2 is a key-regulator within the RAS and counteracts with ACE in two ways: either it metabolizes the vasoconstrictive and pro-inflammatoric Ang 1-8 directly to generate the vasodilatory and anti-proliferative Ang 1-7, or it competes for Ang 1-10 with ACE to form Ang 1-9, a precursor of Ang 1-7[37]. A variety of peptide mediators, relevant in other physiological systems, is processed by ACE2, too, such as apelin-13 and apelin-36, des-Arg9-bradykinin, dynorphin A, neurotensin and casamorphin[38]. Interestingly, some of these mediators might be involved in wound healing and tissue injury[39]. A commercially available ACE2 specific inhibitor is **DX 600** whereas a recent study described differences of the half-maximal inhibitory concentration (IC50) between human and rodent ACE2 *in vitro*[40]. **Diminazene Aceturate, called DIZE** and known as a tryphanocide, is reported being an ACE2 activator and reducing mean arterial pressure, heart muscle mass and myocardial fibrosis[41]. Another synthetic ACE2 activator currently in pre-clinical development stage, **XNT**, protects in monocrotaline-induced pulmonary hypertension rat model against elevated right ventricular systolic pressure, right ventricular hypertrophy, increased pulmonary vessel wall thickness and interstitial fibrosis[42].

Angiotensin 1-9 is a poorly characterized peptide and was believed being also an inert Angiotensin like Angiotensin 1-10 and only the precursor of Ang 1-7. But recent findings suggest that it has physiological effects, too. Increased Ang 1-9 levels in plasma can be observed in patients after a myocardial infarction. Furthermore, the administration of Ang 1-9 was shown to decrease levels of Ang 1-8, inhibits ACE-activity and prevents cardiac myocyte hypertrophy in myocardial infarcted rats[43]. But the molecular effect of Ang1-9 is still unclear. On the one hand it is assumed that Ang 1-9 acts as a competitive inhibitor of ACE, on the other hand a recent study supposes that Ang 1-9 interacts directly with AT_2-receptor, a counterpart of the profibrotic, vasoconstrictive and proliferative AT_1-receptor[44]. But the importance of Angiotensin 1-9 is without doubt, keeping in mind that this peptide is a precursor of Angiotensin 1-7. ACE or NEP cleave two C-terminal amino residues from Ang 1-9 generating Ang 1-7.

Angiotensin 1-7 is a ligand of the G-coupled MAS-receptor[45] and is the biological active counterpart to Ang 1-8. Many actions of the peptide are often reversed to those attributed to Ang 1-8 and have counter-regulatory effects within the RAS. For a long time, Angiotensin 1-7 has been regarded as an inactive product in RAS[46]. But later findings emphasize clearly the importance of Ang 1-7 for the physiology of the heart[47], blood vessels[48] and the kidney[49]. Altered expression and function of ACE2 and thus lower levels of Ang 1-7 are associated with cardiac and vascular disorders[50]. Hence, reduced ACE2 levels could lead to elevated Ang 1-8 levels, which promotes hypertension[51]. Ang 1-7 also antagonizes the Ang 1-8-induced fibrosis and myocardial hypertrophy[47] and causes endothelial depended vasodilation mediated by NO release through interaction with MAS[52].

The G-coupled **MAS receptor** is triggered by Ang 1-7 and abundantly expressed in the brain and testis. Lower levels of expression were also observed in other tissues, such as in the heart, liver, spleen, tongue, kidney and lung[53]. MAS is encoded by its proto-oncogen *Mas*. One of the major pathways of MAS-signaling in the cardiovascular system is the phosphorylation of Akt, which leads to a NO release, but also other factors, such as inhibition of the p38 MAP kinase or activation of the proteinkinase C, are involved in the signaling[54]. Summarized, the activation of MAS through Ang 1-7 shows varying positive effects, such as vasodilation, antiproliferation, it operates antiarrythmogenic and antiangiogenic, increases the renal blood flow and the renal water resorption and modulates the excretion of renal Na^+ [55]. However, therapeutic application of Ang 1-7 is limited due to its short half-life and rapid turnover[25]. Hence, another approach raising levels of Ang1-7 is to increase the catalytic activity or amount of ACE2.

2.1.3. Therapeutic manipulation of the human RAAS

As described above, a misdirected or imbalanced Renin-Angiotensin-Aldosterone-System plays a key role for hypertension and further pathophysiological conditions. Therefore, components of the system are common targets for different therapeutic drugs.

The inhibition of ACE is a promising treatment option against different types of cardiovascular diseases. So, **ACE inhibitors** have been developed for treatment of left ventricular dysfunction, heart failure and hypertension. **Captopril** has been the first orally active ACE-inhibitor, followed by another ACE-inhibitor **Enalapril**. Enalapril is poorer absorbed than Captopril and is administered as a prodrug , which is converted by hepatic esterolysis to its active form, but has the advantage being a more

potent inhibitor of ACE, slower eliminated and does not possess a sulfhydryl group. **Lisinopril**, an ACE-inhibitor of the second generation, is similar to Enalapril, but administered in its active form because of the better oral bioavailability (25 %)[56]. Side-effects of these drugs are dry cough, acute kidney failure and pharmacologic escape mechanisms: treated persons develop a long-term resistance respective tolerance against these inhibitors[57] .

Also the inhibition of AT_1-R is a used treatment of hypertension, congestive heart failure and diabetic nephropathy. **Angiotensin II type 1 receptor blockers (ARBs)** such as **Losartan**, which was firstly introduced in 1995, are competitive and selective inhibitors of the receptor and have fewer side-effects than ACE-inhibitors[57].

Renin got also in focus of physicians in recent years due to its key-regulating function in the RAAS. Increased levels of Renin in blood are biomarkers of primary aldosteronism and are further responsible for hypertension[58]. For this reason, direct and selective inhibitors of Renin are in clinical use for treatment of primary hypertension. **Aliskiren** has been the first in the class of direct Renin inhibitors[9]. Furthermore, determination of plasma-Renin-concentration in blood is crucial for the treatment of primary hypertension.

2.1.4. Recombinant human Angiotensin-Converting Enzyme 2

Recombinant human ACE2 can be a novel clinical treatment approach to complement insufficient endogen ACE2 to raise levels of Ang 1-7 and lower the amount of Ang 1-8, both associated with various physiological protective effects (See above). rhACE2 only contains the extracellular domain (amino residues 1 – 740) without the membrane anchor present in the native enzyme and occurs as a dimer[11]. The recombinant enzyme shows various positive effects: infusion of rhACE2 lowers Ang 1-8 mediated myocardial fibrosis, expression of hypertrophy markers and reduces Ang 1-8 induced superoxide production. Furthermore, rhACE2 partially prevented the development of dilated cardiomyopathy in pressure-overloaded wild-type mice[59]. Additionally, rhACE2 was reported to protect mice from acute respiratory distress syndrome (ARDS) induced by acid aspiration or sepsis. This finding was surprising because ACE2 is also a receptor for the severe acute respiratory syndrome (SARS) coronavirus, a predisposing factor of ARDS[60]. And finally, also the progression of diabetic nephropathy in mice could be reduced by rhACE2[13].

2.1.5. Alternative RAS-associated enzymes

In addition to ACE and ACE2, other enzymes are also able to cleave Angiotensin-peptides. The **CPA, called Carboxypeptidase A,** generates Ang 1-9 from Ang 1-10 like ACE2, **PCP, also known as prolyl carboxypeptidase,** cleaves like ACE2, too, but Ang 1-8. The **PEP, prolyl endopeptidase**, is capable to form the vasodilator Ang 1-7 by degrading the inert Ang 1-10[25]. The physiological relevance of these enzymes is currently under investigation. Unchallenged is the importance of NEP.

Neprilysin (NEP) is part of different signal cascades and involved in the metabolism of a number of regulatory peptides, such as in immune and inflammatory system, or in the Renin-Angiotensin-System. NEP, also called CALLA, CD10, Neutral Endopeptidase or Enkephalinase, is an integral membrane ectopeptidase and belongs to the protein family of M13 zincpeptidases[61]. The enzyme has a single HEXXH active side motive with preference for cleaving on the N-terminal side of hydrophobic residues[62]. NEP is able to form Angiotensin 1-7 in two ways: either it cuts off three amino residue of the inert Angiotensin 1-10, or two amino acids of Ang 1-9 forming Ang 1-7, both with similar turn-over rates[23 63] .

An alternative enzyme to ACE and thus part of the classical RAS-axis is **Chymase**. The ACE-independent formation of Ang 1-8 by the serine proteinase was first observed in the heart. Cardiac mast cells are the major cellular source of this enzyme. Chymase together with Renin is thought to influence the local RAAS in a critical way. This could be the reason why mast cells play a direct role in promoting left ventricular dysfunction in a model of congestive heart failure[25].

Aminopeptidase A and N are also part of the Renin-Angiotensin-System, although with different implications compared to ACE or ACE2. Both are zinc metalloproteases that belong to thermolysin-like enzyme group with significant identity between their amino-acid sequences. The N-terminal Aspartic Acid of Ang 1-8 is cleaved off by Aminopeptidase A forming Ang 2-8. Both, Ang 1-8 and Ang 2-8, are part of the Renin-Angiotensin-System of the brain that participates in the control of blood pressure, vasopressin release and increase water consumption. The binding affinity of these peptides to AT_1-R and AT_2-R is the same[64]. Aminopeptidase N hydrolyzes N-terminal Arginin of Ang 2-8 to generate Ang 3-8, a ligand of IRAP. IRAP, short for insulin regulated aminopeptidase, is a membrane spanning zinc-metallopeptidase and its activity is associated with learning and memory, natriuresis and bloodflow[65].

3. Methods

3.1. Activity Assay

Background

This assay quantifies the enzymatic activity of ACE2 using Mca-Ala-Pro-Lys (Dnp) as substrate. ACE2 cleaves the C-terminal Lys residue and removes the 2,4-dinitrophenyl moiety that quenches the fluorescence of 7-methoxycoumarin moiety resulting in an increased fluorescence which is measured photometrically.[66]

Working procedure

- 1mg of ACE2 Substrate Mca-Ala-Pro-Lys(Dnp)-OH (Thermo) dissolved in 300µl DMSO (Sigma) + 1135µl MES buffer = 1mM
- Working dilution: 1:5 in MES buffer (1ml + 4ml), pre-warmed at 37°C
- Prepare standard curve with APN01 #0308-A in a range between 0.16 and 0.01 µg/ml in MES buffer in U-bottom micro plate (Greiner) in duplicates
- dilute samples in MES buffer to concentration in estimated detectable range in U-bottom micro plate in duplicates
- transfer from the U-bottom plate 50 µl APN 01 standard or samples to flat bottom micro plate (Greiner) for measurement
- add 50µl of substrate working solution (1:5 prediluted as described above) to each sample in wells of flat bottom micro plate
- temper spectrophotometer (Anthos ELISA-reader) to 37 °C
- start immediately measurement following kinetic program “APN01-1-01” at wave lengths 320nm and 430nm (excitation resp. emission)
- increase of fluorescence in unknown samples is read on the standard curve using graph pad and depicted as µg/ml APN01

Buffers / Reagents

MES-buffer	50 mM MES, 300 mM NaCl, 10 µM $ZnCl_2$, 0.01 % Brij-35, pH 6.5

3.2.BiaCore®

Background

Biacore® systems investigate the interaction events between biomolecules by analyzing the surface plasmon resonance (SRP). The SRP-phenomenon is observable in thin conducting layers at an interface between media of different refracting index. In Biacore systems, the media are the sample solutions and the golden sensor chip is the conducting layer. The energy, angel and electric field intensity of incident light, which is reflected by the sensor chip, is measured and differ due to the refractive index of a solution. The refractive index depends on the characteristic and concentration of a solution and the resulting calculated values are called resonance units. (RU) In conclusion, binding events can be followed in real time.

In this experiment, recombinant murine ACE2 was attached on the Dextran-surface of a CM5 sensor chip via covalent immobilization, more precisely amine coupling. The first step is activating the carboxyl-groups of the Dextran-surface by a mixture of 0.2 M EDC and 0.05 M NHS to give reactive succinimide esters. The next step is pH scouting with rmACE2 which depends on the isoelectric point of the enzyme: if the pH of the running solution is below the isoelectric point of the protein, yielding nucleophilic groups, like amine groups of amino acids react spontaneously with the succinimide esters. Non-reacted residues are saturated with Ethanolamine. Then, an antibody-solution against rmACE2 passes over the surface. Resulting binding events can be detected caused by changes of refractive indices of the light. After analyzing the binding events, the chip has to be regenerated to repeat the measurement with other antibodies. This is the process of removing bound antibodies form the sensor chip without destroying the bound protein. Appropriate solutions are HCl, NaOH or glycine-buffer whereas it is recommended to keep in mind that a highly altered pH-value could denature the protein and epitops[67].

The resulting binding-curve is analyzed by software (Biacore® Control Software 2000). The curve is interpolated and by the fitted graph the association (ka = 1/Ms) respective dissociation (kd = 1/s) constant could be determined. Deviation between the calculated and real binding curve are expressed by the value Chi2. To obtain reliable data this value should be below 10.

Biacore® is used to determine the association respective dissociation constant for two different antibodies towards rmACE2: goat anti-mACE2 (R&D) and rabbit anti-mACE2 pAb (Abnova)

Working procedure

- activate dextran surface with EDC and NHS
- pH-scouting
    - set acetate-buffer to pH 4.5, 5.0, 5.5
    - exchange buffer (Microcon®, Millipore) of rmACE2 (40 µg/ml) with acetate-buffer
    - apply rmACE2 on Biacore (BIAcore™ 2000, Running Buffer: HBS-EP Buffer (GE-Healthcare Bio-Sciences), Flow rate: 20 µl/min, 20 °C)
    - read out relative response of binding-activity
- bind rmACE2 with scouted pH
- saturate unbound surface with Ethanolamine
- bind first antibody (20 µl, goat anti-mACE2, 10µg/ml (67 mM))
- wash antibody with glycine-buffer (2x) and 2M NaCl (2x)
- bind second antibody (20 µl, rabbit anti-mACE2,10µg/ml (67 mM))
- wash antibody with glycine-buffer (2x) and 2M NaCl (2x)
- bind first antibody (20 µl, goat anti-mACE2,100µg/ml (670 mM))
- wash antibody with glycine-buffer (2x), 5 mM HCl, 4 M NaCl
- Fit binding curves with Software (Biacore2000 control software)

3.3.CASY®

Background

The CASY®-technology (Schärfe System GmbH) uses an electric field to count living cells. When a cell is exposed to a low voltage electric field, an intact membrane prevents that the electric field goes through the cell, in opposite to a broken or damaged cell wall which is not capable to resist. The cells are solved in CASY® ton (Schärfe System GmbH), an isotonic saline solution, absorbed one by one by a needle, brought to a precision measuring pore and exposed to an electric field. The resistor of the cell membrane provides information about the cell condition, viability, size and concentration of the volume.[68] To confirm the data, determinations are made in triplicates.

Working procedure

- dilute cell suspension 1:100 in CASY ton (Schärfe System GmbH)
- clean CASY-device (Schärfe System GmbH, Modell TTC) 3 times with CASY clean (Schärfe System GmbH) up to
- adjust CASY to measure 3 times with 200 µl
- start measurement
- adjust software to count only cells >20 µM cell radius

3.4.CD-Spectroscopy

Background

CD-Spectroscopy is a method to investigate among other properties the secondary structures of proteins. Getting secondary structural information, the low UV spectrum (185 nm to 260 nm) of linear polarized light is used. The interaction between the analyst and the light vary depending on the structure of the protein. This interaction leads to a different absorbance and speed of the right and the left circulating light as well as their wavelengths and leads to a conversion of linear polarized light to elliptical polarized light which is measured and depicted in a spectrogram. Alpha-helices and beta-sheets show characteristic maxims and minimums in the spectrum (See Figure 2), why an approximate prediction of the global structure can be made by comparing with known proteins respective the proportion of a secondary structure can be assessed[69].

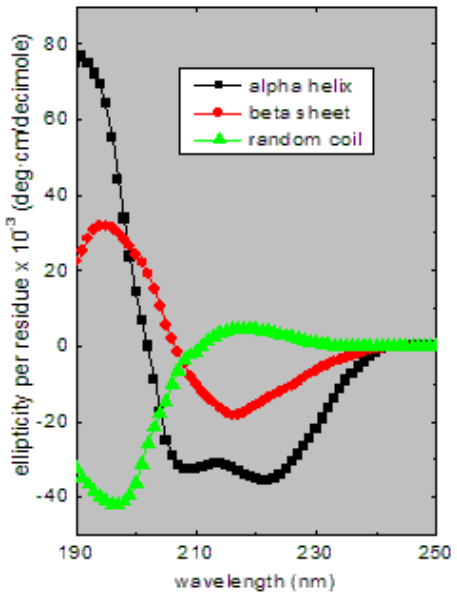

Figure 2: CD-spectrum of characteristic shapes and magnitudes of alpha-helix, betha sheet and random coil. © 2000-2011, Alliance Protein Laboratories Inc.

This method is used to compare the secondary structure of recombinant murine and human ACE2 and to investigate if ACE2s still occur in their native forms after freezing. Both are applied in a round and dust-free cuvette at a concentration of 1mg/ml and measured within 190 nm and 260 nm (J715 Spectropolarimeter JASCO) at RT.

3.5.Purification

3.5.1. Concentrating of CHO-culture supernatants

The rmACE2-transfected CHO-cells are cultivated under serum-free conditions in cell culture roller bottles (Smooth Wall Polystyrene Roller Bottles, Corning) at 37 °C (See 3.6). The supernatants are exchanged two times a week, centrifuged (3000 rpm, 10 min, RT), sterile filtrated (0.22 µM Cellular-Acetate filter, Corning) and stored at 4 °C. When greater amounts of supernatants are reached, they are concentrated by a tangential flow system (Centramate™, Filter: 30 k Omega Centramate, T-series, 0.1 m^2, PALL-Life Science) to a 10 to 15 times reduced volume. A more reduced volume may form precipitates of rmACE2. The filtrate is applied on an anion-exchange-chromatography-column.

3.5.2. Anion-Exchange-Chromatography

Background

The first of the serial purification is the capture step on an anion-exchange chromatography-column (DEAE-Sepharose™ Fast Flow, GE Healthcare Life Science). DEAE is a positively charged resin, which is derivatized with gel matrix beads. Due to the isoelectic point (iP) of the capturing protein and the pH of the buffer, DEAE binds to the negatively charged carboxyl-groups of the protein. To capture a protein it is necessary to bring the pH of the buffer above the iP. In this case, the iP of rmACE2 is approximately at a pH of 5.5, therefore the pH of Buffer A is adjusted to a pH of 7.4. Buffer B, the eluting buffer, is equally composed like Buffer A, but also contains NaCl to elute bounded proteins. Cl^- competes with the carboxyl-groups for the binding sites of DEAE and increases the conductivity of the buffer. Due to the weak binding affinity of DEAE to carboxyl-groups, bounded rmACE2 begins to elute at conductivity of 13 mS/cm.[70]

Working procedure

- dilute concentrate 1:2 with cell culture water
- determine conductivity (<8 mS/cm) and pH (7.4) of concentrate
- equilibrate column (XK50, 245 ml, DEAE-Sepharose™ Fast Flow, GE Healthcare Life Science) with Buffer A (flow rate: 10 ml/min, RT)
- apply concentrate on column with Buffer A
- elute rmACE2 with linear solvent gradient of Buffer B from 0 % to 40 %
- wash column with 100 % Buffer B up to UV280 is back on base line
- determine amount of rmACE2 in side fractions of rmACE2 peak by activity assay (See 3.1)
- pool all fractions with at least 0.5 mg/ml rmACE2

Buffers / Reagents

Buffer A: 50 mM Tris/HCl, 50 µM $ZnCl_2$, pH 7.4
Buffer B: 50 mM Tris/HCl, 50 µM $ZnCl_2$, pH 7.4, 1 M NaCl

3.5.3. Ammonium-sulfate-precipitation

Background

The second step is an ammonium-sulfate-precipitation to precipitate impurities and to increase the ionic strength of rmACE2, which is needed for the next chromatography step. Ammonium sulfate (NH_4SO_4) is solved in the pooled rmACE2 fractions and dissociates in strong ionic compounds. The dissociation products bind large amounts of water molecules and build a big hydrate shell. Due to the concentration of ammonium sulfate, proteins are deprived of its water molecules, which they need to remain solved in solution. Bigger proteins start to interact among each other and precipitate. Also the hydrate shell of rmACE2 is decreased but the enzyme is still capable to stay in solution, although with an increased ionic strength. To avoid local concentration of NH_4SO_4 it is necessary to mix the fractions gently with the stock solution by stirring. The precipitate is harvested by centrifugation and the cleared solvent is applied on a Hydrophobic-Interaction-Chromatography column.[70]

Working procedure

- prepare 3.6 M NH_4SO_4-stock solution(See 3.5.4)
- calculate needed amount of stock solution for a NH_4SO_4 concentration of 1.5 M of the pooled rmACE2 fractions
- mix fractions with stock solution with stirring
- let rest mixed solutions for 30 min at RT
- centrifuge precipitate down (3000 rpm, 10 min, RT)

Buffers / Reagents

Stock solution: 3.6 M NH_4SO_4, 50 mM Tris/HCl, 50 µM $ZnCl_2$, pH 7.4

3.5.4. Hydrophobic Interaction Chromatography

Background

Due to the ammonium sulfate precipitation the ionic strength of rmACE2 is increased. This characteristic is used by Hydrophobic Interaction chromatography. The stationary phase is a phenyl-modified sepharose, which interacts with hydrophobic parts of proteins to bind them. The samples are eluted with lowering salt concentration, which allows the proteins to regenerate their hydrate shell and to lose the ionic strength. The point of elution depends on the individual characteristics of proteins which are utilized to fractionate the sample[70].

Working procedure

- equilibrate column (XK56, 58.4 ml, HIC-Phenyl-Sepharose™ Fast Flow, GE Healthcare Life Science) with Buffer A (flow rate: 8 ml/min, RT)
- apply supernatant of centrifuged ammonium sulfate precipitated solvent on column with Buffer A
- wash out impurities with 100 ml of 60 % of Buffer B
- elute rmACE2 with a linear solvent gradient of Buffer B from 60 % to 100 %
- wash column with 100 % of Buffer B up to UV280 is back on base line
- determine amount of rmACE2 in side fractions of rmACE2 peak by activity assay (See 3.1)
- pool all fractions with amount higher than 0.5 mg/ml rmACE2

Buffers / Reagents

Buffer A: 50 mM Tris/HCl, 50 µM $ZnCl_2$, pH 7.4, 1.5 M NH_4SO_4
Buffer B: 50 mM Tris/HCl, 50 µM $ZnCl_2$, pH 7.4,

3.5.5. Size-Exclusion-Chromatography

Background

The fractionation of the sample only depends on the size of proteins and their hydrodynamic diameter. Small particles are able to remain longer in holes and gaps within the stationary phase of the gel, bigger proteins elute earlier due to their hydrodynamic radius[70].

Due to the peak broadening of this separation method, minimal sample-volumes need to be loaded on of the SEC-column (Superdex™, 200 prepgrade, Biopilot 35/600, GE Healthcare Life Sciences). The pooled rmACE2 fractions of the HIC have to be concentrated to less than 25 ml (See 3.5.1). Side fractions of the rmACE2 peak are investigated by HPLC (See 3.15) to determine impurities more exactly and to decide which fractions of rmACE2 get pooled and stored at -80 °C.

Working procedure

- concentrate pooled HIC fractions to less than 25 ml (See 3.5.1)
- equilibrate column (Superdex™, 200 prepgrade, Biopilot 35/600, GE Healthcare Life Sciences) with SEC-buffer (flow rate: 5 ml/min, RT)
- apply sample volume on the column
- start fractionation when UV214 rises
- determine impurities in side fractions of rmACE2 peak by HPLC (See 3.15)
- pool all clean rmACE2 containing fractions
- filtrate pool (0.22 µM Cellular-Acetate filter, Corning)
- determine amount of rmACE2 by OD_{280} (0.1% absorption equivalent to 1.750 in presence of glycine-buffer)
- fractionate pool in vials (CryoTubes™, Nunc)
- label and store vials at -80°C

Buffers / Reagents

SEC-buffer 100 mM glycine, 150 mM NaCl, 50 µM $ZnCl_2$, pH 7.5

3.6.Cloning/Fermenting/Expression

Preliminary Work / Background

In 2008, members of Apeiron Biologics AG amplified the extracellular domain of murine ACE2 by PCR and cloned the fragment with the restrictions enzymes XhoI/MluI into the pIRES-DHFR vector. The resulting plasmid was called MAD45 and the accuracy of the insert was verified by sequencing.

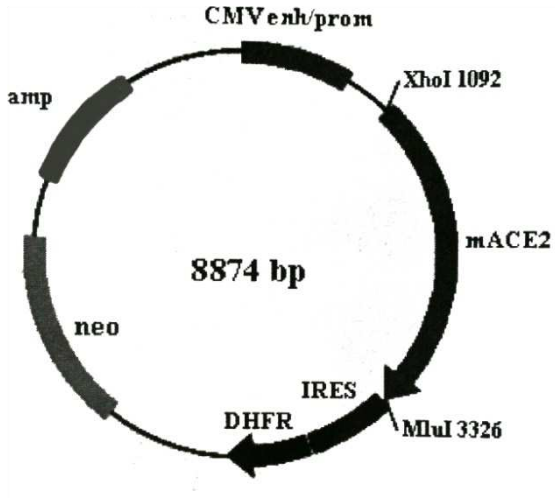

Figure 3: rmACE2 expression plasmid MAD45

CHO-DHFR$^-$ cells were cultured in growth medium (DMEM high glucose, 10 % FCS, 2 mM glutamate, 1 x HT) and split to 96 well plates in a ratio of 1:3 on the day before transfection. MAD 45 was transfected using 0.2 µg plasmid and 1 µl Lipofectin per well. Following transfection cells were transferred to selection medium containing G418 (neomycin) and raising concentrations of MTX (methotrexate) to increase expression levels of rmACE2.

rmACE2 and DHFR are transcribed bicistronic, connected via an Internal Ribosome Entry Site (IRES) (See Figure 3). Depending on to concentration of MTX, a competitive inhibitor of the essential enzyme DHFR (Dihydrofolate reductase), only cells with high levels of rmACE-DHFR-mRNA are viable[71].

Using activity test and Western Blot analysis, cell lines were selected and cultivated until they could be stably passaged twice a week at a ratio of 1:3 or 1:4. Four cell lines with the highest production rate of rmACE2 were frozen and stored at -80 °C.

Working procedure

Four cell lines (2E9, 2H7, 2G12, 7F12) are unfrozen and cultivated in selection medium (DMEM high glucose, 10 % FCS, 2mM glutamate, 0.5 mg/ml G418, 2 µM MTX) in 25 cm^2 flasks (Nunc) at 37 °C, 5 % CO_2-saturation and 90 % humidity. Cell lines are split twice a week with Trypsin/EDTA (0.05 %/0.02 %) in a ratio of 1:3 and are transferred to bigger flasks (75 cm^2 and 225 cm^2). The activity of cells are measured by activity assay (See 3.1) and CASY (See 3.3) When three 225 cm^2 flasks are completely grown, cells are transferred together to one Roller Bottle (Corning®) and fermented up to the Roller Bottle is overgrown, too. Then, culture medium is replaced by serum-free and chemical defined medium (Polymun Scientific). Two times a week, 2/3 of medium is exchanged and stored at 4 °C for further purification (See 3.5).

3.7. Deglycosylation of rmACE2 and rhACE2

Background

Recombinant human and murine ACE2 consist of 740 amino acid residues and the calculated molecular weight of both is nearly the same. But compared to their size, rhACE2 is larger than rmACE2. By deglycosylating the enzymes, further experiments (HPLC, SDS-PAGE, Native-PAGE) may investigate if the size-variety is only due to their different quantities of N-glycosylation sites which are occupied by complex bi-antennary oligosaccharides structures[72]. Recombinant murine ACE2 has 5 N-glycosylation sites, thus is two less than rhACE2.

The glycosidase Peptide: N-Glycosidase F (New England $Biolabs_{INC}$, MW = 36 kDa) from *Flavobacterium meningosepticum*, also known as PNGaseF, hydrolyzes completely all types of N-glycan chains from proteins, when sterically accessible. The enzymes are deglycosylated in its reduced as well as in its native form.

Working procedure (denaturized ACE2s)

- mix 1-20 µg rmACE2/rhACE2 with 1 µl 10x Glycoprotein Denaturing Buffer, ad 10 µl H_2O
- denature enzymes by heating at 100 °C for 10 min
- add 2 µl 10x G7 Reaction Buffer, 2 µl 10 % NP40, 4µl H_2O and 2 µl PNGase F
- incubate reaction volume at 37 °C for 1 hour
- store mixture at -20 °C

Working procedure (native ACE2s)

- mix 1-20 µg rmACE2/rhACE2, 2 µl 10x G7 Reaction Buffer, 2 µl 10 % NP40, 2 µl PNGase F, ad 10 µl H_2O
- incubate reaction volume at 37 °C for 3 hour
- store mixture at -20 °C

3.8. Determination of Sialic Acids

Background

N-linked oligosaccharides are added to newly synthesized polypeptides in the endoplasmatic reticulum (ER) and play a crucial role in protein folding, oligomerization, sorting, and transport[73]. Furthermore, decreased levels of glycosylation are associated with markedly reduced catalytic activity of enzymes[74]. Hence, the amount of glycosylations is important for the quality and activity of enzymes. N-linked glycosylation is predicted to occur at the asparagines (Asn, N) residue of the Asn-Xaa-Ser/Thr consensus sequence of a protein, where Xaa is any amino acid except proline. Glycosylation-sites are occupied by complex bi-antennary sialic acid-structures[72]. rmACE2 carries five consensus sequences, thus the ratio between rmACE2 and sialic acid of a fully glycosylated enzyme is 1 to 10. rhACE2 carries seven consensus sequences, accordingly the ratio is 1 to 14.

This assay determines the ratio between ACE2 and sialic acids by a chromogenic reaction. The sialic acids are cleaved from the enzyme by acid hydrolysis and react subsequently with the resorcinol solution, which contains chopper as chromogenic substance. Due to the content of sialic acid, the solution changes color detectable at an optical density of 580 nm. A standard curve is prepared with six concentrations of N-acetylneuraminic acid.

Working procedure

- prepare Solution A, B and C
- prepare resorcinol solution
- prepare butylacetate-butanol solution
- prepare standard curve in H_2O (100, 80, 60, 40, 20, 0 µg/ml N-acetylneuraminic acid, in triplicates)
- dilute rmACE2 in PBS to concentration of 1 mg/ml and 0.5 mg/ml
- pipette 100 µl of 0.5 mg/ml and 1 mg/ml ACE2 to 10 ml glass tube (in triplicates)
- add 1 ml resorcinol solution
- seal glass tubes with hollow stopper and heat samples at 100 °C for 30 min (water bath)
- chill samples on ice for 5 min and then 10 min at RT
- add 1 ml butylacetate-butanol solution
- vortex mixture for 3 sec
- 2 phases forms within 5 min; upper phase: blue and clear, bottom phase: clear and colorless
- pipette 300 µl of upper phase to quartz cuvette and measure absorbance at OD_{580}
- measure absorbance of standard curve
- content of sialic acids are read on the standard curve using graph pad and depicted as µg/ml
- concentration of sialic acids are correlated to concentration of rmACE2 (0.5 or 1 mg/ml)

Buffers / Reagents

Solution A	2 g Resorcinol, ad 100 g pureH_2O
Solution B	70 g HCl (37 %), ad 111 g pure H_2O
Solution C	2.5 g $CuSO_4$ * 5 H_2O, ad 100 g pure H_2O
Resorcinol solution	10 ml Solution A, 40 ml Solution B, 0,25 ml Solution C, 49,75 ml pure H_2O
butylacetate-butanol solution	24 ml butanol, 96 ml butylacetate
PBS	1.44 g Na_2HPO_4 * 2 H_2O, 0.2 g KH_2PO_4, 23.4 g NaCl, ad 1 L pure H_2O, pH 7.4

3.9. Dynamic Light Scattering

Dynamic Light Scattering is a physical technique to investigate the size distribution profile of small particles in solution. A laser lightens a protein solution in a micro cuvette and hits on small particle. The light is scattered in all directions and the scattered light of different scattering points interferes. By using a coherent and monochromatic laser, the intensity of scattering varies due to a time-dependent fluctuation. This fluctuation is caused by the movement of particles, called Brownian

Motion. Small particles move faster than large particles. This change of light scattering is determined by a detector placed at a 90° angle to the incident laser light. By using an autocorrelation function the translational diffusion coefficient DT can be calculated. This coefficient is necessary to calculate the hydrodynamic radius RH of the molecules in solution. Further, the molecular weight (MW) can be estimated, but this is not recommended: the shape of proteins could be different to the shape definition used to estimate MW what would sophisticate the result. In addition, an assertion can be made concerning disparity and modularity of the protein solution. Monomodal distributions have a baseline range of 1.002 to 0.997. A bimodal protein solution has a range of 1.003 to 1.005. If the baseline is higher, the solution is either multimodal or dusty. The average of ten measurements is summarized to one acquisition. The uniformity of sizes is determined by a monomodal curve fit analysis called Cumulants. The sum of squares (SOS) error statistic is the difference between the measured date and the Cumulants-calculated intensity correlation curves. SOS errors should be lower than 20.00 to be convincing. Less than 5.0 are perfect [75].

Working procedure

- dilute protein solution to 1mg/ml in pure water and centrifuge (micro centrifuge)
- blow out the microcuvette
- pipette protein solution near to surface in microcuvette (avoid impurities)
- start measurement (DynaPro™, Wyatt Technology)
- make a least 20 acquisition
- evaluate data with software (Dynamics)

3.10. IEF-Gel

Background

The isoelectric point (iP) of proteins varies due to their amino acid composition and is the pH-level at which the net electrical charge is zero. An Isoelectric Focusing Gel consists of immobilized ampholyte solutions in an acrylamide gel matrix with a stable pH-gradient ladder. Applied proteins migrate towards the anode through regions of decreasing pH-levels, until it reaches a region of a pH-level corresponding to its iP where the net electrical charge is zero. As a result, proteins are separated on the gel to sharpen bands corresponding to its iPs. This individual characteristic of a protein is used for separating protein solutions, for example by anion- or cation-exchange chromatography[70] (See 3.5.2).

Recombinant murine ACE2 and human ACE2 is applied on an IEF-gel to determine and compare the isoelectic points.

Working procedure

- prepare sample solution
- prepare anode buffer and cathode buffer and chill down to 2 – 8 °C
- fill upper buffer chamber with cathode buffer
- fill lower buffer chamber with anode buffer
- apply sample solution on gel (Novex® pH 3–7 IEF Gel)
- running conditions: 100 V constant for one hour, 200 V constant for one hour, 500 V constant for 30 minutes
- fix gel with 12 % TCA for one hour
- stain gel with Novex Colloidal Blue Staining Kit (Invitrogen™)

Buffers / Reagents

Sample solution	2 µl protein, 3 µl H2O, 5 µl 2x IEF-sample buffer pH 3-7 (Novex)
Anode Buffer	12 ml of 50 x Novex IEF Anode Buffer to 588 ml pure water
Cathode Buffer	20 ml Novex IEF Cathode Buffer pH 3-10 (10x) to 180 ml pure water

3.11. LC-MS/MS quantification of angiotensins (RAS-Fingerprinting®)

Background

The substrate specificity and the sensitivity towards the selective inhibitor DX600 of recombinant murine and human ACE2 are investigated in undiluted human plasma to assess and compare their characteristics under physiological conditions. The resulting concentrations of 10 Angiotensin peptide metabolites are simultaneously measured by a LC-MS/MS based quantification method (RAS-Fingerprinting®).

Working procedure

Anti-coagulated blood collected from healthy volunteers is collected and separated by centrifugation (10 min, 3000 rpm, RT). Recombinant human Renin (Sigma) is added to isolated plasma (E.C. 100 pg/ml) followed by addition of rmACE2 or rhACE2 (E.C. 5 µg/ml). For investigation of the conversion of Ang 1-10 to Ang 1-9, 1 µM Lisinopril (Sigma) was added to the samples in order to prevent degradation of Ang 1-10 by ACE. Plasma is incubated without or with increasing concentrations of DX600 (Sigma). Then, samples are chilled on ice and subjected to LC-MS/MS-analysis.

Working procedure (LC-MS/MS)

Plasma samples are spiked with 100pg/ml stable isotope-labeled internal standards and subjected to solid phase extraction using Sep-Pak cartridges (Waters) according manufacturer's protocol. Following elution and solvent evaporation, samples are reconstituted in 50µl 50% acetonitrile/0.1% formic acid and subjected to LC-MS/MS analysis using a reversed phase analytical column (Luna C18, Phenomenex) using a gradient ranging from 10% acetonitrile/0.1% formic acid to 70% acetonitrile/0.1% formic acid in 9 minutes. The eluate is analyzed in line with a QTRAP-4000 mass spectrometer (AB Sciex) operated in the MRM mode using dwell times of 25 msec at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standards, two different mass transitions are measured. Angiotensin peptide concentrations are calculated by relating endogenous peptide signals to internal standard signals provided that integrated signals achieved a signal-to-noise ratio above 10. The quantification limits for individual peptides are found to range between 1pg/ml and 5pg/ml in undiluted plasma.

3.12. Limulus Amebocyte Lysate (Endochrome™)

Background

The LAL assay detects bacterial endotoxin of gram$^-$ bacteria, Lipopolysaccharide (LPS), which is e.g. a strong activator of monocytes to release immune cytokines. Overproduction of immune cytokines is known to cause septic shocks and multiple organ dysfunction syndromes[76].

The assay bases upon the ability of bacterial endotoxin initiating activation of a proenzyme (a cascade of serine protease enzymes) in LAL. In presence of a colorless substrate (S-2423), it rapidly catalyzes the cleavage of the chromophore, p-nitroaniline (pNA) which produces a yellow color that is measured spectrophotometrically at a wavelength between 405-410 nm[77].

The LAL-assay (Endochrome™, Charles River Endosafe) is used to determine bacterial endotoxin in recombinant murine ACE2 solutions.

Working procedure

- prepare LAL-solution, substrate-buffer-solution
- prepare standard (0.24, 0.12, 0.06, 0.03 EU/ml)
- add 50 µl of undiluted sample/standard in well of microplate
- add 50 µl of LAL-solution and mix and incubate for 17 min at 37 °C
- add 50 µl of substrate-buffer-solution and mix and incubate for 17 min at 37 °C
- stop reaction with 50 µl 40 % Acetic Acid and measure with plate reader at 405 nm

Buffer / Reagents

LAL-solution rehydrate LAL-powder with 1.4 ml LAL-free water
substrate-buffer-solution mixed one volume of substrate solution with one volume of buffer

3.13. Native PAGE

Background

Native PAGE (Polyacrlyamid gel electrophoresis) separates biomolecules in polyacrylamidegels in an electric field under non-reduced conditions. The running distance of a protein depends on its charge, molecular weight and shape. Hence, also dimerizations or multimerizations can be investigated[70]. Protein bands are unspecifically visualized by Coomassie®-Brilliant-Blue Staining[78].

Working procedure

- prepare sample solution
- prepare anode and cathode buffer
- fill upper buffer chamber with cathode buffer
- fill lower buffer chamber with anode buffer
- apply sample solution on gel (NativePAGE™ 3-12% Bis-Tris Gel)
- running conditions: 60 V constant for 20 min, 150 V constant for 80 min
- stain gel with Novex Colloidal Blue Staining Kit (Invitrogen™) (10 min fixing, 3 hours staining)

Buffers / Reagents

Native Running Buffer (20x)	20.9 g BisTris, 17.9 g Tricine, ad 100 ml H_2O
Native Sample Buffer (4x)	2 ml of Native Running Buffer, 4 g Glycerol, ad 10 ml H_2O
Sample solution	2 µl protein, 2.5 µl 4x Native Sample Buffer, 5.5 µl H_2O
Anode Buffer	50 ml of 20x Native Running Buffer to 950 ml H_2O
Cathode Buffer	10 ml of 20x Native Running Buffer, 10 ml of Native PAGE™Cathode Buffer Additive, 180 ml H_2O

3.14. SDS-PAGE

Background

In contrast to Native-PAGE, in SDS-PAGE, proteins are reduced and denatured by heat and reducing substances, like 2-Mercaptoethanol, DTT (Dithiotreithol) and SDS (Sodium dodecyl sulfate). SDS is a detergent, which binds proteins and abolishes their net charge. Hence, the running distance in SDS-PAGE depends only on the molecular weight and size of the protein and not on its form or net charge. [70].

Separated protein bands can be detected either unspecifically by Coomassie®-Brilliant-Blue Staining or immunologically by antibodies (Western Blot). Coomassie®-Brilliant-Blue is the name of a triphenylmethane dye which interacts with hydrophobic amino acid residues to visualize unspecifically proteins[78]. Western Blot uses the specific binding affinity of antibodies to its epitope on molecules. Bound antibodies are further detected by second antibodies, which carry a modification. In this case, horseradish peroxidase is conjugated to the second antibody and catalyses the

chemiluminescent reaction of luminol which is detected and visualized by Biospectrum AC Imaging System.

SDS-PAGE is used to determine impurities of purification steps of rmACE2 by Coomassie® Brilliant Blue Staining and confirm identity of rmACE2 by Western Blot. Furthermore, rhACE2 is also applied on gels to compare both ACE2s concerning the molecular weight under native and deglycosylated conditions (See 3.7).

Working procedure (SDS-PAGE)

- prepare sample solution
- denature by heat (95 °C for 5 min) and centrifuge down (micro centrifuge)
- prepare MOPS-buffer
- apply sample on gel (NuPAGE 4-12 % Bis-Tris Gel)
- running conditions: 20 min 60 V, 60 min 150 V
- either stain gel with Coomassie® Brilliant Blue or make a Western Blot

Working procedure (Coomassie® Brilliant Blue staining)

- stain gel with Novex Colloidal Blue Staining Kit (Invitrogen™) (10 min fixing, 3 hours staining)

Working procedure (Western Blot)

- wash gel 10 min with water, than 10 min with transfer buffer
- rehydrate membrane (Hybond-P™ PVDF Transfer membrane) with MeOH for 10 sek
- membrane together with filter paper (Sandwich 0,45µm Pore Size, Amersham Biosciences) for 10 min in Transferbuffer
- transfer proteins from gel to membrane (Pierce Fast Semi Dry Blotter (50 min with 15 V))
- block membrane 1 hour with 20 ml 5% BSA in TBST
- incubate membrane with 15 ml 0,2µg/ml Goat anti-mACE2 (R&D) over night
- wash 3x with TBST
- 1 hour with Anti-Goat HRP, 0.1 µg/ml
- 3x with TBST
- develop 5 min at RT with developer (SuperSignalWest Pico Chemiluminescent Substrate)
- 5 min exposure, Biospectrum AC Imaging System (LTF-Labortechnik GmbH)

Buffers / Reagents

MOPS-Buffer:	50 ml 20x NuPAGE® MOPS SDS Running Buffer (Invitrogen), 950 ml H_2O
Sample Solution:	0.2 µg protein, 2 µl Lane Marker Reducing Sample Buffer (5x) (Pierce Biotechnology), ad 10 µl H2O
Transferbuffer:	10 ml 10x Pierce Fast Semi Dry Transfer Buffer (Thermo Scientific), 90 ml H2O
TBST:	100mM Tris pH7,5, 150mM NaCl, 1% Tween-20
Developer:	SuperSignalWest Pico Chemiluminescent Substrate

3.15. Size-Exclusion-High-Performance-Liquid-Chromatography

Background

Compared to ordinary chromatography, High Performance Liquid Chromatography (HPLC) uses higher pressure to pump the analyte through a shorter and more densely packed column to reach better separation of samples and higher resolution of the chromatogram, which allows analyzing the purity of enzyme solutions. Size-exclusion-HPLC separates samples according to their size; bigger proteins elute earlier than smaller ones due to their ability to remain between particles in the column. Molecular weight is determined by comparing the retention time of the analyte with the Gel-Filtration-Standard.

Working procedure

Enzymes are analyzed using SEC-HPLC (MAbPac™ SEC-1, 5 µm, 300 Å, 4 X 300 mm, DIONEX) with UV detection at 214 nm with a flow rate of 1 ml/min. Chromatography takes 25 min with Running-Buffer (50 mM $NaPO_4$, 300 mM NaCl, pH 6.8)

3.16. Thermal Shift Assay

Background

This method uses a fluorophore, called Sypro® Orange, which is quenched by water and gets its ability to re-emit light at 620 nm only in presence of hydrophobic or ionic groups. A stable protein in solution has no hydrophobic groups on the surface; hence the fluorophore in solution when

illuminated with 480 nm is not able to re-emit light. The background noise is therefore very low. If the temperature rises, the protein undergoes thermal unfolding and exposes its hydrophobic core region. The hydrophobic rests get bound by Sypro® Orange and the fluorophore is able to re-emit light at 620 nm. The fluorescence data are plotted and the midpoint of TM of the resulting curve is taken as reference to compare proteins about their thermal stability under different conditions[79].

Working procedure

- dilute mACE2 to about 1 mg/ml
- set pH-level of buffer to be tested (with 0.1 M NaOH respective 1:2500 dilution of pure HAc)
- make a buffer change in Centrifugal Filter Devices (Millipore)
    - apply 50 µl of mACE2 solution onto filter and add 200 µl of the buffer
    - centrifuge (4 min/13000 rpm/RT)
    - add again 200 µl of buffer and centrifuge (4 min/13000 rpm/RT)
    - turn around filter and centrifuge the buffer changed concentrate in vial (1 min/1000 rpm/RT
- dilute Sypro Orange Stocksolution (Sigma) in pure water 1:1000
- add 5 µl of diluted Sypro Orange in an optical well
- add 20 µl of pH-varying ACE2s (Triplicate)
- deal optical well and centrifuge down
- use QPCR device (Stratagene™, MX3005P) to measure the fluorescence (492 Ex, 610 Em = FerroxFilter) of thermal increased samples (30 °C to 98 °C)
- differentiate the raw data
- determine the minimum/maximum of the graph (is equivalent to inflection point of Melting Curve) and set data in relation to pH-level

3.17. *In vitro* Turnover of Ang 1-10 and Ang 1-8

Background

ACE2 is described cleaving one amino acid residue from the C-terminal side of Ang 1-10 and Ang 1-8, although the catalytic efficiency of ACE2 is reported being 400-times higher for Ang 1-8 as a substrate

than for Ang 1-10[37]. The cleavage of both is an important factor to decrease levels of Ang 1-8; either by direct conversion, or by cleaving the precursor Ang 1-10 to avoid the formation of Ang 1-8.
By an *in vitro* kinetic assay, the catalytic efficiency of all rmACE2 batches and rhACE2 for Ang 1-10 and Ang 1-8 cleavage followed by reversed-phase HPLC quantification of substrate and product concentration is investigated. The determined substrate turnover rates provide various information: quality and catalytic activity of purified rmACE2 batches can be compared and assessed (See 3.5) and furthermore, differences in the species-specific turnover rate may give new insights into pharmacological differences between murine and human ACE2.

Working procedure (Hydrolysis of Ang 1-10 and Ang 1-8)

- prepare stock solution of Ang 1-10 and Ang 1-8 and determine concentration by OD_{275} (ε=1390 M^{-1} cm^{-1})
- temper heating block at 37 °C
- dilute stock solution of 100 µM in MES-buffer
- dilute rmACE2 / rhACE2 to 1.25 µg/ml in MES-buffer
- pipette 400 µl of substrate solution to eppendorf reagent vial and temper on heating block (in triplicates)
- add 100 µl of enzyme solution to substrate solution
- stop reaction in interval of 10 min by pipetting 100 µl of reaction mix to 20 µl 0.5 M EDTA-solution

Working procedure (RP-HPLC)

The concentrations of product and substrate are determined using reverse-phase HPLC (Source™ 5RPC, 4,6x150mm, Amersham Pharmacia biotech) with an in-line diode array detector at UV detection at 280 nm. The separation is carried out at room temperature with a flow rate of 0.8 ml/min. A linear solvent gradient of 90 % Buffer A and 10 % Buffer B to 100 % Buffer B over 17 and 8 min re-equilibration with 10 % Buffer B was used. Peptide concentrations are calculated via calibration curves of Ang 1-10 and Ang 1-8 (80/65/50/35/20 µM in 83mM EDTA/MES-buffer)

Buffers / Reagents

MES-buffer	50 mM MES, 300 mM NaCl, 10 µM ZnCl2, 0.01 % Brij-35, pH 6.5
Buffer A	0.08 % orthophosphoric acid
Buffer B	40 % acetonitrile in 0.08 % orthophosphoric acid

4. Results

4.1. Recombinant murine ACE2

4.1.1. Expression

Recombinant murine ACE2 was expressed in CHO-cells. In 2008, members of Apeiron Biologics AG transfected CHO cells with MAD45, a rmACE2 expression plasmid, selected the best producing clones and stored them at -80 °C. In the course of the Masters, the four best clones (2H7, 2G12, 7F12, 2E9) were unfrozen and cultivated in selection medium. The production rate of active rmACE2 per day and cell was assessed by activity assay and CASY® (See Table 1).

supernatant	rmACE2 (µg/ml)	Cell Count (CASY®)	active rmACE2 /day*cell (pg)
2H7	11,2	2,10E+06	53,43
2G12	3,3	1,11E+06	29,91
7F12	2,1	9,22E+05	22,80
2E9	14,1	1,87E+06	75,66

Table 1: Production rate of four rmACE2-transfected CHO-cells. Cell counts were determined within 24 hours and the average was correlated to the concentration of rmACE2 determined by activity assay.

All four clones were further cultivated and transferred into fermenting roller bottles. The selection medium was replaced by serum-free and chemical defined medium, which was exchanged twice a week and stored for purification. Within five months, six batches of rmACE2 were purified. The loss of rmACE2 during purification was 80% at the beginning and after adjustment and optimization of each step below 50%.

4.1.2. Purification

Four steps to purity. Supernatants of rmACE2-transfected CHO-cells were concentrated and purified via four serial steps. Firstly, rmACE2 was captured by an anion-exchange-column to DEAE. DEAE binds weakly to rmACE2, wherefore the conductivity of the applied solution must not be higher than 8 mS/cm, otherwise rmACE2 elutes together with other impurities. One out of six DEAE-chromatograms is shown as a representative (See Figure 4).

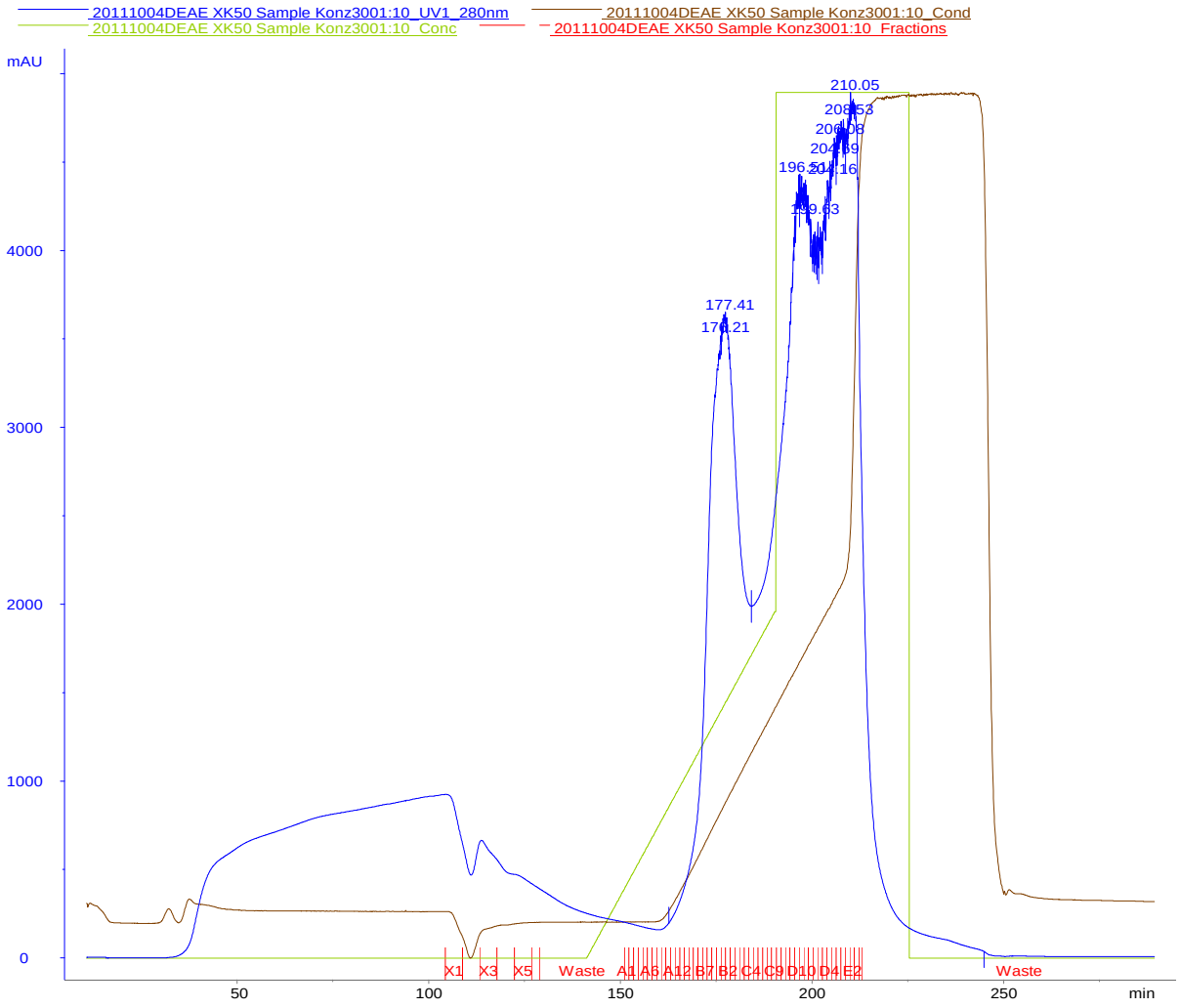

Figure 4: Anion-Exchange-Chromatogram of supernatant of rmACE2-transfected CHO-cells. Supernatants were applied during the first 100 min. After washing of unbound molecules for 40 min, a gradient of Buffer B (green) increases to 40 %, followed by a column-wash with 100 % up to the UV-detection at 280 nm (blue) drops to baseline. Conductivity (brown) follows the concentration of Buffer B with a delay of 15 min. Name of fractions are labeled in red. First peak within 150 and 200 min is rmACE2.

Fractions with a concentration higher than 0.5 mg of rmACE2, determined by activity assay, were pooled and the hydrophobicity of proteins was increased by ammonium sulfate. After centrifugation to remove precipitated proteins, solution was applied on a hydrophobic interaction chromatography column filled with Phenyl-Sepharose. Representative to the six-batches of rmACE2-purification, one chromatogram is shown (See Figure 5).

Figure 5: Hydrophobic-Interaction-Chromatogram of pooled rmACE2-Anion-Exchange-Eluate. 600 ml of DEAE-eluate was applied followed by 150 ml isocratic wash step with 60 % Buffer B (green). With a delay of 50 ml, conductivity (brown) decreases when concentration of Buffer B rises. rmACE2 starts eluting at a Buffer B concentration of 80 %, seen by the second peak of UV280 (blue). During the wash step with 100 % Buffer B, other proteins elute.

To increase purity, fractions in the rear area of the peak were not pooled, although rmACE2 was detectable by activity assay in larger quantities. Pooled rmACE2-fractions were concentrated to reduce volume and finally applied on a Size-Exclusion-Chromatography-Column. Like before, only one out of six chromatograms is shown (See Figure 6).

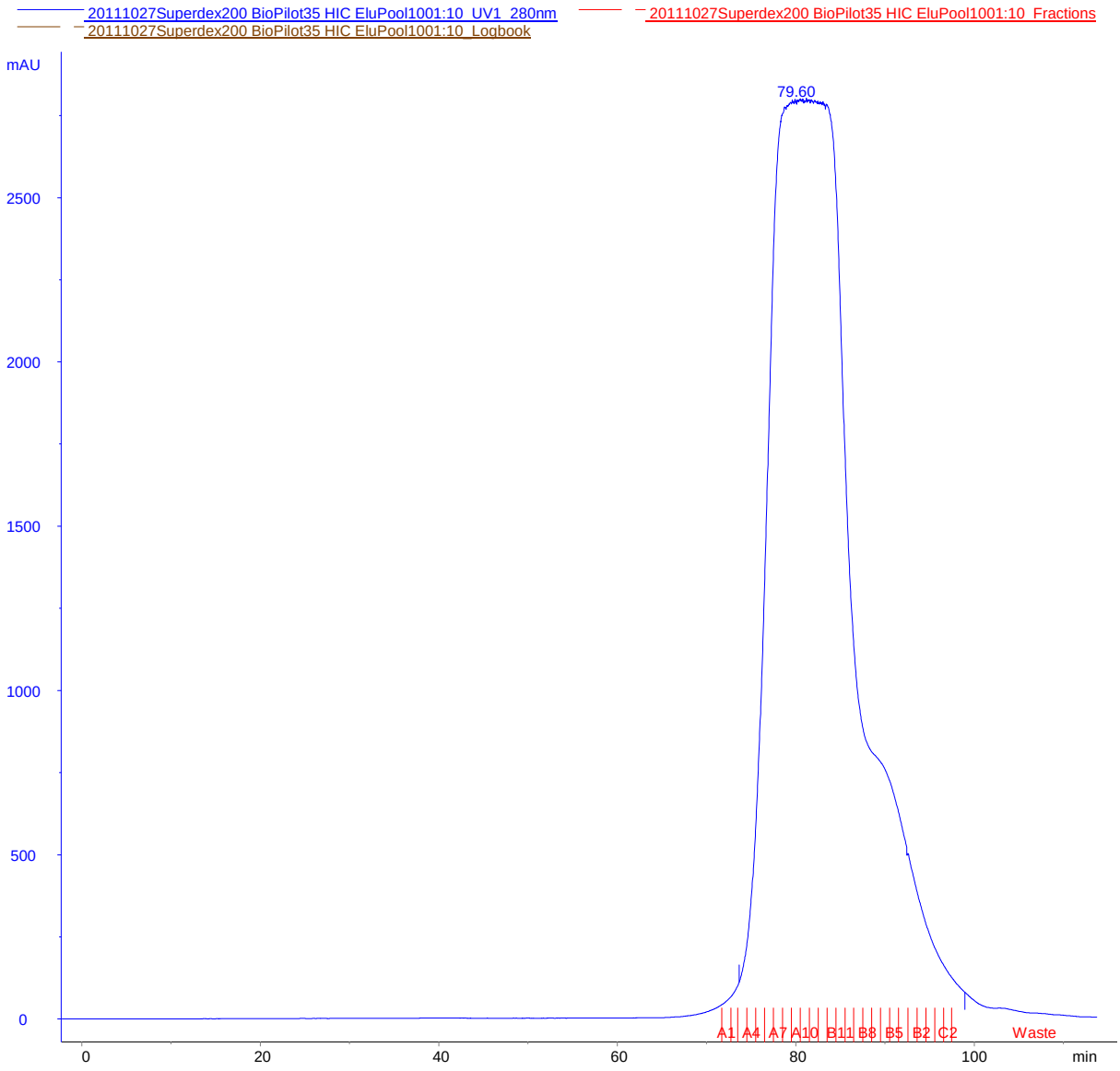

Figure 6: Size-Exclusion-Chromatogram, last step of rmACE2 purification. Reduced, pooled fractions of HIC were applied and rmACE2 starts eluting after 70 min, seen by UV 280 nm (blue), fractionated up to UV 280 nm drops back to baseline. Impurities are visible by a hump at the drop of the peak.

Impurities were detected as humps at the tail of the rmACE2-peak by the chromatogram. To avoid them, undiluted fractions from the rear part of the peak have been investigated more precisely by HPLC at UV 214 nm to decide which are pooled in the final batch (See Figure 7).

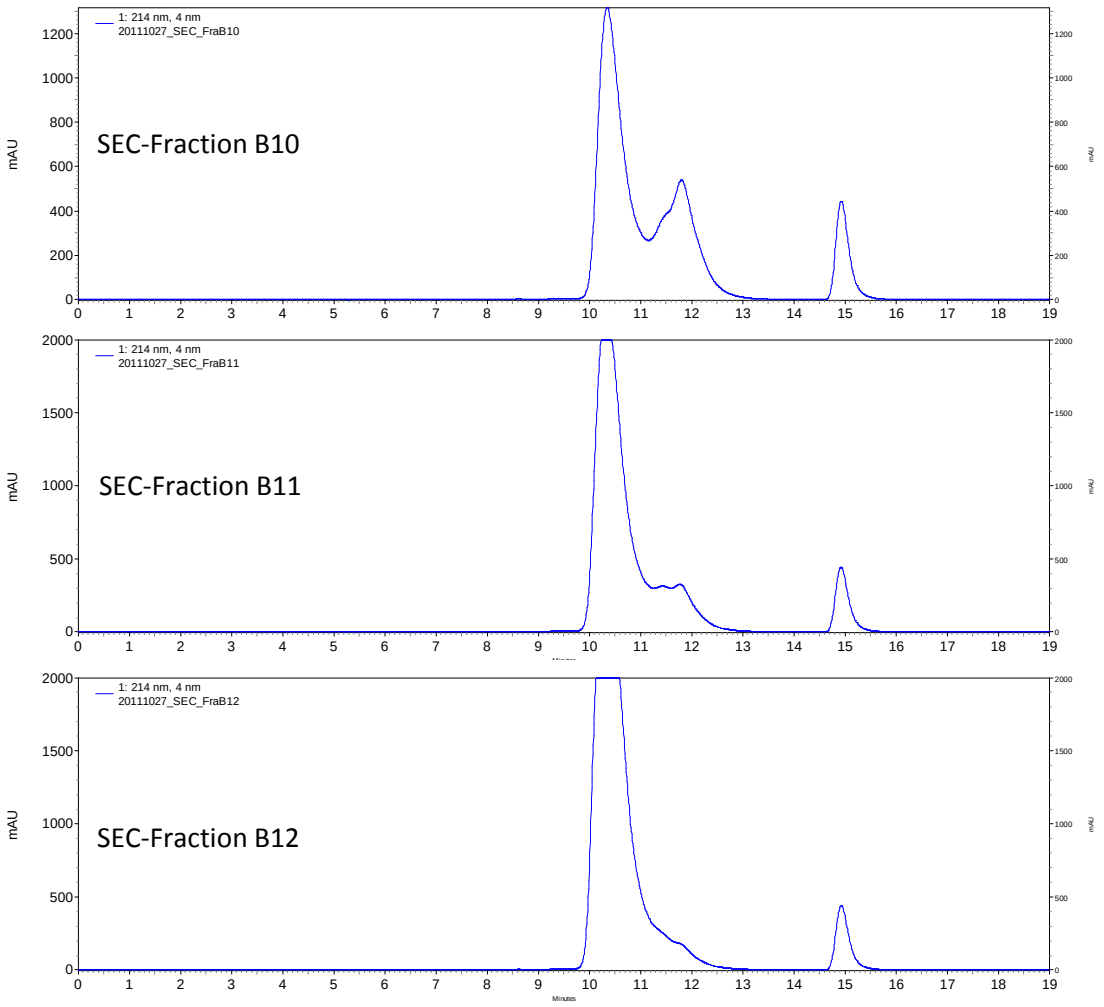

Figure 7: HPLC of the rear-three fractions of rmACE2-peak at SEC. Three fractions of the rear area of the rmACE2-peak of the Size-Exclusion-Chromatogram (See Figure 6) were investigated by HPCL at 214 nm (blue) to decide, which are pooled for the final rmACE2-batch. The biggest peak at a retention time of 9.8 min is rmACE2 and the peak at a retention time of 14.7 min is glycine. Fraction B10 is impure, visible by a second peak connected to rmACE2. Fraction B11 shows fewer impurities, although too much for pooling for the batch. Fraction B12 is the purest, although a little hump is visible, but due to the superimposing concentration of rmACE2 in the batch it is accepted.

Finally, 1:15 diluted rmACE2 batches were also investigated by SEC-HPLC and its purity assessed in percent (See Figure 8).

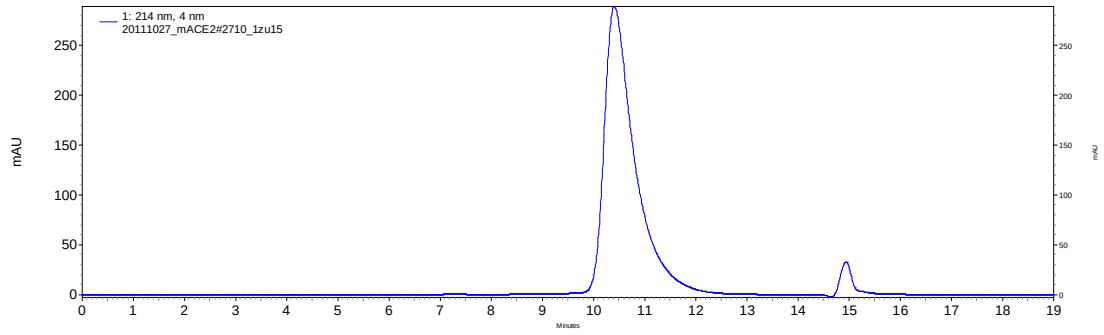

Figure 8: HPLC-chromatogram of rmACE2 batch. The retention time of rmACE2 is 10 min, measured by UV 214 nm (blue). The second peak at 14.6 min indicates glycine, part of the storage-buffer. Purity of rmACE2 is > 99 %.

4.1.3. Purity

Key data of rmACE2 batches. Six batches of rmACE2 were purified, all with similar purity but varying concentrations and amounts. Quantity was determined by OD_{280} (0.1% absorption equivalent to 1.750 in presence of glycine-buffer) and Endotoxin-Units by Limulus Amoebocyte Lysate assay.

	rmACE2 #1706	rmACE2 #0707	rmACE2 #2907	rmACE2 #2608	rmACE2 #0710	rmACE2 #2710
Concentration (mg/ml)	0.75	0.77	2.34	1.72	2.62	2.28
Volume (ml)	27	45	40.5	39	112.5	48
Quantity (mg)	20.25	34.65	94.77	67.16	294.75	109.44
Purity (%)	>99	> 99	> 99	> 99	> 99	> 99
Endotoxin units (EU/mg)	6.1	0.145	< 0.250	< 0.150	0.104	0.130

Table 2: Key Data of all six rmACE2 batches. Concentration was determined by OD_{280}, purity by HPLC and Endotoxin level by LAL-Assay.

All six batches of rmACE2 were further investigated by SDS-PAGE to assess purity and homogeneity.

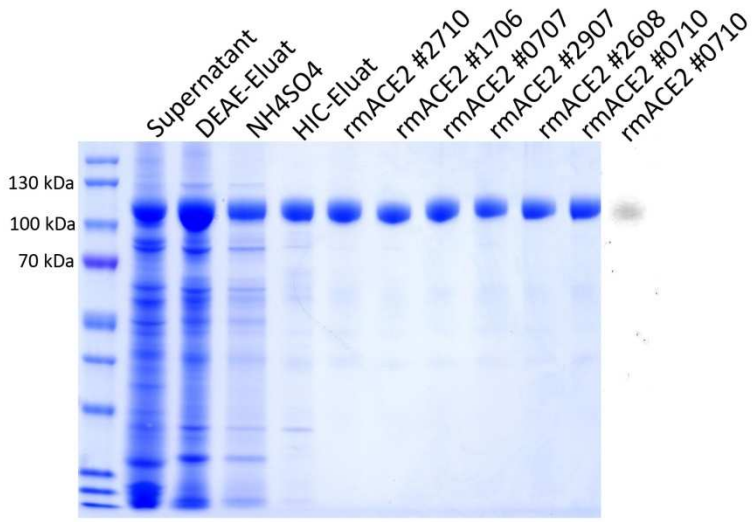

Figure 9: SDS-PAGE of rmACE2 batches. From the left to the right side, elutes of purification were applied, followed by all six rmACE2 batches, stained by Coomassie® Brilliant Blue. On the right hand side, rmACE2 is shown identified by goat anti-rmACE2 antibody.

4.1.4. Stability

Highest stability of rmACE2 in a pH range of 6.0 to 7.5. Recombinant murine ACE2 is stored at -80°C in storage buffer (100mM glycine, 150 mM NaCl, 50 µM $ZnCl_2$, pH 7.5) Due to minor variations within the last step of purification, Size-Exclusion-Chromatography, final pH levels of rmACE2 batches vary in a range of 7.1 to 7.5. These slight variations might cause differences in terms of stability for rmACE2. The melting point, a surrogate marker for stability, is the inflection point of a melting curve, which was determined by the Thermal Shift assay. Representative to the individual measurements in true triplicates (red, blue, green), one melting curve of rmACE2 at a pH-level of 7.5 is shown. (Figure 10, left) The melting points of rmACE2 under different pH-levels, depicted on a graph, revealed the highest stability in a pH range of 6.0 to 7.5 (See Figure 10, right).

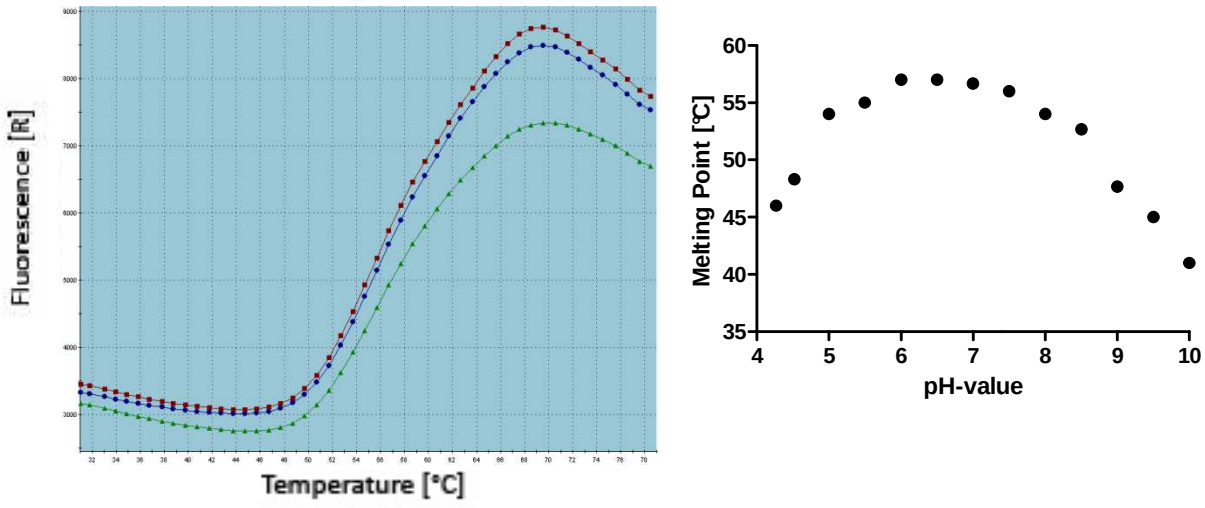

Figure 10: Melting Point of rmACE2 in a pH range within 4.0 to 10. Melting curve (left) was determined by increasing temperatures with Sypro® Orange in excess in a pH range of 4.0 to 10.0. Sypro® Orange interacted with hydrophobic residues of heat-denaturing rmACE2 and started fluorescing which is presented as a melting curve. Representative to the individual measurements in true triplicates (red, blue, green), one melting curve of rmACE2 at a pH-level of 7.5 is shown. Representative for all measurements, the melting curve of rmACE2 in a 7.5 pH-buffer is shown. The inflection point of the melting curve is indicated as melting point which is a surrogate marker for protein. Melting points of rmACE2 under different pH-buffer conditions are given in the graph on the right side.

4.1.5. Affinity to Antibodies

Biacore revealed a higher affinity of the rabbit antibody to rmACE2 than the goat antibody. The affinity of two different antibodies (goat anti rmACE2, monoclonal (R&D), rabbit anti rmACE2 polyclonal (Abnova)) were investigated by Biacore-technology. Therefore, rmACE2 was bound to the sensor chip at a pH-level of 4.5, where the enzyme showed the highest relative response and binding affinity to the activated chip. (Data not shown) The relative response of both antibodies to rmACE2 were investigated and revealed a better affinity of rabbit anti-rmACE2 than goat anti-rmACE2 ($1.78x10^{7}$ vs $4.42*10^{6}$ 1/Ms) and a similar dissociation ($9.99x10^{-4}$ vs. $3.52x10^{-4}$ 1/s). But due to the poor regeneration of the protein and dissociation of the antibodies, only one measurement with the rabbit antibody and two measurements with the goat antibody were realized. It is recommended to repeat the measurement to confirm the data.

4.2.rmACE2 vs. rhACE2

4.2.1. Secondary structure

Secondary structure of rhACE2 and rmACE2 is equal. In 2004, the crystal molecular structure of native human ACE2 was investigated and published and has revealed an enzyme with huge proportion of alpha-helices[80]. In contrast, the structure of murine ACE2 is still unsolved, however it is supposed being equally structured due to a sequence coverage to human ACE2 of 83 %[15]. Confirming this assumption, the secondary structure of both ACE2s were investigated and compared by Circular Dichroism Spectroscopy. It was found that both structures display equally minima in the CD-spectrum (See Figure 11), which is typical for an alpha helical secondary structure (See **Fehler! Verweisquelle konnte nicht gefunden werden.**). And both occur natively even after repeated freezing and unfreezing (Data not shown).

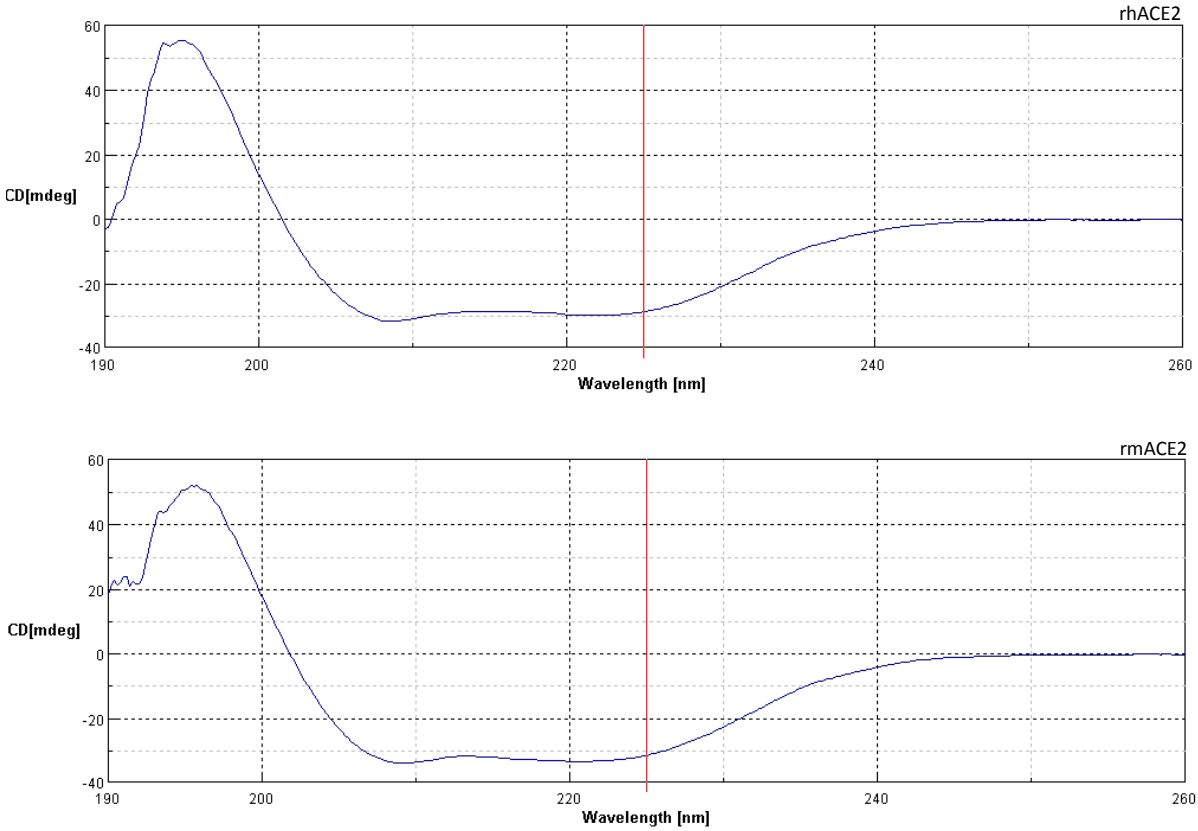

Figure 11: CD-Spectroscopy of rhACE2 and rmACE2. Both enzymes show a similar characteristic spectrogram for a mainly alpha-helical secondary structure.

4.2.2. Size

Although the calculated molecular weights of monomeric rmACE2 and rhACE2 are 85.2 kDa and 85.3 kDa, both recombinant ACE2 give a single band at approximately 180 kDa in Native PAGE (See Figure 12 C). Also the retention time in HPLC (See Figure 12 A) of both enzymes points to the presence of homodimers. In SDS-PAGE (See Figure 12 B), ACE2s give single bands around 100 kDa what can be explained by the reduced status of the enzymes and the cleavage of non-covalent dimer bonds. Interestingly, the retention time and running distance of both ACE2 versions differ in Native PAGE as well as in SDS-PAGE which points to a covalent modification of the enzymes. The mass shift was found to be caused by two additional glycosylation sites in the human enzyme and was removed by the glycosidase PNGaseF from *Flavobacterium meningosepticum* which hydrolyzes completely all types of N-glycan chains from proteins. Both deglycosylated enzymes give bands at the same molecular weight in SDS-PAGE, however the human enzyme was found being completely deglycosylated only when PNGaseF had removed N-glycans under reduced conditions. In contrast, PNGaseF could remove all N-glycans from native and reduced murine ACE2.

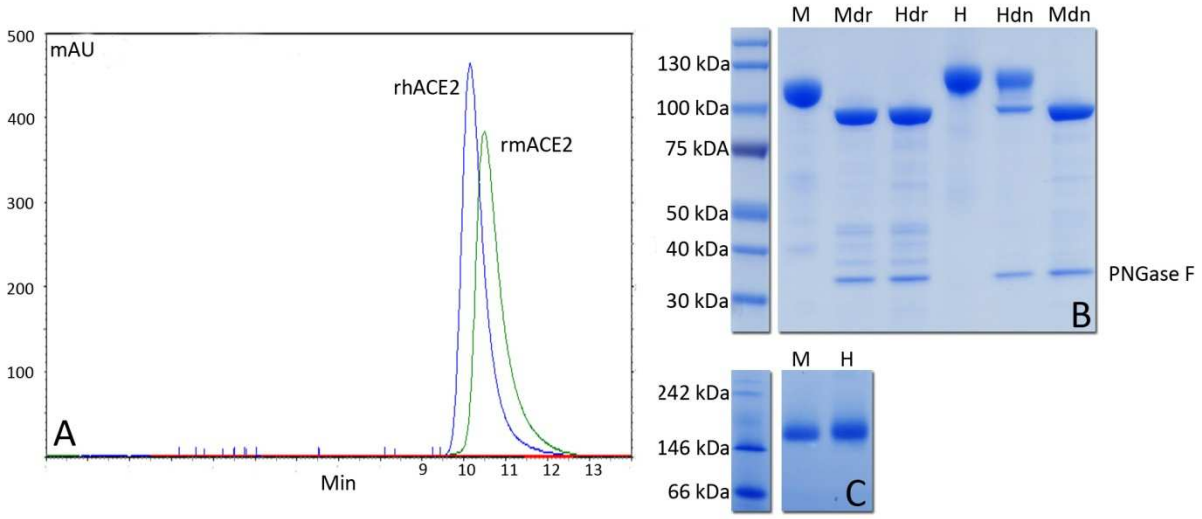

Figure 12: Comparison of rmACE2 and rhACE2 according to size and glycosylation. (A) Equal amounts of rmACE2 (green) and rhACE2 (blue) were analyzed by SEC-HPLC. In-line measurements of the absorbance at 214 nm are given in absorption units. (B) N-glycans of both enzymes were removed under native and reduced condition by PNGaseF (36 kDa) and equal amounts of enzymes were subjected to SDS-PAGE analysis followed by Coomassie® Brilliant Blue staining. (C) Native recombinant murine and human ACE2 were compared by Native PAGE and Coomassie® Brilliant Blue staining. Marker bands are depicted to allow estimation of molecular weights. M: rmACE2, H: rhACE, Mdr: rmACE2 reduced degylcosylated, Hdr: rhACE2 reduced deglycosylated, Mdn: rmACE2 native deglycosylated, Hdn: rhACE2 native deglycosylated

4.2.3. Hydrodynamic radius

rhACE2 exhibits a larger hydrodynamic radius than rmACE2. Both ACE2 versions slightly differ in retention time by size exclusion HPLC and running distance in Native and SDS-PAGE which points to a different hydrodynamic radius due to varying glycosylations. Dynamic Light Scattering provides exact information about hydrodynamic radius, modularity of proteins and calculates roughly the molecular weight. The determined hydrodynamic radius of recombinant murine ACE2 was 6.0 nm compared to 6.6 nm of rhACE2. Further, the calculated molecular weight for both was too high (rhACE2: 276 kDa, rmACE2: 220 kDa), but indicates in-line with other findings that both only occur as dimers (baseline range = 1.000). Furthermore, both protein solutions are nearly free of impurities (SOS < 10), which emphasizes the validity and correctness of measurement, too.

4.2.4. Glycosylation

Glycosylation-sites of ACE2s are occupied to 70 to 90 %. Depending on growth conditions of CHO-cells, the amount of sialic acid, covalently bound to glycosylation-sites of ACE2, varies and is a marker for protein quality and acitivity[74]. Due to seven glycosylation-sites of rhACE2, 14 sialic acids can ideally be bound to rhACE2, the five glycosylation-sites of rmACE2 are occupied by four less. By an assay, which hydrolyses and determines sialic acids, the ratio between rmACE2 and N-glycans was investigated and revealed 70 to 90 % occupied glycosylations-sites, fluctuating slightly between batches. 12.5 sialic acids were covalently bound to rhACE2, which corresponds to a percentage of 85.7 %.

4.2.5. Isoelectric Point

Isoelectic point of rmACE2 is higher compared to rhACE2. The characteristic isoelectric point of a protein depends on its amino acids composition and describes the pH-level in which the net charge of the protein is zero. Isoelectric Focusing is a method to determine the isoelectric point whereas it is not an exact point but a pH-range. The pH range of zero net charge for rmACE2 is approximate within 6.1 to 5.2, in contrast to rhACE2 where the pH-range is lower within 5.5 to 4.8.

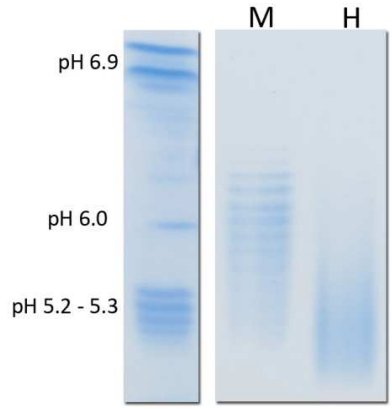

Figure 13: Isoelectric Focusing with rmACE and rhACE2. rmACE2 (M) and rhACE2 (H) were subjected to IEF, followed by Coomassie® Brilliant Blue staining. Marker bands are depicted for estimating the range of the isoelectic point

4.2.6. Catalytic activity *in vitro* (excess of substrates)

Recombinant human and murine ACE2 differ in regard to Ang 1-10 conversion rate *in vitro*. The catalytic activity and substrate turnover of rmACE2 and rhACE2, based on the previously determined concentrations, were also investigated and compared in an *in vitro* system. Defined concentrations of enzymes were co-incubated with human Ang 1-10 and Ang 1-8 in excess, respectively; both represent natural substrates for the enzymes. Increase and decrease of substrates and products, measured by RP-HPLC, were found correlating negatively (See Figure 14), corresponding to the activity of ACE2s. Turnover-rates (k_{cat}) of the enzymes were calculated via the graphical determined product formation and substrate degradation. rhACE2 converts Angiotensin II in a comparable rate to rmACE2, although with a 1.2 higher turnover number (See **Fehler! Verweisquelle konnte nicht gefunden werden.**). According to the conversion rate of Angiotensin I, ACE2s differ in regard to their catalytic degradation efficiencies whereby human ACE2 is 15 fold more effective in cleaving Ang 1-10 to Angiotensin 1-9 than its mouse homologue ($1.8x10^{-2}$ vs. $1.2x10^{-3}$ s^{-1}). But comparison of substrate turnovers reveals that Ang 1-8 is the preferred substrate for both enzymes *in vitro*. rhACE2 cleaves Ang 1-8 (0.77 s^{-1}) with a 42 fold higher turnover number than Ang 1-10, rmACE2 (0.62 s^{-1}) even with a 492 fold higher turnover rate. These findings go in-line with recent experiments revealing these species-different enzymes differ more than known so far.

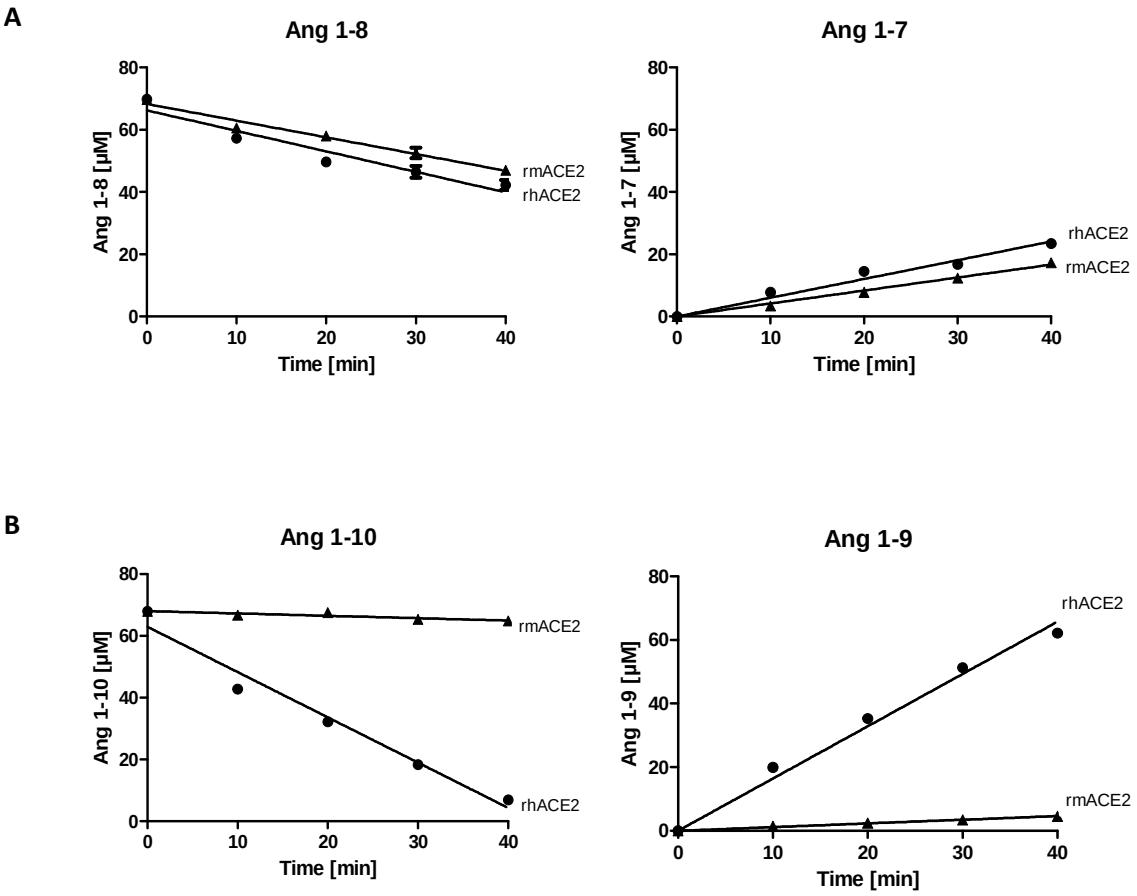

Figure 14: Cleavage and formation of Angiotensin 1-10 and Angiotensin 1-8 by ACE2s *in vitro*. Both ACE2s were co-incubated in MES-buffer with their natural substrates Ang 1-8 (A) and Ang 1-10 (B) in excess (65 µM), respectively, and aliquots were taken every 10 min to determine substrate amount by RP-HPLC. The resulted concentrations are shown in the graph. Ang 1-10 was incubated with a 100 fold higher concentration of ACE2 (125 µg/ml) than Ang 1-8 (1.25 µg/ml). Each time point was measured in true triplicates and standard deviation and the linear regressions of the measured values are shown in the graph.

	k_{cat} [s^{-1}]	
	rhACE2	**rmACE2**
Ang 1-8 ➔ Ang 1-7	0.77	0.62
Ang 1-10 ➔ Ang 1-9	$1.8x10^{-2}$	$1.3x10^{-3}$

Table 3: Ang 1-10 and Ang 1-8-related turnover rates of rhACE2 and rmACE2. The substrate turnover and product formation numbers were determined for the kinetic experiment in Figure 14 (See above). The slope of the linear regression across all points was determined for substrates and products and related to the molar concentration of both enzymes. The resulting values for k_{cat} are given in the table.

4.3.Catalytic activity in undiluted human plasma (RAS-Fingerprinting®)

Comparison of rmACE2 and rhACE2 in human plasma reveals a species-specific turnover of Ang 1-10. Differences in substrate turnover rates of rmACE2 and rhACE2 were investigated under physiological conditions in undiluted fresh human plasma. Therefore, Renin and 5 µg/ml rmACE2 or rhACE2. Following incubation, samples were subjected to LC-MS/MS-analysis. It was found that plasma samples with increased Renin-activity in otherwise healthy conditions, contain high amounts of Angiotensin 1-10 (189 pg/ml) and Angiotensin 1-8 (369 pg/ml) and lower levels of all the other angiotensins (See Figure 15, left). Furthermore, both ACE2s are able to cleave Ang 1-8 very efficiently. However, Ang 1-10-levels were decreased more effectively by recombinant human ACE2 (112 pg/ml, see Figure 15, middle) than by the murine version (174 pg/ml, see Figure 15, right). Furthermore, Ang 1-9 levels were only detectable by rhACE2 which emphasizes the quicker Ang 1-10-related turnover of human ACE2. The ACE2 activity of both angiotensin conversions can be summarized and assessed by its surrogate marker Ang 1-5 which is downstream of Ang 1-9 and Ang 1-7. Despite Ang 1-5 is formed by ACE, the formation of Ang 1-9 and Ang 1-7 which represent substrates for ACE, depends directly and uniquely on ACE2 activity due to the absence of NEP in plasma. Ang 1-5 levels in rhACE2 treated plasma were a 25 % higher than in rmACE2 plasma (254 pg/ml vs. 197 pg/ml).

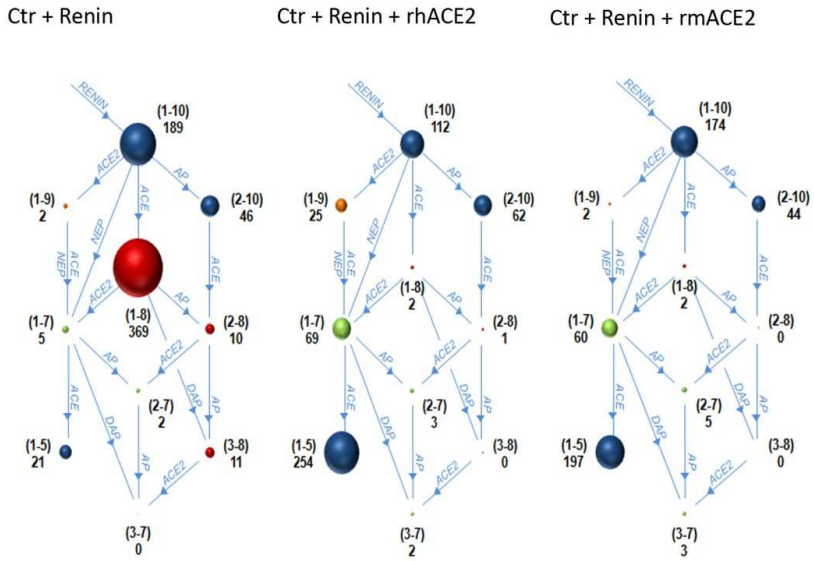

Figure 15: RAS-Fingerprint® of rmACE2 or rhACE2 treated plasma *ex vivo*. 100 pg/ml Renin was added to anti-coagulated blood-plasma (left) and mixed and incubated with rhACE2 (middle) or rmACE2 (right), respectively. The angiotensin-levels of 10 angiotensins were measured by a LC-MS/MS based method (See 3.11) The RAS-Fingerprint® depicts the concentrations (given in pg/ml next to each sphere) of angiotensins by the diameter of the sphere. 0 pg/ml indicate concentrations below quantification limits.) The name respective the amino acid sequence of each Angiotensin is given in brackets beside the sphere. ACE: Angiotensin-Converting-Enzyme; AP: Aminopeptidase; DAP: Di–Aminopeptidase; NEP: Neutral Endopeptidase.

ACE2s in Lisinopril-treated human plasma highlight the species-specific turnover of Ang 1-10. According to the RAS-Fingerprints® above (See Figure 15), the ACE-related degradation of Ang 1-10 and Ang 1-9 was inhibited by Lisinopril to highlight the differing turnover rates. Therefore, ACE2-plasma samples were equally treated as described but with additional Lisinopril. In line with the preceding RAS-Fingerprints® and *in vitro* findings, rhACE2 cleaved Ang 1-10 to Ang 1-9 with higher efficiency than rmACE2 as indicated by the peptide levels depicted in the RAS-Fingerprints® in figureFigure 16. rmACE2 reduced the levels of Ang 1-10 from 710 pg/ml (Data not shown) to 581 pg/ml, contrasted by its human homologue which reduced levels to 118 pg/ml. Accordingly, the concentration of Ang 1-9 rose more effectively by treatment of rhACE2 (412 pg/ml) compared to rmACE2 (40 pg/ml).

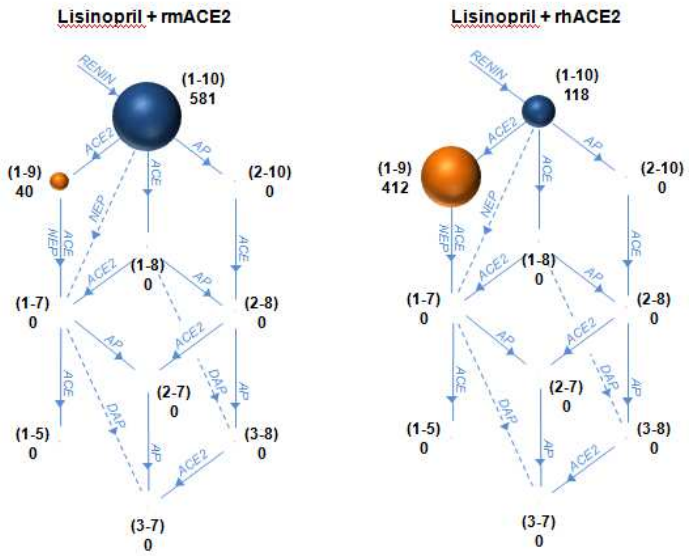

Figure 16: rhACE2 and rmACE2 treated with Lisinopril in plasma *ex vivo*. Anti-coagulated human plasma samples were incubated in the presence of 100 pg/ml Renin, 1 µM Lisinopril and both ACE2s at a final plasma concentration of 5 µg/ml. Angiotensin-levels were measured by LC-MS/MS as described (See 3.11) The RAS-Fingerprint® reflects the concentrations (given in pg/ml next to each sphere) of angiotensins by the diameter of the sphere. 0 pg/ml indicate concentrations below quantification limits. The name respective the amino acid sequence of each angiotensin is given in brackets beside the sphere. ACE: Angiotensin-Converting-Enzyme; AP: Aminopeptidase; DAP: Di–Aminopeptidase; NEP: Neutral Endopeptidase.

Treatment of DX600 reveals different inhibitor-sensitivity of ACE2s in human plasma *ex vivo*. DX600 is a selective ACE2 inhibitor and important for various studies dealing with ACE2 in piglets and rodents. The ability of DX600 to inhibit murine and human ACE2 was investigated under physiological conditions in human plasma *ex vivo*. Therefore, anti-coagulated human blood plasma was treated with 100 pg/ml Renin and 5 µg/ml of both ACE2s, respectively. DX600 was added at increasing concentrations, starting with a 10 fold molar excess of ACE2 up to a 1000 fold molar excess of DX600. Measurement of RAS-Fingerprints® with LC-MS/MS revealed multiple findings: DX600 was not able to decrease the activity of ACE2s in an equimolar ratio (See Figure 17, second from right). 15 µg/ml of DX600 which corresponds to a 100 fold excess of 5 µg/ml enzyme were able to decrease the activity of rhACE2, highlighted by increased amounts of Ang 1-10 and Ang 1-8 and decreased levels of Ang 1-9, Ang 1-7 and Ang 1-5 (See Figure 17 A, right). The catalytic activity of rmACE2 was not affected with an equal DX600 concentration (See Figure 17 B, right), but with a 10 fold higher concentration (Data not shown). As described before, levels of Ang 1-5 are surrogate markers for the activity of ACE2. Therefore, measured Ang 1-5 levels of all DX600 treated plasma samples were related to the control and depicted as percentage in a graph (Figure 18). That revealed a more than 100 fold higher DX600 inhibitor concentration for rmACE2 compared to rhACE2. Ang 1-5 levels were started to decrease in 5 µg/ml rhACE2-plasma samples by a treatment of a 10 fold molar excess of DX600 (1.5 µg/ml). In rmACE2 samples, DX600 had to be more than 100 fold higher concentrated to yield the same effect. Conclusively, murine and human ACE2 differ in terms of catalytic activity as well as inhibitor sensitivity.

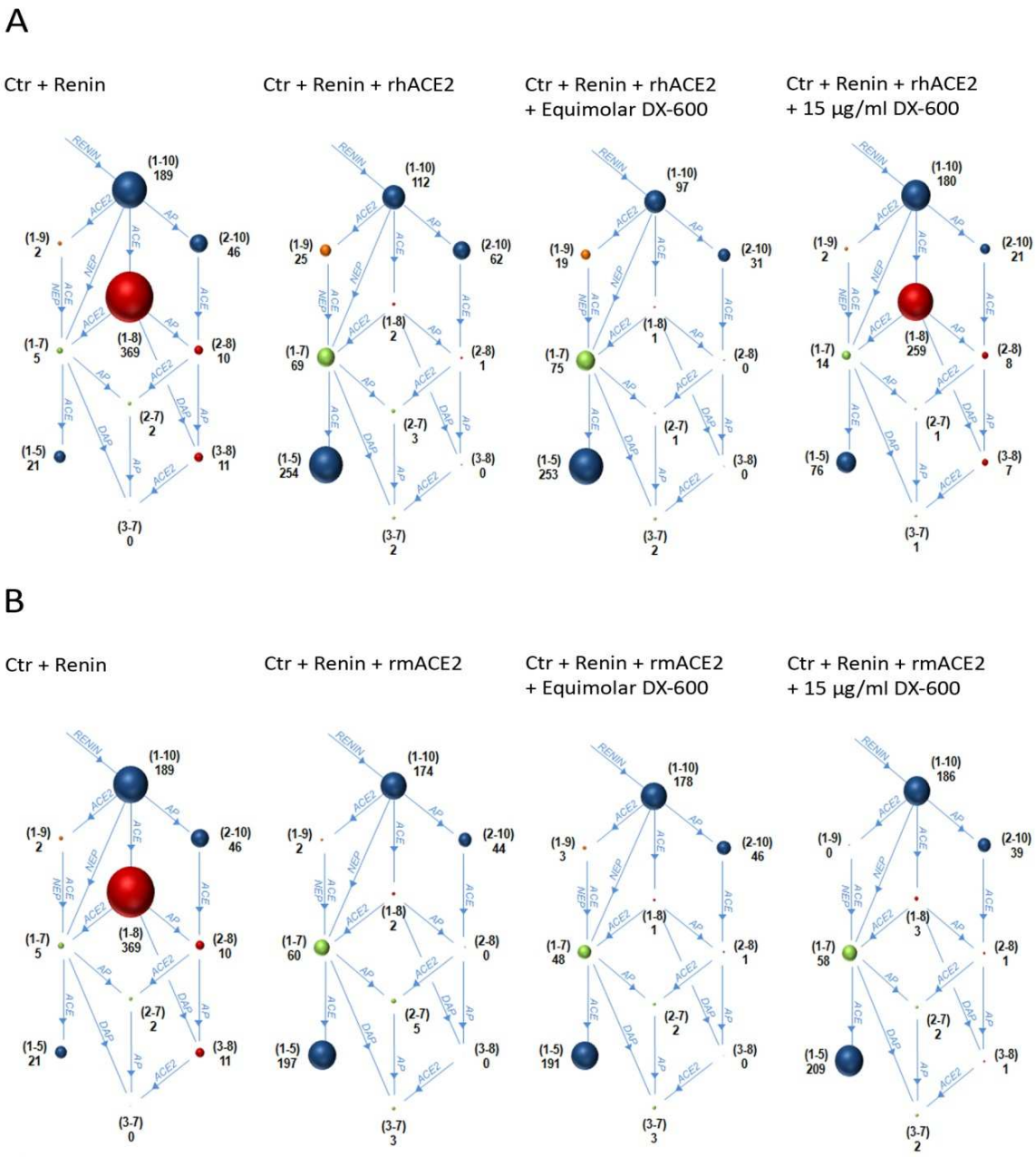

Figure 17: RAS-Fingerprint® of rmACE2 or rhACE2 treated plasma with increasing concentrations of DX600. 100 pg/ml Renin was added to anti-coagulated blood-plasma (A and B, left) and mixed and incubated with rhACE2 (A) or rmACE2 (B), respectively. Increasing amounts of DX600 (from 15 ng to 150 µg) were added to samples followed by analyzes with a LC-MS/MS based method (See 3.11) The RAS-Fingerprint® depicts the concentrations (given in pg/ml next to each sphere) of angiotensins by the diameter of the sphere. 0 pg/ml indicate concentrations below quantification limits.) The experimental setting is given above the RAS-Fingerprint®. The name respective the amino acid sequence of each Angiotensin is given in brackets beside the sphere. ACE: Angiotensin-Converting-Enzyme; AP: Aminopeptidase; DAP: Di–Aminopeptidase; NEP: Neutral Endopeptidase.

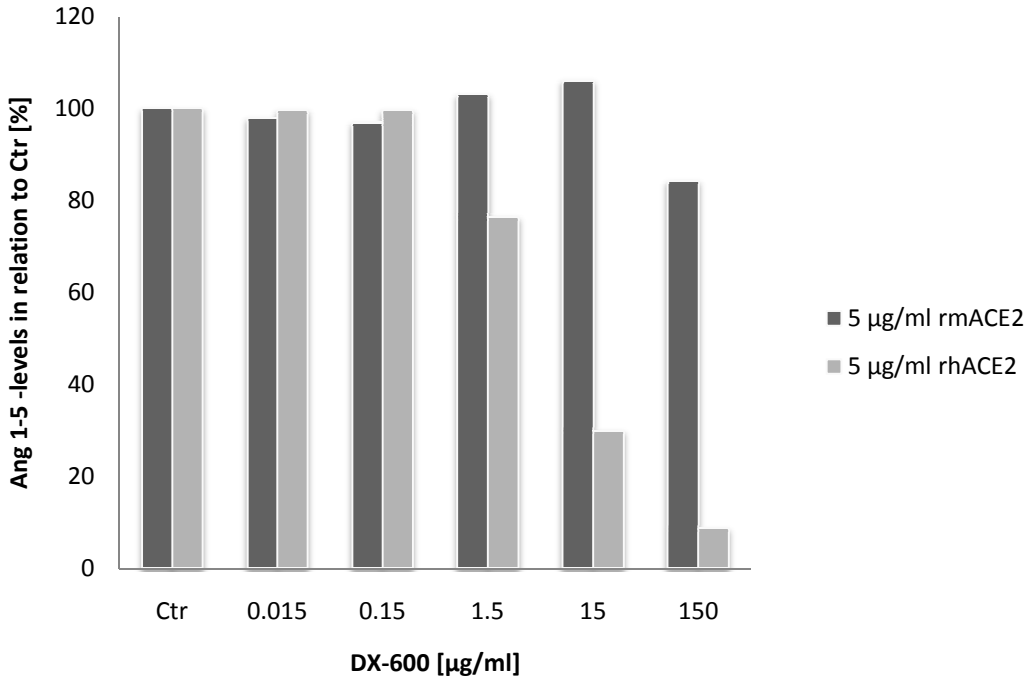

Figure 18: Ang 1-5 concentrations as surrogate markers for ACE2 activity treated with DX600. According to the RAS-Fingerprint® experiment of Figure 17, the graph depicts the Ang 1-5 percentages in relation to Ctr (absence of DX600) correlated to used DX600 concentrations (µg/ml). 0.15 µg/ml DX600 is equimolar to 5 µg/ml of both ACE2s. The dark bar represents rmACE2 treated samples, the light bar mirrors Ang 1-5 concentrations in rhACE2 samples.

5. Discussion

In the course this thesis, rmACE2 was expressed in a CHO-celline under serum-free conditions and purified to homogeneity by serial chromatography steps yielding more than 600 mg of 99% pure enzyme, and characterized in detail by state of the art biochemical and physical methods, for the first time. Fermented cells in roller bottles secreted stable levels of rmACE2 within one and three month before they were exchanged against new-cultivated cells to keep the count of producing bottles on the same level. Six batches of rmACE2 were purified with different yields. Modifications and improvements of purification steps, such as change of buffer-conditions, chromatography steps or additional operations resulted in higher purified concentrations with less loss. The yield could be increased within the six batches from 20 to 50 percent. Except batch #1706 which represents only one thirtieth of yielded rmACE2, all batches are endotoxin free and revealed purity above 99 % determined by HPLC and SDS-Page. rmACE2 occurs as a stable homo-dimer, while no monomeric or other multimeric forms were identified by HPLC or Native-PAGE. Also Dynamic Light Scattering, the most sensitive method to investigate disparity of protein solutions could only detect one form of rmACE2, calculated with a molecular weight near to its dimeric form. In SDS-PAGE, rmACE2 was detected as a monomer due to the presence of detergents, which open the non-covalent bonds between the monomers. By the novel Thermal Shift Assay, implications for the protein stability of varying pH-levels were investigated and resulted in an optimal pH-range for rmACE2-stability within 6.0 to 7.5 in glycine-buffer. Furthermore, the binding activity of two different antibodies towards rmACE2 was investigated to pave the way for further experiments. First results suggest a better binding-affinity for the polyclonal antibody generated in rabbits; however, due to the strong binding action of the antibodies and the poor regeneration of rmACE2 on the chip, too few examinations could be done to ensure the result. Further investigations and repeated measurements are required. The same applies for Isoelectric Focusing: the isoelectric point of rmACE2 could not be accurately determined due to the too-wide range of the standard bands. The range, within the net charge of rmACE2 is zero, was only roughly estimated; however the finding that the isoelectric point of human ACE2 is lower, is undisputed.

Compared to its human homologue, rmACE2 reveals further unexpected differences: Although the calculated molecular weight of both is nearly identical, HPLC, Native and SDS-Page showed a significant mass shift which was found to be caused by two additional glycosylation sites of rhACE2, thus four sialic acids more compared to rmACE2. Human ACE2 was found being occupied by an average of twelve N-glycans per protein which corresponds to a occupation percentage of 85 % . The

percentage of rmACE2 differed between the batches within 70 % to 90 %, thus lied in the range of human ACE2. When all N-glycans were removed, ACE2s showed equal running distances in SDS-PAGE. However, N-glycans of rhACE2 were only completely removed when the glycosidase PNGaseF was applied under reducing conditions. Native deglycosylated rhACE2 showed two bands in SDS-PAGE due to an incomplete removal of N-glycans. Sialic acids of equal amounts of native and reduced rmACE2 could be removed completely in the same treatment time of PNGaseF. This finding may points to different ternary structures of ACE2 by virtue of different accessibilities of N-glycans by PNGaseF. Due to the lack of a published molecular crystal structure of murine ACE2, Circular-Dichroism spectroscopy was used to at least investigate and compare the secondary structure respective the proportion of alpha-helices, beta-sheets or random coils structures. The spectrograms of both ACE2s revealed an equal proportion of alpha-helices and suggested that the sequence coverage of only 83 %[15] does not affect the secondary structure. Another way to rudimentary estimate the ternary structure is a measurement of the hydrodynamic radius using Dynamic Light Scattering. Not surprisingly, the hydrodynamic diameter of rhACE2 was found to be 10 % larger compared to rmACE2, explainable by its additional glycosylations, thus a different ternary structure.

The physiological relevance of ACE2 is based on its ability to cleave various peptides which possess multiple effector functions[37]. However, Angiotensin I and Angiotensin II, both are described to be cleaved by ACE2 *in vitro*[11], are relevant peptides within the Renin-Angiotensin-System. According to the findings of differing ternary structures of recombinant murine and human ACE2, also the substrate turnover rates of Ang 1-10 and Ang 1-8 were determined to investigate if the differing structures may also affect the physiological relevance of the enzymatic functions of the enzymes. Therefore, the Ang 1-10 and 1-8 related-turnover numbers for both ACE2 versions were specified by a well-defined *in vitro* model system (See Figure 14). The conversion rate of ACE2 for Angiotensin I to Ang 1-9 has been described to be slower than for Ang 1-8 why this enzyme reaction was supposed playing a minor role by increasing levels of Ang 1-7 than the direct production through Ang 1-8 cleavage. The *in vitro* assay could confirm previous findings concerning substrate specific turnover rates and determined a 42 higher turnover rate for Ang 1-8 compared to Ang 1-10 by rhACE2. (See Table 3) The calculated k_{cat}-values differ from the ones of recent studies, which could be caused by different buffer conditions. However, nevertheless the qualitative conclusions were internally controlled and comparable. rmACE2 cleaved Angiotensin II with a comparable turnover rate to its human homolog but differed substantially in regard to the turnover number of Angiotensin I. The formation of Ang 1-9 by murine ACE2 was found being 93 % slower compared to human ACE2. This finding might points to a species-specific activation of the alternative RAS-axis.

Only limited conclusions about physiological consequences can be made by *in vitro* experiments. For example, in blood plasma the majority of enzymes of the Renin-Angiotensin-System are present in excess compared to their substrates which is in absolute contrast to the *in vitro* conditions employed. To assess differences between the two enzymes, *in vitro* experiments are indispensible due to their well-defined conditions, but can only hint at implications for the *in vivo* situation. Therefore, the enzymatic activity of both recombinant ACE2s was investigated in their natural environment, which is undiluted human blood plasma. In this environment, substrate molecules are present in femto–molar concentrations, which implicates a molar excess of RAS enzymes. The ACE2 concentration of 5 µg/ml reflects the human *in vivo* plasma condition in regard to circulating enzymes after systemic administration of rhACE2. Angiotensin-levels of RAS were brought to a higher level with additional Renin to emphasis differences in turn-over rates. A novel LC-MS/MS based method allows the simultaneous quantification of multiple angiotensin metabolites down to a low quantification limit of 1 pg/ml for certain peptides. Measured levels are depicted in one single graph, a so-called RAS-Fingerprint®.

The RAS-Fingerprints® of murine and human ACE2 treated plasma confirmed the *in vitro* findings. In opposite to rmACE2, rhACE2 is able to form detectable concentrations of Ang 1-9 by the cleavage of Ang 1-10 (See Figure 16). To optimize conditions for the investigation of this ACE2 mediated reaction, the degradation of Ang 1-10 and Ang 1-9 by ACE was inhibited by Lisinopril (See Figure 16). Granted, when the degradation of Ang 1-9 is prevented, even murine ACE2 is capable to form Ang 1-9 but with a substantial lower efficiency than human ACE2. The catalytic turnover of Ang 1-8 to Ang 1-7 was found being nearly identical, even if Ang 1-7 concentrations in rhACE2 samples were slightly higher. This also goes in line with the previous *in vitro* findings.

The absolute enzyme activity of ACE2 is reflected by observed levels of Ang 1-5, which can be regarded as a cumulative marker for alternative RAS activation. Currently, the biological function of Ang 1-5 is unknown but its formation depends exclusively on ACE2 in plasma. Due to the lack of soluble Neprilysin or other alternative enzymes, only ACE2 is capable to form both peptides of the alternative axis of RAS which are immediately cleaved by ACE to generate Ang 1-5. Thus, Ang 1-5 is a surrogate marker for the absolute activity of ACE2.

The concentrations of Ang 1-5 were measured in ACE2 treated plasma samples with increasing concentrations of DX600, a selective inhibitor of ACE2[40] (See Figure 18). It was found that DX600 is a competitive inhibitor of both ACE2 versions because an equimolar concentration of enzyme and inhibitor did not affect the levels of Ang 1-5 compared to samples in absence of DX600. Interestingly, a ten-fold molar excess of DX600 to rhACE2 starts to lower the levels of Ang 1-5 with a linear

decrease to a thousand-fold molar excess. In contrast, the concentration of Ang 1-5 in rmACE2 plasma samples starts to decrease in presence of a thousand-fold molar excess of DX600, thus the activity of recombinant human ACE2 is initially affected and decreased with a hundred-fold lower DX600 molar excess than murine ACE2. This finding goes in line with a recent *in vitro* study where the calculated IC50 for human ACE2 was 78-folder lower than that for rodent ACE2[40].

To avoid misunderstandings it is essential to emphasize the differences between plasma and whole blood conditions. The Renin-Angiotensin-Aldosterone-System is affected by multiple enzymes, like Angiotensin receptors or Neprilysin. NEP is expressed and attached on cell surfaces of circulating blood cells and leukocytes[81] and is also capable to generate Angiotensin 1-7 and Angiotensin 1-9[37]. Therefore, the differences between both ACE2 homologues were investigated in human plasma to exclude effects of NEP or other membrane bound enzymes. Thus, the published RAS-Fingerprints® reflect RAS-levels in plasma, not in whole blood.

Nevertheless, the physiological implications of the species-specific activity of ACE2 are crucial. The ACE2 mediated formation of Ang 1-9 from Ang 1-10 represents an important step for the establishment of the alternative RAS-axis, which is absolute important during ACE-inhibitor treatments to form Ang 1-7 *in vivo*. Due to the lack of the alternative axis, these findings shall give rise to reconsiderations of ACE-inhibitor studies in mice. Furthermore, Ang 1-9 may also provide physioprotective effects[43], as it was reported in rats and is currently under investigation. Also the used DX600 concentrations in rodent studies shall be reconsidered to avoid false ACE2-activity data.

It is highly recommended to compare the enzymatic activity of murine endogenous and recombinant ACE2 to confirm the findings about the species specificity of the enzyme. Furthermore, the crystal structure of recombinant murine ACE2 and especially its binding pocket shall be investigated. This may provides information about the different Ang 1-10 turnover rate and the catalytic activity in general. Additional murine RAS-enzymes should also be investigated like ACE or NEP. Maybe the lack of ACE2-related degrading of Ang 1-10 is balanced by a higher activity of NEP, respectively by a lower conversion rate of ACE, which leads to similar Angiotensin-levels like in humans. Increased amounts of Ang 1-10 could also compensate the lower turnover of ACE2 by a higher saturation of the binding pocket. Or the different angiotensin-levels affect the physiology of mice in another way compared to human, e.g. caused by a different angiotensin-receptor-density to offset increased angiotensin-levels or a decreased affinity of the receptors. Shortly, many questions are still unanswered. In conclusion, the significance of mice-studies in regard to the Renin-Angiotensin-Aldosterone-System shall be reconsidered and reviewed.

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