Effect of long-acting insulin analogues on human cardiovascular cell models

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Thorsten Hartmann
aus Simmerath

Düsseldorf, Februar 2017

aus dem Deutschen Diabetes-Zentrum
Paul-Langerhans-Gruppe für Integrative Physiologie
Leibniz-Zentrum für Diabetes-Forschung
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Jürgen Eckel
Korreferent: Prof. Dr. Eckhard Lammert
Tag der mündlichen Prüfung: 02.06.2017

NO ONE IS BETTER THAN YOUR BEST, BUT YOUR BEST WILL MAKE YOU BETTER. AND THAT'S GOING TO MAKE US ALL BETTER.

- SEAN CORVELLE

Zusammenfassung

Diabetes Mellitus stellt eine der größten Herausforderungen des Gesundheitssektors dar, mit welcher sich die westliche Welt in den nächsten Jahrzehnten konfrontiert sehen wird. Es wird erwartet, dass die Zahl der Diabetiker rapide ansteigt, was zu großen ökonomischen Belastungen und zu einer Reduzierung der Lebensqualität der Neu-Diabetiker führen wird. Wenn die Krankheit unbehandelt bleibt, kann dies für den Patienten zu lebensbedrohlichen Konsequenzen, wie zum Beispiel Ketoazidose, führen. Zusätzlich kann falscher oder unbehandelter Diabetes zu Langzeitkomplikationen wie Nephropathie, Retinopathie, Neuropathie oder kardiovaskulären Erkrankungen führen. Nur mit adäquater Behandlung der Patienten ist es möglich diese Langzeitkomplikationen zu umgehen. Zwar gibt es zurzeit verschiedene Behandlungsmöglichkeiten für Typ 2 Diabetiker, jedoch kann es sein, dass bei fortschreitendem Krankheitsbild und sich verschlechternder Prognosen eine Behandlung mit Insulinmedikamenten unumgänglich wird. Darüber hinaus ist die Behandlung mit Insulin zurzeit die einzige Möglichkeit Typ 1 Diabetiker zu therapieren.

Insulin wurde im Jahr 1921 von Banting und Best zum ersten Mal isoliert. Kurz darauf wurde die erste erfolgreiche Behandlung eines Diabetikers mit tierischem Insulin durchgeführt. Mitte der 1990er Jahre wurde das erste Insulinanalog (Insulin Lispro) mit einer Veränderung der Primärstruktur zur Behandlung von Diabetikern eingeführt. Darauf folgten weitere Insulinanaloga. Diese lassen sich grob in zwei Klassen unterteilen, schnell wirksame Insulinanaloga und lang wirksame Insulinanaloga. Diese weisen im Vergleich zu regulärem Insulin vorteilhafte pharmakodynamische und pharmakokinetische Eigenschaften auf (schnelle Wirksamkeit vs. lange anhaltender Effekt). Trotz Verbesserungen der pharmakokinetischen und pharmakodynamischen Eigenschaften sind lang wirksame Insulinanaloga nicht vollständig in der Lage, die endogene Insulinsekretion nachzuahmen. Um dies zu erzielen befinden sich zurzeit verschiedene ultra-lang wirksame Insulinanaloga in der (prä-)klinischen Entwicklung.

Durch die Veränderung am Insulinmolekül kann es zu unerwünschten Nebenwirkungen kommen. So konnte für AspB10-Insulin gezeigt werden, dass dieses Insulin eine erhöhte kanzerogene Wirkung hat. Im Fall von Insulin Degludec hat die amerikanische Behörde für Lebens- und Arzneimittel (FDA) die Zulassung für den amerikanischen Markt mit der Begründung eventueller kardiovaskulärer Risiken im Zusammenhang mit der Verwendung

von Insulin Degludec im Jahr 2013 zunächst untersagt. Des Weiteren forderte die FDA eine klinische Studie, die diese Risiken ausschließen soll. Um die molekularbiologischen Grundlagen für die potentiellen Nebenwirkungen von Insulin Degludec zu untersuchen wurde im ersten Teil dieser Thesis die Wirkung von Insulin Degludec auf den Insulin Signalweg und die Funktion von Kardiomyozyten mit Insulin Glargin und regulärem Insulin verglichen. Dazu wurden Kontraktionsparameter in primären Rattenkardiomyozyten sowie die Kontraktionsfrequenz in humanen Kardiomyozyten, als auch der Apoptosesignalweg und die Glukoseaufnahme im Gleichgewichtszustand untersucht. In keinem dieser Parameter konnte ein Unterschied zwischen Insulin Degludec und den anderen verwendeten Insulinen festgestellt werden. Jedoch wurde festgestellt, dass es zu einem signifikant verzögerten Wirkungseintritt im Insulin Signalweg von HL-1 Kardiomyozyten kommt, wenn diese mit Insulin Degludec behandelt wurden. Dies wurde durch die signifikant geringere Bindungsaffinität von Insulin Degludec an den Insulinrezeptor erklärt.

Im zweiten Teil der Thesis wurde die Sicherheit und Wirksamkeit des neu entwickelten ultra-lang wirksamen Insulinanalogs HM12470 in *in vitro* und *in vivo* Experimenten untersucht. Bei HM12470 handelt es sich um ein so genanntes Fc-Fusionsprotein. In den *in vitro* Experimenten wurde eine signifikant geringere Bindungsaffinität von HM12470 zum Insulinrezeptor festgestellt. Die Verweilzeit am Insulinrezeptor unterscheidet sich hingegen nicht im Vergleich zu regulärem Insulin. Darüber hinaus wurde kein Unterschied im Insulinrezeptor-Autophosphorylierungsmuster festgestellt. Einzig auf Grund der geringen Rezeptoraffinität musste die 5-fache Konzentration von HM12470 verwendet werden, um zu vergleichbaren Zeitpunkten Phosphorylierung analysieren zu können. Die Analyse der mitogenen Eigenschaften von HM12470 ergab keinen Unterschied zu regulärem Insulin, selbst wenn hohe Konzentrationen der Insuline (1000 nM) verwendet wurden. Es konnte gezeigt werden, dass die metabolische Wirkung nach Kurzzeit-Stimulation (2 h) im Vergleich zu regulärem Insulin leicht reduziert war. Nach der Langzeit-Stimulation (24 h) konnte jedoch kein Unterschied zwischen beiden Insulinen festgestellt werden. Interessanterweise ist diese Beobachtung auf geringere Insulinrezeptor-Herunterregulierung nach Behandlung mit HM12470 zurückzuführen. Dies ist sogar der Fall, wenn eine 5-fach erhöhte Konzentration von HM12470 verwendet wurde. Um die Insulinrezeptor-Regulierung weiter zu untersuchen, wurde ein Desensitisierungs/Resensitisierungs-Modell entwickelt. Darin wurden die Zellen für fünf Tage mit 500 nM des jeweiligen Insulins behandelt und anschließend für bis zu zwei Tage in insulin-freiem Medium weiter kultiviert. Bei dieser Versuchsreihe konnten weitere Beweise gefunden werden, dass HM12470 die behandelten Zellen nicht dauerhaft

desensitisiert. Nach fünf-tägiger Behandlung war der Insulinsignalweg in HM12470 behandelten Zellen signifikant beeinträchtigt, nach zwei Tagen ohne Insulinbehandlung zeigten die HM12470 behandelten Zellen eine nahezu vergleichbare Insulinwirkung wie die Kontrollzellen. Darüber hinaus war die Insulinwirkung auf diese Zellen signifikant besser als auf Zellen welche zuvor mit regulärem Insulin behandelt wurden. In den *in vitro* Experimenten konnte gezeigt werden, dass HM12470 seine Wirkung in allen Zielorganen von männlichen Wistar-Ratten erreicht. Zusätzlich wurde gezeigt, dass HM12470 eine Serum-Halbwertzeit von 44.1 h in Ratten aufweist und in der Lage ist, den Blutglukosespiegel in $^{db/db}$Mäusen bis zu sieben Tage nach einer einzelnen Injektion zu normalisieren.

Zusammenfassend konnte in dieser Thesis kein negativer Effekt von Insulin Degludec auf den Insulinsignalweg und die Funktion von Kardiomyozyten gezeigt werden. Zusätzlich konnte in dieser Thesis eine umfassende *in vitro* und *in vivo* Studie des Insulinanalog HM12470 präsentiert werden. Dabei zeigte sich, dass HM12470 ein substantiell verlängertes pharmakokinetisches Profil und ein sicheres metabolisches/mitogenes Profil aufweist und zusätzlich die einzigartige Eigenschaft besitzt, Zellen selbst in hohen Konzentrationen nicht irreparabel zu desensitisieren. Diese prä-klinischen Daten liefern Hinweise für die Sicherheit und Effektivität von HM12470, welche nun in klinischen Studien noch nachgewiesen werden müssen.

Summary

Diabetes mellitus is one of the largest health issues the western society is facing in the coming decades. The number of diabetic patients is expected to increase rapidly, leading to high economic burdens and to decreased quality of life for the patients themselves. These patients face life threatening consequences, such as ketoacidosis, if the disease is not treated. Furthermore, long-time consequences, such as nephropathy, retinopathy, neuropathy and cardiovascular disease can occur if the patients are not treated properly. With sufficient treatment only, diabetic patients have a chance to circumvent most of the complications associated with diabetes. Today, several therapy options for the treatment of type 2 diabetes mellitus are available. However, ultimately exogenous insulin supplementation might become necessary to prevent hyperglycaemia. Furthermore, the supplementation of exogenous insulin is the only treatment option for type 1 diabetes mellitus patients to date.

The first isolation of insulin was conducted by Banting and Best in 1921. Shortly after, the first diabetic patient was successfully treated with exogenous insulin supplementation. In the mid-1990s insulin lispro, the first insulin analogue with modification in its primary structure was introduced and several others followed. These insulin analogues are mainly divided into two classes: rapid- and long-acting insulin analogues, which exhibit favourable pharmacokinetic/pharmacodynamic profiles compared to regular insulin (faster onset vs. longer duration of action). However, the pharmacokinetic/pharmacodynamic profiles of long-acting insulin analogues do not mimic endogenous insulin secretion yet and novel ultra-long-acting insulin analogues are currently in (pre-)clinical development.

Furthermore, the introduced modifications can lead to undesired side-effects as could be shown for insulin AspB10, which shows increased cancerogenicity. For insulin degludec the American Food and Drug Administration raised concerns about potential cardiovascular side-effects and therefore requested a dedicated cardiovascular outcome trial. To analyse the molecular basis of insulin degludecs potential side-effects, the first part of this thesis was to compare insulin degludec to insulin glargine and regular insulin in regard of signalling and function on cardiomyocytes. Therefore contractility parameters of adult rat ventricular myocytes, beating-rate of human cardiomyocytes, as well as apoptotic signalling and glucose uptake under steady-state conditions were analysed. In none of these parameters a difference between the different analogues was observed. However, the onset of action in HL-1 cells was

significantly slower with insulin degludec, which was explained by the significantly lower binding affinity of insulin degludec towards the insulin receptor found in this study.

In the second part of the thesis *in vitro* and *in vivo* studies regarding the safety and efficacy of the novel ultra-long-acting insulin analogue HM12470, an Fc-fusion protein, were conducted. In the *in vitro* studies we found significantly lower binding affinities towards the insulin receptor with HM12470, but similar off-rates compared to regular insulin. No difference in the insulin receptor autophosphorylation pattern was detected, even though a 5-fold higher insulin concentration had to be used to observe onset of signalling after short-term treatment. The mitogenic potency of HM12470 was found to be comparable to regular insulin even if high concentrations (1000 nM) were used. The metabolic potency was slightly reduced after short-term stimulation (2 h). However, after long-term stimulation (24 h) with HM12470 comparable metabolic potency was observed. Interestingly, this observation was due to significantly less receptor down-regulation in the cells treated with HM12470, even with a 5-fold higher concentration. In another experiment the insulin receptor downregulation was analysed in a desensitisation/resensitisation model. Cells were treated for 5 days with 500 nM of the respective insulin. Subsequently, cells were cultivated for up to 48 h in the absence of insulin. Here further evidence was found, that HM12470 did not irreversibly desensitise the cells. After 5 days of treatment the insulin signalling was significantly reduced. After 48 h of recovery, the cells treated with HM12470 showed insulin signalling nearly comparable to the control cells. Furthermore, HM12470 treated cells showed significantly increased signalling compared to insulin treated cells at this time point. In the *in vivo* experiments it was demonstrated that HM12470 is able to induce insulin signalling in all major insulin target tissues in male Wistar rats. Additionally, HM12470 has a serum half-life of up to 44.1 h in rats and glucose lowering effects of up to 7 days in $^{db/db}$mice after single subcutaneous injection.

In conclusion, this thesis provides no evidence for harmful effects of insulin degludec with regard to cardiomyocyte signalling and function. Furthermore, this thesis provides a comprehensive pre-clinical study of the insulin analogue HM12470. HM12470 exhibits a substantially prolonged pharmacokinetic profile with a safe metabolic/mitogenic profile and the unique feature of absence of irreversible insulin signalling desensitisation. Thus providing pre-clinical evidence for safety and efficacy of HM12470, which needs to be proven in clinical trials.

Table of Contents

List of Tables and Figures

Abbreviations

AA	amino acid
ADA	American Diabetes Association
APAF-1	apoptotic protease activating factor-1
ARVM	adult rat ventricular myocytes
BAD	BCL-xL/BCL-2 associated death promoter
BAX	BCL-2 associated X-protein
BIL	basalinsulin PEGlispro
BMI	body mass index
CNS	central nervous system
Con-Ins G1	minimized insulin found in venom of Cone snails
CPE	carboxypeptidase E
CTLA-4	cytotoxic T lymphocyte-associated protein 4
CV	cardiovascular
CVB	Coxsackievirus B
CVD	cardiovascular disease
Cyt-*c*	cytochrome-*c*
DPP4	dipeptidyl peptidase 4
EASD	European Association for the Study of Diabetes
EMA	European Medical Agency
FcRn	neonatal Fc-receptor
FDA	Food and Drug Administration
GAD65	glutamic acid decarboxylase 65
GFR	glomerular filtration rate
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLUT4	glucose transporter 4
GOx	glucose oxidase
GRB	growth factor receptor-bound
HbA1c	glycated haemoglobin
HCAEC	human coronary artery endothelial cells
HCASMC	human coronary artery smooth muscle cells
HLA	human leucocyte antigen
HSA	human serum albumin
HSkMC	human skeletal muscle cells
I-A2	insulinoma-associated antigen-2
IAA	insulin auto-antibody
IDeg	insulin degludec
IDet	insulin detemir
IFG	impaired fasting glucose
IFIH1	interferon-induced helicase 1
IGF-1R	insulin like growthfactor-1 receptor
IgG	immunoglobulin G
IGla	insulin glargine
IGlaM1	active metabolite of insulin glargine
IGT	impaired glucose tolerance
IL2RA	interleukin-2 receptor-alpha
Ins 115	insulin 115
Ins	regular insulin
IR	insulin receptor
IRI	ischemia reperfusion injury
IRS	insulin receptor substrate
LCFA	long-chain fatty acids
MACE	major adverse cardiac events
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
M-IR	membrane bound insulin receptor preparation
NPH	neutral protamine Hagedorn insulin
PBA	phenylboronic acid
PC1/2	proprotein convertase 1/2
PD	pharmacodynamics
PDK1	phosphoinositide-dependent protein kinase-1
PEG	polyethylene glycol
PFK1	6-phosphofructo-2-kinase
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PK	pharmacokinetics
PKB	protein kinase B
PLN	phospholamban
PPAR-γ	peroxisome proliferator-activated receptor γ
PTPN22	protein tyrosine phosphatase, non-receptor type 22
rER	rough endoplasmatic reticulum
RHI	regular human insulin
ROS	reactive oxygen species
s.c.	subcutaneous
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SGLT2	sodium-glucose-cotransporter 2

S-IR	solubilised insulin receptor preparation
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TET	transendothelial transport
WHO	World Health Organisation
ZDF rats	Zucker Diabetic Fatty rats
ZnT8	zinc transporter 8
β_2m	β_2-microglobulin

1 Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disorder where the body cannot produce enough insulin or cannot properly use the insulin it produces [1] leading to an increased blood glucose level, known as hyperglycaemia. Diabetes has become one of the largest health issues worldwide with 415 million adults (20 – 79 years) and more than 0.5 million children and adolescents (<15 years) suffering from this disease [2]. These numbers are expected to grow rapidly, up to 642 million adults until 2040 and an annual increase of roughly 3 % in children [2-5]. Furthermore, 318 million people show impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) and are considered pre-diabetic with a high risk of developing diabetes mellitus. The number of people being pre-diabetic is estimated to increase up to 481 million in 2040 [2].

Diabetes can be divided into two main types: 1) Type 1 diabetes mellitus (T1DM) and 2) Type 2 diabetes mellitus (T2DM) and is diagnosed when patients show clinical symptoms (e.g. polyurea, polydipsia or dry mouth, lack of energy, sudden weight loss) and match at least one of the diagnostic criteria described in Table 1.

Table 1: Laboratory criteria for diagnosis of diabetes. Adapted from the WHO [6, 7]

Diagnostic criterion	Diagnostic value
Fasting plasma glucose	≥ 7.0 mmol/L
Two hour plasma glucose	≥ 11.1 mmol/L
Random venous plasma glucose	≥ 11.1 mmol/L
Glycated haemoglobin	≥ 6.5 %

1.1.1 Type 1 Diabetes Mellitus

Even though the exact etiology of T1DM is not fully understood, it is generally considered as an autoimmune disease leading to β-cell death and subsequently to absolute insulin deficiency and chronic-hyperglycaemia. Therefore, patients with T1DM require life-long substitution of insulin to survive.

This autoimmune disorder is triggered by environmental factors in genetically susceptible individuals [8]. Several genetic alleles have been found which are correlated with

development of T1DM. The allele with the strongest correlation is the human leucocyte antigen (*HLA*) region [9-11]. Other alleles associated with the development of T1DM are the insulin gene region [12, 13], protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) [14, 15], interleukin-2 receptor-alpha gene (*IL2RA*) region [16, 17] and the gene encoding for cytotoxic T lymphocyte-associated protein 4 (*CTLA-4*) [18, 19]. Another locus discovered is the gene which encodes for interferon-induced helicase 1 (*IFIH1*) [20]. The *IFIH1* locus is especially interesting, since it plays a crucial role in innate immunity against viral dsRNA of picorna viruses [21] and infection with Coxsackievirus B (CVB), a picorna virus, which seems to be linked to development of T1DM. This could be a potential link between genetic susceptibility and environmental factors.

In general, viral infection of islet cells seems to come closest to represent a causal relationship to T1DM development [22, 23]. It could be shown that islets from recent-onset T1DM patients show significant increased virus infection compared to the control group [24, 25] and that anti-CVB4 antibodies recognize autoantibodies in T1DM patients, which subsequently leads to apoptosis of β-cells [26]. Other proposed environmental factors are bacteria, cow's milk and lack of vitamin D. For bacteria it could be shown that antibiotics and probiotics, at least indirectly, influence T1DM development by altering the gut microbiome [27]. Low levels of vitamin D metabolites are found in T1DM patients around clinical onset [28] and a decreased incidence is observed in people with genetic predisposition by increased vitamin D intake [29]. However, for cow's milk the literature is contradictory. While a cross-reactivity of serum antibodies of T1DM patients between albumin and ICA-1, a β-cell surface protein, was found in 1992 [30] the Diabetes Autoimmunity Study in the Young (DAISY) trial did not find any causal consequence [31]. It is believed that immunity to cow's milk proteins is rather a general defect in mucosal immunity than a risk factor for itself [32].

Since T1DM is an autoimmune disease, several autoantibodies have been identified which lead to progression and manifestation of T1DM. The main autoantigens are insulinoma-associated antigen-2 (I-A2), insulin (IAA or micro IAA), glutamic acid decarboxylase 65 (GAD65) and zinc transporter 8 (ZnT8). In the BABYDIAB trial it was found that just a small percentage of children expressing only one of those autoantibodies progresses to T1DM [33, 34]. Furthermore, in the European Nicotinamide Diabetes Intervention Trial (ENDIT) 62 % of the study participants expressing multiple autoantibodies progress to T1DM [35], confirming the results from the Diabetes Prevention Trial-1 (DPT-1)

trial [36]. Therefore, the number of autoantibodies seems to play a more important role than the specificity of the autoantibodies in the development and progression of T1DM.

1.1.2 Type 2 Diabetes Mellitus

The major form of diabetes is T2DM, which accounts for 90-95 % of all diabetic people. T2DM has another etiology than T1DM. Instead of destruction of β-cells leading to insulin deficiency, T2DM is rather characterised by insulin resistance in the major target organs (e.g. skeletal muscle, adipose tissue, liver) combined with relative insulin deficiency [37]. In later stages of the disease the pancreatic β-cells fail to produce enough insulin to cope with hyperglycaemia.

As in T1DM, genetic susceptibility plays an important role in the development of T2DM. With different genetic approaches more than 80 common genetic variants associated with the disease were identified [38]. Almost all of those candidate genes play a role in regulation of insulin secretion and only a minor part is involved in insulin sensitivity [39]. However, the increase in risk of developing T2DM associated with these variants varies between 5-40 %. Furthermore, only a minority of observed heritability is associated with these loci [38, 40]. Thus, environmental factors are the main causes for the development of T2DM. Those are, but not limited to, excess body fat especially in the abdominal area, sedentary lifestyle and a poor diet with high intake of unsaturated fatty acids and processed sugar [2, 41]. For example studies could show that the likelihood of developing T2DM is positively correlated with the body mass index (BMI) and highly increased in morbidly obese (BMI ≥ 35) people [42, 43].

1.1.3 Consequences of Diabetes

Diabetes leads to decreased quality of life and is accompanied by an enormous economic burden. Healthcare expenditure is 2 - 3 fold higher in diabetic patients compared to non-diabetic people [44-46]. Acute, life threatening complications are diabetic ketoacidosis due to exceptionally high hyperglycaemia or coma triggered by hypoglycaemia. Long-term risks are mainly triggered by micro- and macroangiopathies due to a constant hyperglycaemic state [47]. In microangiopathies small blood vessels are damaged, which can lead to nephropathy, retinopathy and neuropathy. In macroangiopathies large blood vessels undergo pathological changes (e.g. atherosclerosis) leading to cardiovascular disease (CVD).

Nephropathy due to diabetes is the main cause of end stage renal failure in industrialised countries [48]. Additionally, end stage nephropathy is a major risk factor for the development of CVD [49, 50]. Early in the disease progression the kidney undergoes hypertrophy mainly

due to increase of the proximal tubule mass [51]. Consequently, reabsorption of glomerular filtrate is increased leading to an increase in glomerular filtration rate (GFR) [52]. This in turn decreases the excretion of several substances, such as glucose, fatty acids, proteins and amino acids. Ultimately the progression leads to renal failure and can lead to death if left untreated.

Retinopathy is caused by lesions within the retina and the main reason for blindness in adults [53, 54]. This disease is slowly progressing and most diabetic patients develop retinal lesions 20 years after diagnosis [54-56]. In early stages, the so called non-proliferative state, hyperglycaemia leads to pericyte death and basement membrane thickening, which alters vascular permeability [53]. After progression to the proliferative state, neovascularisation and macula edema occurs, resulting in visual impairment and can ultimately lead to loss-of-vision.

Neuropathy develops in more than half of all diabetics [57] and diabetics have a lifetime risk of up to 15 % for lower extremity amputation [58]. Furthermore, it is the primary cause for impaired wound healing, erectile dysfunction and cardiovascular dysfunction in diabetes. Neuron size seems to play an important role in diabetic neuropathy. Longer nerves show early loss of conduction velocity and loss of nerve terminals. This primarily leads to loss of sensation and tingling observed in feet and hands [58]. However, not only motoneurons but the autonomic nervous system is influenced too. This leads to orthostatic hypotension, resulting from loss of ability to properly adjust heart rate and vascular tone. Furthermore, nerves innervating the intestines can be affected, leading to nausea, bloating and diarrhoea [58].

CVD summarises several disorders including atherosclerosis, myocardial infarction, stroke and impaired cardiac functionality. CVD is the most common cause of death in people suffering from diabetes [2]. For example, the risk of diabetic patients to develop myocardial infarction is equivalent to non-diabetics with history of myocardial infarction [59] and threefold increased compared to the general population [60]. Atherosclerosis develops due to atherosclerotic plaque formation, where several cell types and processes are involved. Plaque formation is associated with the risk of blocking the vessel or disruption of the plaque with thrombus formation, leading to the aforementioned consequences (e.g. stroke, myocardial infarction). Another risk is damage to cardiac tissue due to diabetic cardiomyopathy, which is characterised by diastolic dysfunction [61]. With progression, diabetic cardiomyopathy may result in diastolic heart failure due to myocardial stiffening, hypertrophy and neuronal abnormalities [58]. In the group of patients with diastolic heart failure, diabetic individuals are overrepresented [62].

Taken together, diabetic people can and will suffer from a variety of pathophysiological processes if the disease is not treated properly. This leads to the need of sufficient therapy or prevention approaches to stop or reverse damage inflicted by diabetes.

1.1.4 Diabetes Therapy

For T1DM, clinical trials mostly focus on immune therapies to normalise immune dysregulation or induce tolerance against specific antigens [63] with insufficient effect to date. Only few studies tried to elaborate induction of β-cell regeneration itself [64, 65]. In animal studies different combinations of immunotherapies were tested [66-68], which were not successful in reversal of T1DM, due to immense adverse effects of high anti-CD3 antibody doses used. Combination therapies conducted in humans mostly did not decrease insulin requirement and hypoglycaemic events [69]. Other approaches are islet cell transplantation, which bears limitations due to lack of donors and the need of immunosuppression [70] or insulin producing stem cells and reprogrammed cells [71, 72]. Even though huge efforts are currently undertaken to tackle T1DM progression and onset, to date the only successful therapy is substitution with exogenous insulin to prevent death.

Since destruction of β-cells is not the main cause for the development of T2DM, there are several treatment options available maintaining glycaemic levels close to those of non-diabetics. In a consensus statement jointly published by the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD), intervention strategies for T2DM patients are suggested [73]. As the first step they recommend initiating lifestyle intervention with increased activity targeting weight loss. This leads to improved glycaemic control in type 2 diabetics by improving or resolving the risk factor overweight/obesity [74, 75]. However, patient compliance strongly limits the effect of lifestyle intervention therapies. If patients do not reach glycated haemoglobin (HbA1c) goals, or are not able to lose or maintain weight, metformin should be additionally introduced into the therapy plan. The exact mechanism of how metformin improves glycaemic control is not fully understood yet. However, it is known that metformin inhibits complex 1 of the mitochondrial respiratory chain and thereby blocking gluconeogenesis [76]. Additionally, hepatic glucose production is inhibited via differential gluconeogenic gene expression [76]. This leads to lower hepatic glucose output accompanied with lower fasting plasma glucose level.

During progression of the disease additional drugs may be required to prevent hyperglycaemia. The ADA and EASD recommend using sulfonylurea or insulin for further treatment [73]. Sulfonylureas enhance insulin secretion via binding to its receptor which is part of the potassium channel complex on β-cells [77] and thereby leading to reduced blood glucose levels. Insulin analogues themselves are designed to mimic endogenous insulin secretion and compensate for unsatisfactory insulin output by β-cells. The advantage of insulin analogues is the ability to intensify therapy in case HbA1c aims are still not met.

Other classes of anti-diabetic drugs, not covered in the favourable treatment regimen by the ADA and EASD are: glinides, alpha-glucosidase inhibitors, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, pramlintide, colesevelam, glucagon-like peptide-1 (GLP-1) receptor agonists, dipeptidyl peptidase 4 (DPP4) inhibitors, bromocriptine and sodium-glucose-cotransporter 2 (SGLT2) inhibitors. Similar to sulfonylureas, glinides increase insulin secretion [78]. PPAR-γ agonists improve insulin sensitivity [79] partly via remodelling of fat depots [80]. The remaining substance classes can be divided into three groups, depending on their site of action. 1) Drug action in the gastro-intestinal tract, like GLP-1 receptor agonists [81] and DPP4 inhibitors [81]. 2) Drug action in the kidney, which is shown for SGLT2 inhibitors [82, 83] and 3) drug action in the CNS as shown for the dopamine-receptor agonist bromocriptine [84, 85].

Another, very promising yet invasive technique to treat T2DM is bariatric surgery. Several studies could show that bariatric surgery is a powerful tool which can resolve clinical and laboratory signs of T2DM for up to more than 2 years after surgery. During this time medication was not needed anymore [86, 87]. The metabolic effect could be shown for both, morbidly obese patients (BMI > 35) (82 % of patients in first two years and 62 % for more than 2 years following surgery) [86] and obese patients (BMI 30 - 35) (85.3 % off medication) [87].

1.2 Insulin

1.2.1 Structure and Processing

The first isolation of insulin from pancreatic tissue was done in 1921 by Banting and Best [88]. Pancreatic extracts were used shortly after to treat the first diabetic patients. It took many years and development in biological techniques to identify the structure and processing steps involved in maturation of insulin. Insulin was the first protein ever for which the whole primary amino acid (AA) sequence was determined. Sanger discovered the two-chained

structure of insulin in 1955 [89, 90]. Insulin consists of an A- and B-chain linked by two disulphide bridges between CysA7 – CysB7 and CysA20 – CysB19. An additional disulphide bridge links CysA6 – CysA11. The A-chain consists of 21 AA and the B-chain of 30 AA. Over a decade later evidence was found that insulin has a single-chained precursor molecule, which was named proinsulin [91, 92], connecting both chains via the so called C-peptide, a peptide consisting of 31 AA. Even later an additional precursor molecule, preproinsulin, was discovered [93-95].

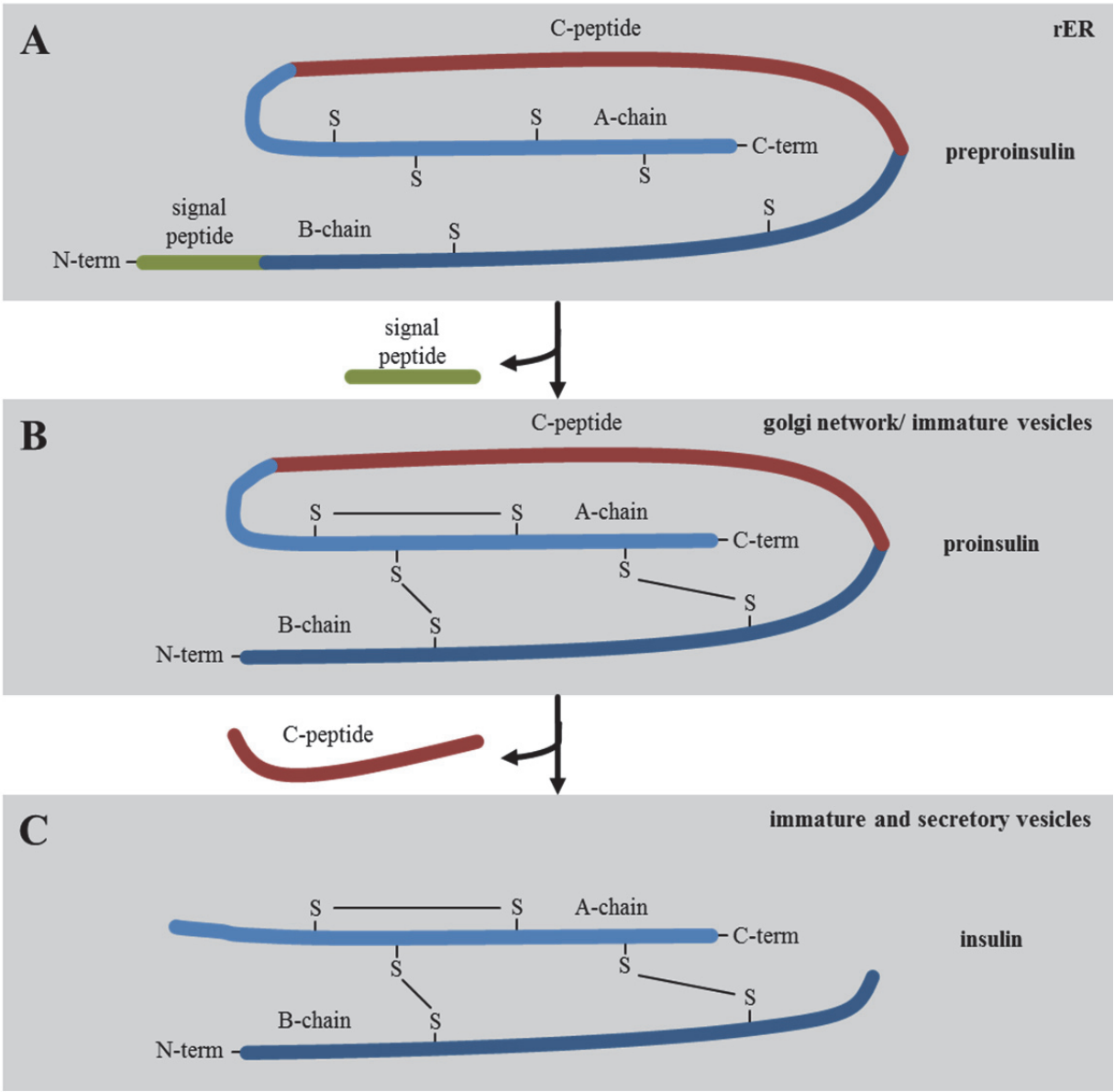


Figure 1: Schematic overview of insulin maturation.
Insulin is translated as two precursor molecules, **(A)** preproinsulin and **(B)** proinsulin. **(A)** Preproinsulin is translated into the rER via the signal peptide (green) which is cleaved off inside rERs lumen. **(B)** Proinsulin is transported via the golgi network and stored into immature, secretory vesicles. In these vesicles the C-peptide (red) is cleaved off. **(C)** The mature insulin consisting of an A-chain (light blue) and a B-chain (dark blue) is stored in secretory vesicles until exocytosis. rER = rough endoplasmatic reticulum, N-term = N-terminus, C-term = C-terminus

Preproinsulin is synthesized on the membrane of the rough endoplasmatic reticulum (rER) as a single-chained molecule consisting of insulins A- and B-chain, the characteristic C-peptide of proinsulin and a 24 AA signal peptide on the N-terminus of the molecule. The signal peptide facilitates penetration of the nascent preproinsulin molecule into rERs lumen. After transition into the lumen the signal peptide is cleaved and preproinsulin thereby converted to proinsulin (Fig. 1A-B). The newly formed proinsulin is then transported from the rER to the *cis*-golgi network by an ATP-dependent pathway [96]. Subsequently, proinsulin is transported stepwise to the *trans*-golgi network where it is stored in immature clathrin-coated secretory vesicles [97, 98]. Conversion of proinsulin to insulin is facilitated in these immature clathrin-coated vesicles [99-101], where the C-peptide is cleaved off (Fig. 1B-C) by proprotein convertase 1 and 2 (PC1/PC2) and carboxypeptidase E (CPE) under acidic conditions [102].

In the maturing exocytotic vesicles acidic pH-values are found together with high concentrations of insulin and Zn^{2+}-ions [103] which leads to formation of densely packed insulin hexamers [104, 105]. These vesicles are then stored in the β-cell until stimulating agents lead to exocytosis or until these vesicles are degraded by crinophagy.

1.2.2 Signalling and Function

Once released into the blood-stream the hexamers dissolve first to dimers and subsequently to the active, monomeric form of insulin. Major target-organs affected by insulin are skeletal muscle, liver and adipose tissue. Additionally, insulin affects other organ systems as well (e.g. heart and brain). In general, insulin action leads to glucose uptake into the tissue, stimulation of glycogen and fatty acid synthesis and furthermore to inhibition of gluconeogenesis and lipolysis [106]. Moreover, insulin affects a variety of processes in other organs, such as contractility and beating-rate of the heart [107, 108]. These processes are regulated by a complex signalling network (for schematic overview see Fig. 2).

In the first step insulin needs to bind to the insulin receptor (IR). The IR is a member of the tyrosine kinase receptor family and consists of two heterodimers which are linked via two disulphide bridges. Each heterodimer contains an α- and a β-subunit, whereas the α-subunits are located extracellular, the β-subunit has three domains: the extracellular domain, the transmembrane domain and the cytosolic domain [109]. Insulin binds to the α-subunit leading to conformational changes of the IR and thereby activation of the tyrosine kinase. This leads to auto-phosphorylation of the cytosolic domain of the β-subunit (e.g. auto-phosphorylation

of Tyr^{972}, $Tyr^{1146,}$ Tyr^{1150} and Tyr^{1151}). Activation of the phosphorylation sites leads to downstream phosphorylation of various substrates, such as insulin receptor substrates (IRS), Cbl and Shc isoforms [106]. The insulin signalling pathway is split into two major arms; the metabolic posphoinositide 3-kinase (PI3K) pathway and the mitogenic mitogen-activated protein kinase (MAPK) pathway. In the metabolic pathway, PI3K is recruited by IRS proteins via the regulatory subunit p85 and catalyses phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP_3) via its catalytic subunit p110. PIP_3 in turn facilitates recruitment of protein kinase B (PKB), also known as Akt, and phosphoinositide-dependent protein kinase-1 (PDK1) to the plasma membrane. Due to the close proximity of PDK1 and Akt, the latter is phosphorylated at the Thr^{308} residue [110] and is subsequently phosphorylated at the Ser^{473} residue via the RICTOR-mTOR complex [111]. Full activation of Akt regulates a plethora of downstream targets which lead to the aforementioned effects (e.g. glucose uptake, glycogen synthesis, fatty acid synthesis) as well as promoting anti-apoptotic effects, protein synthesis and differential gene expression among other processes [110].

In the mitogenic pathway, Shc and IRS-1 recruit members from the growth factor receptor-bound (GRB) protein family [112, 113]. The GRB proteins lead to activation of different proteins in the Ras-Raf-MAPK pathway, ultimately leading to activation and translocation of Erk 1/2 into the nucleus and thereby promoting cellular growth [112]. However, the growth promoting effect of insulin at physiological concentrations is much less compared to other growth factors, such as IGF-1 or EGF.

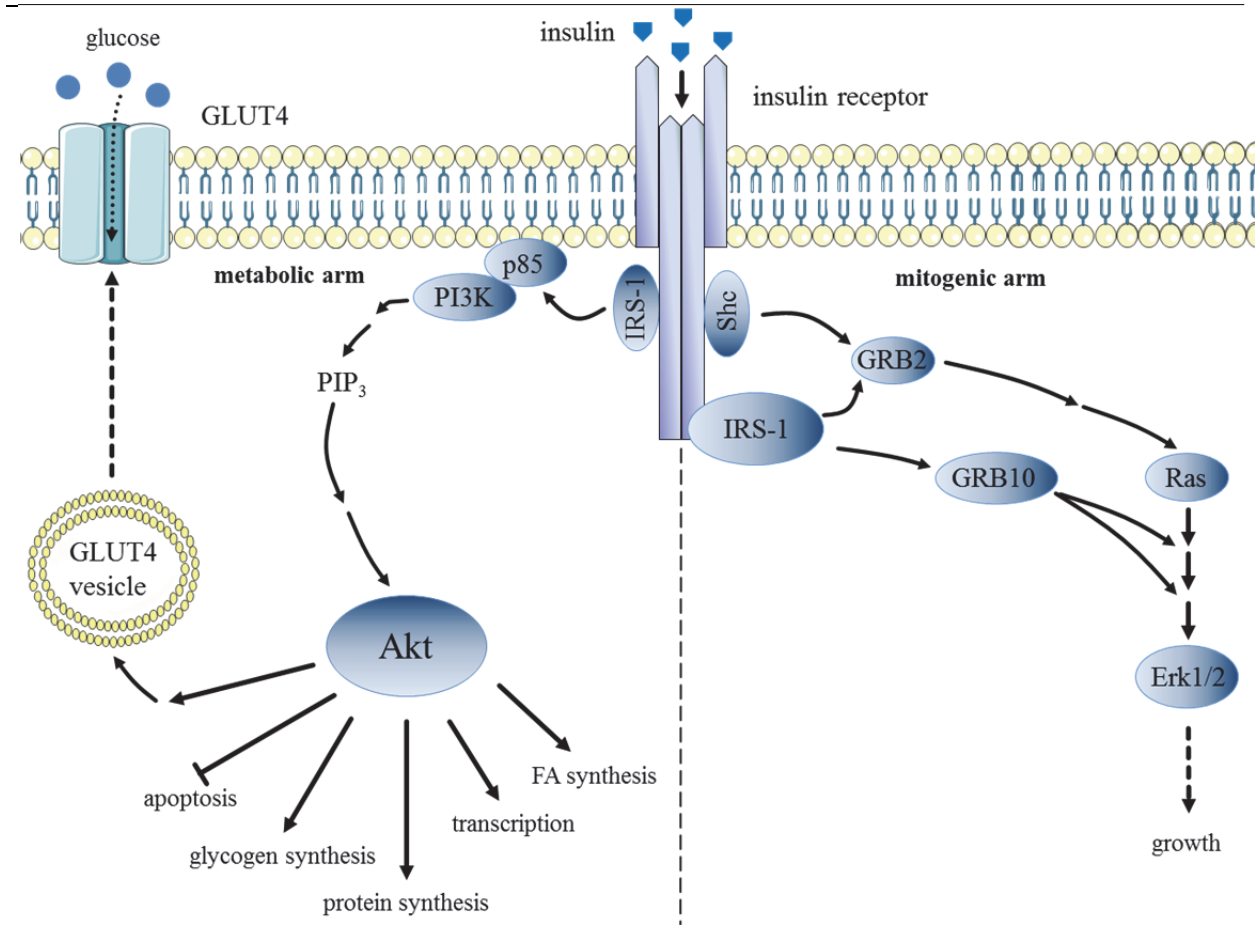


Figure 2: Schematic overview of crucial steps in insulin signalling.
After binding of insulin to the insulin receptor, the signalling cascade leads to various physiological effects. In the metabolic arm (left side) receptor autophosphorylation leads to activation of IRS proteins, which in turn activates PI3K via the regulatory subunit p85. PI3K leads to activation of Akt via the second messenger PIP_3. Akt in turn triggers several processes, most importantly GLUT4 vesicle translocation to the cell membrane, leading to glucose uptake into the cells. In the mitogenic arm (right side) insulin receptor autophosphorylation activates Shc proteins and IRS-1, leading to activation of proteins from the GRB family. The activation of GRBs leads to subsequent activation of the mitogen activated protein kinase pathway (e.g. Ras and Erk1/2) initiating cell growth. Adapted from [114].

1.3 Insulin Analogues

Postprandial serum insulin concentrations rise and reach a peak after 30-45 min and subsequently the concentrations decline and are back to basal level after 2 – 3 h. Ideally subcutaneous (s.c.) administered insulin or insulin analogues would mimic this time-action-profile.

Early modifications of insulin did not meet these requirements. The first slow-release insulin was developed in 1936 using animal insulin together with protamine, a protein isolated from fish sperm, called neutral protamine Hagedorn (NPH) insulin [115]. Protamine reduces solubility of insulin and leads to small insulin crystal depots at the injection site [115, 116].

However, the depots vary in size and therefore the absorption kinetics varies tremendously [117]. The inter- and intra-patient variability is further increased due to failure in resuspension of the NPH insulin solution prior to administration [118, 119]. Onset of action of NPH insulins are between 2-4 h with a peak in serum levels at 4-10 h and maximum duration of up to 18 h [120]. Thus it requires administration twice daily and pharmacokinetic (PK) and pharmacodynamic (PD) characteristics can lead to hypoglycaemia, which is a common issue for NPH recipients [121].

In 1978 Genentech developed the first synthetic human insulin using *Escherichia coli* [122]. This insulin was used to develop regular human insulin (RHI) and human NPH insulin in 1982 by the Eli Lilly laboratories. Even though RHI is not further modified, s.c. injection does not mimic physiological pattern since it shows slower peak serum level and prolonged elevated serum insulin level compared to healthy individuals [123]. The onset of action is seen after 30-60 min, peak serum concentrations are reached between 2-6 h and the duration of action is up to 8 h [120]. Consequently, this leads to postprandial hyperglycaemia and subsequent increased risk of hypoglycaemia. Postprandial hyperglycaemia can only be avoided, if RHI is injected around 60 min prior to the meal leading to low patient compliance [124].

These problems could not be overcome until the development of the first “real” insulin analogues, defined as modifications introduced into the primary structure of the native human insulin molecule, which emerged in the 1990s. Subsequently, various modified insulin analogues were tested in laboratories, which led to the discovery of several AA important for IR-binding. These AA are: GlyA1, IleA2, ValA3, TyrA19, LeuB6, ValB12, GlyB23 and PheB24 [125]. Researchers were then able to modify the insulin in a way that those insulins showed either rapid onset of action or extended duration of action, leading to the two categories of insulin analogues: rapid-acting insulin analogues or prandial insulin analogues and long-acting insulin analogues or basal insulin analogues.

1.3.1 Rapid-Acting Insulin Analogues

The general design principle of rapid-acting insulin analogues is accelerating the onset of action by faster absorption due to minimising dimer- and hexamer-formation. For dimerization the region B26-B30 of the insulin molecule is important [126]. Therefore, changes in this region were introduced to design rapid-acting insulin analogues.

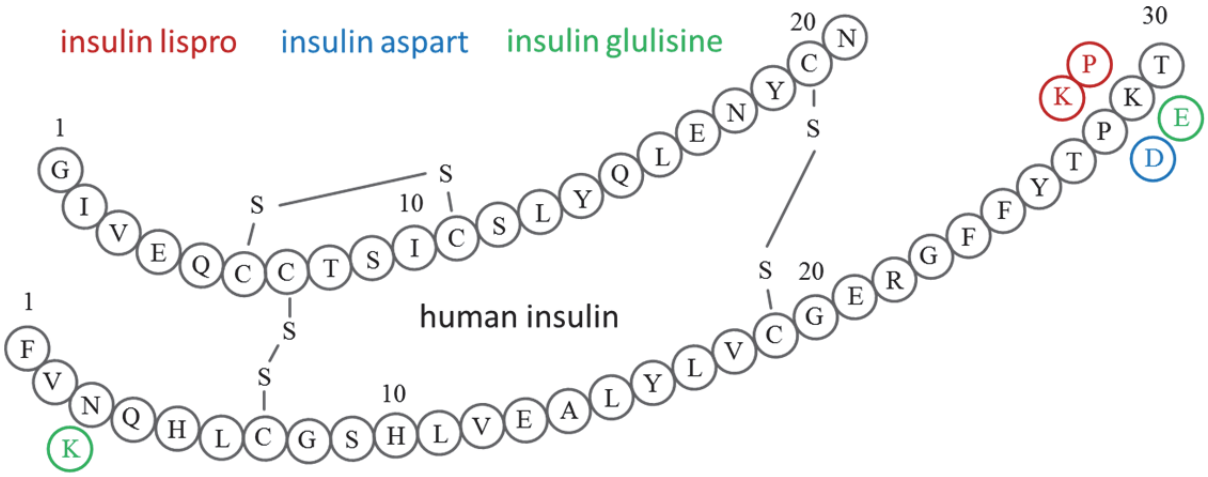


Figure 3: Modifications of rapid acting insulin analogues compared to human insulin.
Insulin lispro (red) shows a reversal of the amino acids proline and lysine at position B28 and B29. Insulin aspart (blue) single substitution is aspartic acid instead of proline at position B28. Insulin glulisine (green) is modified on position B3, where asparagine is substituted by lysine, and position B29, where lysine is substituted by glutamic acid. These changes lead to faster onset of action observed in all three analogues. Adapted from [127].

Insulin lispro (Eli Lilly)

Insulin lispro was the first rapid-acting insulin analogue available and was approved in 1996. In insulin lispro the primary structure shows a reversal of the AA at position B28 and B29 (ProB28,LysB29 -> LysB28,ProB29), naming the molecule insulin lispro (Fig. 3). This modification causes decreased dimer formation and thereby leading to the desired effects of faster absorption, higher peak serum level and a shorter duration of action compared to regular human insulin [128]. Onset of action starts within 15 min, peak serum levels are reached around 1h after injection and duration of action is 2-4 h [128, 129]. Importantly, the affinity for IR is similar compared to human insulin. It is noteworthy, that the insulin-like growthfactor-1 receptor (IGF-1R) affinity is slightly increased, but lispro does not show increased mitogenicity [130]. The rapid onset and high peak serum level are leading to improved postprandial glucose level and lower rates of hypoglycaemic events between two meals compared to RHI [131-133].

Insulin aspart (Novo Nordisk)

Insulin aspart was approved in 2000 for clinical use, and the second rapid-acting insulin analogue available. In insulin aspart ProB28 is substituted with the charged AA aspartic acid (AspB28) (Fig. 3), which leads to reduced self-dimerization [134]. IR and IGF-1R interaction kinetics are comparable to RHI [135] and IGF-1R receptor affinity is slightly lower [136]. Comparable metabolic efficacy to RHI after intravenous injection could be shown [137]. The PK/PD profile of insulin aspart is similar to insulin lispro [138-140] and thereby showing

non-inferiority of insulin aspart compared to insulin lispro. With the given properties the postprandial glucose level are lower with aspart [139], even when the analogue is injected directly before the meal compared to RHI [141]. Expectedly, lower hypoglycaemic events were observed between two meals with insulin aspart [142].

Insulin glulisine (Sanofi-Aventis)

Insulin glulisine was approved in 2004 for clinical use. Substitution of AsnB3 with LysB3 and LysB29 with GluB29 is introduced into the insulin molecule (Fig. 3). The substitution at position B29 weakens dimerization of insulin, but does not fully prevent it [143] and thereby desensitizing glulisine to denaturation after injection [144]. Insulin glulisine is the only rapid-acting insulin analogue with a zinc-free vehicle, preventing hexameric aggregation of the molecule. Furthermore, the isoelectric point is lowered to pH 5.1, increasing solubility at injection site [145], leading to slightly improved PK/PD profiles compared to insulin lispro [145]. It shows the ability to achieve good postprandial glycaemic control even when it is injected directly prior or 15 min after the meal [146]. Safety and efficacy could be shown in clinical trials with T1DM and T2DM patients [147, 148].

1.3.2 Long-Acting Insulin Analogues

In contrast to rapid-acting insulin analogues, the design principle for long-acting insulin analogues is delaying release and absorption of monomeric insulin molecules and ideally increasing circulatory half-life. Currently, three different approaches are used to achieve slow absorption and increased half-life. These approaches are: shifting the isoelectric point towards a neutral pH leading to precipitation of the drug at injection site (insulin glargine (IGla)), fatty acid-acylation of insulin enabling binding to human serum albumin (HSA) and thereby increasing the half-life (insulin detemir (IDet) and insulin degludec (IDeg)), or increasing hydrodynamic size of the molecule to hamper absorption and excretion (basalinsulin PEGlispro (BIL)).

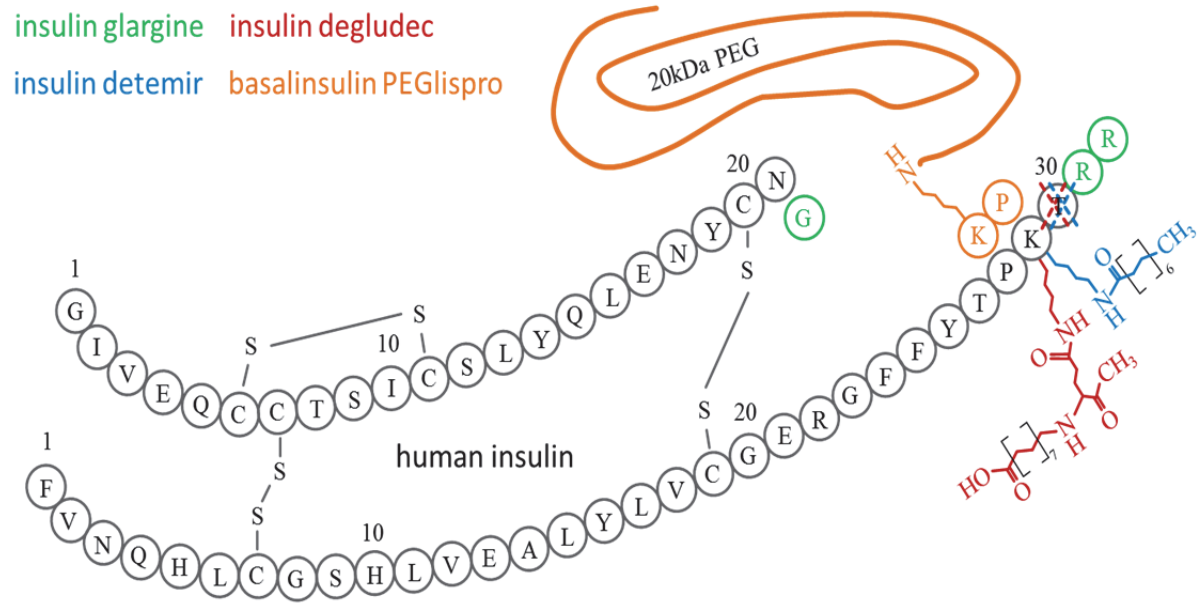


Figure 4: Modifications of long-acting insulin analogues compared to human insulin.
Insulin glargine (green) shows a substitution of the amino acids asparagine with glycine at position A21 and addition of two arginine residues at position B31 and B32. Insulin degludec (red) has a deletion of threonine at position B30 (red X) and a C16 fatty acid attached via a glutamic acid spacer to lysine at position B29. Insulin detemir (blue) has the same deletion as insulin degludec (blue X) with a C14 fatty acid attached to lysine at position B29. Basalinsulin PEGlispro (orange) shows the reversal of proline and lysine at position B28 and B29 as seen in insulin lispro, with a 20-kDa polyethylene glycol residue attached to lysine at position B28. These changes lead to different modes of action, however all modifications lead to the desired long duration of action in all shown analogues. PEG = polyethylene glycol. Adapted from [127].

Insulin glargine (Sanofi-Aventis)

IGla was first approved in 2000 for clinical use in type 1 and type 2 diabetics and the first available long-acting insulin analogue. In IGla the B-chain is elongated by two arginine residues and asparagine at position A21 is substituted with glycine (GlyA21,ArgB31,ArgB32 human insulin) (Fig. 4). The modifications lead to a shift in the isoelectric point from pH 5.4 to nearly neutral pH values (6.7), leading to subsequent precipitation after s.c. injection and the formation of a depot with slow dissociation of monomeric insulin molecules [149]. Compared to regular insulin (Ins) there are no differences in respect of IR binding, IR autophosphorylation and signalling in muscle cells [150]. The duration of action is up to 24 h after single s.c. injection without showing pronounced peak serum levels, coming closer to mimicking basal insulin secretion compared to NPH insulin [149, 151]. However, in some patients twice-daily dosing is needed to achieve sufficient glycaemic control [152]. Better glycaemic control with less frequent hypoglycaemia could be shown in large, multicentre clinical trials compared to NPH insulin [121, 153-155]. Interestingly, up-concentration of IGla from 100 U/ml to 300 U/ml extended the duration of action up to 32 h [156] and

significantly reduced hypoglycaemic events [157], most likely due to increased hexamer formation and a bigger depot resulting in a decreased surface with slower absorption.

Insulin detemir (Novo Nordisk)

IDet was first approved in 2004 and was the first insulin analogue with fatty acid-acylation. The native AA structure is altered by deletion of ThrB30 and addition of a C14 myristic fatty acid residue to LysB29 [158] (Fig. 4). The fatty acid residue leads to self-association as dihexamers after injection and reversible binding to serum albumin at injection site. Thereby delaying absorption and build-up of a floating depot in the circulation resulting in extended serum half-life [159]. Interestingly, IDet seems not to interfere with other albumin-binding drugs [160]. Furthermore, it could be shown that the delayed absorption leads to a flat peak-to-trough ratio [161, 162]. It shows slightly shorter mean duration of action in T1DM (21.5h) compared to IGla [161], but no difference between both in T2DM with residual endogenous insulin secretion [163]. However, the PREDICTIVE trial showed that many patients require twice-daily dosing [164]. It was shown that IDet exhibits less intrapatient variability compared to NPH insulin and IGla [165], which is favourable since this leads to more reliable results in HbA1c reduction and hyper- and hypoglycaemia. The safety and efficacy of IDet was demonstrated in several large clinical studies [166, 167] with less frequent hypoglycaemic events compared to NPH insulin for both T1DM and T2DM [168, 169]. *In vitro* receptor binding studies (IR/IGF-1R), metabolic and mitogenic potency are lower with IDet compared to human insulin and IGla [136].

Insulin degludec (Novo Nordisk)

IDeg was first approved in 2012 in Japan, followed by approval in 2013 in the EU and 2015 in the US. The molecule is altered by deletion of ThrB30 and acetylation of a C16 fatty diacid via a glutamic spacer (Fig. 4). These modifications enable formation of high-molecular-weight fibrils, as well as albumin-binding. IDeg forms stable dihexameric structures in the presence of zinc and phenol and upon diffusion of phenol it will form stable multihexameric fibrils [170, 171] and subsequent, gradual diffusion of zinc at the terminal hexamers leads to destabilisation and dissociation of dimers, which then dissociate to the monomeric form [170]. IR affinity is around 5-fold lower compared to human insulin and reduced mitogenic potential compared to Ins was observed [172]. In glucose clamp studies with T1DM patients the duration of action was shown to be at least 42 h at steady-state-conditions [173] and variation of glucose lowering effect was 4 times lower vs. IGla with more even distribution over 24 h [174]. Safety and efficacy was shown in several trials for

T1DM and T2DM patients, with a lower frequency of hypoglycaemic events compared to IGla [175]. Thus IDeg is the first "real" once-daily insulin with the additional benefit of flexible injection intervals in both, T1DM and T2DM [176, 177].

Basalinsulin PEGlispro (Eli Lilly)

For protracted absorption of BIL the rapid-acting insulin analogue lispro is covalently linked with a 20-kDa polyethylene glycol (PEG) chain at LysB28 (Fig. 4), thus increasing the hydrodynamic size. BIL shows reduced IR binding affinity, with slower association and faster dissociation rate and decreased mitogenic potency compared to human insulin and AspB10, respectively [178, 179]. The half-life in T2DM patients was shown to be 2-3 days and furthermore BIL shows a flat PK profile [180]. Interestingly, BIL demonstrates relative hepatopreferential action and thereby mimicking physiological secretion better than other basal analogues [181], since the secretion of insulin into the portal vein leads to 2-3 fold higher insulin concentrations in the liver compared to the periphery. Additionally, trials in T1DM and T2DM patients showed lower nocturnal hypoglycaemic episodes in patients receiving either BIL vs IGla, but more overall hypoglycaemia [182, 183].

1.3.3 Undesired Side-Effects of Insulin Modification

Besides the benefits of insulin modification, these modifications could result in unwanted or even harmful side effects. Since IR and IGF-1R display more than 50 % sequence homology and the ability to form IR/IGF-1R heterodimers [184], changes introduced into the AA sequence of insulin could increase affinity for the mitogenic IGF-1R. Alterations in B10 and B26-30 region for example could be shown to alter the affinity towards the IGF-1R [185]. The prime example for unwanted side-effects of insulin analogues is the analogue AspB10 insulin. However, potential unwanted side-effects are also shown in different studies for IGla, IDeg and BIL.

AspB10 Insulin – The Super-Mitogenic Insulin

AspB10 insulin was the first analogue proposed for clinical use as rapid-acting insulin analogue [186]. Studies demonstrated twice as fast absorption compared to RHI [187]. Increased affinity for IR and IGF-1R, decreased dissociation from IR and prolonged cellular processing [188-190] were observed for this analogue which consequently leads to enhanced metabolic effects. However, the mitogenic potency of AspB10 insulin is largely increased as well [150]. In studies with a rat breast cancer model increased cancerogenicity was observed, leading to discontinuation of the development for clinical use.

Insulin glargine – Increased Mitogenic Potency?

Epidemiological studies have shown an association between the use of IGla and increased cancer risk [191-193]; however, definite conclusion of a possible causal relationship could not be drawn from these studies. Nonetheless, those observations seem to be underlined by several *in vitro* studies, showing that the addition of arginine at position B31 and B32 enhance binding affinity of insulin towards the IGF-1R. It could be shown that IGla exhibits increased affinity for the IGF-1R and shows an increased mitogenic potency *in vitro* [136]. Furthermore, correlation of increased mitogenic potency and IGF-1R and/or IRS-1 level in human fibroblasts and smooth muscle cells [194] and increased proliferation in the MCF-7 breast cancer cell line, with high IGF-1R level was demonstrated [195]. However, other *in vitro* studies did not confirm these results. The varying results could be obtained due to study design with different cell lines [196].

Further evidence against increased cancer risk by using IGla was the discovery of rapid metabolisation of IGla to the so called insulin glargine metabolite 1 (IGlaM1) after injection. In IGlaM1 both arginine residues at position B31 and B32 are cleaved off from the molecule [197]. Subsequently, it was shown that IGlaM1 exhibits a mitogenic potency similar to that of human insulin [198]. Additionally, the importance of IGF-1R signalling with IGla treatment was challenged by *in vivo* experiments showing no difference between IGla and Ins [199] and furthermore with a non-metabolisable IGla analogue no IGF-1R signalling was detectable *in vivo* [200, 201]. Finally the long term trial ORIGINE and the follow up trial (ORIGINALE) did not show a difference between treatment with IGla and incidence of cancer in humans [202, 203]. Thus IGla seems to be safe to use in regard to cancerogenicity in humans.

Insulin degludec – Negative Cardiovascular Side Effects?

Due to tissue specific insulin signalling [204] it is possible for insulin analogues to exhibit a differential effect compared to human insulin, even though they do not show different receptor interactions (IR/IGF-1R). In 2013 the American Food and Drug Administration (FDA) requested a dedicated cardiovascular outcome trial for IDeg (DEVOTE trial) [205] since their re-analysis of data from several phase III clinical studies (non-dedicated cardiovascular (CV) outcome studies) conducted with IDeg showed an increased CV risk and increased major adverse cardiac events and unstable angina pectoris (MACE+). In this analysis the FDA found consistent trends towards harm with IDeg treatment in MACE+. Novo Nordisk subsequently conducted the DEVOTE trial and in 2015 the FDA conditionally

approved IDeg since the interim-analysis of the DEVOTE trial showed non-inferiority to IGla in respect to CV risk and thus ruling out potential harm for patients treated with IDeg.

Basalinsulin PEGlispro – Hepatopreferential Effect Harming the Liver?

The desired hepatopreferential effect of BIL seems to have a negative impact on different liver markers. In the IMAGINE trials significantly increased triglycerides, aminotransferase level and liver fat content were measured [182, 183, 206]. Even though the underlying mechanism is not yet elucidated, these results were leading to the discontinuation of development of BIL by Eli Lilly.

1.4 Future Developments of Insulin Analogues

Even though tremendous improvements in the last two decades in the treatment of diabetes have been made, the need to improve and refine therapy options is still relevant. Various approaches to improve PK/PD profiles and administration routes are currently under investigation.

1.4.1 Ultra-Rapid Insulin Analogues

Current therapeutics show improved PK/PD profiles compared to traditional treatment regiments. However, available rapid-acting insulin analogues do not fully mimic physiological response to rise in glucose level (delayed time-to-peak, longer duration of action) and ideally are needed to be injected a certain amount of time prior to the meal. The development of ultra-rapid insulin analogues with faster time-to-peak profiles and a shorter duration would be beneficial in terms of mimicking physiological insulin response.

Current approaches are mainly focused on refining application methods and vehicles instead of developing novel analogues. Examples are the increase of blood flow by mild heating of injection site, which showed more rapid absorption of insulin after s.c. injection [207]. Intradermal injection of insulin lispro with microneedles is another approach with an alternative route of application [208]. The same is true for inhalation of Technosphere® insulin, which is applied by the Technosphere® Insulin Inhalation System [209]. The company Biodel is using novel excipients in the injection solution to increase monomeric insulin in the injection solution [210, 211]. Novo Nordisk also uses two novel excipients in the injection solution to increase stability of monomers and faster uptake (nicotinamide and arginine) [212]. The BioChaperone technology is used to enhance insulin blood penetration by increasing solubility and stabilising the insulin monomer [213].

Interestingly, in 2015 an article was published by Safavi-Hemami et al [214] presenting minimized insulin (Con-Ins G1) used as a venom component by Cone snails. Even though Con-Ins G1 lacks essential AA for binding to the IR receptor it shows rapid induction of hypoglycaemia in the Cone snails' prey [214]. These findings could potentially lead to development of ultra-rapid insulin analogues [215].

1.4.2 Ultra-Long Insulin Analogues

To achieve a more convenient therapy for the patients it would be preferable to have insulin analogues with extended PK and very flat PD profiles. There are several approaches to achieve extended duration of action in development at the moment.

PE0139 insulin (PhaseBio) has recently completed clinical phase I (NCTC02148861) and recruitment for a phase IIa clinical study is currently ongoing (NCT02581657). The human insulin structure is modified by fusing the ELP1-120 biopolymer developed by PhaseBio to the A-chain at position 21 [216]. PE0139 insulin shows potential for weekly administration and low peak-to-trough ratio was confirmed in the phase I clinical trial [217].

AB101 insulin (AntriaBio) is currently in the preclinical phase. In AB101 insulin a small PEG-residue is attached to human insulin, which is encapsulated in biodegradable poly-lactic, poly-glycolic (PLGA) microspheres leading to long duration of action and low peak-to-trough ratio [218].

The long acting insulin 287 (LAI287) (Novo Nordisk) completed two phase I clinical trials in T1DM (NCT01730014) and in T2DM (NCT02148861) and healthy volunteers. However, properties of LAI287 are undisclosed and no further data regarding the structure are published by Novo Nordisk.

HM12470 (Hanmi Pharmaceuticals) is currently recruiting patients for phase I clinical study (NCT02302443). For HM12470 the Long Acting Protein/Peptide Discovery Platform Technology (LAPSCOVERY) is used to link a non-glycosylated Fc-fragment via a short PEG-linker to the modified Insulin 115, resulting in a 56 kDa insulin molecule. Preclinical studies have shown promising results with a long duration of action and favourable PK/PD profiles [219, 220].

1.4.3 Future Developments in Insulin Therapy

Besides the development of even faster and longer-acting insulin analogues, researchers are investigating approaches to develop so called smart insulins which should be able to prevent hyperglycaemia without risk of inducing hypoglycaemia.

Researcher from the Massachusetts Institute of Technology (MIT) demonstrated a technique which mimics insulin response as seen in healthy individuals after glucose challenge. Therefore, the researchers linked different phenylboronic acid (PBA) moieties via an aliphatic, albumin binding linker to lysine at position B29 [221]. In this approach PBA is used as a glucose sensor, enabling activation of the bound insulin after binding of glucose to PBA [221].

Another interesting approach is the use of microneedle-array patches which are loaded with hypoxia-sensitive vesicles [222]. These vesicles are filled with insulin and the glucose oxidase (GOx) enzyme. GOx is thereby used as the glucose sensor, leading to oxidation of glucose and thereby generating hypoxic conditions. Subsequently, hypoxia triggers dissociation of the vesicles and thereby releasing the stored insulin. Experiments in a mouse model of T1DM could show fast and safe release of insulin upon rising glucose levels [222].

1.5 Objectives

The tremendous progress of insulin therapy for treatment of diabetes and potential harmful consequences of insulin modification draws attention of researchers to analyse the effects of those modified insulins.

Potential harm of IDeg in regard of CV risk together with tissue specific action of insulin sparked the interest of our workgroup to elucidate potential differences in the signalling and function of IDeg compared to other insulins in different cardiac cell models.

Therefore, the first objective was to compare IDeg, Ins and IGla as well as its active metabolite IGlaM1 in regard to contractility parameters in adult rat ventricular myocytes (ARVM), glucose uptake in HL-1 cells, effect on H_2O_2 induced apoptosis in H9c2-E2 cells and beating-rate in human Cor.4U® cells. Furthermore, potential differences in crucial signalling steps of the insulin signalling pathway were evaluated in ARVM and HL-1 cells.

The second objective of the thesis was the comprehensive and extensive evaluation of the potential once-weekly ultra-long acting insulin analogue HM12470. Therefore, extensive

in vitro and *in vivo* studies were conducted. The ability to induce crucial steps in insulin signalling (e.g. autophosphorylation of the IR, subsequent dissociation, Akt activation, glucose uptake) was assessed. Additionally, potential mitogenic effects were evaluated. Furthermore, potential organ specificity in rats and endothelial passage in human coronary artery endothelial cells were analysed.

2 Published articles

2.1 Study 1: Effect of the long-acting insulin analogues glargine and degludec on cardiomyocyte cell signalling and function

Hartmann T, Overhagen S, Ouwens DM, Raschke S, Wohlfart P, Tennagels N, Wronkowitz N, Eckel J. Cardiovasc. Diabetol. (2016) **15**:96

Effect of the long-acting insulin analogues glargine and degludec on cardiomyocyte cell signalling and function

Thorsten Hartmann [1], Sabrina Overhagen [2], D. Margriet Ouwens [2,3,4], Silja Raschke [1], Paulus Wohlfart [5], Norbert Tennagels [5], Nina Wronkowitz [1], Jürgen Eckel [1*]

[1]Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, 40225 Düsseldorf, Germany

[2]Institute for Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, 40225 Düsseldorf, Germany

[3]German Center for Diabetes Research (DZD), 85764 München-Neuherberg, Germany

[4]Department of Endocrinology, Ghent University Hospital, 9000 Ghent, Belgium

[5]R&D Diabetes Division, Sanofi-Aventis Deutschland GmbH, 65929 Frankfurt, Germany

[*]corresponding author

Address for correspondence:

Prof. Dr. Jürgen Eckel

German Diabetes Center

Paul-Langerhans-Group for Integrative Physiology

Auf'm Hennekamp 65,

D-40225 Düsseldorf, Germany

Tel: +49 211 3382561, Fax: +49 211 3382697

Email: eckel@uni-duesseldorf.de

Keywords: insulin glargine, insulin degludec, cardiac action, insulin analogues

Abstract

Background: The effects of insulin on cardiomyocytes, such as positive inotropic action and glucose uptake are well described. However, in vitro studies comparing long-acting insulin analogues with regard to cardiomyocyte signalling and function have not been systematically conducted.

Methods: Insulin receptor binding was assessed using membrane embedded and solubilised insulin receptor preparations. Insulin signalling was analysed in adult rat ventricular myocytes (ARVM) and HL-1 cardiac cells. Inotropic effects were examined in ARVM and the contribution of Akt to this effect was assessed by specific inhibition with triciribine. Furthermore, beating-rate in Cor.4U® human cardiomyocytes, glucose uptake in HL-1 cells, and prevention from H_2O_2 induced caspase 3/7 activation in cardiac cells overexpressing the human insulin receptor (H9c2-E2) were analysed. One-way ANOVA was performed to determine significance between conditions.

Results: Insulin degludec showed significant lower insulin receptor affinity in membrane embedded insulin receptor preparations. In HL-1 cardiomyocytes, stimulation with insulin degludec resulted in a lower Akt(Ser^{473}) and Akt(Thr^{308}) phosphorylation compared to insulin, insulin glargine and its active metabolite M1 after 5- and 10-min incubation. After 60-min treatment, phosphorylation of Akt was comparable for all insulin analogues. Stimulation of glucose uptake in HL-1 cells was increased by 40-60 %, with a similar result for all analogues. Incubation of electrically paced ARVM resulted for all insulins in a significantly increased sarcomere shortening, contractility- and relaxation velocity. This positive inotropic effect of all insulins was Akt dependent. Additionally, in Cor.4U® cardiomyocytes a 10-20% increased beating-rate was detected for all insulins, with slower onset of action in cells treated with insulin degludec. H9c2-E2 cells challenged with H_2O_2 showed a 5-fold increase in caspase 3/7 activation, which could be abrogated by all insulins used.

Conclusions: In conclusion, we compared for the first time the signalling and functional impact of the long-acting insulin analogues insulin glargine and insulin degludec in cardiomyocyte cell models. We demonstrated similar efficacy under steady-state conditions relative to regular insulin in functional endpoint experiments. However, it remains to be shown how these results translate to the in vivo situation

Introduction

In addition to skeletal muscle, liver and adipose tissue, the heart with 10,000 – 100,000 expressed insulin receptors per cardiomyocyte [1] must be considered as an additional major organ affected by insulin (Ins). In cardiomyocytes, Ins modulates glucose transport, metabolism, protein synthesis, hypertrophy, contractility, beating-rate, and apoptosis [1-3].

Long-acting insulin analogues are designed to deliver a constant basal insulin supply throughout the day via the subcutaneous route resulting in improved fasting blood glucose and overall glycaemic control while reducing the risk of hypoglycaemia [4-6]. Insulin glargine (IGla) has one amino acid exchange in the A-chain and two additional arginine residues at the B-chain, resulting in an altered isoelectric point that leads to local precipitation after subcutaneous injection [7, 8]. From this depot, IGla is slowly dissolved followed by immediate biotransformation into the active metabolite M1 (IGlaM1) [9, 10]. For insulin degludec (IDeg) it has been proposed that its protracted action results from slow dissolution of subcutaneous multi-hexamer assemblies [11]. However

the structure of the side chain attached to this molecule (n-16 fatty acid) [11] suggests that the protraction mode may be similar to that of insulin detemir, which binds to human serum albumin (HSA) via its fatty acid side chain, thereby protracting its duration of action by providing a 'floating depot' with the consequence of a reduced biological availability [12-14].

Besides the benefits of insulin analogue modification, modifying the insulin molecule may lead to an altered activation profile such as receptor signalling or pharmacodynamics and pharmacokinetics. Recently the effects of insulin analogues on the cardiovascular system gained considerable interest. So far, finalised clinical data is only available for IGla, whereas for IDeg the investigation is still ongoing. Results from the Outcome Reduction With Initial Glargine Intervention (ORIGIN) trial proved non-inferiority of IGla compared to standard care treatment in regard to cardiovascular effects (Trial Number NCT00069784) [15]. Just recently the interim analysis of the dedicated cardiovascular outcome trial for IDeg (DEVOTE) (Trial Number NCT01959529), requested in 2013 by the American Food and Drug Administration (FDA) [16] suggested non-inferiority to IGla, leading to approval of IDeg for the US market with a post marketing commitment to provide further non-inferiority data in major adverse cardiovascular events. [17]. These clinical trials were designed to compare systemic metabolic effects of the analogues in patients. However, strong evidence for tissue-specific action of insulin exists [18] and to the best of our knowledge, there are no in vitro studies published which elucidate and compare the effect of different long-acting insulin analogues on cardiomyocyte cell models. Thus, data regarding the signalling and function of insulin analogues in cardiomyocyte cell models might shed light on potential differences of these drugs in relation to their cardiac action. Therefore, we compared the impact of IGla, IGlaM1, and IDeg to Ins in regard to signalling, contractility and anti-apoptotic potency in HL 1 cardiomyocytes, ARVM, H9c2-E2, and Cor.4U® cells. Our data show a very similar cardiomyocyte action profile for both IGla and IDeg, at least under steady-state conditions.

Material & Methods

Cell culture

The cardiac mouse cell line HL-1, a cell line derived from the AT-1 mouse atrial cardiomyocyte tumour lineage [19], was kindly provided by Dr. W.C. Claycomb (Louisiana State University, New Orleans, LA, USA). HL-1 cells were cultivated in Claycomb medium containing 10 % fetal calf serum (FCS), 100 µM norepinephrine and 4 mM L-glutamine (all from Sigma-Aldrich, Munich, Germany) on gelatine/fibronectin coated plates. H9c2 cells (ATCC CRL-1446), stably transfected with the human insulin receptor in our laboratory [20] (H9c2-E2) were cultivated in DMEM, low glucose containing 10 % FCS, 1% non-essential amino acids and 600 µg/ml G418 (all from Invitrogen, Carlsbad, CA, USA). Commercially available iPS-derived human cardiomyocytes (Cor.4U®) (Axiogenesis, Cologne, Germany) were cultured in Cor.4U® Complete Medium containing 10 % FCS on fibronectin coated 96-well E-Plate (Acea Biosciences, San Diego, CA, USA). The medium was changed twice daily. All cells were incubated at 37 °C with 5 % CO2 in a humidified incubator.

Competition binding experiments on membrane embedded and solubilised insulin receptor preparations

Isolation of insulin receptor (IR) embedded plasma membranes (M-IR) and competition binding experiments were performed as previously described [21].

Briefly, CHO-cells overexpressing the IR were collected and re-suspended in ice-cold 2.25 STM buffer (2.25 M sucrose, 5 mM Tris HCl pH 7.4, 5 mM $MgCl_2$, Complete Protease Inhibitor) and disrupted using a Dounce homogenizer followed by sonication. The homogenate was overlaid with 0.8 STM buffer (0.8 M sucrose, 5 mM Tris HCl pH 7.4, 5 mM $MgCl_2$, Complete Protease Inhibitor) and ultra-centrifuged for 90 minutes at 100,000 g. Plasma membranes at the interface were collected and washed twice with phosphate buffered saline (PBS). The final pellet was re-suspended in dilution buffer (50 mM Tric-HCl pH 7.4, 5 mM $MgCl_2$, Complete Protease Inhibitor) and again homogenised with a Dounce homogenizer. Competition binding experiments were performed in a binding buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% BSA, Complete Protease Inhibitor, adjusted to pH 7.8) in 96-well microplates. In each well 2 µg isolated membrane were incubated with 0.25 mg wheat germ agglutinin polyvinyltoluene polyethylenimine scintillation proximity assay (SPA) beads. Constant concentrations of [^{125}I]-labelled human insulin (100 pM) and various concentrations of respective unlabelled insulin (0.001 – 1000 nM) were added for 12 hours at room temperature (23 °C). The radioactivity was measured at equilibrium in a microplate scintillation counter (Wallac Microbeta, Freiburg, Germany).

Binding on a freshly solubilised IR preparation (S-IR) was performed as previously described [22] with some modifications. Aliquots of membranes were incubated at 4 °C for 30 min in a solubilisation buffer (20mM HEPES-NaOH, 100mM NaCl, 10mM $MgSO_4$, 1% (w/v) n-Dodecyl-ß-D-maltoside (Sigma-Aldrich, Munich, Germany), adjusted to pH 7.8 and Complete TM Protease Inhibitor cocktail). Thereafter, ultra-centrifugation was performed at 100,000 g for 30 mins and 4°C to remove non solubilised debris. Protein concentration in the supernatant was adjusted to 0.15 mg/ml with binding buffer (100mM HEPES-NaOH, 100mM NaCl, 10mM $MgSO_4$, 0.025% (v/v) Tween-20, adjusted to pH 7.8 and Complete TM Protease Inhibitor cocktail). To Streptavidin SPA beads (5 mg in 1000 ml binding buffer), 50 µl of an anti-IR alpha-antibody 83-7 (Abcam, Cambridge, UK) was added. After incubation for 30 min, SPA beads were once washed and finally re-suspended in 500 µl binding buffer. A solution of solubilised receptor (1 ml, 0.15mg/ml) was added and incubated for further 60 min, before washing and resuspension in 1.5 ml. Subsequently, 100 µl re-suspended IR-Antibody-SPA beads (containing 10 µg total protein) were mixed with 50 µl [^{125}I]-labelled insulin tracer (100 pM) and 50 µl non-radioactive insulin (0.001 – 1000 nM), incubated for 12 hours at room temperature (23 °C) under shaking, centrifuged for 2 min and measured in the scintillation counter (Wallac Microbeta, Freiburg, Germany).

Effect of insulin and insulin analogues on contractility of primary adult rat ventricular cardiomyocytes

Adult rat ventricular cardiomyocytes (ARVM) were isolated from wild-type Lewis rats (Lew/Crl) as previously described [23]. ARVM were cultivated three hours in Medium 199 with Hanks' Balanced Salts containing 5 mM Creatin, 2 mM Carnitine and 5 mM Taurine supplemented with 10 % FCS and 1 % Insulin/Transferrin/Selene on laminin coated dishes (ibidi GmbH, Martinsried, Germany). Subsequently, ARVM were cultivated over-night in DMEM/F12 containing 33 µM biotin and 17 µM pantothenate (Invitrogen, Carlsbad, CA, USA). Prior to measurement, ARVM were pre-incubated for 5 min with 100 nM of Ins (porcine insulin, Cat. No.: I5523, Sigma-Aldrich, Munich, Germany), IGla, IGlaM1 or IDeg (provided by Sanofi-Aventis, Frankfurt a.M., Germany) in modified Tyrodes solution: 125 mM NaCl; 1.2 mM KH_2PO_4; 2.6 mM KCl; 1.2 mM

$MgSO_4$*$7H_2O$; 1 mM $CaCl_2$*$2H_2O$; 10 mM Glucose; 10 mM HEPES; adjusted to pH = 7.4 prior to measurement. Furthermore, untreated ARVM or ARVM treated with 10 nM isoproterenol (Sigma-Aldrich, Munich, Germany) were immediately measured. ARVM were paced with bipolar pulses in a contractility and fluorescence system (IonOptix, Milton, MA, USA) at 15 V, 1 Hz, 0.5 ms, at 37 °C for up to 10 minutes and 10-14 contractions of at least 10 rod shaped ARVM per condition were recorded. Sarcomeric shortening, shortening rate and re-lengthening rate were calculated using the IonWizard software (IonOptix, Milton, MA, USA). To determine the role of Akt for the positive inotropic effect of insulin and the analogues, ARVM were pre-treated with 10 µM of the specific Akt inhibitor triciribine (Sigma-Aldrich, Munich, Germany) for 30 min in contraction buffer. Afterwards, ARVM were treated as described above. After 30 minute treatment with 10 µM triciribine, ARVM viability was assessed by incubating the cells with 0.1 % trypan blue in PBS for 5 minutes. Microscopic pictures were taken randomly with at least 10 pictures per condition. As a positive control 200 µM H_2O_2 was utilised. For each condition at least 400 cells were counted per experiment.

Immunoblotting

ARVM and HL-1 cells were treated as indicated and lysed in buffer containing 50 mM HEPES (pH 7.4) (PromoCell, Heidelberg, Germany), 1% Triton X-100 (Sigma-Aldrich, Munich, Germany), PhosSTOP and CompleteTM Protease Inhibitor cocktail (Roche, Basel, Switzerland). After incubation for 2 h at 4 °C, the suspension was centrifuged at 10,000 g for 15 min. 5 µg protein sample of the total cell lysate was separated by SDS/PAGE (10% gel) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% (w/v) non-fat dried skimmed milk powder and incubated overnight with anti-phospho Akt(Ser^{473}) antibody, anti-phospho Akt(Thr^{308}), anti-GAPDH antibody (all Cell Signalling Technology, Danvers, MA, USA) or anti-tubulin antibody (Abcam, Cambridge, UK). After washing, membranes were incubated with appropriate horseradish peroxidase-coupled secondary antibody and processed for enhanced chemiluminescence (ECL) detection using Immobilion horse radish peroxidase (HRP) substrate (Millipore, Darmstadt, Germany). Signals were visualised and evaluated on a VersaDoc 4000 MP Bio-Rad Laboratories work station and analysed by Quantity One analysis software (version 4.6.7) (both Bio-Rad Laboratories, Hercules, CA, USA).

Impedance measurement in human Cor.4U® cells

Cor.4U® cardiomyocytes were seeded at a density of 30,000 cells per well. From day 3 on the cells were cultured in Iscove's Basal Medium containing 1 % GlutaMAX supplement (both Life Technologies, Carlsbad, CA, USA) and 2 µg/ml Ciprobay (Bayer, Leverkusen, Germany). Treatment with 500 nM of the designated insulin or analogue or 100 nM isoproterenol was started at day 4 after seeding. Impedance of each well was measured with the RTCA Cardio xCELLigence Analyser [24] (Acea Biosciences, San Diego, CA, USA) during the whole experiment. Beating-rate and Cell index were analysed by RTCA Cardio Software (Acea Biosciences, San Diego, CA, USA).

Glucose uptake in HL-1 cardiomyocytes

HL-1 cells were seeded at a density of 400,000 cells per well in a 12-well plate. Glucose uptake was measured in serum-starved HL-1 cells, either kept untreated or exposed for 60 min to 200 nM insulin and

the analogues, respectively. Subsequently 0.12 mM deoxy D glucose (Sigma-Aldrich, Munich, Germany) with 0.055 mCi 2-deoxy-D-[^{14}C]glucose (PerkinElmer, Waltham, MA, USA) was added to the cells. After 10 min incubation the uptake was terminated by repeated washing with ice cold PBS. Afterwards, the cardiomyocytes were lysed with lysis buffer containing 1% SDS and 200 mM NaOH. Incorporated glucose was measured by scintillation counting of the cell lysates in a liquid scintillation counter (Beckman Coulter, Pasadena, CA, USA). Values were corrected for non-specific uptake as measured by incubation with L-[^{14}C]glucose (PerkinElmer, Waltham, MA, USA).

Caspase 3 activity Assay

To assess the anti-apoptotic effect of insulin and its analogues, H9c2-E2 cells were seeded in a density of 5,000 cells per well of a 96-well plate. The next morning cells were treated with 100 nM of the respective insulin either in the presence or absence of 800 µM of H_2O_2 for 2 h. Caspase 3/7 activity was then measured by the Caspase-Glo® 3/7 Assay System (Promega, Madison, Wisconsin, USA) as described in the manual. After 2 h incubation period caspase 3/7 activity was analysed by measuring the luminescence in an Infinite 200 plate reader (Tecan, Männedorf, Switzerland).

Statistical analysis

Results are expressed as mean values ± SEM of at least three independent experiments. For statistical analysis Graphpad Prism v5.00 (Graphpad Software, San Diego, CA, USA) was used. One-way ANOVA was performed to determine significance between conditions, with level of significance chosen at $p<0.05$. In case of IC_{50} determinations for binding, a non-parametric Kruskal-Wallis testing was performed, again with level of significance chosen at $p<0.05$.

Results

Insulin degludec shows lower binding affinity and slower onset of insulin signalling

First we compared the binding affinities of Ins, IGlaM1 and IDeg with a competitive binding assay using M-IR (Fig. 1A) and S-IR (Suppl. Fig. 1). The analysis revealed an IC_{50} value of 0.83 ± 0.07 nmol/L for Ins in M-IR binding assays (Table 1). The IC_{50} of IGlaM1 and IDeg were 1.8- and 23.6-fold higher compared to Ins, reflecting a substantial lower binding affinity for IDeg. However, in S-IR binding assays we observed higher binding affinities for all tested insulins. The IC_{50} value for Ins was 0.58 ± 0.10 nmol/L (Suppl. Table 1). Under these conditions binding of IDeg improved with a 5.4-fold higher IC_{50} compared to Ins. Next, to compare the activation of the insulin signalling cascade by Ins, IGla, IGlaM1 and IDeg, we assessed the Ser^{473} phosphorylation of Akt by Western blot analysis in HL-1 cardiomyocytes (Fig. 1B-D). The time-course revealed a significantly lower Akt(Ser473) phosphorylation level after 5 min and 10 min treatment with IDeg, compared to Ins and the other insulin analogues at 200 nM. After 60 min treatment IDeg showed a similar Akt(Ser^{473}) phosphorylation level compared to Ins and IGlaM1; however, it was still significantly lower compared to IGla (Fig. 1D). The same kinetic was observed for the phosphorylation of Akt(Thr^{308}) (Suppl. Fig. 2).

Positive inotropic effect of insulin and long-acting insulin analogues

To confirm a full activation of Akt in ARVM under experimental conditions, we first analysed the Akt(Ser^{473}) and Akt(Thr^{308}) activation after 10 minutes

incubation with insulin and the different analogues. We observed a comparable Akt response under all conditions (Fig. 2A and Suppl. Fig. 3). Next, we compared the positive inotropic effect of the different insulin analogues to Ins in freshly isolated ARVM. ARVM pre-treated with 100 nM Ins, IGla, IGlaM1 and IDeg for 5 min, respectively showed a significant increase in sarcomeric shortening (~2.5-fold), which describes the total sarcomeric shortening per sarcomere in µm (Fig. 2B). Departure- (~2-fold) (Fig. 2C) and return velocity (~3-fold) (Fig. 2D), which describes the shortening rate and re-lengthening rate of single sarcomeres in µm/s, are significantly increased compared to the control situation (Fig. 2). As can be seen from the data, an equipotent positive inotropic effect was observed for all analogues.

The positive inotropic effect of insulin and long-acting insulin analogues is Akt dependent

To assess the role of Akt in the insulin-induced inotropic effect, we treated ARVM with the specific Akt-inhibitor triciribine. Triciribine in a concentration of 10 µM did not affect cell viability as determined by trypan blue staining (Suppl. Fig. 4). After 30 min pre-incubation with triciribine insulin signalling (Fig. 3A and Suppl. Fig. 3) was completely abolished with all tested insulins. In line with the signalling data, the positive inotropic effect of insulin and the different insulin analogues was completely abolished at the level of all cardiac contraction parameters, such as sarcomeric shortening (Fig. 3B), departure velocity (Fig. 3C) and return velocity (Fig 3D).

Both insulin glargine and insulin degludec increase the beating rate of human cardiomyocytes

To investigate long-term effects of insulin and its analogues on the beating rate of human iPSC-derived cardiomyocytes (Cor.4U®) we used an impedance-based measurement approach. To determine baseline values, beating rate prior to substance application was analysed. In all measured conditions the mean beating-rate was around 30 beats per minute (bpm) (Fig. 4A). Since we did not observe any effects with 100 nM of the respective insulins, we used 500 nM for the treatment of the Cor.4U® cells. After substance application Ins, IGla and IGlaM1 reach their maximum beating-rate after 10 min, whereas IDeg reaches its maximum with a delay after 20 min (Fig.4B) and subsequently, the increased beating-rate remained stable for up to 6 hours (Fig. 4C). For better comparison of the increased beating-rate in each condition we calculated the area under the curve for the whole measurement (Fig. 4D). The analysis revealed a significant increase in the beating-rate for Ins, IGla, IGlaM1 and IDeg.

Both insulin glargine and insulin degludec increase glucose-uptake to the same extent as regular insulin

Since glucose metabolism is important for cardiomyocyte function, we compared the potency of Ins, IGla, IGlaM1 and IDeg to stimulate glucose uptake in HL 1 cells. Glucose uptake was significantly increased after 200 nM of IGla, IGlaM1 and IDeg treatment (between 1.46- and 1.58-fold) and no difference between the insulins has been detected (Fig. 5).

Anti-apoptotic efficiency of insulin glargine and insulin degludec is comparable to regular insulin

Next we evaluated the anti-apoptotic potency of the long-acting insulin analogues in the rat cardiomyocyte cell line H9c2 overexpressing the human insulin receptor (H9c2-E2). For this purpose, we treated the cells with a combination of either insulin or the different analogues in the presence of H_2O_2, and subsequently measured caspase 3/7 activation. After exposure to H_2O_2 we found a significant induction of caspase

3/7 activity in H9c2-E2 cell (4.8-fold) (Fig. 6). However, the combination of Ins and H2O2 decreased caspase 3/7 activity up to 70.3 % compared to H_2O_2 treatment alone, and was not significant anymore. A similar effect was observed for all tested insulin analogues.

Discussion

Treatment of diabetic patients with insulin analogues has been shown to provide a more efficient, reproducible, and convenient therapy than regular insulin. The analogues may vary from insulin with respect to metabolic potency, stability or onset, and duration of action that is achieved by either sequence or secondary structural modifications. These changes may lead to an altered functional profile, emphasizing the importance of examining all steps in the action of an insulin analogue in vitro and in vivo. Regarding the effects of insulin analogues in cardiomyocyte cell models no published data is available. Therefore, we compared the long acting insulin analogues IGla and IDeg in cardiac cell models. Using this in vitro setting, we could show the absence of any difference in functional cardiac endpoint measurements and insulin signalling between the long-acting insulin analogues insulin glargine and degludec under steady state conditions.

Since Akt is a major element of the insulin signalling pathway, we first analysed the activation of Akt in ARVM and HL-1 cells after treatment with the different insulin analogues. Although our results show a slower onset of Akt activation in HL-1 cells treated with IDeg for 5- and 10 minutes compared to the other insulins (Fig. 1B & C), we did not observe a difference in Akt activation in HL-1 cells treated for 60 minutes and acutely treated ARVM (Fig. 1D & Fig. 2A). We speculate that a possible explanation for the slower onset of Akt phosphorylation in HL-1 cells might be the low binding affinity of IDeg towards the insulin receptor. In receptor binding studies using S-IR of both isoforms, the binding affinity for IDeg was found to be 13-15 % relative to human insulin [25]. The results from our indirect binding assays with the S-IR show a similar binding affinity of IDeg with ~18.6 % relative to human insulin. However, it should be noted that S-IR displays a less complex construct compared to M-IR. In these preparations the IR is surrounded by lipids and protein complexes. In M-IR preparations IDeg showed a binding affinity of ~4 % compared to Ins and ~13 % compared to IGlaM1. It could be possible that the fatty acid residue attached to IDeg interacts with other components of the membrane, and therefore leading to a reduced binding affinity towards the IR. The very low binding affinity of IDeg in M-IR (Fig. 1A) could be an explanation for the slower onset of action observed in HL-1 and Cor.4U® cells. The results obtained in M IR preparations with Ins and IGlaM1 and IDeg in S-IR are comparable to previously published data [21, 25].

Insulin-mediated Akt activation in the myocardium triggers a variety of processes, like glucose uptake, modification of calcium signalling and anti-apoptotic effects [26]. Even though under physiological conditions the main energy source for the heart is fatty acids, about one third is derived from glucose [27] and with increasing blood glucose and insulin level, glucose becomes the favoured substrate in the heart [2]. Therefore, we next analysed the ability of IGla and IDeg to stimulate glucose uptake under steady-state conditions in HL-1 cells, since we observed a full Akt activation with IDeg after 60 minutes. Under these conditions we observed no difference between IGla and IDeg in the insulin stimulated glucose uptake compared to Ins. We therefore conclude that IGla and IDeg are equipotent in regulating cardiac glucose consumption, at least under steady-state conditions.

Another important aspect of insulin function in cardiomyocytes is the positive inotropic effect which was already described in the 1920s by Visscher and Müller [28] and is due to an increased excitation-contraction coupling, which in turn is controlled by entry and release of Ca^{2+} from the sacroplasmatic reticulum (SR). Using insulin and the insulin analogues we observed a similar positive inotropic effect, as shown by comparable increased sarcomeric shortening, departure- and return-velocity in ARVM. Furthermore, we could show that the positive inotropic effect in ARVM is completely Akt dependent. The Akt dependency of cardiomyocyte contraction was shown previously by Graves et al. in HL-1 cells, where inhibition of Akt activation leads to decreased total $[Ca^{2+}]_i$, intracellular Ca^{2+} transients and membrane I_{Ca} [29]. Furthermore, Reinartz et al. [30] recently showed that Akt1 and Akt2 knockdown affected phosphorylation of proteins involved in regulation of heart contraction as well as relaxation and regulation of heart rate. Additionally, proteins involved in Ca^{2+} release and re-entry into the SR are affected (e.g. CaMKII or phospholamban, a direct target of Akt) [30, 31], which could be a possible explanation for the complete abrogation of the positive inotropic effect after Akt-inhibition. Insulin increases the beating rate of cardiac muscle, but the underlying mechanism is controversially discussed in the literature. While some groups found evidence for insulin to directly increase the beating-rate in vivo [32, 33], others claimed that insulin acts on the nervous system and thereby leads to beta-adrenergic stimulation of the heart [34, 35]. In our in vitro experiments with Cor.4U® cells we observed a slight but significant increase in beating-rate of Cor.4U® cardiomyocytes for up to 6 hours. As observed in HL-1 signalling, we measured a slower onset of action with IDeg. However, under steady state conditions no differences between the different analogues could be detected. Together with the results from the ARVM contraction experiments we conclude that the increase in beating rate of cardiomyocytes is at least partly independent of nervous system activity and directly affected by insulin itself.

Furthermore, insulin is known to reduce the damage of ischemia/reperfusion injury (IRI) in vivo as well as in vitro [36-39]. This damage is induced by massive production of reactive oxygen species (ROS) [40]. While diabetes per se is a risk factor for ischemic heart disease, the damage inflicted by IRI is even worse in diabetic patients [41]. Therefore, we aimed to mimic IRI in H9c2-E2 cardiomyocytes by challenging the cells with H_2O_2 with subsequent measurement of caspase 3/7 activity. To elucidate the potency of IGla and IDeg in prevention of caspase 3/7 activation during ROS treatment, we treated part of the cells with a combination of H_2O_2 and the respective insulin. With our results we were able to reproduce the protective effect of Ins during IRI and furthermore we were able to show that both, IGla and IDeg, have the same potency to prevent caspase 3/7 activation as Ins .

Conclusion

In conclusion, the long-acting insulin analogues IGla and IDeg show no major differences in several cardiomyocyte in vitro models regarding insulin signalling, contractility parameters, beating rate, glucose uptake, and protection from oxidative stress induced caspase 3/7 activation under steady-state conditions. However, for IDeg we observed a slower onset of action in Akt phosphorylation in HL-1 cells as well as slower response to IDeg in human Cor.4U® cardiomyocytes. Additionally, we observed very low binding affinities of IDeg in M-IR preparations. Whether these effects translate to the complex in vivo situation needs further evaluation.

List of abbreviations

ARVM	Adult rat ventricular myocytes
ANOVA	Analysis of variance
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
DMEM	Dulbecco's Modified Eagle Medium
ECL	enhanced chemiluminescence
FCS	fetal calf serum
FDA	American Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H9c2-E2	H9c2 cells overexpressing the human insulin receptor
HRP	horse radish peroxidase
HSA	human serum albumin
IDeg	insulin degludec
IGla	insulin glargine
IGlaM1	active metabolite of insulin glargine
Ins	regular insulin
IR	Insulin receptor
IRI	ischemia/reperfusion injury
M-IR	membrane embedded insulin receptor
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
S-IR	solubilised insulin receptor
SDS/PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SPA	scintillation proximity assay
SR	sacroplasmatic reticulum
TBS	tris-buffered saline

Competing interests

Paulus Wohlfart and Norbert Tennagels are employees of Sanofi. Jürgen Eckel received funding by Sanofi.

Authors' contributions

TH: data collection and analysis, study design, drafted the manuscript. SO: data collection, critical revision of the manuscript. MO: critical revision of the manuscript. SR: study design, critical revision of the manuscript. PW: data collection and analysis, study design, critical revision of the manuscript. NT: study design, critical revision of the manuscript. NW: data analysis, study design, critical revision of the manuscript. JE: study design, critical revision of the manuscript

Acknowledgements

The assistance of Dr. Marcel Blumensatt in setting up the HL-1 cardiomyocyte experiments, as well as the help of Pia Fahlbusch and Daniela Herzfeld de Wiza in isolating the ARVM and the assistance of Anika Duenbostell in culturing the Cor.4U® cells are gratefully acknowledged and highly appreciated. This study was funded by Sanofi.

Tables and Figures

Table 1: Summary of competition binding assay using M-IR

Insulin	IR affinity IC_{50} (nmol/L)	p-value vs. Ins
Ins	0.83 ± 0.07	-
IGlaM1	2.15 ± 0.61	0.2350
IDeg	19.59 ± 1.10	<0.0001

Data represent means ± SEM. All insulins were tested in at least four independent experiments on different days. Binding values within a single experiment were obtained in quadruplicates per insulin and averaged for each experiment. Regular insulin (Ins), active metabolite of glargine (IGlaM1), insulin degludec (IDeg)

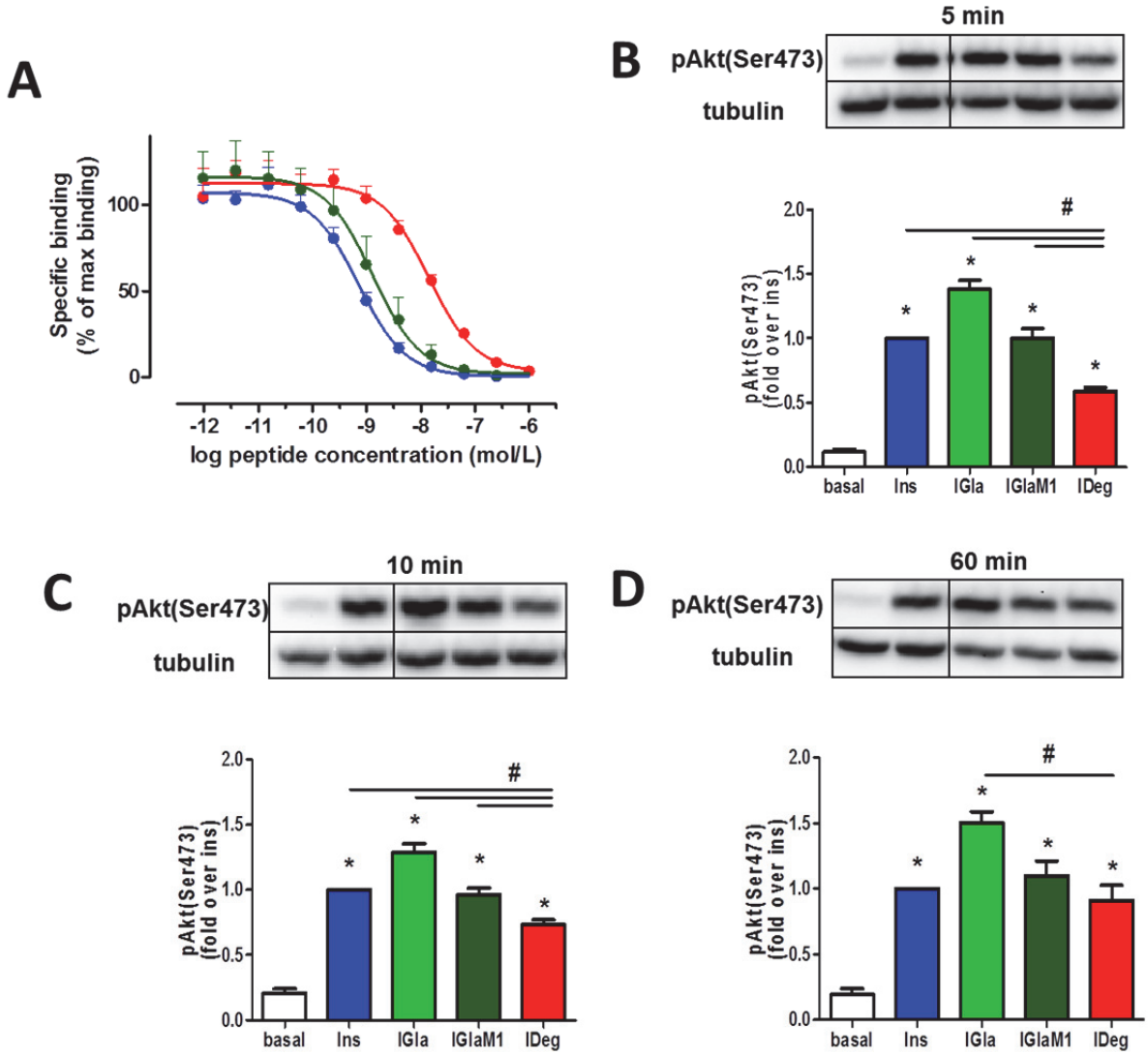


Figure 1: Binding affinity and Akt signalling of long-acting insulin analogues. (A) Membrane embedded insulin receptor preparations were used to analyse binding of Ins, IGlaM1 and IDeg in a competition binding assay, as described in [21]. Percentage of binding is normalised to maximum binding of [^{125}I]-labelled human insulin. Data represent mean values ± SEM, n = 4-5. **(B-D)** HL-1 cells were used to assess the onset of insulin action by treatment with 200 nM for 5 **(B)**; 10 **(C)** or 60 min **(D)** with insulin or insulin analogues. Phosphorylation of Akt(Ser^{473}) was assessed by Western blot analysis. Data are normalised to tubulin levels. Representative blots are shown. Data represent mean values ± SEM, n = 4 5, *p<0.05 vs. basal, #p<0.05 vs. IDeg. Regular insulin (Ins), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

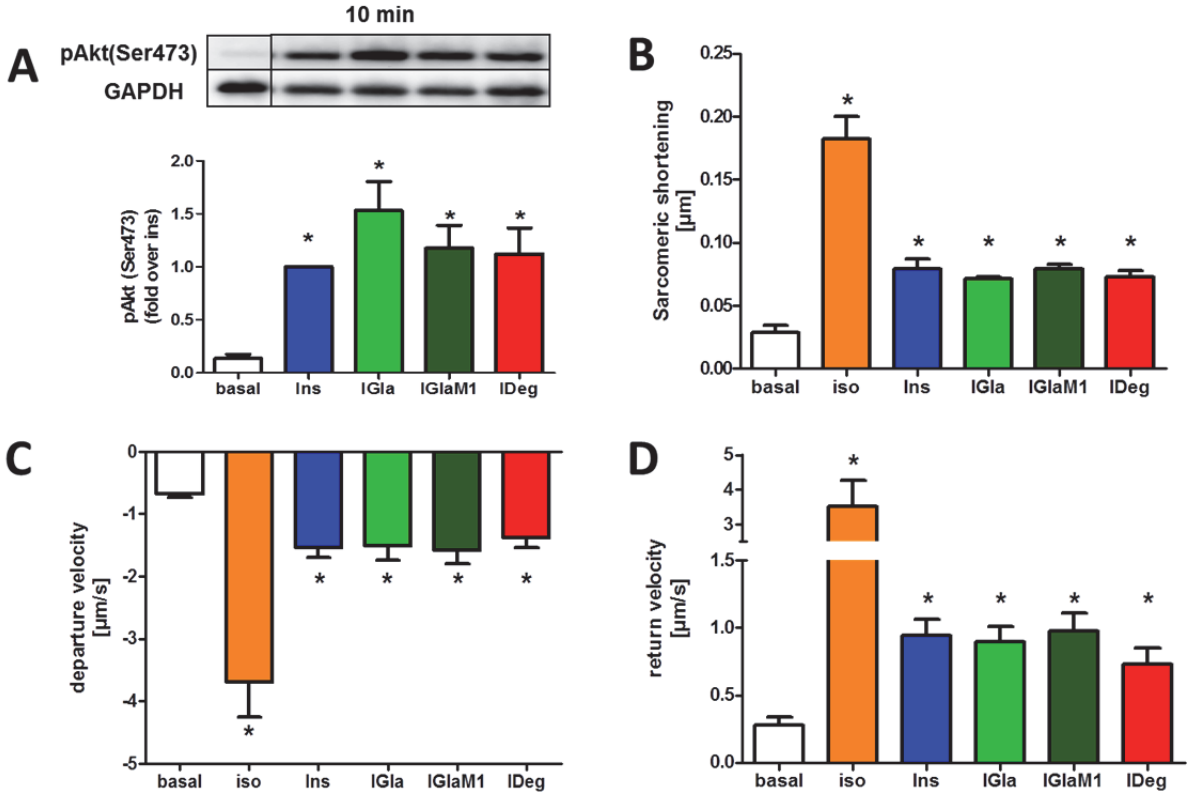


Figure 2: Effect of insulin and insulin analogues on Akt signalling and cardiac contraction in ARVM. Adult rat ventricular cardiomyocytes (ARVM) were treated for 10 min with 100 nM insulin or insulin analogues to investigate the insulin signalling pathway in these cells **(A)**. ARVM were isolated by enzymatic digestion, starved overnight and treated with 100 nM insulin or insulin analogues or 10 nM isoproterenol as positive control. ARVM were paced at 1 Hz, 15 V, 0.5 ms with the IonOptix Myopacer Cell Stimulator System to assess the sarcomeric shortening **(B)**, departure velocity **(C)** and return velocity **(D)**. In each condition at least 10 contractions of 10-14 cardiomyocytes were measured under steady-state conditions. Data represent mean values ± SEM, n=4, *p<0.05 vs. basal. Regular insulin (Ins), isoproterenol (iso), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

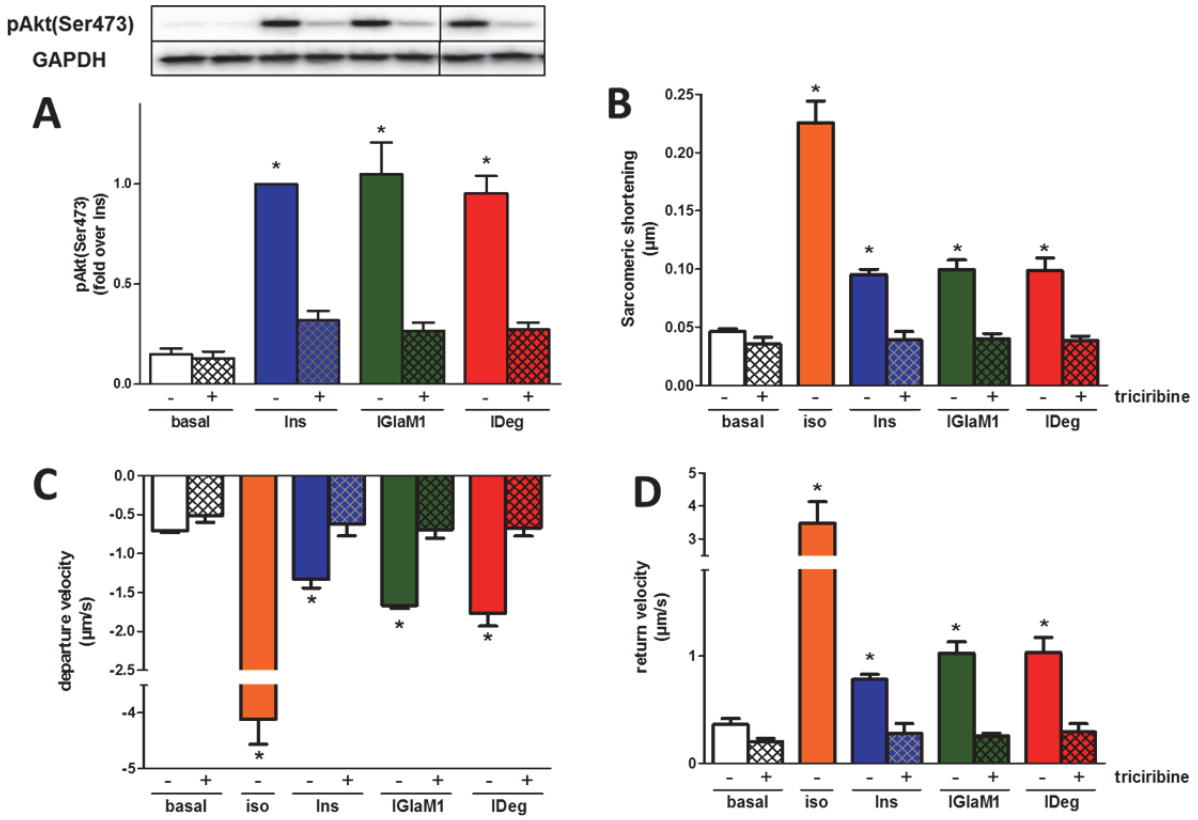


Figure 3: Impact of Akt inhibition on insulin-induced cardiac contraction. Adult rat ventricular cardiomyocytes (ARVM) were analysed either without pre-treatment (blank bars) or pre-treated with 10 µM of the specific Akt-inhibitor triciribine (filled bars) for 30 min. Subsequently, ARVM were treated with either 100 nM insulin or insulin analogues for 10 minutes to investigate the insulin signalling pathway after triciribine treatment **(A)**. Furthermore, ARVM were treated with 100 nM of insulin or insulin analogues or 10 nM isoproterenol and paced at 1 Hz, 15 V, 0.5 ms with the IonOptix Myopacer Cell Stimulator System to assess the sarcomeric shortening **(B)**, departure velocity **(C)** and return velocity **(D)**. In each condition at least 10 contractions of 10-14 cardiomyocytes were measured. Data represent mean values ± SEM, n=3-5, *p<0.05 vs. basal (treated). Regular insulin (Ins), isoproterenol (iso), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

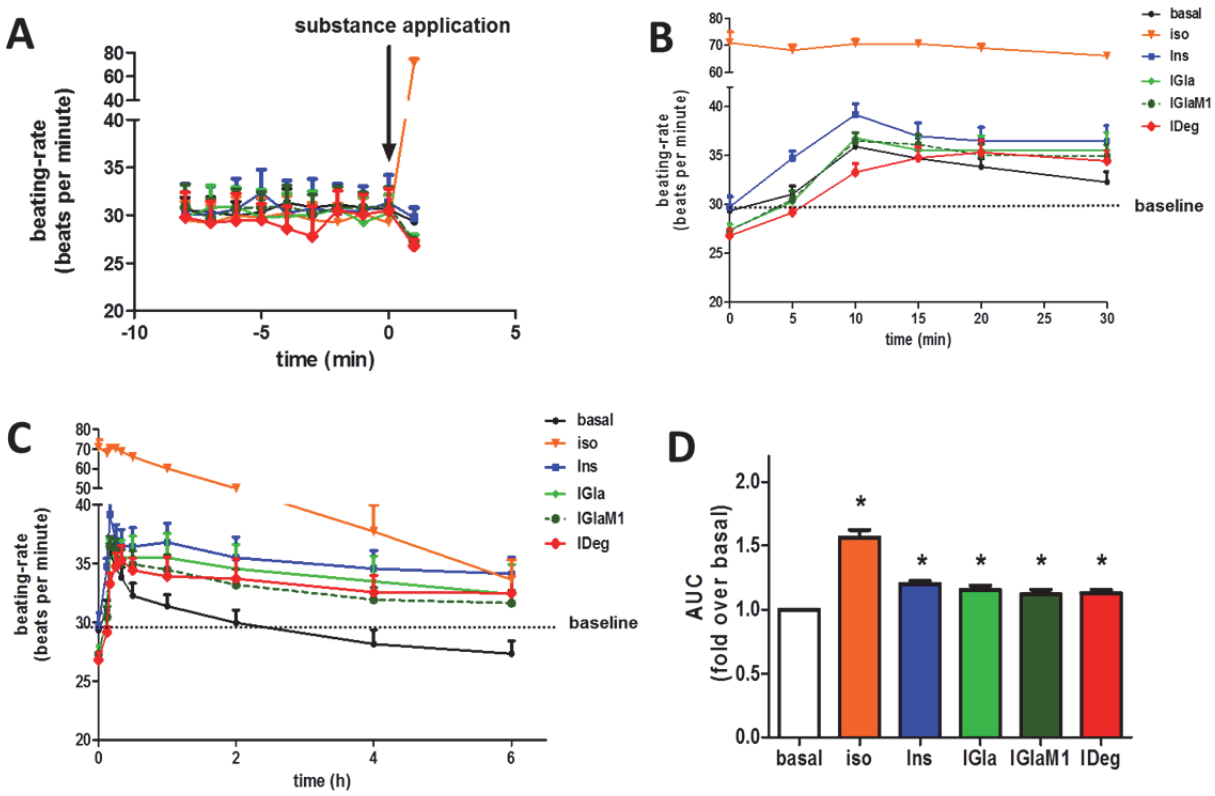


Figure 4: Effect of insulin glargine and insulin degludec on the beating-rate of human iPSC-derived cardiomyocytes. Commercially available human iPSC-derived cardiomyocytes (Cor.4U®) were used to assess the long-term effect of insulins on the beating-rate **(A-D)**. Experiments were performed at day 4 after seeding. Cells were treated with 500 nM of insulin or insulin analogues or 100 nM isoproterenol, respectively. The beating-rate was measured for 6 hours. **(A)** Basal beating-rate prior to substance application. **(B)** Beating-rate of the first 30 minutes after substance application. **(C)** 0.5 - 6 hours time course of beating-rate in Cor.4U® cells after substance application. **(D)** Area under the curve for the 6 hours beating-rate measurement. Data represent mean values ± SEM, n=4, *p<0.05 vs. basal. Regular insulin (Ins), isoproterenol (iso), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

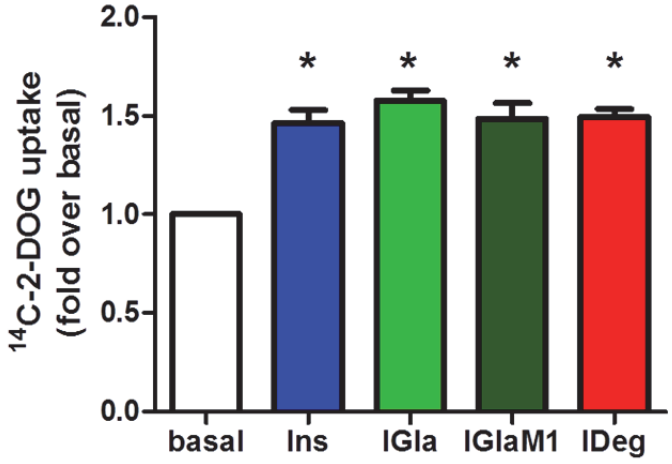


Figure 5: 2-deoxy-D-glucose uptake in HL-1 cells after insulin stimulation with regular insulin and long-acting insulin analogues. HL-1 cells were used to assess the effect of insulin, insulin glargine, the active metabolite M1 and insulin degludec on 2-deoxy-D-glucose uptake. The cells were pre-treated with 200 nM of the indicated insulin for 1 hour. Subsequently, the cells were exposed to radioactive labelled mix containing 2-deoxy-D-glucose and 2-deoxy-D-[1-^{14}C]glucose for 10 min at 37 °C. Data represent mean values ± SEM, n=4, *p<0.05 vs. basal. Regular insulin (Ins), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

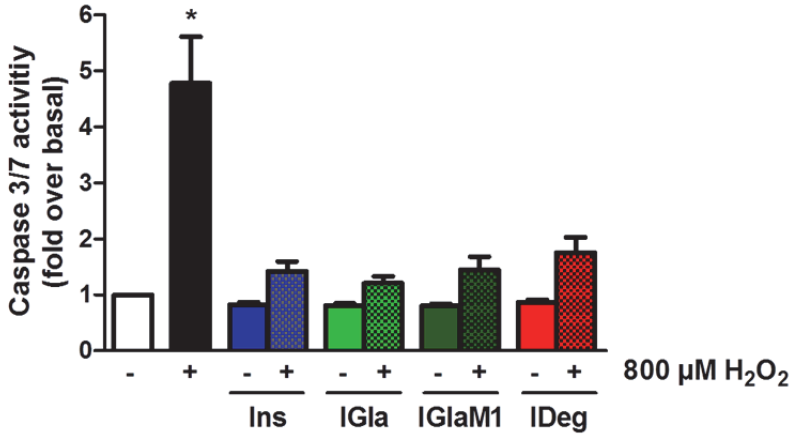


Figure 6: Anti-apoptotic potency of long-acting insulin analogues in the presence of H_2O_2. H9c2 cardiomyocytes overexpressing the human insulin receptor (H9c2-E2 cells) were treated for 4 hours with 100 nM of insulin and insulin analogues in the presence or absence of 800 µM H_2O_2 to evaluate the cardio protective effects of insulin and its analogues. Caspase 3/7 activity was measured using the Promega Caspase 3/7 Glo Assay. Each condition was performed in quadruplicates. Data represent mean values ± SEM, n=5-6, *p<0.05 vs. basal. Regular insulin (Ins), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

Reference List

1. Muniyappa R, Montagnani M, Koh KK, Quon MJ: Cardiovascular actions of insulin. EndocrRev 2007, 28(5):463-491.

2. Bertrand L, Horman S, Beauloye C, Vanoverschelde JL: Insulin signalling in the heart. CardiovascRes 2008, 79(2):238-248.

3. Klein LJ, Visser FC: The effect of insulin on the heart : Part 1: Effects on metabolism and function. NethHeart J 2010, 18(4):197-201.

4. (ADA) ADA: Standards of medical care in diabetes--2008. Diabetes Care 2008, 31 Suppl 1:S12-S54.

5. Rodbard HW, Gough S, Lane W, Korsholm L, Bretler DM, Handelsman Y: Reduced risk of hypoglycemia with insulin degludec versus insulin glargine in patients with type 2 diabetes requiring high doses of Basal insulin: a meta-analysis of 5 randomized begin trials. EndocrPract 2014, 20(4):285-292.

6. Rosenstock J, Dailey G, Massi-Benedetti M, Fritsche A, Lin Z, Salzman A: Reduced hypoglycemia risk with insulin glargine: a meta-analysis comparing insulin glargine with human NPH insulin in type 2 diabetes. Diabetes Care 2005, 28(4):950-955.

7. Heinemann L, Linkeschova R, Rave K, Hompesch B, Sedlak M, Heise T: Time-action profile of the long-acting insulin analog insulin glargine (HOE901) in comparison with those of NPH insulin and placebo. Diabetes Care 2000, 23(5):644-649.

8. Rosskamp RH, Park G: Long-acting insulin analogs. Diabetes Care 1999, 22 Suppl 2:B109-B113.

9. Kuerzel GU, Sandow J, Seipke G, Lang AM, Maas J, Skrzipczyk HJ: Kinetic and metabolite profile of insulin glargine (LANTUS®). Diabetologia 2001, 44.

10. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T, Bernhardt E: Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. CurrMedResOpin 2003, 19(1):34-40.

11. Jonassen I, Havelund S, Hoeg-Jensen T, Steensgaard DB, Wahlund PO, Ribel U: Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin. PharmRes 2012, 29(8):2104-2114.

12. Hamilton-Wessler M, Ader M, Dea M, Moore D, Jorgensen PN, Markussen J, Bergman RN: Mechanism of protracted metabolic effects of fatty acid acylated insulin, NN304, in dogs: retention of NN304 by albumin. Diabetologia 1999, 42(10):1254-1263.

13. Kurtzhals P, Havelund S, Jonassen I, Kiehr B, Larsen UD, Ribel U, Markussen J: Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. BiochemJ 1995, 312 (Pt 3):725-731.

14. Myers SR, Yakubu-Madus FE, Johnson WT, Baker JE, Cusick TS, Williams VK, Tinsley FC, Kriauciunas A, Manetta J, Chen VJ: Acylation of human insulin with palmitic acid extends the time action of human insulin in diabetic dogs. Diabetes 1997, 46(4):637-642.

15. Gerstein HC, Bosch J, Dagenais GR, Diaz R, Jung H, Maggioni AP, Pogue J, Probstfield J, Ramachandran A, Riddle MC et al: Basal insulin and cardiovascular and other outcomes in dysglycemia. NEnglJMed 2012, 367(4):319-328.

16. Endocrinologic and Metabolic Drugs Advisory Committee Meeting - Insulin Degludec and Insulin Degludec/Aspart [http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/dr

ugs/endocrinologicandmetabolicdrugsadvisorycommittee/ucm330923.pdf]

17. FDA approves two new drug treatments for diabetes mellitus [http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm464321.htm]

18. Rask-Madsen C, Kahn CR: Tissue-specific insulin signaling, metabolic syndrome, and cardiovascular disease. ArterioscIerThrombVascBiol 2012, 32(9):2052-2059.

19. Claycomb WC, Lanson NA, Jr., Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ, Jr.: HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. ProcNatlAcadSciUSA 1998, 95(6):2979-2984.

20. Uhlig M, Passlack W, Eckel J: Functional role of Rab11 in GLUT4 trafficking in cardiomyocytes. MolCell Endocrinol 2005, 235(1-2):1-9.

21. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R, Tennagels N: In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites. PLoSOne 2010, 5(3):e9540.

22. Hansen BF, Glendorf T, Hegelund AC, Lundby A, Lutzen A, Slaaby R, Stidsen CE: Molecular characterisation of long-acting insulin analogues in comparison with human insulin, IGF-1 and insulin X10. PloS one 2012, 7(5):e34274.

23. Eckel J, Pandalis G, Reinauer H: Insulin action on the glucose transport system in isolated cardiocytes from adult rat. BiochemJ 1983, 212(2):385-392.

24. Scott CW, Zhang X, Abi-Gerges N, Lamore SD, Abassi YA, Peters MF: An impedance-based cellular assay using human iPSC-derived cardiomyocytes to quantify modulators of cardiac contractility. ToxicolSci 2014, 142(2):331-338.

25. Nishimura E, Sørensen AR, Hansen BF, Stidsen CE, Olsen GS, Schaäffer L, al. e: Insulin degludec: A new ultra-long, basal insulin designed to maintain full metabolic effect while minimizing mitogenic potential. Diabetologia 2010, 53:S388-S389.

26. Sussman MA, Volkers M, Fischer K, Bailey B, Cottage CT, Din S, Gude N, Avitabile D, Alvarez R, Sundararaman B et al: Myocardial AKT: the omnipresent nexus. Physiol Rev 2011, 91(3):1023-1070.

27. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC: Myocardial fatty acid metabolism in health and disease. Physiol Rev 2010, 90(1):207-258.

28. Visscher MB, Muller EA: The influence of insulin upon the mammalian heart. JPhysiol 1927, 62(4):341-348.

29. Graves BM, Simerly T, Li C, Williams DL, Wondergem R: Phosphoinositide-3-kinase/akt - dependent signaling is required for maintenance of [Ca(2+)](i), I(Ca), and Ca(2+) transients in HL-1 cardiomyocytes. JBiomedSci 2012, 19:59.

30. Reinartz M, Raupach A, Kaisers W, Godecke A: AKT1 and AKT2 induce distinct phosphorylation patterns in HL-1 cardiac myocytes. JProteomeRes 2014, 13(10):4232-4245.

31. Catalucci D, Latronico MV, Ceci M, Rusconi F, Young HS, Gallo P, Santonastasi M, Bellacosa A, Brown JH, Condorelli G: Akt increases sarcoplasmic reticulum Ca2+ cycling by direct phosphorylation of phospholamban at Thr17. JBiolChem 2009, 284(41):28180-28187.

32. Jacobsen F, Christensen NJ: Stimulation of heart rate by insulin: uninfluenced by beta-adrenergic receptor

blockade in rabbits. ScandJClinLab Invest 1979, 39(3):253-256.

33. Keen HL, Brands MW, Alonso-Galicia M, Hall JE: Chronic adrenergic receptor blockade does not prevent hyperinsulinemia-induced hypertension in rats. AmJHypertens 1996, 9(12 Pt 1):1192-1199.

34. Anderson EA, Hoffman RP, Balon TW, Sinkey CA, Mark AL: Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. JClinInvest 1991, 87(6):2246-2252.

35. Siani A, Strazzullo P, Giorgione N, De LA, Mancini M: Insulin-induced increase in heart rate and its prevention by propranolol. EurJClinPharmacol 1990, 38(4):393-395.

36. Ji L, Fu F, Zhang L, Liu W, Cai X, Zhang L, Zheng Q, Zhang H, Gao F: Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress. AmJPhysiol EndocrinolMetab 2010, 298(4):E871-E880.

37. Shi YF, Liu N, Li YX, Song CL, Song XJ, Zhao Z, Liu B: Insulin protects H9c2 rat cardiomyoblast cells against hydrogen peroxide-induced injury through upregulation of microRNA-210. Free RadicRes 2015, 49(9):1147-1155.

38. Wong VW, Mardini M, Cheung NW, Mihailidou AS: High-dose insulin in experimental myocardial infarction in rabbits: protection against effects of hyperglycaemia. JDiabetes Complications 2011, 25(2):122-128.

39. Xing W, Yan W, Fu F, Jin Y, Ji L, Liu W, Wang L, Lv A, Duan Y, Zhang J et al: Insulin inhibits myocardial ischemia-induced apoptosis and alleviates chronic adverse changes in post-ischemic cardiac structure and function. Apoptosis 2009, 14(9):1050-1060.

40. Braunersreuther V, Jaquet V: Reactive oxygen species in myocardial reperfusion injury: from physiopathology to therapeutic approaches. CurrPharmBiotechnol 2012, 13(1):97-114.

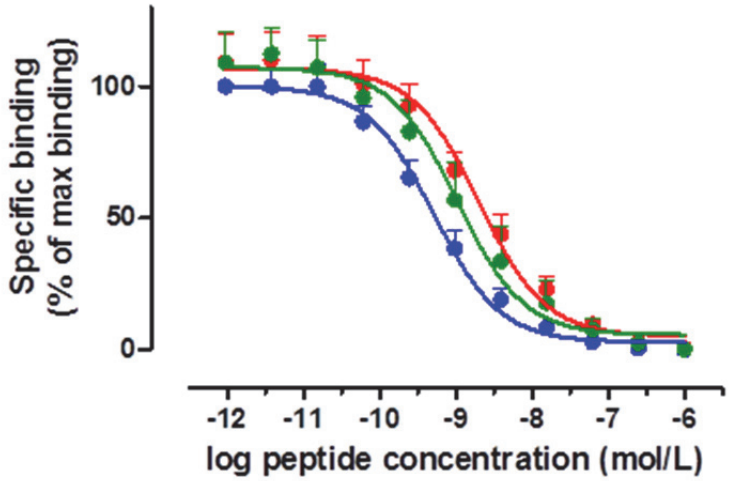
41. Ferdinandy P, Schulz R, Baxter GF: Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. PharmacolRev 2007, 59(4):418-458.

Supplemental Material

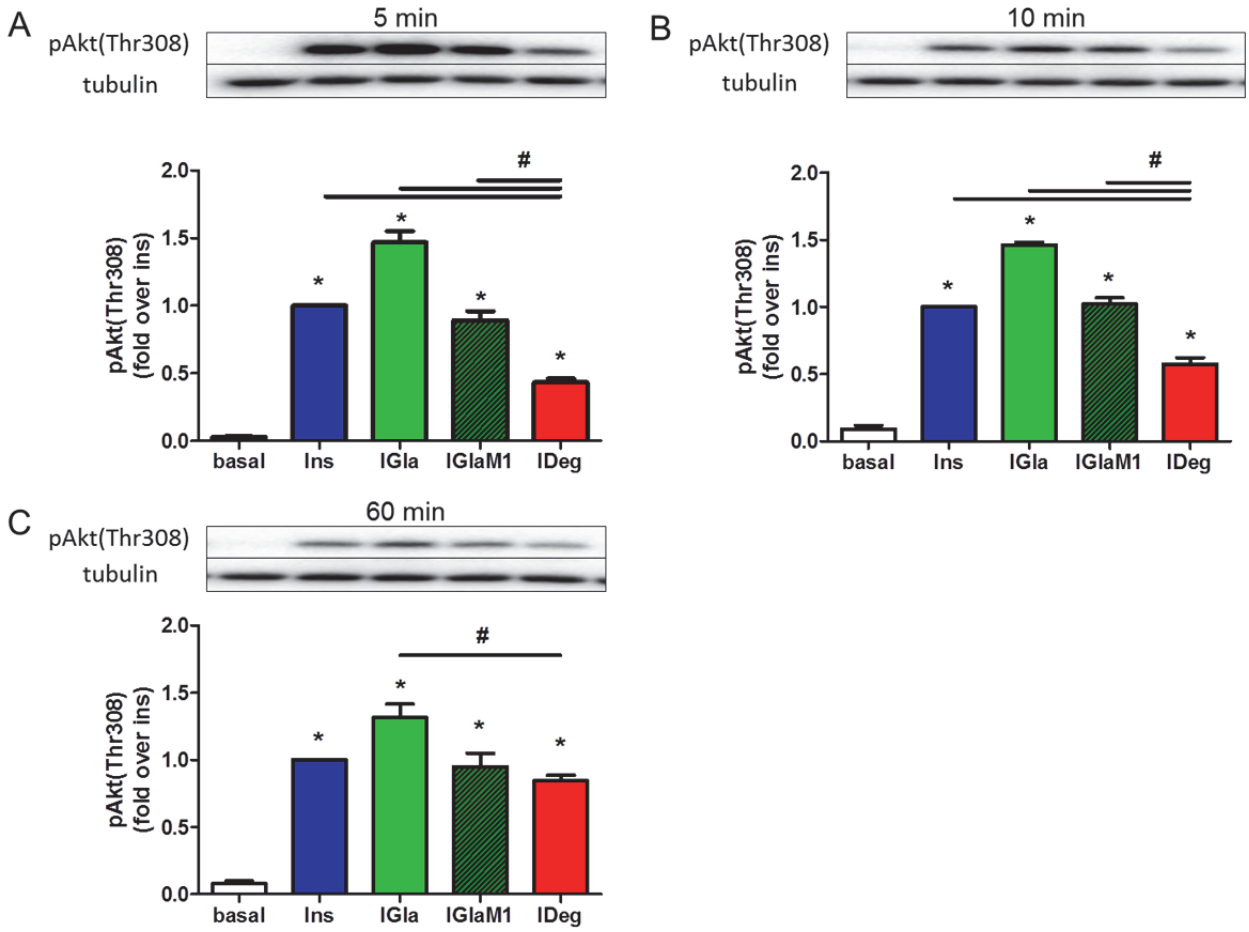
Supplementary Table 1: Summary of competition binding assay using S-IR

Insulin	IR affinity IC_{50} (nmol/L)	p-value vs. Ins
Ins	0.58 ± 0.10	-
IGlaM1	2.09 ± 0.74	0.10
IDeg	3.12 ± 0.42	<0.01

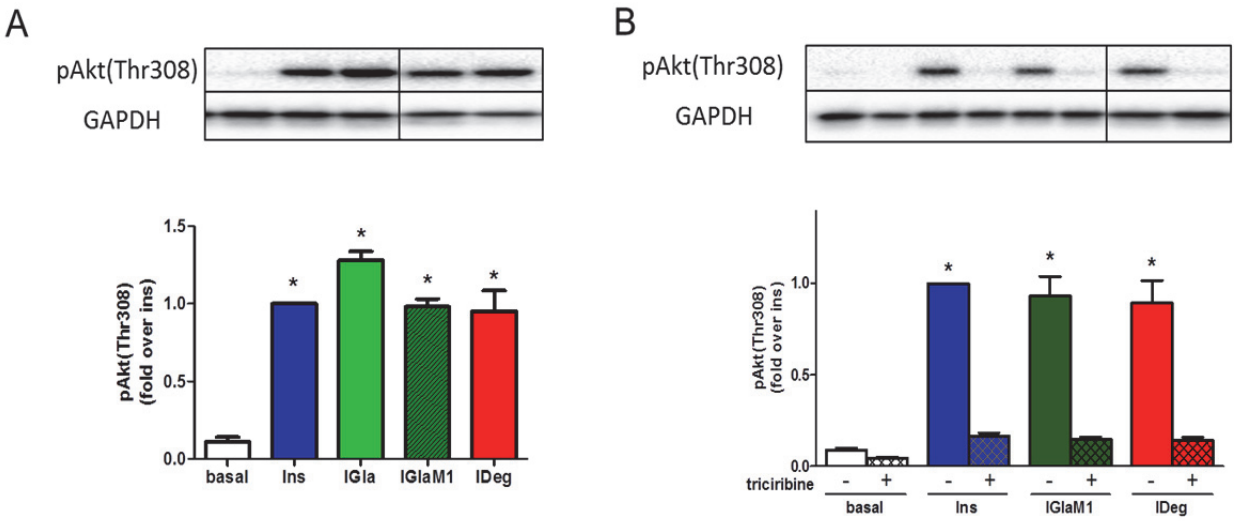
Data represent means ± SEM. All insulins were used at least six times on different days. IC_{50} were obtained in quadruplicates per insulin and averaged for each experiment. Regular insulin (Ins), active metabolite of glargine (IGlaM1), insulin degludec (IDeg)



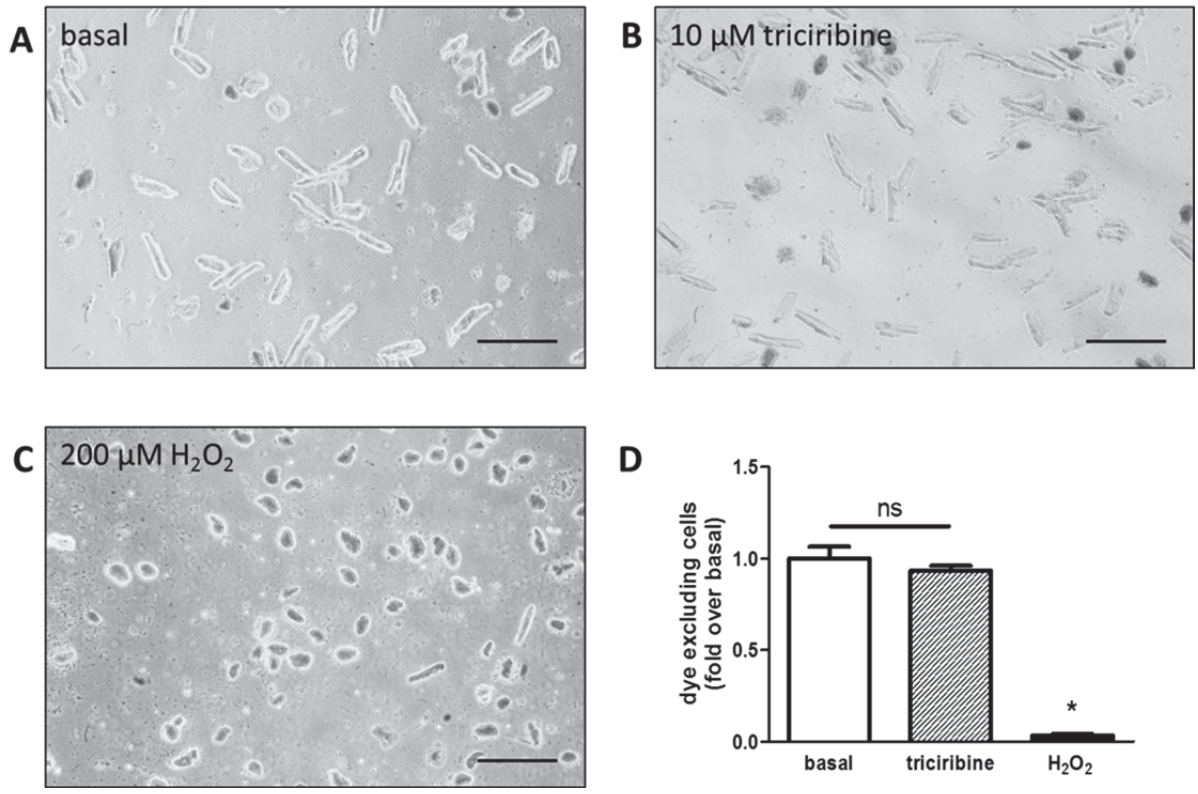
Supplementary Figure 1: Competition binding assay using solubilised insulin receptor. Solubilised insulin receptor preparations were used to analyse binding of Ins, IGlaM1 and IDeg in a competition binding assay. Percentage of binding is normalised to maximum binding of [^{125}I]-labelled human insulin. Data represent mean values ± SEM, n = 6-7.



Supplementary Figure 2: **Phosphorylation of Akt(Thr^{308}) in HL-1 cells**. **(A-C)** HL-1 cells were used to assess the onset of insulin action by treatment with 200 nM for 5 **(A)**; 10 **(B)** or 60 min **(C)** with insulin or insulin analogues. Phosphorylation of Akt(Thr^{308}) was assessed by Western blot analysis. Data are normalised to tubulin levels. Representative blots are shown. Data represent mean values ± SEM, n = 3, *p<0.05 vs. basal, #p<0.05 vs. IDeg. Regular insulin (Ins), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).



Supplementary Figure 3: Phosphorylation of Akt(Thr^{308}) in adult rat ventricular myocytes. (A) Adult rat ventricular myocytes (ARVM) were used to assess the onset of insulin action by treatment with 100 nM for 10 min with insulin or insulin analogues. **(B)** ARVM were analysed either without pre-treatment (blank bars) or pre-treated with 10 µM of the specific Akt-inhibitor triciribine (filled bars) for 30 min. Subsequently, ARVM were treated with either 100 nM insulin or insulin analogues for 10 minutes to investigate the insulin signalling pathway after triciribine treatment. Phosphorylation of Akt(Thr^{308}) was assessed by Western blot analysis. Data are normalised to GAPDH levels. Representative blots are shown. Data represent mean values ± SEM, n = 4-5, *p<0.05 vs. basal. Regular insulin (Ins), insulin glargine (IGla), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg)



Supplementary Figure 4: Cell viability assay after triciribine treatment. Adult rat ventricular cardiomyocytes (ARVM) were stained with 0.1 % trypan blue in PBS for 5 minutes after treatment with 10 µM triciribine, subsequently random bright field images were taken and viable (white) and dead (blue) cells were quantified. **(A-C)** Representative bright field pictures of basal conditions **(A)**, 10 µM triciribine treatment **(B)** and 200 µM H_2O_2 treatment **(C)**. At least 400 cells per condition per experiment were counted. Scale bar = 200 µM. **(D)** Quantification of living and dead cells. Data represent mean values ± SEM, n = 3, *p<0.05 vs. basal.

2.2 Study 2: ^{LAPS}Insulin 115: A novel ultra-long-acting basal insulin with a unique action profile

Wronkowitz N, **Hartmann T**, Görgens S, Dietze-Schroeder D, Indrakusuma I, Choi IY, Park SH, Lee YM, Kwon SC, Kang Y, Hompesch M, Eckel J., Diabetes, Obesity and Metabolism (2017) submitted

^{LAPS}Insulin115: A novel ultra-long-acting basal insulin with a unique action profile

Running title: ^{LAPS}**Insulin115: A novel basal insulin analogue**

Nina Wronkowitz[1], Thorsten Hartmann[1], Sven Görgens[1], Daniela Dietze-Schroeder[1], Ira Indrakusuma[1], In Young Choi[2], Sung Hee Park[2], Young-Mi Lee[2], Se Chang Kwon[2], Yeonjoo Kang[3], Marcus Hompesch[3], and Juergen Eckel[1]

[1]Paul-Langerhans-Group, German Diabetes Center, Düsseldorf, Germany

[2]Hanmi Pharmaceutical, Co., Ltd., Seoul, South Korea

[3]Profil Institute for Clinical Research, Chula Vista, CA, USA

Address for correspondence:

Prof. Dr. Jürgen Eckel

German Diabetes Center

Auf'm Hennekamp 65

D-40225 Düsseldorf, Germany

Tel: +49 211 3382561, Fax: +49 211 3382697

Email: eckel@uni-duesseldorf.de

Abstract: 234 words

Main body: 3466 words

Abstract

Aims: Developing a safe, while more convenient long-acting basal insulin for diabetic patients is still an unmet medical need. Thus, we conducted a comprehensive pre-clinical study of the novel ultra-long acting insulin analogue ^{LAPS}Insulin115.

Methods:
Pharmacokinetic/pharmacodynamic studies comparing ^{LAPS}Insulin115 with other basal insulins were conducted in $^{db/db}$mice. Insulin signaling in the major target organs was analysed by Western blot after single subcutaneous injection in wildtype male Wistar rats. Using *in vitro* assays we analyzed transendothelial transport, insulin receptor interaction, mitogenic and metabolic properties of ^{LAPS}Insulin115. Furthermore, insulin receptor downregulation after long-term exposure to high concentrations of ^{LAPS}Insulin115 was analyzed using an *in vitro* desentization/resensitization model.

Results: The novel Fc-conjugated insulin derivative ^{LAPS}Insulin115 showed an extensively prolonged pharmacokinetic and pharmacodynamic profile in rodents. Despite its size of 59 kDa, ^{LAPS}Insulin115 passes the vascular endothelial barrier and induces insulin signaling in all major target tissues in rats. *In vitro*, ^{LAPS}Insulin115 showed a very slow onset of action due to its reduced insulin receptor affinity; however, after long-term stimulation it was equipotent in respect to its metabolic potency and showed no increased mitogenic action when compared to regular insulin. Remarkably, under conditions of chronic exposure, ^{LAPS}Insulin115 does not induce irreversible desensitization of target cells, most likely due to much less prominent insulin receptor downregulation.

Conclusion: Thus, ^{LAPS}Insulin115 exhibits a unique *in vivo* and *in vitro* profile and thereby represents an excellent candidate for a once-weekly insulin analogue.

Introduction

Basal insulin analogues are designed to mimic a more physiological pattern of endogenous insulin secretion and to cover the daily basal insulin needs with the goal to decrease the risk of hypoglycaemia. Therefore, basal insulin analogues ideally present a flat pharmacokinetic and pharmacodynamic profile throughout the day, as well as a long half-life to reduce frequent dosing. Insulin degludec and insulin glargine come closer to the ideal of creating a peakless basal insulin [1-3], and have to be injected once-daily. The approach to create a hepato-preferential insulin analogue with an increased half-life for up to 24-45 h by PEGylation of insulin lispro [4], was stopped at the end of clinical phase III due to an increase in liver fat in the IMAGINE trials [5]. Further formats of basal insulin analogues are under development to reduce injection frequency and, thus, to create a more convenient therapy and to improve life quality of diabetic patients.

To extend the half-life of proteins or peptides while maintaining their therapeutic potential, several recombinant techniques and chemical strategies have been developed. A variety of different approaches have used genetic fusion of pharmacologically active proteins to the immunoglobulin derived Fc-fragment, a naturally long-half-life protein (reviewed in [6]). These so called Fc-fusion proteins have been made over the past 35 years to prolong the half-life of proteins or peptides and 11 of them have been approved for marketing by the FDA [7]. In contrast to other structural modifications such as PEGylation, the extended half-life of Fc-fusion proteins occurs not only due to slower renal clearance of larger sized molecules [8], but also by endothelial recycling via the neonatal Fc receptor (FcRn) (reviewed in [9]).

Based on this knowledge, HANMI pharmaceuticals has developed a next

generation long-acting technology, namely LAPSCOVERY™ (Long acting protein/peptide discovery) (Jung SY, U.S. Patent US7968316 B2, 2011). This technology consists of a carrier in form of an aglycosylated human immunoglobulin G4 fragment (LAPS-carrier), and a biological drug moiety. Both are connected via a flexible 3.4 kDa PEG linker to minimize steric hindrance. Aglycosylation of IgG4 completely removed the residual effector function, thereby minimizing the immunoglobulin-mediated response known for protein-based drugs [10,11]. This LAPS-carrier was conjugated to Insulin115, a novel insulin analogue with increased serum stability (Jung SY, Patent Pending WO2015108398A1, 2015), resulting in ^{LAPS}Insulin115.

The aim of the present study was to assess the action profile of ^{LAPS}Insulin115 using both in vivo and in vitro approaches. We report a novel basal insulin analogue that exhibits substantially prolonged pharmacokinetic and pharmacodynamic profiles in rodents and activates insulin receptor (IR) signaling in all major target tissues. In vitro, ^{LAPS}Insulin115 is characterized by a very slow onset of action, a similar off-rate and a less and reversible IR downregulation compared to insulin. Therefore, ^{LAPS}Insulin115 represents an excellent candidate for once-weekly administration.

Methods

Insulin molecules

As regular insulin, porcine (Sigma-Aldrich, St. Louis, USA) and human insulin (Humulin-R, Eli Lilly and Company, Indianapolis, USA) was used. ^{LAPS}Insulin, ^{LAPS}Insulin115 and Insulin115 was kindly provided by Hanmi Pharmaceuticals (Seoul, South Korea). Insulin degludec was obtained from pharmacy. 20K-PEG Insulin was manufactured by Hanmi Pharmaceuticals (Seoul, South Korea). ^{125}I-labeled insulin was purchased from Phoenix Pharmaceuticals (Burlingame, USA). ^{125}I-labeled IGF-1 was obtained from Perkin Elmer (Massachusetts, USA).

Receptor signaling in rats

All experiments were performed with approval of the local government committee, the ‘‘Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen’’. Male Wistar rats were obtained by Charles River (HsdCpb:WU) (Charles River, Sulzfeld, Germany) and were used at 10 weeks of age. Animals were food deprived for at least for 8 h prior sacrifice. Rats received a single subcutaneous injection of 60 nmol/kg ^{LAPS}Insulin115 diluted in saline, or saline alone (control group). After indicated time points rats were anaesthetized with 1.5% isoflurane, sacrificed by decapitation, blood samples were collected, and skeletal muscle (M. gastrocnemius), liver and visceral adipose tissue were removed and snap frozen. Subsequently, tissues were lysed and processed as described before [12].

Trans-endothelial passage

$5x10^4$ hCAEC were seeded in the upper compartment of a transwell system (24-well, pore size 0.4 µm, Corning Incorporated Life Sciences, New York, USA) as described before [13], and were maintained in Endothelial Cell Medium MV (PromoCell, Heidelberg, Germany) containing 20% FCS for 6 d. Then, medium was switched to Endothelial Cell Medium MV containing 20% heat-inactivated FCS and cells in the upper compartment were exposed to 100 nM regular insulin or ^{LAPS}Insulin115. Regular insulin or ^{LAPS}Insulin115 concentrations in the lower compartment indicating trans-endothelial passage were measured in a time frame for up to 24 h. During the experiments, confluence of the endothelial monolayer was checked by phalloidin-staining and by the transfer of a 3 kDa FITC-dextran (ThermoFisher Scientific, Waltham, USA) as described before [13].

In vitro analysis of growth promoting activity

DNA synthesis in hCASMC was monitored as described before [14]. To assess the cell proliferative potency of the different insulin molecules in Saos-2 and MCF-7 cells [^{3}H]-thymidine incorporation into DNA was measured. Starved cells were incubated with the different insulin molecules for 19 or 15 h for Saos-2 or MCF-7 cells, respectively. After incubation, medium was removed and cells were washed with assay media described in cell culture section. [^{3}H]-thymidine solution (0.125 µCi per well as final concentration) was added to the cells and incubated for 6 h. Afterwards, cells were detached by 1xTE-buffer, transferred to unifilter-96 GF/C plate (Perkin Elmer, Massachusetts, USA) and dried for 4 h at 55°C. After adding MicroScint O solution (Perkin Elmer, Massachusetts, USA), β energy was measured by scintillation counter (Perkin Elmer, Topcounter) and EC_{50} value of insulin samples were calculated from 4-parameter logistic regression.

Analysis of protein abundance and phosphorylation

Cell lysis, SDS-PAGE and Western blot analysis was performed as described before [14]. Primary antibodies for anti-phospho-Akt(Ser473), anti-total-Akt, anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204), anti-phospho-IR beta (Tyr1150/1151 and Tyr1146) (Cell Signalling Technology, Frankfurt, Germany), anti-phospho-IR beta (Tyr972) (Abcam, Cambridge, United Kingdom), anti-IR (β-subunit) (Merck Millipore, Schwalbach, Germany), anti-α-tubulin (Merck Millipore, Schwalbach, Germany), anti-β-actin (Abcam, Cambridge, United Kingdom) and corresponding secondary HRP-coupled antibodies (Promega, Mannheim, Germany) were used.

RNA isolation, cDNA synthesis and qRT-PCR

RNA isolation, cDNA synthesis and qRT-PCR was performed as described before [14]. mRNA expression levels were determined with predesigned Quantitect Primer Assays for IR (QT02396128) and β-Actin (QT01680476).

Presentation of data and statistics

Data are expressed as mean ± S.E.M. Unpaired two-tailed Student's t-test or one-way ANOVA (post hoc test: Bonferroni) were used to determine statistical significance. All statistical analyses were done using Prism (GraphPad, La Jolla, CA, USA) considering a p-value < 0.05 as statistically significant.

Results

^{LAPS}Insulin115 exhibits a prolonged pharmacokinetic and pharmacodynamic profile compared to ^{LAPS}Insulin

The long-acting basal insulin ^{LAPS}Insulin115 consists of the novel insulin analogue Insulin115 conjugated to the non-glycosylated FC region of a human immunoglobulin G4 fragment via a small PEG linker and is developed for once-weekly administration (Suppl. Fig. 1a). After subcutaneous injection to rats, the half-life of ^{LAPS}Insulin115 is 2.4-fold extended compared to ^{LAPS}Insulin (human insulin conjugated to LAPS-carrier via PEG linker) and even 7-fold and 15-fold elevated in comparison to the other long-acting insulin analogues 20 kDa PEG-Insulin and insulin degludec, respectively (Suppl. Fig. 1b). To assess the glucose lowering efficacy of ^{LAPS}Insulin115, fasting blood glucose levels were measured after a single subcutaneous administration in diabetic $^{db/db}$mice. ^{LAPS}Insulin115 was found to normalize fasting blood glucose levels in a dose-dependent manner for more than 7 days, whereas blood glucose levels after ^{LAPS}Insulin administration started to increase after 3 days already (Suppl. Fig. 1c). Furthermore, to achieve a similar reduction in HbA1c in $^{db/db}$mice a 4-fold lower concentration of

^{LAPS}Insulin115 is sufficient compared to ^{LAPS}Insulin (Suppl. Fig. 1d).

^{LAPS}Insulin115 crosses the endothelial barrier and induces insulin signaling in all target tissues

Since the endothelium plays a pivotal role in drug distribution in vivo, we analyzed whether ^{LAPS}Insulin115 is able to cross the endothelial barrier via an in vitro permeability assay as described before [13]. Confluency of human coronary artery endothelial cells (hCAEC) was determined visually by phalloidin-staining and quantitatively by transfer of a 3 kDa FITC-dextran (Suppl. Fig. 2). After 4 and 8 h, ^{LAPS}Insulin115 transport across the endothelial layer was significantly less efficient compared to regular insulin (Fig. 1a). However, after 24 h the same amount of ^{LAPS}Insulin115 and regular insulin passes the endothelial layer, most likely due to the absence of IR downregulation under these conditions (Fig. 1a and Suppl. Fig. 2). Furthermore, the induction of insulin signaling in the major insulin-sensitive tissues was investigated in rats. 8 h and 24 h after a single subcutaneous administration of 60 nmol/kg ^{LAPS}Insulin115, a concentration of 45 nmol/l and 137 nmol/l of ^{LAPS}Insulin115 was detected in blood plasma, respectively (Fig. 1b). In line with that, blood glucose levels were significantly decreased after 24 h (Fig. 1c). At the signaling level, liver and skeletal muscle showed a significantly induced IR phosphorylation 8 h after ^{LAPS}Insulin115 administration, with the liver showing the strongest response. However, after 24 h we found a significantly increased IR phosphorylation in all major insulin-sensitive tissues (Fig. 1d,e). Further downstream, we observed a significant Akt phosphorylation after 24 h in all analyzed tissues (Fig. 1f,g).

^{LAPS}Insulin115 interacts with the insulin receptor similar to regular insulin

First we measured the binding affinity of the different insulins to the IR and the IGF-1 receptor (Fig. 2a). The unconjugated Insulin115 showed a binding affinity to the IR and the IGF-1 receptor comparable to regular insulin. LAPS conjugation reduces the binding affinity to the IR to 1.7 % compared to regular insulin, whereas binding to the IGF-1 receptor was not detectable anymore. Additionally, we assessed the dissociation-rate of ^{LAPS}Insulin115 in H9c2-E2 myocytes, overexpressing the human IR (Fig. 2b,c). After 30 min pre-incubation with ^{LAPS}Insulin115, we observed a similar amount of ^{125}I-labeled insulin bound to the cells compared to regular insulin, indicating a comparable dissociation-rate for both insulins. In contrast, pre-incubation with the insulin analogue AspB10, which has a prolonged occupancy time on the receptor, leads to reduced binding of ^{125}I-labeled insulin (Fig. 2b), which is significantly decreased when compared to ^{LAPS}Insulin115 and regular insulin (Fig. 2c). Next, we assessed the IR phosphorylation pattern of ^{LAPS}Insulin115 after treatment of H9c2-E2 cells. To assure full action of ^{LAPS}Insulin115 we used a 5-fold higher concentration of ^{LAPS}Insulin115, which acutely showed a similar downstream signaling compared to 100 nM regular insulin (data not shown). At all three tyrosine phosphorylation sites, we did not detect any difference between ^{LAPS}Insulin115 and regular insulin after 10 min exposure (Fig. 2d-g).

^{LAPS}Insulin115 has a similar mitogenic potency compared to regular insulin

To exclude any carcinogenicity we analyzed the mitogenic potency of ^{LAPS}Insulin115 in cancer cell lines. In Saos-2 and MCF-7 cells, predominantly expressing the IGF-1 receptor, ^{LAPS}Insulin115 induces proliferation only by 5% compared to regular insulin (Fig. 3a). Additionally, we investigated the mitogenic potency of ^{LAPS}Insulin115 in human coronary artery smooth muscle cells (hCASMC), since smooth muscle cell proliferation is a hallmark in the development of atherosclerosis. After

short-term stimulation with the different insulin molecules, we could observe a significant Erk1/2 activation with insulin AspB10 only (Fig. 3c). After 24 h no increased ERK1/2 phosphorylation was detectable for any insulin (Fig. 3d). Consistent with the signaling data, only insulin AspB10 significantly induced proliferation using low (100 nM) and high (1000 nM) concentrations of the different insulins (Fig. 3e,f).

After long-term stimulation, ^{LAPS}Insulin115 exhibits a similar metabolic potency compared to regular insulin

Focusing on the metabolic potency, we analyzed the impact of ^{LAPS}Insulin115 on Akt activation and glucose uptake in human skeletal muscle cells. We did not observe an acute Akt activation with ^{LAPS}Insulin115 in contrast to the other insulin molecules (Fig. 4b). However, after long-term stimulation we could not detect any significant difference between regular insulin and ^{LAPS}Insulin115 anymore (Fig. 4c). A similar pattern was observed for the induction of glucose uptake in hSkMC. In the acute setting, the EC_{50} value of LAPSInsulin115 revealed a 20% potency to induce glucose uptake compared to regular insulin (Fig. 4d). However, after long-term exposure the ^{LAPS}Insulin115-induced glucose uptake was not significantly different from regular insulin anymore due to a drop down of the regular insulin-induced glucose uptake (Fig. 4e). To determine why regular insulin is less effective after 24 h exposure, IR protein abundance was assessed in both, short- and long-term setting. After 24 h the IR protein level was tremendously decreased in the regular insulin and Insulin115 treated cells. In contrast, treatment with 100 nM ^{LAPS}Insulin115 did not induce IR downregulation. Even 500 nM LAPSInsulin115 only slightly reduced the IR protein level, which was still significantly higher compared to regular insulin and Insulin115 (Fig. 4f-h).

Less insulin receptor downregulation by ^{LAPS}Insulin115 leads to improved insulin signaling after chronic exposure compared to regular insulin

To further elaborate on the absence of IR downregulation by ^{LAPS}Insulin115, we generated a time-course treating hCASMC with 100 nM of the different insulin molecules for up to 48 h. After 12 h the IR was substantially and comparable downregulated by regular insulin, Insulin115 and AspB10 but not by ^{LAPS}Insulin115. This absence of downregulation of the IR remained unaltered up to 48 h treatment (Fig 5b). To investigate whether the decreased IR downregulation is beneficial for maintaining insulin signaling under chronic exposure to high insulin concentrations, we established a cell culture model of insulin desensitization/resensitization in hCASMC (Fig. 5c). To assure ^{LAPS}Insulin115-induced downregulation of the IR, we chronically exposed the cells to 500 nM ^{LAPS}Insulin115 or regular insulin and observed a significantly decreased insulin-stimulated Akt phosphorylation in both conditions. However, the insulin signaling in the ^{LAPS}Insulin115 treated cells recovered almost completely within 48 h and was significantly improved compared to the regular insulin treated cells after 24 and 48 h recovery (Fig. 5e). The reduced Akt activation or the difference between the ^{LAPS}Insulin115 and the regular insulin exposed cells could not be explained by a reduction of total Akt levels (Fig 5f). In accordance to insulin signaling, chronic exposure leads to a significant reduction of the IR protein level in both conditions. However, in the ^{LAPS}Insulin115 treated cells the IR recovers completely within 48 h. In contrast, the IR protein level of the regular insulin treated cells recovers only to 45% within 48 h and was significantly decreased compared to the ^{LAPS}Insulin115 treated cells after 24 h and 48 h recovery (Fig. 5g). Interestingly, in both conditions the IR mRNA levels were not altered after

the chronic exposure as well as during the recovery phase (Fig. 5h).

Discussion

We present a comprehensive *in vivo* and *in vitro* characterization of the novel weekly insulin ^{LAPS}Insulin115. In rodents, ^{LAPS}Insulin115 showed a 15-fold extended serum half-life compared to the long-acting insulin degludec and a blood glucose lowering efficiency for up to 7 days. After entering the circulation, ^{LAPS}Insulin115 crosses the endothelial barrier comparable to regular insulin and induces insulin signaling in all major target tissues. *In vitro*, we studied the precise mechanism of ^{LAPS}Insulin115 action and found an equipotent metabolic and mitogenic potency after long-term stimulation compared to insulin. Remarkably, in an *in vitro* model of chronic exposure, ^{LAPS}Insulin115 does not desensitize target cells irreversibly in contrast to insulin most likely due to less IR downregulation.

This is the first comprehensive characterization of a weekly insulin based on Fc-technology, however other basal insulin analogues with the potential for once-weekly dosing are currently under development (e.g. PE0139 developed by PhaseBio [clinical phase I completed (Trial Number: NCT01835730)], Insulin 287 developed by Novo Nordisk [clinical phase I completed (Trial Number: NCT02148861)], AB101 developed by AntriaBio (preclinical phase [15])). While the modification of Insulin 287 is undisclosed, the once-weekly potential of PE1039 and AB101 results from genetic fusion of native insulin to an elastin-like peptide (ELP) polymer and from PEGylation of human insulin encapsulated into poly-lactic, poly-glycolic (PLGA) microspheres, respectively.

Since the basal insulin PEGlispro (BIL) gained considerable interest as a hepato-preferential insulin analogue most likely due to its hydrodynamic size leading to trapping in the sinusoidal space [16], we investigated whether this might also play a role for ^{LAPS}Insulin115. Beside an elevated hepatic glucose uptake induced by BIL, Moore and colleagues showed an increase in insulin signaling in the liver compared to human insulin [16]. Here, we demonstrate that ^{LAPS}Insulin115 with a molecular size of 59 kDa is able to cross an endothelial layer consisting of hCAEC in vitro indicating that ^{LAPS}Insulin115 absorption does not need a wide fenestration of the endothelium known for hepatic sinusoidal capillaries. Most importantly, 24 h after ^{LAPS}Insulin115 injection we observed a comparable insulin signaling in liver, skeletal muscle and adipose tissue in vivo suggesting that ^{LAPS}Insulin115 reaches all major target tissues without any preference.

It is well known that diabetes contributes to the progression of atherosclerosis and increases incidence of cancer; thus, it is essential to analyze the mitogenic potency of novel long-acting insulin analogues. The recombinant insulin analogue AspB10 was already in clinical development when it was linked to carcinogenic activity due to an increased IGF-1 receptor affinity and a sustained activation of the IR [17,18]. Regardingly, we show that ^{LAPS}Insulin115 has a very low binding affinity to the IR and no detectable affinity to the IGF-1 receptor compared to AspB10 as well as regular insulin. Additionally, ^{LAPS}Insulin115 has a faster dissociation rate of the IR compared to AspB10, suggesting a lower mitogenic potency. This was confirmed by measuring proliferation in hCASMC. Here, we observed no significant induction of proliferation by ^{LAPS}Insulin115 even with very high concentrations (1000 nM). Furthermore, in cancer cell lines with high expression of the IGF-1 receptor, ^{LAPS}Insulin115 induced proliferation only to 5% compared to insulin. Therefore, ^{LAPS}Insulin115 is not exhibiting an increased mitogenic activity and does not provide any indication for a pro-cancerogenic and pro-atherogenic potency.

The metabolic activity of ^{LAPS}Insulin115 was assessed by analyzing insulin signaling and glucose uptake in hSkMC. In line with other insulin analogues, such as insulin glargine and detemir [19], we found a lower metabolic potency of ^{LAPS}Insulin115 (~20%) compared to regular human insulin. However, in line with the pharmacodynamics data *in vivo*, stimulation of insulin signaling and glucose uptake by ^{LAPS}Insulin115 *in vitro* showed a flat and prolonged profile of action in contrast to regular insulin. Remarkably, the difference in the action profile of these two insulin molecules *in vitro* results most likely from a reduced IR downregulation by ^{LAPS}Insulin115 (see Fig. 5). Taken together, the metabolic action profile *in vitro* and the pharmacokinetic and pharmacodynamic data *in vivo* show that ^{LAPS}Insulin115 fulfills the requirements of a basal insulin analogue with a low peak-to-through ratio.

It is known that insulin analogues induce IR downregulation in proportion to their capacity to bind to the receptor [20]. In line with that, we do not observe a significant downregulation of the IR with 100 nM ^{LAPS}Insulin115 in three different cell types (hCAEC, hSkMC and hCASMC). However, when we increased the concentration to 500 nM, which showed equal receptor occupancy for regular insulin and ^{LAPS}Insulin115 in our dissociation assay, we observed a significant higher IR protein level in the ^{LAPS}Insulin115 treated hSkMC. Thus, it is more likely that the reduced IR downregulation by ^{LAPS}Insulin115 results from an altered processing of the insulin/receptor complex due to the increased molecular size.

Whether the reduced IR downregulation might have an effect on insulin responsiveness under conditions of chronic exposure was investigated by measuring insulin sensitivity of cells treated with high concentrations of regular insulin or ^{LAPS}Insulin115 for 5 days. In our in vitro model we observed that the chronic treatment by ^{LAPS}Insulin115 or regular insulin leads to insulin resistance in both conditions. However, insulin signaling in ^{LAPS}Insulin115 treated cells recovers almost completely in contrast to regular insulin treated cells. There are extensive studies in humans providing very similar results to those we obtained in our *in vitro* model. These studies investigated the IR level in monocytes prepared from peripheral blood and found a reduction of IR level after chronically elevated insulin levels due to overeating and obesity [21-24], and a return to normal receptor concentrations upon prolonged exposure to normal insulin levels, which can take up to several weeks [23]. Although monocytes are not considered as target cells for the metabolic action of insulin, previous studies have shown that changes in insulin binding to monocytes reflect alterations in IRs at target tissues such as liver and adipose tissue [23,25-28]. Taking this into account, our data indicate that ^{LAPS}Insulin115 treatment might lead to a faster recovery after treatment-associated increased insulin levels and therefore to an improvement in insulin sensitivity. However, to confirm our data long-term clinical studies in humans are required.

In conclusion, we report a comprehensive *in vivo* and *in vitro* profile of the novel weekly insulin ^{LAPS}Insulin115. This insulin shows several unique properties, such as a substantially prolonged pharmacokinetic and –dynamic profile, a very slow onset of action, no increased mitogenic potency, and most importantly it does not irreversibly desensitize target cells under chronic conditions, making ^{LAPS}Insulin115 an excellent candidate for a once-weekly insulin derivative.

Acknowledgement

The assistance of Dr. Henrike Sell in setting up the *in vivo* insulin signalling experiments, the technical assistance of Andrea Cramer, and the secretarial assistance of Birgit Hurow (Paul-

Langerhans-Group, German Diabetes Center) are acknowledged and highly appreciated.

N.W., T.H., J.E., I.Y.C. S.H.P. Y.M.L. and S.C.K. designed experiments. N.W., D.D.-S., T.H., I.I., S.W.G., and S.H.P. conducted experiments; N.W., T.H., S.W.G., I.Y.C., S.H.P. analysed the data, N.W., T.H., S.W.G., J.E., I.Y.C., Y.K., and M.H. interpret the data N.W. and T.H. wrote the manuscript. All authors critically revised the manuscript.

This study was funded by Profil Institute for Clinical Research.

Figures

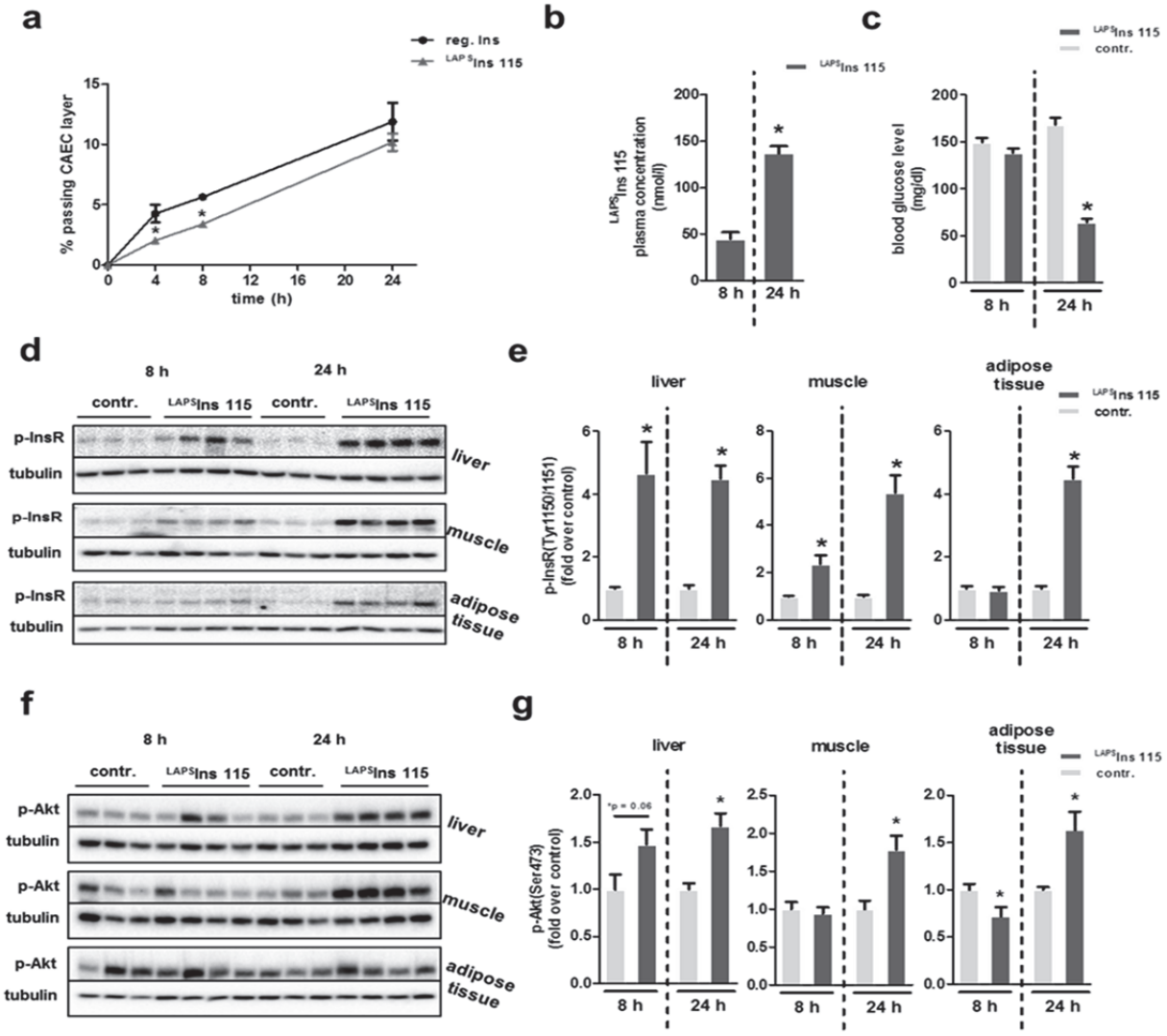


Figure 1: Endothelial passage and tissue-specific signaling of ^{LAPS}Insulin115. (a) Human coronary artery endothelial cells (hCAEC) were seeded on matrigel-coated polyethylene terephthalate (PET) filters of a transwell system. After reaching full confluency, cells in the upper compartment were exposed to 100 nM regular insulin or ^{LAPS}Insulin115. The amount of both insulins in the lower compartment were determined by ELISA. (n=3-4; mean±S.E.M.). **(b-g)** 8 h and 24 h after ^{LAPS}Insulin115 (60 nmol/kg) injection, Wistar rats were sacrificed, blood samples were taken and organs were isolated. **(b)** Plasma concentrations of ^{LAPS}Insulin115 were assessed by ELISA (n=6-8; mean±S.E.M.) **(c)** Blood glucose levels were determined with a One Touch Ultra Glucometer (n=6-8; mean±S.E.M.; *p<0.05 compared to control). **(d-g)** Organs were lysed and total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against the phosphorylated insulin receptor(Tyr$^{1150/1151}$) and Akt(Ser^{473}). **(d,f)** Representative Western blots and (e,g) respective quantifications are shown. Data are tubulin normalized (n=6-8; mean±S.E.M.; *p<0.05 compared to corresponding control). Ins, insulin; InsR, insulin receptor; Tyr, tyrosine; contr., control; -cells, without cells.

a

Test materials	Receptor binding (%)	
	Insulin receptor	IGF-1 receptor
reg. Ins	100	0.6
IGF-1	0.6	100
Asp^{B10}	248	2.5
Ins 115	86	0.5
^{LAPS}Ins 115	1.7	not detectable

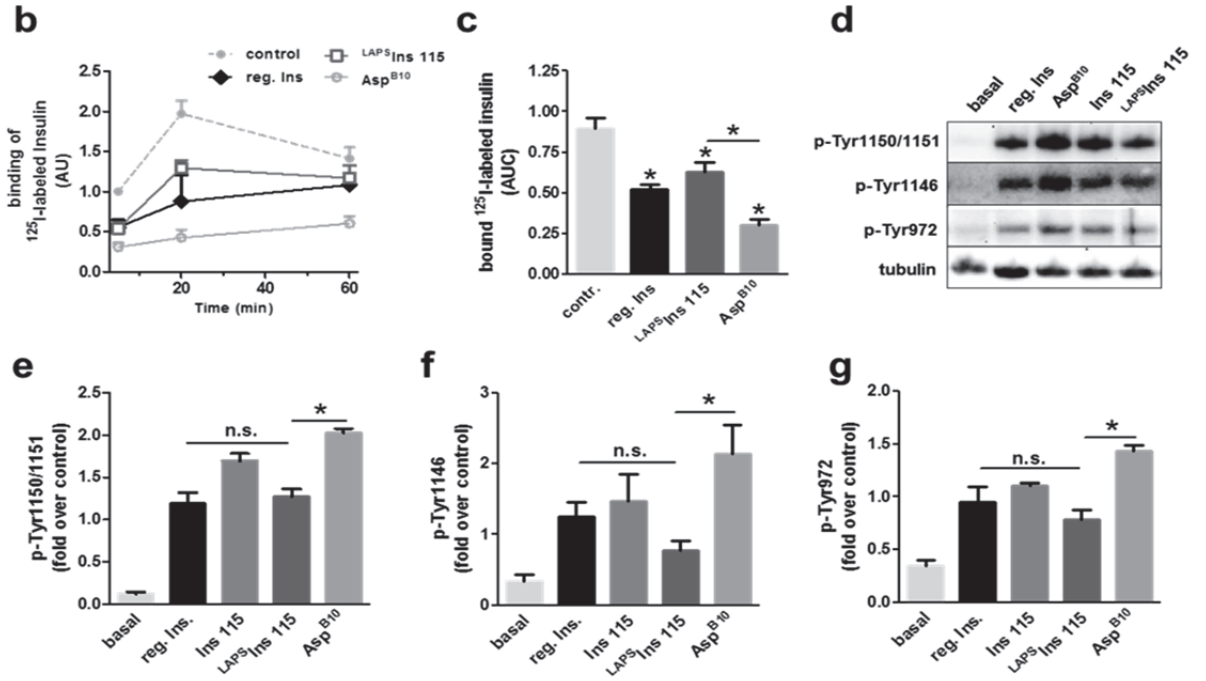


Figure 2: Insulin receptor interaction of ^{LAPS}Insulin115. (a) Binding affinity of the test materials was measured by competition of ^{125}I-labeled insulin or ^{125}I-labeled IGF-1 binding in a scintillation proximity assay (SPA) (n=3). **(b)** H9c2-E2 cells, overexpressing the human insulin receptor, were equilibrated with 500 nM of the different insulin molecules, washed, and ^{125}I-labeled regular insulin was then added for different time points to allow receptor binding. Insulin AspB10 was used as a positive control (n=3; mean±S.E.M.). **(c)** Calculated area under the curve from **(b)** reflecting the dissociation-rate of the different insulin molecules (n=3; mean±S.E.M.; *p<0.05 compared to control or as indicated). **(d-g)** After starvation in serum- and insulin-free medium for 2 h, H9c2-E2 cells were stimulated with the different insulin molecules for 10 min. Regular insulin, insulin AspB10 and Insulin115 were used in a concentration of 100 nM and ^{LAPS}Insulin115 was given in a concentration of 500 nM. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody to the phosphorylated insulin receptor tyrosine site 1150/1151, 1146 and 972. **(d)** Representative Western blots and **(e-g)** respective quantifications are shown. Data are tubulin normalized (n=3-4; mean±S.E.M.; *p<0.05 compared to control or as indicated). Ins, insulin; Tyr, tyrosine; reg. Ins, regular insulin; contr., control; AU, arbitrary unit; AUC, area under the curve.

a

Test materials	Mitogenic potency (%)	
	Saos-2	MCF-7
Reg. Ins	100	100
IGF-1	6,255	1,527
Asp^{B10}	756	912
Ins 115	119	88
^{LAPS}Ins 115	5.3	5.4

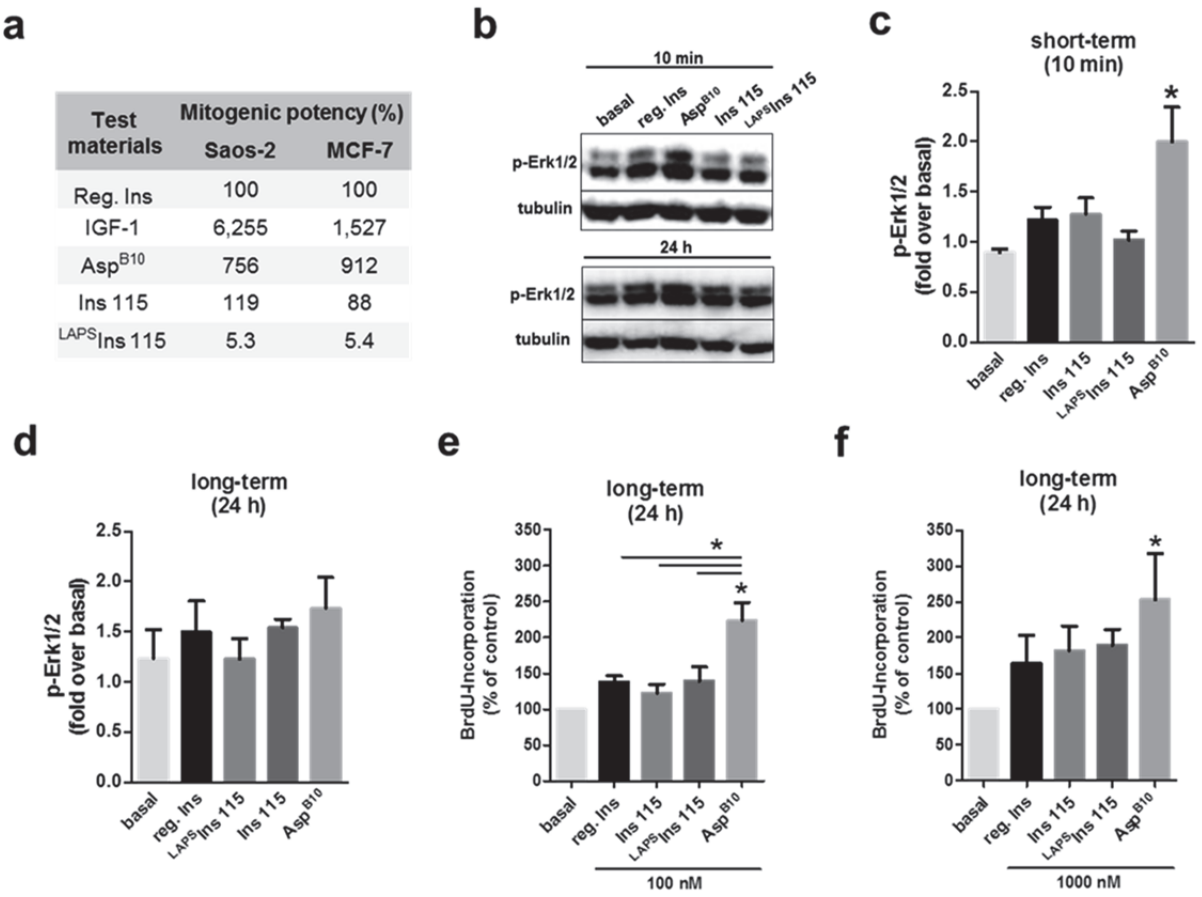


Figure 3: Mitogenic potency of ^{LAPS}Insulin115. (a) The mitogenic potency was determined by [^{3}H]-thymidine incorporation into DNA. After 24 h starvation, Saos-2 and MCF-7 cells were exposed to the different Insulin molecules. EC_{50} values were calculated and regular insulin was taken as 100% (n=3). **(b-d)** Human coronary artery smooth muscle cells (hCASMC) were treated short-term (10 min) or long-term (24 h) with 100 nM of the indicated insulins. Erk1/2 phosphorylation was assessed by Western blot analysis as described before. **(b)** Representative Western blots and **(c,d)** respective quantifications are shown. Data are tubulin normalized (n=5-6; mean±S.E.M.; $p<0.05$ compared to basal). **(e,f)** Proliferation of hCASMC was determined by measuring the incorporation of BrdU into DNA. Cells were treated with indicated insulins in a concentration of 100 nM **(e)** or 1000 nM **(f)** for 24 h. Data are expressed relative to the basal control value taken as 100% (n=6; mean±S.E.M.; *$p<0.05$ compared to basal or as indicated). Ins, insulin; reg. Ins, regular insulin; BrdU, Bromodeoxyuridine.

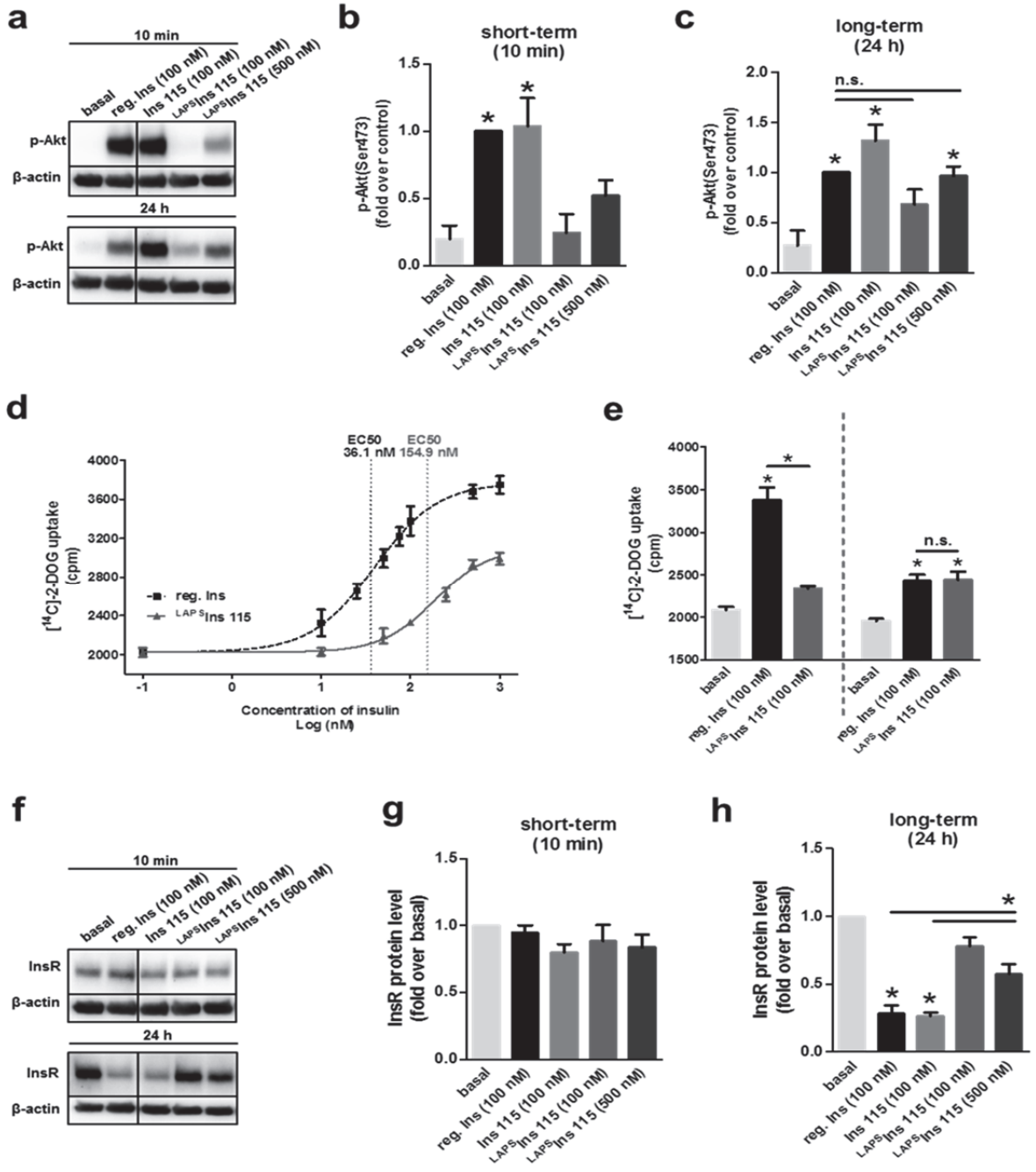


Figure 4: Metabolic potency of ^{LAPS}Insulin115. (a-c) Human skeletal muscle cells (hSkMC) were treated short-term (10 min) or long-term (24 h) with 100 or 500 nM of the indicated insulins. Akt(Ser^{473}) phosphorylation was assessed by Western blot analysis as described before. **(a)** Representative Western blots and **(b,c)** respective quantifications are shown. Data are β-actin normalized (n=4, mean±S.E.M.; *p<0.05 compared to basal or as indicated). **(d)** Concentration-response curve of regular insulin and ^{LAPS}Insulin for stimulating glucose uptake in hSkMC. Glucose uptake was measured by ^{14}C-labeled 2-deoxy-D-glucose (2-DOG) for 2 h. **(e)** Cells were stimulated 30 min (left panel) or 22 h (right panel) with 100 nM regular insulin or ^{LAPS}Insulin115. Glucose uptake was measured as stated before (n=3; mean±SED; *p<0.05 compared to basal or as indicated). **(f-h)** hSkMC were treated short-term (10 min) or long-term (24 h) with 100 or 500 nM of the indicated insulins. Insulin receptor protein level was assessed by Western blot analysis as described before. **(f)** Representative Western blots and **(g,h)** respective quantifications are shown. Data are β-actin normalized (n=4, mean±S.E.M.; *p<0.05 compared to basal or as indicated). Ins, insulin; InsR, insulin receptor; reg. Ins, regular insulin; 2-DOG, 2-deoxy-D-glucose; cpm, counts per minute.

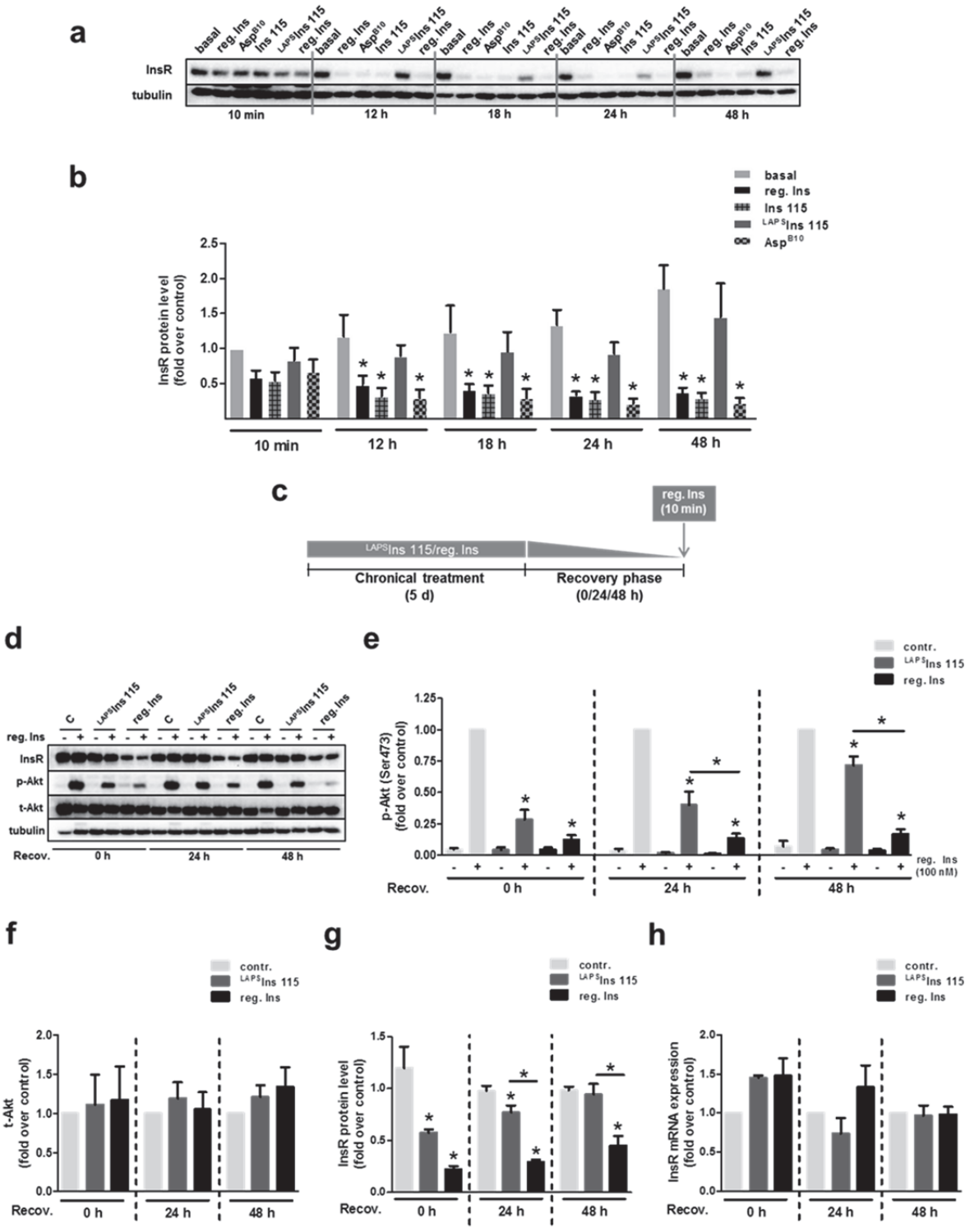


Figure 5: Effect of chronic exposure to ^{LAPS}Insulin115 on the insulin signaling cascade. **(a,b)** HCASMC were treated with 100 nM of the indicated insulins for up to 48 h. Insulin receptor protein level was assessed by Western blot analysis as described before. **(a)** Representative Western blot and **(b)** respective quantification is shown. Data are tubulin normalized (n=5; mean±S.E.M.; $p<0.05$ compared to basal). **(c)** Experimental design of the insulin desensitization/resensitization cell culture model. **(d-h)** HCASMC were exposed to 500 nM ^{LAPS}Insulin115 or regular insulin for 5 days, followed by a recovery phase in serum- and insulin-free medium for indicated time period. Afterwards cells were acutely (10 min) stimulated with 100 nM regular insulin. **(d-g)** Akt(Ser^{473}) phosphorylation, total Akt and insulin receptor protein abundance were assessed by Western blot analysis. **(d)** Representative

Western blots and **(e-g)** respective quantifications are shown. Data are tubulin normalized (n=3-4, mean±S.E.M.; *p<0.05 compared to corresponding control or as indicated). **(h)** Insulin receptor mRNA level was quantified by real-time PCR and normalized to the level of β-actin. Data are expressed relative to control (n=3-4, mean±S.E.M.). Ins, insulin; InsR, insulin receptor; reg. Ins, regular insulin; contr., control; recov., recovery.

Reference List

1. Owens DR, Matfin G, Monnier L: Basal insulin analogues in the management of diabetes mellitus: What progress have we made? Diabetes Metab Res Rev 2014;30:104-119

2. Becker RH, Dahmen R, Bergmann K, Lehmann A, Jax T, Heise T: New insulin glargine 300 Units . mL-1 provides a more even activity profile and prolonged glycemic control at steady state compared with insulin glargine 100 Units . mL-1. Diabetes Care 2015;38:637-643

3. Gough SC, Harris S, Woo V, Davies M: Insulin degludec: overview of a novel ultra long-acting basal insulin. Diabetes Obes Metab 2013;15:301-309

4. Sinha VP, Choi SL, Soon DK, Mace KF, Yeo KP, Lim ST, Howey DC: Single-dose pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 in healthy subjects. J Clin Pharmacol 2014;54:792-799

5. Hirose T: Development of new basal insulin peglispro (LY2605541) ends in a disappointing result. Diabetology International 2016;7:16-17

6. Rath T, Baker K, Dumont JA, Peters RT, Jiang H, Qiao SW, Lencer WI, Pierce GF, Blumberg RS: Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. Crit Rev Biotechnol 2015;35:235-254

7. Strohl WR: Fusion Proteins for Half-Life Extension of Biologics as a Strategy to Make Biobetters. BioDrugs 2015;29:215-239

8. Kontermann RE: Strategies for extended serum half-life of protein therapeutics. Curr Opin Biotechnol 2011;22:868-876

9. Roopenian DC, Akilesh S: FcRn: the neonatal Fc receptor comes of age. Nat Rev Immunol 2007;7:715-725

10. Rosenberg AS: Immunogenicity of biological therapeutics: a hierarchy of concerns. Dev Biol (Basel) 2003;112:15-21

11. Barbosa MD: Immunogenicity of biotherapeutics in the context of developing biosimilars and biobetters. Drug Discov Today 2011;16:345-353

12. Burghoff S, Flogel U, Bongardt S, Burkart V, Sell H, Tucci S, Ikels K, Eberhard D, Kern M, Kloting N, Eckel J, Schrader J: Deletion of CD73 promotes dyslipidemia and intramyocellular lipid accumulation in muscle of mice. Arch Physiol Biochem 2013;119:39-51

13. Martins-Green M, Petreaca M, Yao M: An assay system for in vitro detection of permeability in human "endothelium". Methods Enzymol 2008;443:137-153

14. Wronkowitz N, Gorgens SW, Romacho T, Villalobos LA, Sanchez-Ferrer CF, Peiro C, Sell H, Eckel J: Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. Biochim Biophys Acta 2014;1842:1613-1621

15. Roberts BK, Wang X, Rosendahl MS, Mantripragada S: The In Vitro and In Vivo Pharmacology of AB101, a Potential Once-Weekly Basal Subcutaneous Insulin. Diabetes 2015;64 [Suppl. 1]:A 25

16. Moore MC, Smith MS, Sinha VP, Beals JM, Michael MD, Jacober SJ, Cherrington AD: Novel PEGylated basal insulin LY2605541 has a preferential hepatic effect on glucose metabolism. Diabetes 2014;63:494-504

17. Tennagels N, Werner U: The metabolic and mitogenic properties of basal insulin analogues. Arch Physiol Biochem 2013;119:1-14

18. Hansen BF, Danielsen GM, Drejer K, Sorensen AR, Wiberg FC, Klein HH, Lundemose AG: Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. Biochem J 1996;315 (Pt 1):271-279

19. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C, Trub T: Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. Diabetes 2000;49:999-1005

20. Kosmakos FC, Roth J: Insulin-induced loss of the insulin receptor in IM-9 lymphocytes. A biological process mediated through the insulin receptor. J Biol Chem 1980;255:9860-9869

21. Archer JA, Gorden P, Gavin JR, III, Lesniak MA, Roth J: Insulin receptors in human circulating lymphocytes: application to the study of insulin resistance in man. J Clin Endocrinol Metab 1973;36:627-633

22. Archer JA, Gorden P, Roth J: Defect in insulin binding to receptors in obese man. Amelioration with calorie restriction. J Clin Invest 1975;55:166-174

23. Bar RS, Gorden P, Roth J, Kahn CR, De MP: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding, and dieting. J Clin Invest 1976;58:1123-1135

24. Bar RS, Harrison LC, Muggeo M, Gorden P, Kahn CR, Roth J: Regulation of insulin receptors in normal and abnormal physiology in humans. Adv Intern Med 1979;24:23-52

25. Soll AH, Goldfine ID, Roth J, Kahn CR: Thymic lymphocytes in obese (ob-ob) mice. A mirror of the insulin receptor defect in liver and fat. J Biol Chem 1974;249:4127-4131

26. DeFronzo RA, Soman V, Sherwin RS, Hendler R, Felig P: Insulin binding to monocytes and insulin action in human obesity, starvation, and refeeding. J Clin Invest 1978;62:204-213

27. Olefsky JM: Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J Clin Invest 1976;57:1165-1172

28. Olefsky JM, Jen P, Reaven GM, Alto P: Insulin binding to isolated human adipocytes. Diabetes 1974;23:565-571

29. Gorgens SW, Eckardt K, Elsen M, Tennagels N, Eckel J: Chitinase-3-like protein 1 protects skeletal muscle from TNFalpha-induced inflammation and insulin resistance. Biochem J 2014;459:479-488

30. Uhlig M, Passlack W, Eckel J: Functional role of Rab11 in GLUT4 trafficking in cardiomyocytes. Mol Cell Endocrinol 2005;235:1-9

Supplementary Material

Generation of Insulin 115 and ^{LAPS}Insulin 115

To obtain the Insulin 115 gene, site-directed mutagenesis PCR was performed with the native human insulin gene as template DNA, followed by cloning into pET-22b (+) plasmid vector (Merck). Insulin 115 gene was transformed into the BL21DE3 Escherichia coli strain and overexpressed in inclusion bodies (IB). Harvested cells were lysed and disrupted using a Microfluidizer (ACTechnology). Pellets were collected and then dissolved in glycine buffer containing 6 M urea. The dissolved IB was slowly diluted 3–10 fold for refolding into a native tertiary structure with disulfide bridges. Fully regenerated proinsulin was converted to insulin by photolytic enzymes, and finally purified using three steps of column chromatography.

To conjugate Insulin 115 to the LAPS-carrier, Insulin 115 was mixed with homo bi-functional aldehyde PEG (MW 3,400 kDa) in the presence of sodium cyanoborohydride. Upon completion of the modification reaction, mono-PEGylated peptides (proteins) were purified using ion exchange chromatography. For conjugation, human aglycosylated Fc-fragment was added to the PEGylated Insulin 115 at established optimal condition. For site specificity, the reaction was performed under highly specific conditions (pH 5.5–8.5) with sodium cyanoborohydride. LAPS-carrier-conjugated Insulin 115 was purified by a process including hydrophobic interaction chromatography and anion exchange chromatography.

Cell culture

Human skeletal muscle cells (hSkMC) of healthy Caucasian donors were supplied as proliferating myoblasts (5x 10^5 cells) and were cultured as described previously (29). Shortly, myoblasts were seeded in 6-well culture dishes at a density of $1x10^5$ cells per well and cultured in a-modified DMEM/F12 medium containing skeletal muscle cell growth medium supplement pack (Promocell, Heidelberg, Germany) up to near confluence. The cells were then differentiated and fused by culture in a modified DMEM for 4 days and used for experiments.

Human coronary artery smooth muscle cells (hCASMC) were obtained from PromoCell (Heidelberg, Germany), Tebu-bio (Le Perray-en-Yvelines, France) and Lonza (Basel, Switzerland). Cells from four different donors were supplied as proliferating cells and kept in culture according to the manufacturer's protocol. For all experiments, subconfluent cells of passage five were used. Cells were seeded in six-well culture dishes at a density of $2x10^5$ cells per well and cultured in DMEM medium containing 10% FCS. Cells were allowed to attach for 24 h and then serum-starved for further 24 h.

Human coronary artery endothelial cells (hCAEC) were obtained from PromoCell (Heidelberg, Germany) and were cultured in Endothelial Cell Medium MV (PromoCell, Heidelberg, Germany), containing 20% FCS. For all experiments cells of passage four were used.

Human osteosarcoma cells (Saos-2) were obtained from ATCC (cat. #HTB-85) and were maintained in growth medium (McCoy's 5A with 15% FBS and 1% Pen/Strep). For an assay, $1x10^4$ cells per well were seeded in 96-well plate and allowed to attach for 24 h. Afterwards cells were washed once and starved for 4 h with assay medium (McCoy's 5A with 0.5% BSA and 1% Pen/Strep).

Human breast adenocarcinoma (MCF-7) cells were obtained from ATCC (cat#HTB-22) and were maintained in growth medium (RPMI1640 with 10% FBS and 1% PS). For an assay, $1x10^4$ cells per well were seeded in growth medium to 96-well plate and allowed to attach for 24 h.

Afterwards cells were washed once and starved for 15 h with assay medium (phenol red free RPMI 1640 including 1% Pen/Strep).

H9c2 cells obtained from ATCC (cat. #CRL-1446) were stably transfected with the human insulin receptor in our laboratory (H9c2-E2) (30). Cells were cultivated in DMEM (low glucose, 10% FCS, 1% non-essential amino acids and 600 µg/ml G418 (all from Invitrogen, Carlsbad, CA, USA)). Cells were seeded in 6-well culture dishes at a density of $2.5x10^5$ cells per well. Cells were cultured until reaching full confluency and then serum-starved for 2 h in DMEM low glucose.

Measurement of the different insulin molecules

Concentrations of regular insulin and ^{LAPS}Insulin 115 were determined with the Insulin Quantikine ELISA Kit (RnD Systems, Minneapolis, MN, USA). Insulin was detected according to the manufacturer´s protocol. For detection of ^{LAPS}Insulin 115, a horse radish peroxidase (HRP)-conjugated mouse anti-human IgG4 (Alpha Diagnostics Inc, San Antonio, TX, USA) was used as secondary antibody. Concentrations of 20K-PEG Insulin and insulin degludec were measured with iso-Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Binding studies

The binding of human insulin, IGF-1, insulin AspB10, Insulin 115, and ^{LAPS}Insulin 115 to insulin receptor or IGF-1 receptor was determined by competition of ^{125}I-labeled insulin or ^{125}I-labeled IGF-1 binding in a scintillation proximity assay (SPA), respectively. Membranes were isolated from insulin receptor or IGF-1 receptor overexpressing CHO cells by one-step sucrose gradient method. Membranes (2 µg for insulin receptor and 20 µg for IGF-1 receptor) were incubated with 250 µg WGA PVT SPA beads (Perkin Elmer, Massachusetts, USA), 250 pM of ^{125}I-labeled insulin or ^{125}I-labeled IGF-1 and diluted insulin samples. The radioactivity was measured after 4 h incubation at room temperature by using scintillation counter (Topcount NTX , Perkin Elmer, Massachusetts, USA).

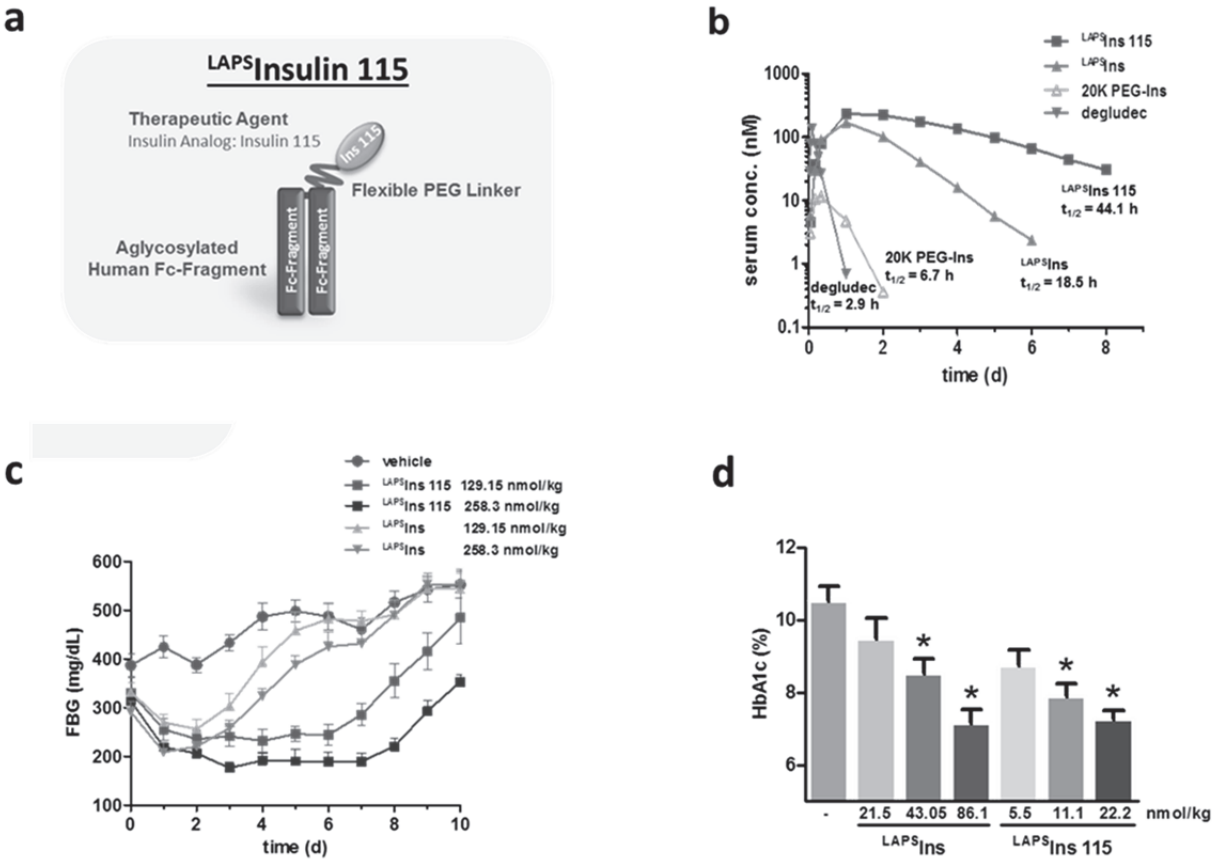
Dissociation studies

Medium was changed directly before H9c2-E2 cells were exposed to 500 nM regular insulin, ^{LAPS}Insulin 115 or AspB10 for 30 min. Untreated cells were used as control. Subsequently, cells were washed twice with ice cold PBS to remove unbound insulin. 0.2 µCi of ^{125}I-labeled insulin diluted in warm DMEM was added to each well and cells were kept at 37°C for 5, 20 or 60 min to allow binding of ^{125}I-labeled insulin. Cells were washed again with ice cold PBS and lysed with 1 M NaOH for 30 min. Lysates were counted in a 1282 Compugamma CS Universal Gamma Counter (LKB Wallac, Victoria, Australia). Unspecific binding was determined by incubating the cells with 1 µM unlabeled regular insulin in combination with 0.2 µCi of ^{125}I-labeled insulin.

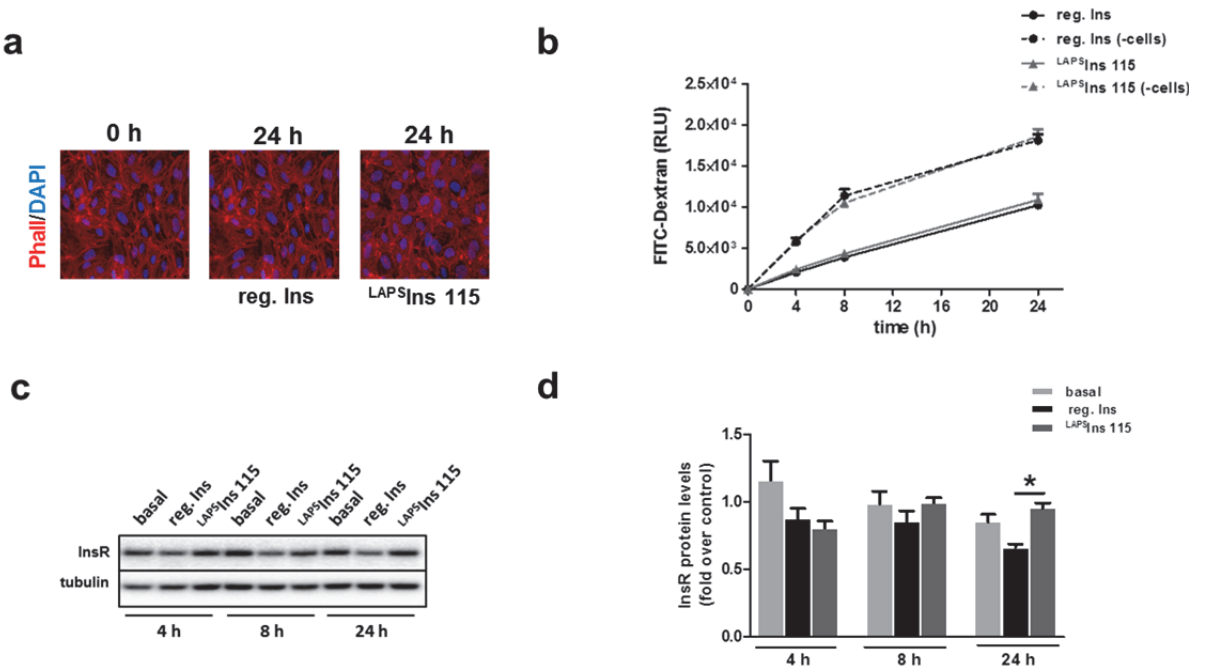
Glucose Uptake

Differentiated human skeletal muscle cells were either short-term (30 min) or long-term (22 h) stimulated with regular insulin or ^{LAPS}Insulin 115 (concentration as indicated). Then, 0.25 µCi 2-deoxy-D-glucose (2-DOG) was added to each well and uptake was measured for 2 h. The experiment was terminated by repeated washing with ice-cold 0.25 µM cytochalasin B and cells were then lysed with 1 M NaOH. The radioactivity of the lysates was counted in a liquid scintillation counter (Beckman). Values were corrected for non-specific uptake as measured after incubation with L-[^{14}C] glucose (Perkin Elmer, Massachusetts, USA).

Supplementary Figures



Supplementary Figure 1: Pharmacokinetics and pharmacodynamics of ^{LAPS}Insulin115. **(a)** Schematic overview of the Long Acting Peptide/Protein Discovery Technology (LAPSCOVERY™) used to generate ^{LAPS}Insulin115. **(b)** After single subcutaneous administration (^{LAPS}Insulin115/^{LAPS}Insulin: 65.1 nmol/kg; 20K PEG-Insulin: 65.1 nmol/kg; insulin degludec: 55.8 nmol/kg) to rats, blood samples were collected at indicated time points, followed by analyzing serum concentrations of the different insulin molecules by ELISA (n=3-5; mean±S.E.M.). **(c)** After a single subcutaneous administration of ^{LAPS}Insulin115 or ^{LAPS}Insulin to $^{db/db}$mice, blood samples were collected at indicated time points after 4 h fasting, and blood glucose levels were measured with a One Touch Ultra Glucometer (n=7; mean±S.E.M.). (d) ^{LAPS}Insulin115 and ^{LAPS}Insulin were subcutaneously administrated every other day (Q2D) to $^{db/db}$mice. Blood samples were collected after 4 weeks of repeated dosing and HbA1c was determined by DCA vantage analyzer (n=6; mean±S.E.M.; *p<0.05 compared to vehicle). Ins, insulin; 20K PEG-Ins, 20 kDa PEG-Insulin; reg., regular.



Supplementary Figure 2: Validation of the in vitro endothelial permeability assay. (a,b) Human coronary artery endothelial cells (hCAEC) were seeded on matrigel-coated polyethylene terephthalate (PET) filters of a transwell system. Cells were cultured 6 d in the upper compartment of the transwell system until they reach full confluency. **(a)** To assure full confluency of the endothelial monolayer, cells were stained against phalloidin (red) and with nuclear dye, DAPI (blue), at the starting (0 h) and end (24 h) point of the experiment. **(b)** The unspecific transport of a 3 kDa FITC dextran was measured to compare the confluence in every well of each condition (with and without cells), (n=4, mean±S.E.M.). **(c,d)** HCAEC were treated with 100 nM of the indicated insulins for up to 24 h. Insulin receptor protein level was assessed by Western blot analysis as described before. **(a)** Representative Western blot and **(b)** respective quantification is shown. Data are tubulin normalized (n=5-6; mean±S.E.M.; $p<0.05$ compared to basal). Scale bar, 50 µm **(a)**. Ins, insulin; reg. Ins, regular insulin; phall, phalloidin; -cells, without cells.

2.3 Contribution Statement

Effect of the long-acting insulin analogues glargine and degludec on cardiomyocyte cell signalling and function

Hartmann T, Overhagen S, Ouwens DM, Raschke S, Wohlfart P, Tennagels N, Wronkowitz N, Eckel J. Cardiovasc. Diabetol. (2016) **15**:96

Impact factor (2016): 4.534

Contribution	**Total:**	**90.8 %**
	Conceived/designed experiments:	85 %
	Performed experiments:	85 %
	Analysed data:	90 %
	Contributed to discussion:	95 %
	Wrote the manuscript:	100 %
	Reviewed/edited manuscript	90 %
	Author: 1st author	

^{LAPS}Insulin 115: A novel ultra-long-acting basal insulin with a unique action profile

Wronkowitz N, **Hartmann T**, Görgens S, Dietze-Schroeder D, Indrakusuma I, Choi IY, Park SH, Lee YM, Kwon SC, Kang Y, Hompesch M, Eckel J. Diabetes Metab. Obes. (2017) submitted

Impact factor (2016): 6.198

Contribution	**Total:**	**40 %**
	Conceived/designed experiments:	35 %
	Performed experiments:	45 %
	Analysed data:	45 %
	Contributed to discussion:	25 %
	Wrote the manuscript:	20 %
	Reviewed/edited manuscript	70 %
	Author: 2nd author	

3 Discussion

3.1 Effects of Insulin and Long-Acting Insulin Analogues in Cardiomyocytes

Traditionally the liver, skeletal muscle and adipose tissue are considered as main targets of insulin. However, other tissues, such as the central nervous system (CNS) and the cardiovascular system, are affected by insulin and its signalling as well [223, 224]. The heart expresses 10,000 – 100,000 IR per cardiomyocyte [223] and shows similarities in insulin action with the aforementioned tissues. Those effects are increased glucose transport, modification of metabolism, protein synthesis, and inhibition of apoptosis [107, 108, 223]. Additionally, insulin triggers (cardio)myocyte-specific effects (e.g. increased contractility and beating-rate due to enhanced Ca^{2+} signalling) [223]. Furthermore, since the FDA raised concerns about potential cardiovascular side-effects of IDeg (as described in chapter 1.3.3) and the lack of availability of *in vitro* studies comparing the molecular effects of long-acting insulin analogues on cardiomyocyte cell models, a comprehensive *in vitro* study comparing IGla, its active metabolite IGlaM1 and IDeg in insulin signalling and important functional readout experiments, were performed.

The binding affinity of insulin analogues towards the IR is important for triggering insulin signalling. Since binding data of IDeg are scarce with only one published poster presentation at the EASD 2010 [172], binding affinities of the analogues in two different binding competition assays were analysed. One using solubilised IR preparations (S-IR), as used in the previously published study and the other using membrane embedded IR preparations (M-IR). Interestingly, the two assays showed different receptor affinities. We did not observe a difference in S-IR preparations between the analogues (Study 1, Supplementary Figure 1) with IGlaM1 showing an IC_{50} of 2.09 ± 0.74 nmol/L and IDeg showing an IC_{50} of 3.12 ± 0.42 nmol/L (Study 1, Supplementary Table 1), which is similar to previously published data [172]. However, when the IC_{50} values in a competition binding assay using M-IR preparations were compared, a tremendous right shift of the inhibitory curve was observed when IDeg was used, compared to Ins and IGlaM1 (Study 1, Figure 1A). In these experiments Ins and IGlaM1 showed similar results as seen in the S-IR preparations. However, IDeg showed a more than 6-fold increased IC_{50} compared to the experiments using S-IR preparations (Study 1, Table 1). A potential explanation for the lower affinity of IDeg in

M-IR preparations is the complex environment of phospholipids and other membrane embedded proteins besides the IR. This could lead to interaction between the fatty acid attached to the B-chain of IDeg and the surrounding of the IR.

The heart requires a high amount of energy to maintain its contraction/relaxation cycle. It consumes about 3.5 – 5 kg ATP per day, which is produced by the heart itself. [225]. The heart relies on oxidative metabolism, reflected by a high mitochondria content which accounts for up to 30 % of the cardiac cell volume [226]. In the fasting state, most of the energy is produced via β-oxidation of long-chain fatty acids (LCFA) [227] and only one fifth of the energy is produced by glucose oxidation [108]. However, in postprandial conditions with high insulin level, glucose becomes the favoured substrate for hearts energy production [108]. This effect is partly dependent on glucose uptake via GLUT4 translocation triggered by insulin signalling [228]. As depicted in Figure 2 the central molecule for insulins metabolic effects is Akt [110]. Thus, it is important for insulin analogues to exhibit a comparable Akt activation as it is observed with Ins. In this study Akt phosphorylation kinetics of IGla, IGlaM1 and IDeg in comparison to Ins *in vitro* in different cardiomyocyte cell models was compared. Stimulation of freshly isolated adult rat ventricular myocytes (ARVM) with 100 nM of the various insulins showed no difference between IGla, IGlaM1, IDeg and Ins on both phosphorylation sites of Akt (Thr^{308} and Ser^{473}) (Study 1, Figure 2A and Supplementary Figure 3A). Interestingly, a slower Akt activation with IDeg was observed, compared to the other insulins when HL-1 cardiomyocytes were treated with 200 nM of the respective insulins (Study 1, Figure 1B-D and Supplementary Figure 2). While IDeg showed significantly lower Akt activation after 5- and 10 min stimulation compared to Ins, IGla and IGlaM1 was comparable to Ins. Only after 60 min IDeg reached a comparable level of Akt activation to Ins and IGlaM1. However, it was still significantly lower compared to IGla. The slower Akt kinetics observed by IDeg can be explained by the lower binding affinity which is described above. The slightly higher Akt activation by IGla can be explained by its higher IGF-1R affinity, as described in chapter 1.3.3. Accordingly, the full activation of Akt by all insulin analogues after 60 min in HL-1 cells led to comparable glucose uptake in these cells. The glucose uptake was between 1.46- to 1.58-fold increased with the insulins used (Study 1, Figure 5). The rather mild effects of insulin in inducing glucose uptake can be explained by high expression levels of GLUT1 compared to relatively low expression levels of GLUT4 in HL-1 cells [229]. Since GLUT1 is insulin-independent and accounts for glucose uptake under basal conditions, GLUT4 responds to insulin stimulation [230]. Thus, an already high glucose

uptake in basal conditions can be expected and the fold-increase after insulin stimulation cannot be as strong as observed in other cells [231].

Another common effect of insulin in several tissues, and highly important in the heart, is the inhibition of apoptosis by insulin. The ability of insulin to reduce damage inflicted by ischemia followed by reperfusion (IRI) was shown in several *in vivo* and *in vitro* studies [232-235]. Damage due to IRI is triggered by the production of reactive oxygen species (ROS) after supply with oxygen is restored [236]. Overproduction of ROS leads to mitochondrial damage and release of several pro-apoptotic molecules (e.g. Cytochrome-*c* (Cyt-*c*)) [237]. Mitochondrial membrane integrity relies largely on the anti-apoptotic proteins of the BCL-2 protein family, BCL-2 and BCL-xL [238]. BCL-xL prevents translocation of the pro-apoptotic molecule BCL-2-associated X-protein (BAX) to the mitochondrial membrane and thereby preventing BAXs activation [239]. The pro-apoptotic BCL-xL/BCL-2 associated death promoter (BAD) binds BCL-xL preventing binding of BAX by BCL-xL and thus promoting apoptosis [240]. Released Cyt-*c* is able to bind to apoptotic protease activating factor-1 (APAF-1) and dATP to form the so called apoptosome [241], which activates the initiator caspase-9. Caspase-9 will then activate the effector caspase-3 and -7 initiating apoptotic proteolysis [242]. The underlying mechanism of Akt's protective effect is the ability to phosphorylate BAD [240] at AA position Ser^{136} [243] and thereby inactivating BAD. In our experimental setup we used H_2O_2 to mimic ROS overproduction and to induce apoptosis. H_2O_2 is commonly used as exogenous ROS and it was shown in cardiomyocytes to induce BAD/BAX translocation and subsequently caspase-3 activation [244]. Accordingly to the similar Akt activation under steady-state conditions no difference with the insulin analogues compared to Ins in the conditions used (100 nM of the respective insulin, 2 h incubation with Ins and 800 µM H_2O_2) was observed (Study 1, Figure 6).

For analysis of the heart-specific effects of insulin, the ability to induce positive inotropic effects on ARVM and the beating-rate of commercially available human derived induced pluripotent stem cells (Cor.4U®) was observed. Differences in cardiomyocyte contractility are facilitated by differential calcium cycling, a process called excitation-contraction coupling. Depolarisation of the cardiomyocyte membrane leads to Ca^{2+} influx via L-type Ca^{2+}-channels. Increased cytoplasmic Ca^{2+} concentration activate ryanodine receptors located on the sarcoplasmic reticulum enhancing Ca^{2+} influx. Ca^{2+} mediates increased contractility by binding to contractile proteins. Relaxation of the cardiomyocyte is enabled by reuptake of cytoplasmic Ca^{2+} into the sarcoplasmic reticulum via the sarcoplasmic/endoplasmic reticulum

calcium ATPase (SERCA). Fast reuptake of Ca^{2+} into the sarcoplasmic reticulum enables stronger contractility in the next excitation-contraction coupling cycle. Both, influx and reuptake are regulated by Akt activity [110]. Decreased Akt activity was shown to reduce SERCA activity and phospholamban (PLN) phosphorylation [245], whereas cardiac overexpression of Akt enhanced Ca^{2+} influx and reuptake [246, 247]. In this study comparable effects of both, IGla and IDeg compared to Ins in regard to increased contractility parameter. Sarcomeric shortening, return- and departure velocity in ARVM as well as beating-rate was significantly increased with each of the used insulins (Study 2, Figure 2 and Figure 4). In the Cor.4U® cells a slightly slower kinetics was observed in IDeg treated cells to reach its maximal beating-rate (20 min with IDeg vs. 10 min with IGla, IGlaM1 and Ins) (Study 2, Figure 4). An explanation for this observation is the lower binding affinity of IDeg, as described previously. Additionally, the role of Akt in the increase in contractility in paced ARVMs was demonstrated. After inhibition of insulin signalling via the specific Akt inhibitor Triciribine the positive inotropic effect was completely abrogated in all tested conditions (Study 1, Figure 3) without influence on the cells viability (Study 1, Supplementary Figure 3), which is in line with the aforementioned observations by others that myocardial Akt plays a pivotal role in this process [245, 247, 248].

Summarising, no difference between the long-acting insulin analogues IGla and IDeg were observed in *in vitro* models of functional cardiac assays. The only exception is the lower binding affinity of IDeg towards the receptor, compared to IGlaM1 and Ins and accordingly slower Akt phosphorylation kinetics in HL-1 cells.

3.1.1 Effects of Lower Insulin Analogue Concentrations on Insulin and Apoptosis Signalling in Cardiomyocytes

Since no differences with supra-physiological level of IGla, IGlaM1 and IDeg compared to Ins was observed, we wanted to analyse if lower insulin concentrations show a difference between the analogues. We chose to analyse the anti-apoptotic effect of the analogues in H9c2-E2 cells with 1 nM instead of 100 nM of the respective insulins, which is closer to physiological level of the analogues (Serum level of IGla: ~50 – 200 pM [165, 249-251]; serum level of IDeg: ~3.000 – 4.000 pM [252-254]). Furthermore, we used the IR overexpressing cell line H9c2-E2 generated in our lab [255], since we wanted to analyse apoptosis signalling via Western blot and regular cells are not as sensitive to low concentrations of insulin [256, 257].

First the insulin and apoptosis signalling were analysed by Western blot analysis in a time-course up to 2 h (Fig. 5). Analysis of pAkt(Ser^{473}) revealed significant activation of Akt with all insulins used. However, IDeg showed a 2.12-fold activity compared to basal, whereas Ins and IGlaM1 showed nearly twice the values compared to basal (3.52- and 3.39-fold, respectively) after 10 min incubation. Subsequently, the phosphorylation decreases with significant values ranging between 1.75- and 1.46-fold compared to basal after 60 min and are nearly back to basal Akt phosphorylation after 120 min (Fig. 5A). The lower Akt phosphorylation values for IDeg at the 10 minute time-point could be a reflection of the lower binding affinity of IDeg towards the insulin receptor [258], as well as the lower concentrations used. These values are reflected in significant phosphorylation of Bad with all treatments. Surprisingly, these values are in a very similar range with values between 1.81-fold for Ins, 1.69-fold for IGlaM1 and 1.51-fold for IDeg (Fig. 5B). However, no difference between basal conditions and treatment with insulin or analogues can be observed at the 60 min and 120 min time-points. The rapid decline in insulin signalling with low concentrations of long-acting insulin analogues could be due to the IR overexpression. The higher the IR levels of a cell are, the more rapid is the degradation of insulin – and therefore insulin analogues – since the degradation is dependent on IR internalisation [259].

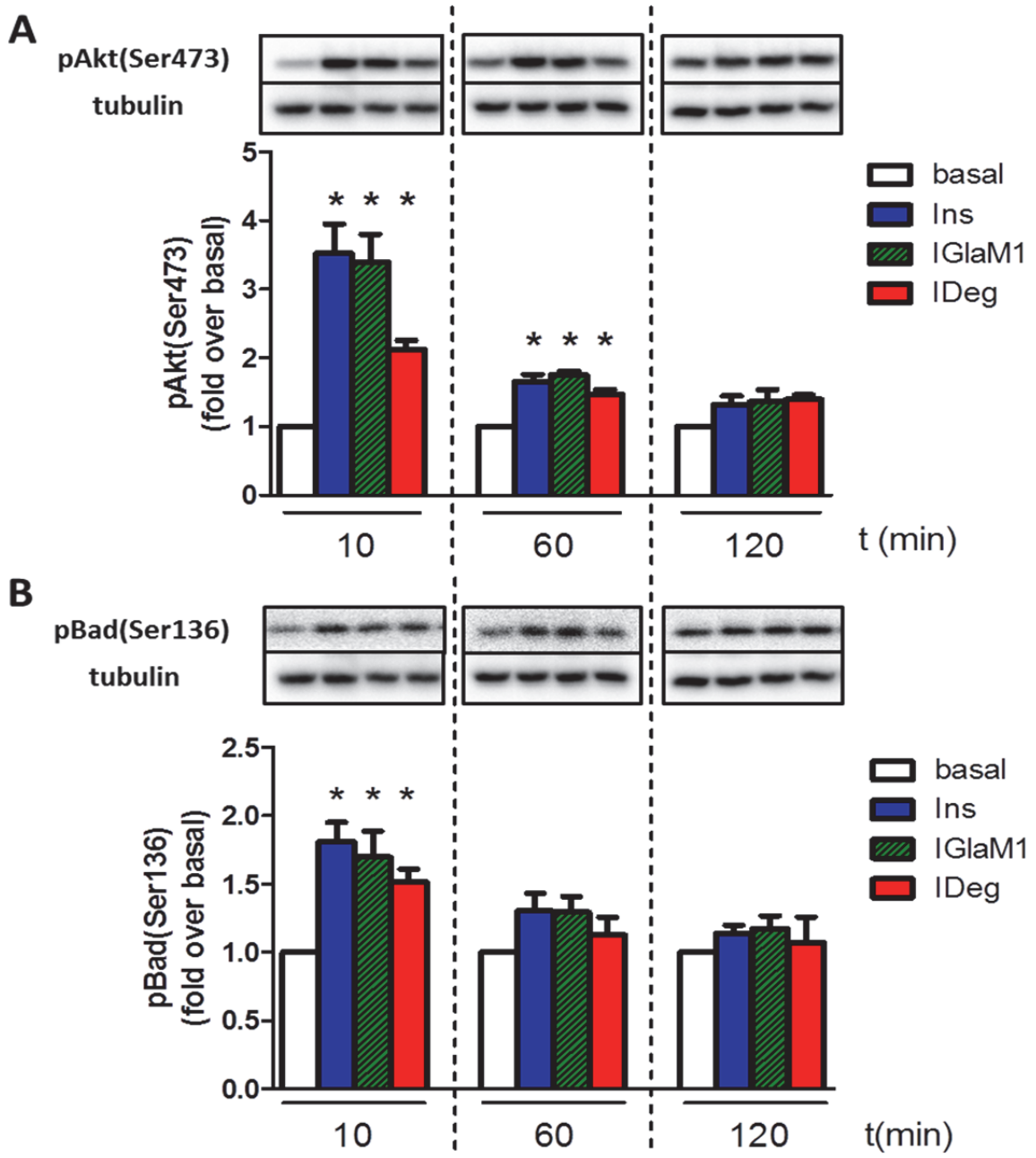


Figure 5: Akt and Bad signalling of low long-acting insulin analogues concentrations.
H9c2 cardiomyocytes overexpressing the human insulin receptor (H9c2-E2 cells) were treated for the indicated time-points with 1 nM of insulin and insulin analogues to evaluate the cardio protective effects of insulin and its analogues. Caspase 3/7 activity was measured using the Promega Caspase 3/7 Glo Assay. **(A)** Phosphorylation of Akt(Ser^{473}) and **(B)** Bad(Ser^{136}) was assessed by Western blot analysis. Data are normalised to tubulin levels. Representative blots are shown. Data represent mean values ± SEM, n = 4-7, *p<0.05 vs. basal. Regular insulin (Ins), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

In line with the signalling data, no difference in caspase 3/7 activation between H_2O_2 treatment alone and H_2O_2 treatment together with 1 nM of the indicated insulin was observed. The caspase 3/7 activity of the cells treated for 2 h with 800 µM H_2O_2 alone are 2.78-fold

increased, and is non-significantly reduced in the treatment conditions with H_2O_2 and Ins, IGla and IDeg (2.31-fold; 2.30-fold and 2.42-fold, respectively) (Fig. 6). Therefore, we conclude that even with nearly physiological insulin level no difference between the insulin analogues exists, at least in regard to apoptotic signalling.

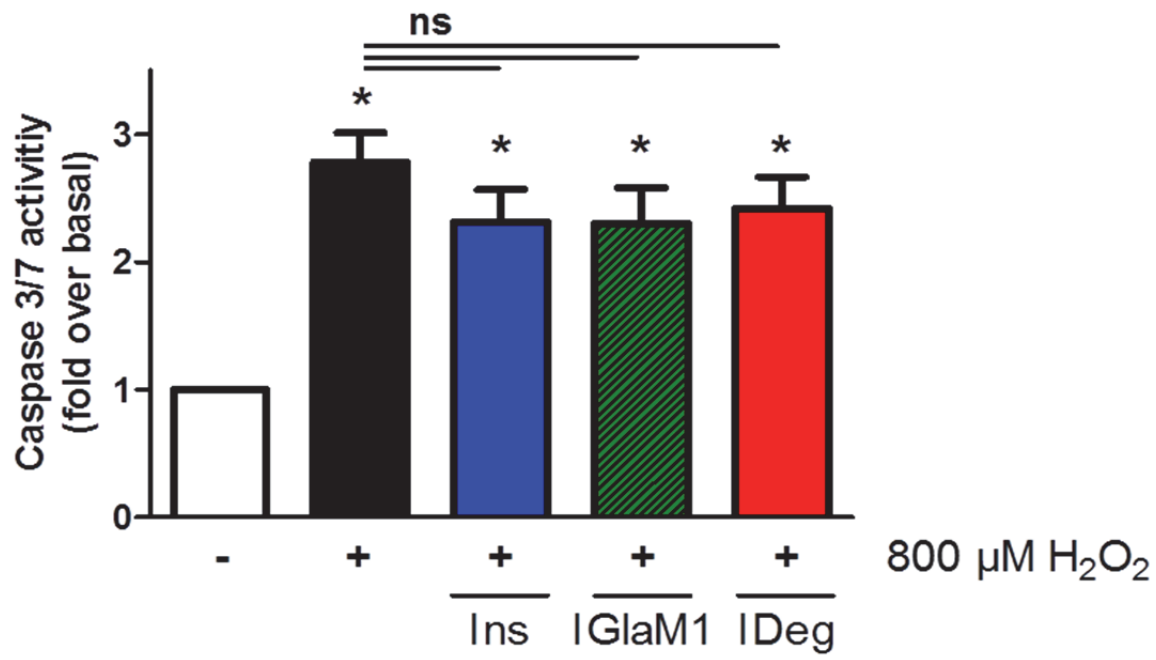


Figure 6: Anti-apoptotic potency of low doses of long-acting insulin analogues in the presence of H_2O_2.
H9c2 cardiomyocytes overexpressing the human insulin receptor (H9c2-E2 cells) were treated for 2 hours with 1 nM of insulin and insulin analogues in the presence or absence of 800 µM H_2O_2 to evaluate the cardio protective effects of insulin and its analogues. Caspase 3/7 activity was measured using the Promega Caspase 3/7 Glo Assay. Each condition was performed in quadruplicates. Data represent mean values ± SEM, n=6, *p<0.05 vs. basal. Regular insulin (Ins), active metabolite of insulin glargine (IGlaM1), insulin degludec (IDeg).

3.1.2 Limitations of Study 1

The underlying mechanism for the substrate specificity switch described by Randle et al. [227] is a complex interaction between LCFA utilisation and glucose utilisation. Briefly, with low blood glucose level available LCFA are transported into the cell via fatty acid transporter (FAT)/CD36 and used in mitochondria via β-oxidation to generate energy. During β-oxidation Acetyl-CoA and NADH accumulate, leading to inhibition of pyruvate dehydrogenase. Furthermore, β-oxidation leads to accumulation of citrate in the cytosol, which in turn inhibits glucose uptake and 6-phosphofructo-2-kinase (PFK1). These inhibitory mechanisms cause rerouting of pyruvate to gluconeogenesis and glucose-6-phosphate to glycogen synthesis [260]. In turn, as soon as blood glucose level and insulin concentrations rise and thereby

increased glucose uptake via insulin-dependent GLUT4 occurs, glucose oxidation increases. Increase in glucose utilisation leads to increased malonyl-CoA concentration in the cytoplasm. Malonyl-CoA in turn inhibits transport of LCFA into the mitochondria and thereby inhibiting β-oxidation [260]. The substrate utilisation is highly regulated in healthy conditions. However, during diabetes reliance on fatty acid metabolism is increased due to inability of regular glucose uptake [261]. In later stages of diabetes availability of LCFA exceeds the utilisation leading to lipid accumulation in the cardiomyocyte. This accumulation increases intracellular ceramide levels which can induce apoptosis and ROS formation [262], thus called lipotoxicity [263]. Furthermore, chronic hyperglycaemia desensitises the heart for insulin signalling , as is well known for other tissues and chronic hyperglycaemia leads to generation of ROS [264]. ROS formation above a certain threshold affects mitochondrial function, Ca^{2+} homeostasis and induces apoptosis [265-267]. This situation is therefore named glucotoxycity. Since we did not observe significant differences between IGla and IDeg in regard of metabolic and functional readouts in healthy cardiomyocytes it would be interesting to compare the effects of both analogues either *in vitro* with cardiomyocytes from diabetic model animals, or in *in vivo* models of diabetes such as the $^{db/db}$mouse or Zucker Diabetic Fatty (ZDF) rats.

3.1.3 Outlook of Study 1

The data have shown that there is no difference between the long-acting insulin analogues IGla and IDeg *in vitro* in regard to signalling and endpoint experiments under steady-state-conditions. This was observed using supraphysiological levels as well as nearly physiological levels of the respective insulins. However, it is unclear how these data translate into the clinical setting and lack of evidence cannot be described as evidence of lack. There might be differences being observable with other experimental approaches. However, another evidence for the safety of IDeg in regard to the cardiovascular system is the fact that the FDA approved IDeg in September 2015 after interim results of the requested CV trial (DEVOTE) were presented to the committee [268]. It will be interesting to see these results once they are published. Nonetheless, the case of IDeg showed the importance of CV data in the approval process of new insulin drugs. Pre-clinical *in vitro* studies could prove first insights into the CV effects of newly developed insulin analogues and should be considered as standard test in *in vitro* testing by the companies.

3.2 Preclinical Testing of the Novel Insulin Analogue HM12470

3.2.1 LAPSCOVERY™ Technology

LAPSCOVERY™ is a technology platform developed by Hanmi Pharmaceuticals and is an acronym for **L**ong **A**cting **P**rotein/Peptide Di**scovery** Platform Technology. With this technology Hanmi Pharmaceuticals is able to develop biopharmaceuticals with extended half-life. [269]. Unmodified peptides or proteins often show unsatisfying PK properties, due to renal clearance or degradation by peptidases [270]. GLP-1 for example is a native peptide with a short half-life (1-2 minutes), due to rapid degradation by DPP-4 [270]. Hanmi Pharmaceuticals was able to increase the serum half-life with the LAPSCOVERY™ technology of their GLP-1 analogue CA-Exendin-4 up to 180 h [271]. The LAPSCOVERY™ technology consists of a biological, such as CA-Exendin-4 or Insulin 115 [272], coupled to an aglycosylated, human immunoglobulin G4 (Fc-)fragment via a short PEG-linker [269, 273, 274]. This composition greatly enhances the serum half-life of the coupled drug by reducing vascular endothelial clearance due to neonatal Fc-receptor (FcRn) recycling [275] and renal clearance due to a hydrodynamic size above the renal threshold [276]. Site specific coupling of the active compound to the Fc-fragment via the PEG-linker enables the specific design of drugs while minimising stereological hindrance at the receptor site at the same time [273].

3.2.2 Pre-Clinical Testing of HM12470

In drug development it is of utmost importance to generate comprehensive data with both, *in vitro/in vivo* (pre-clinical) studies and clinical studies. This is underlined by the FDAs as well as the European Medical Agency (EMA) demand of preclinical data of novel anti-diabetic drugs [277, 278]. The FDA specifically states that certain *in vitro* tests, such as insulin signalling, mitogenesis, receptor affinities, dissociation from the receptor and IR autophosphorylation are useful tools in development of novel insulin analogues [277]. Therefore, in collaboration with Hanmi Pharmaceuticals we conducted preclinical *in vitro* and *in vivo* testing of the novel insulin analogue HM12470, also known as SAR440067 or ^{LAPS}Insulin 115. As recommended by the FDA we analysed PK/PD properties of HM12470, as well as IR autophosphorylation, insulin signalling, receptor interaction and metabolic and mitogenic action of the compound.

Since long-acting insulin analogues are designed to mimic basal endogenous insulin secretion, it is important to develop an analogue which exhibits a peakless PK profile without a pronounced peak-to-trough ratio. While IDeg and IGla are close to a peakless profile, they are

not completely peakless [279, 280]. For HM12470 the daily peak-to-trough ratio is predicted to be ~1.1 and a weekly ratio is predicted to be 1.6 under steady-state conditions [281]. The weekly injection frequency would be highly favourable for diabetic patients requiring insulin therapy, especially if it is able to demonstrate less frequent hypoglycaemic episodes [282, 283]. PK experiments demonstrate a serum half-life of 44.1 h for HM12470 in rats, which is more than 15 times longer compared to IDeg (2.9 h) after a single subcutaneous injection. Additionally, a single subcutaneous injection in $^{db/db}$mice demonstrates the ability to normalise FBG values for up to 7 days and the ability to lower HbA1c values (Study 2, Fig. 1). Interestingly, the undisclosed modification of Insulin 115 exhibits a beneficial effect beyond the coupling of the insulin molecule to the Fc-fragment. This is demonstrated in a 2.4-fold extended serum half-life compared to ^{LAPS}Insulin (Ins coupled to the Fc-fragment) (18.5 h), more than twice the duration to regulate FBG and a 4-fold lower concentration of HM12470 needed to significantly reduce HbA1c, compared to ^{LAPS}Insulin (Study 2, Fig. 1). The increased half-life could be explained by *in vitro* data, showing significantly increased stability of the insulin analogue Insulin 115 (Ins 115) compared to Ins, as well as increased stability of HM12470 compared to ^{LAPS}Insulin in the presence of HepG2 cells for up to 48h (4.3-fold and 1.2-fold, respectively) [284]. Similar results could be shown in human serum samples, where Ins 115 shows ~7.4-fold increased stability compared to Ins, and HM12470 has a 1.4-fold increased stability compared to ^{LAPS}Insulin after 8 days of incubation [284].

Since the receptor interaction of HM12470 plays a pivotal role in generating the insulin signalling as well as mitogenic effects, we analysed the binding affinity of HM12470 towards the IR and IGF-1R as well as IR dissociation and autophosphorylation of the IR. HM12470 exhibits a nearly 60-fold lower binding affinity compared to Ins towards the IR which is due to the Fc-fusion, since Ins 115 does not show a remarkably reduced IR affinity (Study 2, Fig. 3a). Analysing the IGF-1R affinity of HM12470 did show results below the detection limit (Study 2, Fig. 3a). This leads to a lower mitogenic potency of HM12470 in Saos-2 and MCF-7 cells as well as in human coronary artery smooth muscle cells (HCASMC) even with very high insulin analogue concentrations (Study 2, Fig. 4). Both results are very important in clinical safety of insulin analogues, since increased mitogenicity can lead to development of cancer [285] and in case of the HCASMC proliferation of those is the indicator for development of atherosclerosis, a common co-morbidity in diabetic patients [286].

Since insulin is produced in the β-cells of the pancreas and the pancreas releases its hormones into the portal vein, leading directly towards the liver, the concentration of insulin in this

tissue is approximately 2-fold higher compared to tissues in the periphery due to IR-mediated clearance in the liver [287, 288]. Due to the peripheral application of exogenous insulin the concentration in peripheral tissue is higher than the concentration of endogenous insulin would be, since the exogenous insulin is not cleared by the liver first. This leads to equal insulin concentrations in the periphery and liver, leading to relative peripheral hyperinsulinemia [289, 290]. BIL was the only clinically advanced insulin analogue demonstrating a hepatopreferential action after subcutaneous injection [181]. However, as stated in paragraph 1.3.3 the undesired side-effects on liver enzymes led to its discontinuation. To analyse if HM12470 shows hepatopreferential activity we investigated endothelial transfer rates compared to Ins in an *in vitro* permeability assay using human coronary endothelial cells (HCAEC), which do not show a wide fenestration as seen in liver endothelial cells. Even though HM12470 has a size of 59 kDa, it was able to pass the endothelial monolayer showing significantly reduced transendothelial transport (TET) at 4 h and 8 h compared to Ins (Study 2, Fig. 2a). After 24 h no difference between both treatments could be observed, which could potentially be explained by lack of insulin receptor downregulation (Study 2, Fig. 2a & Supplemental Fig. 1c-d), since TET is (partly) dependent on the IR for trafficking through the cell [291-293]. Furthermore, we analysed the insulin signalling pathway in insulins major target tissues (liver, skeletal muscle and adipose tissue) after single subcutaneous injection of 60 nmol/kg BW in wildtype Male Wistar rats at two different time points (8 h and 24 h). The results show activation of the insulin signalling in all tissues after 24 h on both IR phosphorylation, as well as Akt phosphorylation (Study 2; Fig. 2d-g). Interestingly, liver and muscle already show significantly increased IR phosphorylation after 8 h, while the IR in adipose tissue is not phosphorylated at this time-point. On Akt level, the liver shows a clear trend towards activation in the HM12470 treated animals after 8 h ($p<0.06$) while both other tissues do not show an effect at this time point (Study 2, Fig. 2f-g). Therefore, it can be assumed that HM12470 does not show hepatopreferential action, but induces signalling in all classical major target tissues.

The metabolic *in vitro* potency, determined by glucose uptake in differentiated human skeletal muscle cells (HSkMC) of HM12470 was found to be ~4-fold lower compared to Ins (Study 2, Fig. 5d), which is in line with published data about other insulin analogues [136]. When the cells were treated under acute conditions (10 min) with equimolar concentrations, HM12470 showed significantly less glucose uptake compared to Ins. However, under chronic conditions (24 h) no difference was found (Study 2, Fig. 5e). Interestingly, the insulin response declined to the level of glucose uptake by cells treated with HM12470 under chronic conditions. The

reason for this was found to be significantly less IR downregulation after chronic treatment with HM12470 compared to Ins and Ins 115, even with high concentrations of HM12470 (Study 2, Fig. 5f-g). This effect was investigated in further detail by establishing an *in vitro* model with chronic exposure of HCSMC, where the cells were treated with 100 nM of Ins, Ins 115, HM12470 or AspB10 for up to 2 days. It could be demonstrated that even after 2 days of exposure to the various insulins, HM12470 does not show receptor downregulation, compared to all other insulins (Study 2, Fig. 6a-b). Even in a model of chronic treatment (5 days) followed by a recovery phase (up to 2 days) with high insulin concentrations (500 nM) HM12470 shows recovery of IR level, whereas cells treated with Ins do not recover their IR level (Study 2, Fig. 6g). This leads to recovered insulin signalling in cells treated with HM12470 (Study 2, Fig. 6e). In monocyte samples prepared from human blood the investigators found similar results under chronic hyperinsulinemia due to overeating and obesity [294-297] and recovery, which needs up to several weeks, when insulin levels return back to normal level [295]. In the experiments with the chronic exposure/recovery model it was shown that the IR mRNA expression level are not elevated in both conditions (Study 2, Fig. 6h) leading to the assumption of another mechanism responsible for the lower IR downregulation in cells treated with HM12470. For the GLP-1R agonist created using the LAPSCOVERY™ technology, efpeglenatide (^{LAPS}CA-exendin-4) it was shown that rapid dissociation leads to less receptor internalisation and therefore to high efficacy even with low receptor affinity [298, 299]. However, with HM12470 a difference in the dissociation rate compared to Ins was not observed (Study 2, Fig. 2c), which contradicts this hypothesis. Therefore, it would be interesting to further investigate the mechanism behind the faster IR recovery with HM12470 in studies investigating IR internalisation.

Nonetheless, taken together the results of this study provide a comprehensive characterisation of the novel insulin analogue HM12470 with interesting properties. This drug could provide the first weekly insulin analogue if it demonstrates to be safe in long-term clinical studies. Furthermore, the lower impact of HM12470 on the IR internalisation could provide this analogue with a high efficacy, similar to efpeglenatide.

3.2.3 Potential Role of FcRn in HM12470 Kinetics and Recycling

An important factor for Fc-fusion proteins not analysed in this study is the interaction between FcRn and the Fc-fragment of these fusion proteins or IgG. FcRn is a heterodimeric protein consisting of one MHC-class-1-like heavy chain and a non-covalently linked β_2-microglobulin (β_2m) light chain. Even though the heavy chain with its three extracellular

domains, a transmembrane domain and a short cytoplasmic tail [300-302] shares high sequence homology with MHC-1 molecules, it is unable to present antigens due to a concealed peptide-binding groove [300, 301]. Instead of antigen presentation the role of FcRn is the recycling of IgG and albumin. FcRn transports internalised IgG and albumin back to the cells surface [275, 303] and thereby extending the serum half-life of both proteins tremendously. In FcRn knockout-mice the serum half-life of IgG is more than 6-fold reduced [304] and a mutation in the b_2m gene observed in two human individuals leads to hypercatabolism of IgG [305, 306]. The binding of IgG to FcRn only happens under acidic conditions which are found in (sorting) endosomes. Under physiological conditions found in blood or interstitial fluid, the bound cargo is released by FcRn. The binding of IgG occurs via the Fc-fragment, and thus Fc-fusion proteins are recycled by FcRn too (for overview see Figure 7) [307-310]. As stated above, this process is one reason for the long serum half-life of HM12470. One well known process facilitated by FcRn is the transport of IgG from the mothers' side of the syncytiotrophoblasts to the foetus' side, ensuring passive immunity of the foetus [275]. This process implies that HM12470 could be transported from a diabetic mother to the foetus as well, with unknown risks for the foetus. Beside the placenta FcRn is expressed in a variety of tissues and cell types, such as vascular endothelium, lungs or intestines [275]. The vascular endothelium is the main site to protect Fc-fusion proteins from catabolism [311]. In experiments with human placental endothelial cells it was found that both, exocytosis back into the blood as well as transcytosis of IgG through the endothelial cells occurs [312]. FcRn-mediated transcytosis of HM12470 is a possible explanation for the observation that HM12470 undergoes TET in a similar rate as Ins despite having a much lower binding affinity towards the IR, which usually facilitates insulin transcytosis [313]. Therefore, it would be interesting to repeat the TET experiments with FcRn knockout endothelial cells to see if the TET kinetics change, confirming this hypothesis. Another interesting observation is FcRn-expression in the upper airways epithelium of humans [314]. In *in vivo* tests with monkeys, Bitonti et al. were able to demonstrate that an Fc-erythropoietin fusion protein (Fc-epo) was efficiently absorbed after inhalation [315]. These results could be confirmed in a human phase I clinical trial [316]. The role of FcRn in this process was demonstrated in the *in vivo* study using a mutated Fc-form which exhibits a much lower binding affinity towards the FcRn. Application of this mutated Fc-epo showed significantly poorer absorption compared to the native Fc-epo [315]. This application form could therefore provide a non-invasive route of administration for HM12470. However, to test the safety and efficacy of this administration several *in vivo* experiments as well as early clinical phase studies would

be needed. Nonetheless, since FcRn plays a major role in the catabolism of HM12470, further experiments evaluating the role of FcRn-HM12470-interaction, especially in regard to TET, should be conducted.

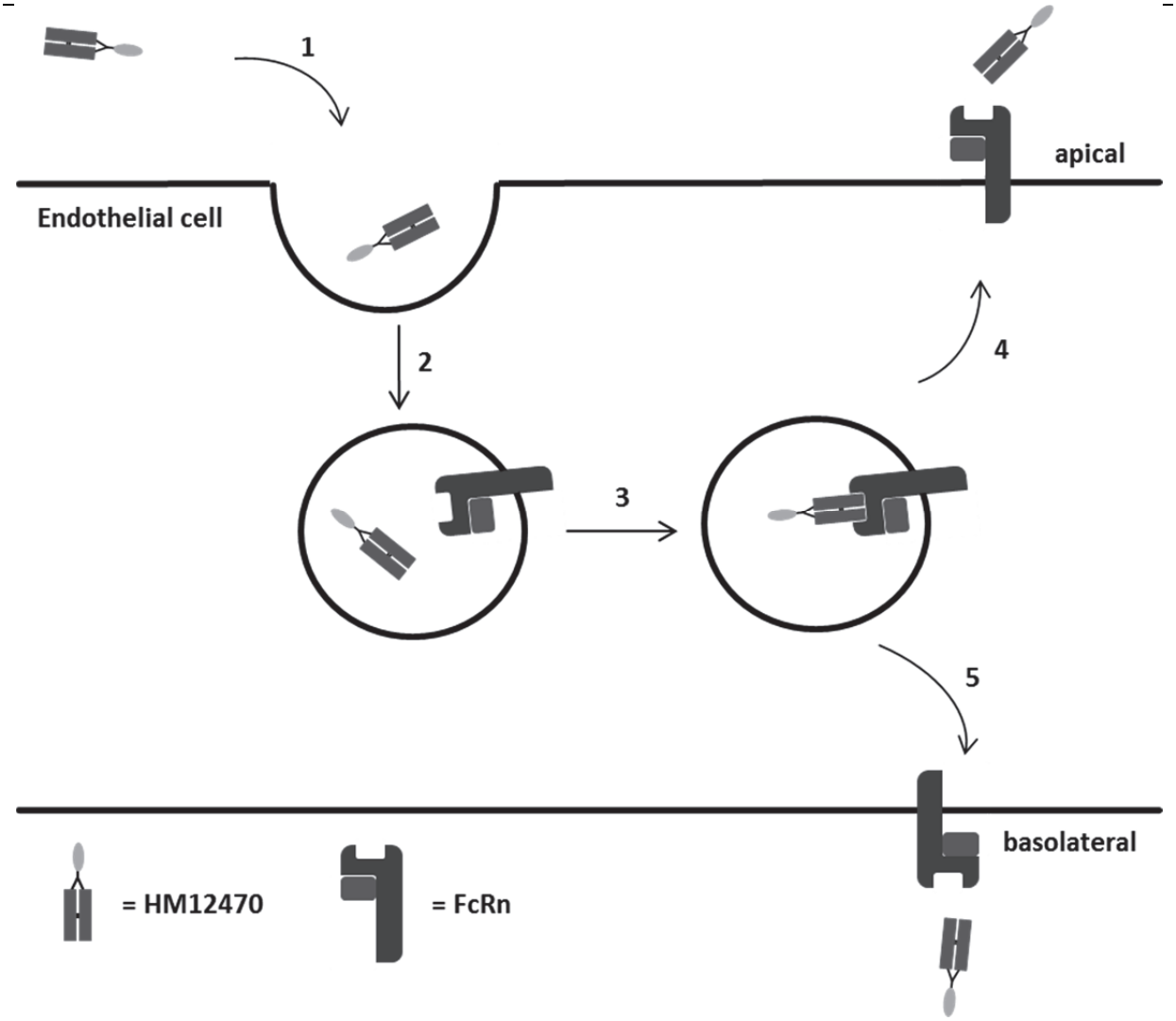


Figure 7: Schematic overview of putative interaction of HM12470 with the FcRn.
When HM12470 undergoes endocytoses either through pinocytosis or potential receptor mediated mechanisms **(1)** the endocytotic vesicle fuses with endosomes **(2)** resulting in acidification of the vesicle. The acidic milieu enables binding of the Fc-fragment to the FcRn, which only occurs at pH < 6.5 **(3)**. From there on are two potential pathways which can either lead to exocytosis back into the blood stream **(4)** or to transport into the interstitial fluid **(5)**. In both cases the binding affinity rapidly decreases due to neutral pH-values leading to release of HM12470 from the FcRn. FcRn = neonatal Fc-receptor. Adapted from [275].

3.2.4 Is a Weekly Insulin Analogue Useful in Clinical Practice?

To date there are no published data of clinical studies with potential weekly insulin analogues. Therefore, no experience with accumulation of those ultra-long-acting insulin analogues is available and clinicians may question the risk of hypoglycaemia due to potential insulin

accumulation after initiating insulin therapy with these analogues. Heise and Meneghini [317] discussed the difference between the so called insulin stacking and therapeutic accumulation [317]. They state that accumulation is usually discussed in the context of rapid-acting insulin analogues and that undesired accumulation is often observed after injection of correctional bolus insulin while previous injections still show insulin effects, increasing the risk of hypoglycaemia [317]. In contrast to rapid-acting insulin analogues, the accumulation of (ultra-)long-acting insulin analogues until steady-state conditions are reached, however, is a desired effect of this class of insulins [317]. At steady-state condition the clearance of the insulin analogue and amount of injected insulin reach equilibrium. That implies that no further accumulation of insulin is possible, as long as the injected dose is not increased. In Figure 8 theoretical serum insulin concentrations are calculated based on the assumption of injection of 10 U of the respective analogue and daily injection of IGla, IDeg and BIL and weekly injection of HM12470. In Figure 8 maximum serum level after the respective injection are shown considering different serum half-lives and disregarding inter-patient variability at the same time [317, 318]. Half-lives chosen for the calculation are 12 h (IGla) [173], 25 h (IDeg) [173], 72 h (BIL) [180] and 132 h (HM12470) [220]. The prediction reveals that the higher the half-life and the higher the injection frequency, the higher the accumulated serum insulin concentrations under steady-state conditions. Furthermore, it will take longer for an analogue with a longer serum half-life to reach steady-state levels. Steady-state conditions with IGla are achieved after ~5 days, whereas IDeg needs twice as long and with BIL a steady-state is achieved after ~28 days. However, if the injection frequency is only once per week, as it would be desirable for HM12470 in combination with the very long half-life of HM12470, steady-state conditions would be achieved after 4-5 weeks with serum insulin level in the range between IGla (13.3 U) and IDeg (20.5 U) (Fig. 8). Additionally, results from clinical studies with the long-acting insulin analogues IGla, IDet, IDeg and BIL do not provide a reason for concern since all showed less or comparable hypoglycaemic events compared to NPH (IGla, IDet) [319, 320] or IGla (IDeg, BIL) [182, 321-323]. Thus, there is no evidence at the moment supporting undesired insulin accumulation or unintentional overdose with weekly insulin analogues.

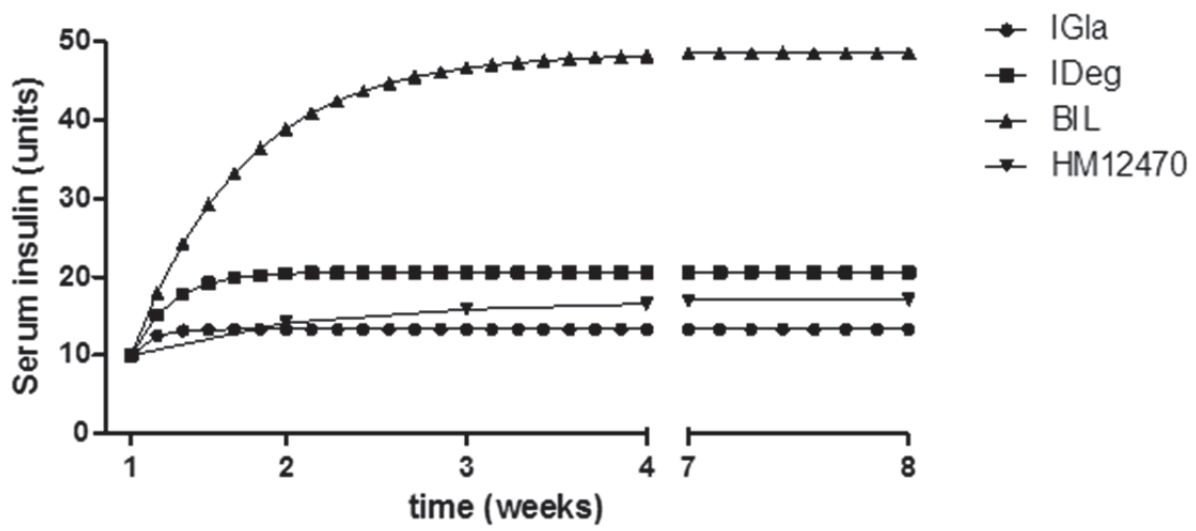


Figure 8: Theoretical insulin accumulation based on (predicted) serum half-life of different insulin analogues in humans.
Stacking of serum insulin is calculated based on daily (IGlar, IDeg, BIL) or weekly (HM12470) injection of 10 U of the respective analogue considering the different serum half-life of each analogue. The different serum half-lives are as followed: IGlar = ~12 h; IDeg = ~25 h; BIL = ~72 h and prediction for HM12470 = ~132 h. The presented graphs are theoretically calculated maximum serum levels and disregard inter-patient variability. IGla = insulin glargine, IDeg = insulin degludec, BIL = basal insulin PEGlispro. Adapted from [318].

Nonetheless, a serious problem could arise due to intentional overdose with HM12470. Literature regarding overdose with long-acting insulin analogues is scarce but some case reports are published reporting overdose with IGla [324-326] and IDet [327]. For IDeg no study reporting overdose is published yet. Management of insulin overdose is usually by carbohydrate supplementation either intravenous or oral [327-329]. However, the longer the half-life and thereby the effect of the insulin the longer the hypoglycaemic period will last and the longer hospitalisation is needed. A severe overdose with HM12470 with its predicted half-life of ~132 h could present a problem for the clinicians. Besides a prolonged period of carbohydrate load, surgical excision of the injection site could reduce the maximum efficacy [329, 330]. Other approaches to shorten the half-life could be the washout of HM12470. Due to the interaction of Fc-coupled proteins with the FcRn blocking this receptor leads to catabolism of circulating HM12470. To date three ways to increase catabolism of Fc-coupled proteins exist. One way is intravenous overload with Fc-fragments or IgG, which will compete for binding to the FcRn and thereby leading to saturation of the recycling process and increased catabolism [331-333]. Two other approaches are blocking of the interaction of HM12740 and the FcRn with either antibodies against the FcRn [334-336] or administration of recombinant Fc-fragments with increased binding affinity towards the FcRn (so called

"Abdegs") [337]. Both strategies would prevent binding HM12470 to the FcRn and thereby inhibit its recycling, leading to increased excretion and catabolism.

3.2.5 Outlook of Study 2

The pre-clinical *in vitro* and *in vivo* studies presented show very interesting and promising results for the potential once-weekly basal insulin analogue HM12470, if safety can be shown in clinical studies. Especially the reduced receptor downregulation could provide the molecule with unique and beneficial features. However, the role of FcRn in the interaction with HM12470 could provide an interesting target for further scientific experiments. Furthermore, it should be considered that prediction of PK/PD data based on animal studies could have limited significance, since the biology of the FcRn in non-primates is different. The human FcRn binds specifically to human, rabbit and guinea pig IgG, whereas mouse FcRn binds promiscuous to a broad variety of IgG [338]. Even in transgenic mice expressing the human FcRn, which are the best small animal model to study novel Fc-fusion proteins to date [304] have limitations. As human FcRn does interact with endogenous mouse IgG, the serum level of endogenous IgG are very low in this model and therefore do not represent conditions in humans [275]. Nonetheless, a first in human clinical phase I study is currently conducted to analyse safety, tolerability PK and PD of HM12470 (NCT02302443). This study will answer remaining questions and the outcome of this trial will be of high interest.

4 References

1. DeFronzo RA FE, Zimmet P, Alberti G: **International Textbook of Diabetes Mellitus**, 4th Edition edn: Wiley-Blackwell; 2015.
2. **IDF Diabetes Atlas, 7 ed.** In.: International Diabetes Federation; 2015.
3. Group DP: **Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999**. *Diabet Med* 2006, **23**(8):857-866.
4. Group SfDiYS, Liese AD, D'Agostino RB, Jr., Hamman RF, Kilgo PD, Lawrence JM, Liu LL, Loots B, Linder B, Marcovina S *et al*: **The burden of diabetes mellitus among US youth: prevalence estimates from the SEARCH for Diabetes in Youth Study**. *Pediatrics* 2006, **118**(4):1510-1518.
5. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G, Group ES: **Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study**. *Lancet* 2009, **373**(9680):2027-2033.
6. **Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia**. In. Geneva, Switzerland: World Healt Organisation (WHO), International Diabetes Federation (IDF); 2006.
7. **Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus**. In. Geneva, Switzerland: World Health Organisation (WHO); 2011.
8. Atkinson MA, Eisenbarth GS: **Type 1 diabetes: new perspectives on disease pathogenesis and treatment**. *Lancet* 2001, **358**(9277):221-229.
9. Concannon P, Rich SS, Nepom GT: **Genetics of type 1A diabetes**. *N Engl J Med* 2009, **360**(16):1646-1654.
10. Nerup J, Platz P, Andersen OO, Christy M, Lyngsoe J, Poulsen JE, Ryder LP, Nielsen LS, Thomsen M, Svejgaard A: **HL-A antigens and diabetes mellitus**. *Lancet* 1974, **2**(7885):864-866.
11. Singal DP, Blajchman MA: **Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus**. *Diabetes* 1973, **22**(6):429-432.
12. Bennett ST, Lucassen AM, Gough SC, Powell EE, Undlien DE, Pritchard LE, Merriman ME, Kawaguchi Y, Dronsfield MJ, Pociot F *et al*: **Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus**. *Nat Genet* 1995, **9**(3):284-292.
13. Lucassen AM, Julier C, Beressi JP, Boitard C, Froguel P, Lathrop M, Bell JI: **Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR**. *Nat Genet* 1993, **4**(3):305-310.
14. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, MacMurray J, Meloni GF, Lucarelli P, Pellecchia M *et al*: **A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes**. *Nat Genet* 2004, **36**(4):337-338.
15. Smyth D, Cooper JD, Collins JE, Heward JM, Franklyn JA, Howson JM, Vella A, Nutland S, Rance HE, Maier L *et al*: **Replication of an association between the lymphoid tyrosine phosphatase locus (LYP/PTPN22) with type 1 diabetes, and evidence for its role as a general autoimmunity locus**. *Diabetes* 2004, **53**(11):3020-3023.
16. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, Bourget K, Plagnol V, Field S, Atkinson M *et al*: **Large-scale genetic fine mapping and**

genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. *Nat Genet* 2007, **39**(9):1074-1082.

17. Qu HQ, Montpetit A, Ge B, Hudson TJ, Polychronakos C: **Toward further mapping of the association between the IL2RA locus and type 1 diabetes**. *Diabetes* 2007, **56**(4):1174-1176.
18. Nistico L, Buzzetti R, Pritchard LE, Van der Auwera B, Giovannini C, Bosi E, Larrad MT, Rios MS, Chow CC, Cockram CS *et al*: **The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry**. *Hum Mol Genet* 1996, **5**(7):1075-1080.
19. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G *et al*: **Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease**. *Nature* 2003, **423**(6939):506-511.
20. Smyth DJ, Cooper JD, Bailey R, Field S, Burren O, Smink LJ, Guja C, Ionescu-Tirgoviste C, Widmer B, Dunger DB *et al*: **A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region**. *Nat Genet* 2006, **38**(6):617-619.
21. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ *et al*: **Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses**. *Nature* 2006, **441**(7089):101-105.
22. Tauriainen S, Oikarinen S, Oikarinen M, Hyoty H: **Enteroviruses in the pathogenesis of type 1 diabetes**. *Semin Immunopathol* 2011, **33**(1):45-55.
23. Oikarinen M, Tauriainen S, Oikarinen S, Honkanen T, Collin P, Rantala I, Maki M, Kaukinen K, Hyoty H: **Type 1 diabetes is associated with enterovirus infection in gut mucosa**. *Diabetes* 2012, **61**(3):687-691.
24. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG: **The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes**. *Diabetologia* 2009, **52**(6):1143-1151.
25. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, Mosca F, Boggi U, Muda AO, Del Prato S *et al*: **Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients**. *Proc Natl Acad Sci U S A* 2007, **104**(12):5115-5120.
26. Bason C, Lorini R, Lunardi C, Dolcino M, Giannattasio A, d'Annunzio G, Rigo A, Pedemonte N, Corrocher R, Puccetti A: **In type 1 diabetes a subset of anti-coxsackievirus B4 antibodies recognize autoantigens and induce apoptosis of pancreatic beta cells**. *PLoS One* 2013, **8**(2):e57729.
27. Vaarala O, Atkinson MA, Neu J: **The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity**. *Diabetes* 2008, **57**(10):2555-2562.
28. Littorin B, Blom P, Scholin A, Arnqvist HJ, Blohme G, Bolinder J, Ekbom-Schnell A, Eriksson JW, Gudbjornsdottir S, Nystrom L *et al*: **Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS)**. *Diabetologia* 2006, **49**(12):2847-2852.
29. Mathieu C, Gysemans C, Giulietti A, Bouillon R: **Vitamin D and diabetes**. *Diabetologia* 2005, **48**(7):1247-1257.
30. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK, Dosch HM: **A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus**. *N Engl J Med* 1992, **327**(5):302-307.
31. Rewers M, Norris JM, Eisenbarth GS, Erlich HA, Beaty B, Klingensmith G, Hoffman M, Yu L, Bugawan TL, Blair A *et al*: **Beta-cell autoantibodies in infants and**

toddlers without IDDM relatives: diabetes autoimmunity study in the young (DAISY). *J Autoimmun* 1996, **9**(3):405-410.
32. Harrison LC, Honeyman MC: **Cow's milk and type 1 diabetes: the real debate is about mucosal immune function**. *Diabetes* 1999, **48**(8):1501-1507.
33. Schlosser M, Banga JP, Madec AM, Binder KA, Strebelow M, Rjasanowski I, Wassmuth R, Gilliam LK, Luo D, Hampe CS: **Dynamic changes of GAD65 autoantibody epitope specificities in individuals at risk of developing type 1 diabetes**. *Diabetologia* 2005, **48**(5):922-930.
34. Achenbach P, Bonifacio E, Koczwara K, Ziegler AG: **Natural history of type 1 diabetes**. *Diabetes* 2005, **54 Suppl 2**:S25-31.
35. Bingley PJ, Gale EA, European Nicotinamide Diabetes Intervention Trial G: **Progression to type 1 diabetes in islet cell antibody-positive relatives in the European Nicotinamide Diabetes Intervention Trial: the role of additional immune, genetic and metabolic markers of risk**. *Diabetologia* 2006, **49**(5):881-890.
36. Redondo MJ, Babu S, Zeidler A, Orban T, Yu L, Greenbaum C, Palmer JP, Cuthbertson D, Eisenbarth GS, Krischer JP *et al*: **Specific human leukocyte antigen DQ influence on expression of antiislet autoantibodies and progression to type 1 diabetes**. *J Clin Endocrinol Metab* 2006, **91**(5):1705-1713.
37. Kahn CR: **Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes**. *Diabetes* 1994, **43**(8):1066-1084.
38. Fuchsberger C, Flannick J, Teslovich TM, Mahajan A, Agarwala V, Gaulton KJ, Ma C, Fontanillas P, Moutsianas L, McCarthy DJ *et al*: **The genetic architecture of type 2 diabetes**. *Nature* 2016, **536**(7614):41-47.
39. McCarthy MI: **Genomics, type 2 diabetes, and obesity**. *N Engl J Med* 2010, **363**(24):2339-2350.
40. Stancakova A, Laakso M: **Genetics of Type 2 Diabetes**. *Endocr Dev* 2016, **31**:203-220.
41. Kahn SE, Cooper ME, Del Prato S: **Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future**. *Lancet* 2014, **383**(9922):1068-1083.
42. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS: **Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001**. *JAMA* 2003, **289**(1):76-79.
43. Field AE, Coakley EH, Must A, Spadano JL, Laird N, Dietz WH, Rimm E, Colditz GA: **Impact of overweight on the risk of developing common chronic diseases during a 10-year period**. *Arch Intern Med* 2001, **161**(13):1581-1586.
44. Koster I, von Ferber L, Ihle P, Schubert I, Hauner H: **The cost burden of diabetes mellitus: the evidence from Germany--the CoDiM study**. *Diabetologia* 2006, **49**(7):1498-1504.
45. Yang W, Zhao W, Xiao J, Li R, Zhang P, Kissimova-Skarbek K, Schneider E, Jia W, Ji L, Guo X *et al*: **Medical care and payment for diabetes in China: enormous threat and great opportunity**. *PLoS One* 2012, **7**(9):e39513.
46. Chatterjee S, Riewpaiboon A, Piyauthakit P, Riewpaiboon W, Boupaijit K, Panpuwong N, Archavanuntagul V: **Cost of diabetes and its complications in Thailand: a complete picture of economic burden**. *Health Soc Care Community* 2011, **19**(3):289-298.
47. Coccheri S: **Approaches to prevention of cardiovascular complications and events in diabetes mellitus**. *Drugs* 2007, **67**(7):997-1026.
48. Gilbertson DT, Liu J, Xue JL, Louis TA, Solid CA, Ebben JP, Collins AJ: **Projecting the number of patients with end-stage renal disease in the United States to the year 2015**. *J Am Soc Nephrol* 2005, **16**(12):3736-3741.

49. Chronic Kidney Disease Prognosis C, Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, Coresh J, Gansevoort RT: **Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis**. *Lancet* 2010, **375**(9731):2073-2081.
50. Groop PH, Thomas MC, Moran JL, Waden J, Thorn LM, Makinen VP, Rosengard-Barlund M, Saraheimo M, Hietala K, Heikkila O *et al*: **The presence and severity of chronic kidney disease predicts all-cause mortality in type 1 diabetes**. *Diabetes* 2009, **58**(7):1651-1658.
51. Seyer-Hansen K, Hansen J, Gundersen HJ: **Renal hypertrophy in experimental diabetes. A morphometric study**. *Diabetologia* 1980, **18**(6):501-505.
52. Vallon V, Blantz RC, Thomson S: **Glomerular hyperfiltration and the salt paradox in early [corrected] type 1 diabetes mellitus: a tubulo-centric view**. *J Am Soc Nephrol* 2003, **14**(2):530-537.
53. Frank RN: **Diabetic retinopathy**. *N Engl J Med* 2004, **350**(1):48-58.
54. Hirai FE, Tielsch JM, Klein BE, Klein R: **Ten-year change in vision-related quality of life in type 1 diabetes: Wisconsin epidemiologic study of diabetic retinopathy**. *Ophthalmology* 2011, **118**(2):353-358.
55. Roy MS, Klein R, O'Colmain BJ, Klein BE, Moss SE, Kempen JH: **The prevalence of diabetic retinopathy among adult type 1 diabetic persons in the United States**. *Arch Ophthalmol* 2004, **122**(4):546-551.
56. Kempen JH, O'Colmain BJ, Leske MC, Haffner SM, Klein R, Moss SE, Taylor HR, Hamman RF, Eye Diseases Prevalence Research G: **The prevalence of diabetic retinopathy among adults in the United States**. *Arch Ophthalmol* 2004, **122**(4):552-563.
57. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ: **Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K**. *Diabetes Care* 2011, **34**(10):2220-2224.
58. Forbes JM, Cooper ME: **Mechanisms of diabetic complications**. *Physiol Rev* 2013, **93**(1):137-188.
59. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M: **Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction**. *N Engl J Med* 1998, **339**(4):229-234.
60. Domanski M, Mitchell G, Pfeffer M, Neaton JD, Norman J, Svendsen K, Grimm R, Cohen J, Stamler J, Group MR: **Pulse pressure and cardiovascular disease-related mortality: follow-up study of the Multiple Risk Factor Intervention Trial (MRFIT)**. *JAMA* 2002, **287**(20):2677-2683.
61. Kannel WB, Hjortland M, Castelli WP: **Role of diabetes in congestive heart failure: the Framingham study**. *Am J Cardiol* 1974, **34**(1):29-34.
62. Liu JE, Palmieri V, Roman MJ, Bella JN, Fabsitz R, Howard BV, Welty TK, Lee ET, Devereux RB: **The impact of diabetes on left ventricular filling pattern in normotensive and hypertensive adults: the Strong Heart Study**. *J Am Coll Cardiol* 2001, **37**(7):1943-1949.
63. van Belle TL, Coppieters KT, von Herrath MG: **Type 1 diabetes: etiology, immunology, and therapeutic strategies**. *Physiol Rev* 2011, **91**(1):79-118.
64. Suarez-Pinzon WL, Lakey JR, Rabinovitch A: **Combination therapy with glucagon-like peptide-1 and gastrin induces beta-cell neogenesis from pancreatic duct cells in human islets transplanted in immunodeficient diabetic mice**. *Cell Transplant* 2008, **17**(6):631-640.
65. Griffin KJ, Thompson PA, Gottschalk M, Kyllo JH, Rabinovitch A: **Combination therapy with sitagliptin and lansoprazole in patients with recent-onset type 1**

diabetes (REPAIR-T1D): 12-month results of a multicentre, randomised, placebo-controlled, phase 2 trial. *Lancet Diabetes Endocrinol* 2014, **2**(9):710-718.

66. Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, Santamaria P, Bluestone JA, Brillantes AM, Herold KC: **Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of beta-cells**. *Endocrinology* 2007, **148**(11):5136-5144.
67. Ablamunits V, Henegariu O, Hansen JB, Opare-Addo L, Preston-Hurlburt P, Santamaria P, Mandrup-Poulsen T, Herold KC: **Synergistic reversal of type 1 diabetes in NOD mice with anti-CD3 and interleukin-1 blockade: evidence of improved immune regulation**. *Diabetes* 2012, **61**(1):145-154.
68. Perl S, Perlman J, Weitzel RP, Phang O, Hsieh MM, Tisdale J: **Addition of rapamycin to anti-CD3 antibody improves long-term glycaemia control in diabetic NOD mice**. *PLoS One* 2013, **8**(6):e67189.
69. Pozzilli P, Maddaloni E, Buzzetti R: **Combination immunotherapies for type 1 diabetes mellitus**. *Nat Rev Endocrinol* 2015, **11**(5):289-297.
70. Bruni A, Gala-Lopez B, Pepper AR, Abualhassan NS, Shapiro AJ: **Islet cell transplantation for the treatment of type 1 diabetes: recent advances and future challenges**. *Diabetes Metab Syndr Obes* 2014, **7**:211-223.
71. Aguayo-Mazzucato C, Bonner-Weir S: **Stem cell therapy for type 1 diabetes mellitus**. *Nat Rev Endocrinol* 2010, **6**(3):139-148.
72. Orlando G, Gianello P, Salvatori M, Stratta RJ, Soker S, Ricordi C, Dominguez-Bendala J: **Cell replacement strategies aimed at reconstitution of the beta-cell compartment in type 1 diabetes**. *Diabetes* 2014, **63**(5):1433-1444.
73. Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B, American Diabetes A, European Association for Study of D: **Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes**. *Diabetes Care* 2009, **32**(1):193-203.
74. Look ARG, Pi-Sunyer X, Blackburn G, Brancati FL, Bray GA, Bright R, Clark JM, Curtis JM, Espeland MA, Foreyt JP *et al*: **Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial**. *Diabetes Care* 2007, **30**(6):1374-1383.
75. Hadden DR, Montgomery DA, Skelly RJ, Trimble ER, Weaver JA, Wilson EA, Buchanan KD: **Maturity onset diabetes mellitus: response to intensive dietary management**. *Br Med J* 1975, **3**(5978):276-278.
76. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F: **Cellular and molecular mechanisms of metformin: an overview**. *Clin Sci (Lond)* 2012, **122**(6):253-270.
77. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JPt, Boyd AE, 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA: **Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion**. *Science* 1995, **268**(5209):423-426.
78. Akiyoshi M, Kakei M, Nakazaki M, Tanaka H: **A new hypoglycemic agent, A-4166, inhibits ATP-sensitive potassium channels in rat pancreatic beta-cells**. *Am J Physiol* 1995, **268**(2 Pt 1):E185-193.
79. Yki-Jarvinen H: **Thiazolidinediones**. *N Engl J Med* 2004, **351**(11):1106-1118.
80. Miyazaki Y, Mahankali A, Matsuda M, Mahankali S, Hardies J, Cusi K, Mandarino LJ, DeFronzo RA: **Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients**. *J Clin Endocrinol Metab* 2002, **87**(6):2784-2791.

81. Drucker DJ, Nauck MA: **The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes**. *Lancet* 2006, **368**(9548):1696-1705.
82. Liu JJ, Lee T, DeFronzo RA: **Why Do SGLT2 inhibitors inhibit only 30-50% of renal glucose reabsorption in humans?** *Diabetes* 2012, **61**(9):2199-2204.
83. Wright EM, Loo DD, Hirayama BA: **Biology of human sodium glucose transporters**. *Physiol Rev* 2011, **91**(2):733-794.
84. Bass J, Takahashi JS: **Circadian integration of metabolism and energetics**. *Science* 2010, **330**(6009):1349-1354.
85. Holt RI, Barnett AH, Bailey CJ: **Bromocriptine: old drug, new formulation and new indication**. *Diabetes Obes Metab* 2010, **12**(12):1048-1057.
86. Buchwald H, Estok R, Fahrbach K, Banel D, Jensen MD, Pories WJ, Bantle JP, Sledge I: **Weight and type 2 diabetes after bariatric surgery: systematic review and meta-analysis**. *Am J Med* 2009, **122**(3):248-256 e245.
87. Fried M, Ribaric G, Buchwald JN, Svacina S, Dolezalova K, Scopinaro N: **Metabolic surgery for the treatment of type 2 diabetes in patients with BMI <35 kg/m2: an integrative review of early studies**. *Obes Surg* 2010, **20**(6):776-790.
88. Banting FG, Best CH: **The internal secretion of the pancreas. 1922**. *Indian J Med Res* 2007, **125**(3):251-266.
89. Ryle AP, Sanger F, Smith LF, Kitai R: **The disulphide bonds of insulin**. *Biochem J* 1955, **60**(4):541-556.
90. Sanger F: **Chemistry of insulin; determination of the structure of insulin opens the way to greater understanding of life processes**. *Science* 1959, **129**(3359):1340-1344.
91. Steiner DF, Oyer PE: **The biosynthesis of insulin and a probable precursor of insulin by a human islet cell adenoma**. *Proc Natl Acad Sci U S A* 1967, **57**(2):473-480.
92. Steiner DF, Cunningham D, Spigelman L, Aten B: **Insulin biosynthesis: evidence for a precursor**. *Science* 1967, **157**(3789):697-700.
93. Yip CC, Hew CL, Hsu H: **Translation of messenger ribonucleic acid from isolated pancreatic islets and human insulinomas**. *Proc Natl Acad Sci U S A* 1975, **72**(12):4777-4779.
94. Lomedico PT, Saunders GF: **Preparation of pancreatic mRNA: cell-free translation of an insulin-immunoreactive polypeptide**. *Nucleic Acids Res* 1976, **3**(2):381-391.
95. Permutt MA, Routman A: **Proinsulin precursors in isolated rat pancreatic islets**. *Biochem Biophys Res Commun* 1977, **78**(3):855-862.
96. Howell SL: **Role of ATP in the intracellular translocation of proinsulin and insulin in the rat pancreatic B cell**. *Nat New Biol* 1972, **235**(55):85-86.
97. Kelly RB: **Pathways of protein secretion in eukaryotes**. *Science* 1985, **230**(4721):25-32.
98. Moore HP: **Factors controlling packaging of peptide hormones into secretory granules**. *Ann N Y Acad Sci* 1987, **493**:50-61.
99. Orci L: **The insulin factory: a tour of the plant surroundings and a visit to the assembly line. The Minkowski lecture 1973 revisited**. *Diabetologia* 1985, **28**(8):528-546.
100. Orci L, Ravazzola M, Amherdt M, Madsen O, Vassalli JD, Perrelet A: **Direct identification of prohormone conversion site in insulin-secreting cells**. *Cell* 1985, **42**(2):671-681.
101. Steiner DF, Michael J, Houghten R, Mathieu M, Gardner PR, Ravazzola M, Orci L: **Use of a synthetic peptide antigen to generate antisera reactive with a proteolytic**

processing site in native human proinsulin: demonstration of cleavage within clathrin-coated (pro)secretory vesicles. *Proc Natl Acad Sci U S A* 1987, **84**(17):6184-6188.

102. Davidson HW: **(Pro)Insulin processing: a historical perspective**. *Cell Biochem Biophys* 2004, **40**(3 Suppl):143-158.
103. Hutton JC: **The insulin secretory granule**. *Diabetologia* 1989, **32**(5):271-281.
104. Bradbury JH, Ramesh V: **1H n.m.r. studies of insulin. Assignment of resonances and properties of tyrosine residues**. *Biochem J* 1985, **229**(3):731-737.
105. Grant PT, Coombs TL, Frank BH: **Differences in the nature of the interaction of insulin and proinsulin with zinc**. *Biochem J* 1972, **126**(2):433-440.
106. Saltiel AR, Kahn CR: **Insulin signalling and the regulation of glucose and lipid metabolism**. *Nature* 2001, **414**(6865):799-806.
107. Klein LJ, Visser FC: **The effect of insulin on the heart : Part 1: Effects on metabolism and function**. *Neth Heart J* 2010, **18**(4):197-201.
108. Bertrand L, Horman S, Beauloye C, Vanoverschelde JL: **Insulin signalling in the heart**. *Cardiovasc Res* 2008, **79**(2):238-248.
109. Lee J, Pilch PF: **The insulin receptor: structure, function, and signaling**. *Am J Physiol* 1994, **266**(2 Pt 1):C319-334.
110. Sussman MA, Volkers M, Fischer K, Bailey B, Cottage CT, Din S, Gude N, Avitabile D, Alvarez R, Sundararaman B *et al*: **Myocardial AKT: the omnipresent nexus**. *Physiol Rev* 2011, **91**(3):1023-1070.
111. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: **Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex**. *Science* 2005, **307**(5712):1098-1101.
112. Sasaoka T, Ishiki M, Sawa T, Ishihara H, Takata Y, Imamura T, Usui I, Olefsky JM, Kobayashi M: **Comparison of the insulin and insulin-like growth factor 1 mitogenic intracellular signaling pathways**. *Endocrinology* 1996, **137**(10):4427-4434.
113. He W, Rose DW, Olefsky JM, Gustafson TA: **Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains**. *J Biol Chem* 1998, **273**(12):6860-6867.
114. **Insulin Receptor Signaling Pathway** [https://www.cellsignal.com/contents/science-pathway-research-cellular-metabolism/insulin-receptor-signaling-pathway/pathways-irs]
115. Hagedorn HC, Jensen BN, Krarup NB, Wodstrup I: **Landmark article Jan 18, 1936: Protamine insulinate. By H.C. Hagedorn, B.N. Jensen, N.B. Krarup, and I. Wodstrup**. *JAMA* 1984, **251**(3):389-392.
116. Soeborg T, Rasmussen CH, Mosekilde E, Colding-Jorgensen M: **Absorption kinetics of insulin after subcutaneous administration**. *Eur J Pharm Sci* 2009, **36**(1):78-90.
117. Soeborg T, Rasmussen CH, Mosekilde E, Colding-Jorgensen M: **Bioavailability and variability of biphasic insulin mixtures**. *Eur J Pharm Sci* 2012, **46**(4):198-208.
118. Jehle PM, Micheler C, Jehle DR, Breitig D, Boehm BO: **Inadequate suspension of neutral protamine Hagendorn (NPH) insulin in pens**. *Lancet* 1999, **354**(9190):1604-1607.
119. Lucidi P, Porcellati F, Marinelli Andreoli A, Carriero I, Candeloro P, Cioli P, Bolli GB, Fanelli CG: **Pharmacokinetics and Pharmacodynamics of NPH Insulin in Type 1 Diabetes: The Importance of Appropriate Resuspension Before Subcutaneous Injection**. *Diabetes Care* 2015, **38**(12):2204-2210.

120. White JR, Jr., Campbell RK, Hirsch IB: **Novel insulins and strict glycemic control. Analogues approximate normal insulin secretory response**. *Postgrad Med* 2003, **113**(6):30-36.
121. Yki-Jarvinen H, Dressler A, Ziemen M, Group HOEsS: **Less nocturnal hypoglycemia and better post-dinner glucose control with bedtime insulin glargine compared with bedtime NPH insulin during insulin combination therapy in type 2 diabetes. HOE 901/3002 Study Group**. *Diabetes Care* 2000, **23**(8):1130-1136.
122. Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, Hirose T, Kraszewski A, Itakura K, Riggs AD: **Expression in Escherichia coli of chemically synthesized genes for human insulin**. *Proc Natl Acad Sci U S A* 1979, **76**(1):106-110.
123. Heinemann L, Starke AA, Hohmann A, Berger M: **Timing between the subcutaneous administration of insulin and consumption of a carbohydrate rich meal**. *Horm Metab Res Suppl* 1992, **26**:137-139.
124. Heinemann L: **Do insulin-treated diabetic patients use an injection-meal-interval in daily life?** *Diabet Med* 1995, **12**(5):449-450.
125. Pullen RA, Lindsay DG, Wood SP, Tickle IJ, Blundell TL, Wollmer A, Krail G, Brandenburg D, Zahn H, Gliemann J *et al*: **Receptor-binding region of insulin**. *Nature* 1976, **259**(5542):369-373.
126. Baker EN, Blundell TL, Cutfield JF, Cutfield SM, Dodson EJ, Dodson GG, Hodgkin DM, Hubbard RE, Isaacs NW, Reynolds CD *et al*: **The structure of 2Zn pig insulin crystals at 1.5 A resolution**. *Philos Trans R Soc Lond B Biol Sci* 1988, **319**(1195):369-456.
127. Varewijck AJ, Janssen JA: **Insulin and its analogues and their affinities for the IGF1 receptor**. *Endocr Relat Cancer* 2012, **19**(5):F63-75.
128. Howey DC, Bowsher RR, Brunelle RL, Woodworth JR: **[Lys(B28), Pro(B29)]-human insulin. A rapidly absorbed analogue of human insulin**. *Diabetes* 1994, **43**(3):396-402.
129. Torlone E, Fanelli C, Rambotti AM, Kassi G, Modarelli F, Di Vincenzo A, Epifano L, Ciofetta M, Pampanelli S, Brunetti P *et al*: **Pharmacokinetics, pharmacodynamics and glucose counterregulation following subcutaneous injection of the monomeric insulin analogue [Lys(B28),Pro(B29)] in IDDM**. *Diabetologia* 1994, **37**(7):713-720.
130. Slieker LJ SK: **Modifications in the 28-29 position of the insulin B-chain alter binding to the IGF-1 receptor with minimal effect on insulin receptor binding**. *Diabetes* 1991, **40 (Suppl. 1)**:A168.
131. Pfutzner A, Kustner E, Forst T, Schulze-Schleppinghoff B, Trautmann ME, Haslbeck M, Schatz H, Beyer J: **Intensive insulin therapy with insulin lispro in patients with type 1 diabetes reduces the frequency of hypoglycemic episodes**. *Exp Clin Endocrinol Diabetes* 1996, **104**(1):25-30.
132. Anderson JH, Jr., Brunelle RL, Koivisto VA, Pfutzner A, Trautmann ME, Vignati L, DiMarchi R: **Reduction of postprandial hyperglycemia and frequency of hypoglycemia in IDDM patients on insulin-analog treatment. Multicenter Insulin Lispro Study Group**. *Diabetes* 1997, **46**(2):265-270.
133. Brunelle BL, Llewelyn J, Anderson JH, Jr., Gale EA, Koivisto VA: **Meta-analysis of the effect of insulin lispro on severe hypoglycemia in patients with type 1 diabetes**. *Diabetes Care* 1998, **21**(10):1726-1731.
134. Brange J, Ribel U, Hansen JF, Dodson G, Hansen MT, Havelund S, Melberg SG, Norris F, Norris K, Snel L *et al*: **Monomeric insulins obtained by protein engineering and their medical implications**. *Nature* 1988, **333**(6174):679-682.

135. Drejer K: **The bioactivity of insulin analogues from in vitro receptor binding to in vivo glucose uptake**. *Diabetes Metab Rev* 1992, **8**(3):259-285.
136. Kurtzhals P, Schaffer L, Sorensen A, Kristensen C, Jonassen I, Schmid C, Trub T: **Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use**. *Diabetes* 2000, **49**(6):999-1005.
137. Kang S CF, Ara J, Owens DR, Peter JR: **Insulin analogues and human insulin: near-equivalent in vivo biological activity in healthy males in spite of widely different in vitro potencies**. *Diabetes* 1991, **40 (Suppl. 1)**:A243.
138. Heinemann L, Weyer C, Rauhaus M, Heinrichs S, Heise T: **Variability of the metabolic effect of soluble insulin and the rapid-acting insulin analog insulin aspart**. *Diabetes Care* 1998, **21**(11):1910-1914.
139. Lindholm A, McEwen J, Riis AP: **Improved postprandial glycemic control with insulin aspart. A randomized double-blind cross-over trial in type 1 diabetes**. *Diabetes Care* 1999, **22**(5):801-805.
140. Mudaliar SR, Lindberg FA, Joyce M, Beerdsen P, Strange P, Lin A, Henry RR: **Insulin aspart (B28 asp-insulin): a fast-acting analog of human insulin: absorption kinetics and action profile compared with regular human insulin in healthy nondiabetic subjects**. *Diabetes Care* 1999, **22**(9):1501-1506.
141. Raskin P, Guthrie RA, Leiter L, Riis A, Jovanovic L: **Use of insulin aspart, a fast-acting insulin analog, as the mealtime insulin in the management of patients with type 1 diabetes**. *Diabetes Care* 2000, **23**(5):583-588.
142. Home PD, Lindholm A, Hylleberg B, Round P: **Improved glycemic control with insulin aspart: a multicenter randomized double-blind crossover trial in type 1 diabetic patients. UK Insulin Aspart Study Group**. *Diabetes Care* 1998, **21**(11):1904-1909.
143. Becker RH: **Insulin glulisine complementing basal insulins: a review of structure and activity**. *Diabetes Technol Ther* 2007, **9**(1):109-121.
144. Ahmad A, Uversky VN, Hong D, Fink AL: **Early events in the fibrillation of monomeric insulin**. *J Biol Chem* 2005, **280**(52):42669-42675.
145. Becker RH, Frick AD: **Clinical pharmacokinetics and pharmacodynamics of insulin glulisine**. *Clin Pharmacokinet* 2008, **47**(1):7-20.
146. Rave K, Klein O, Frick AD, Becker RH: **Advantage of premeal-injected insulin glulisine compared with regular human insulin in subjects with type 1 diabetes**. *Diabetes Care* 2006, **29**(8):1812-1817.
147. Garg SK, Rosenstock J, Ways K: **Optimized Basal-bolus insulin regimens in type 1 diabetes: insulin glulisine versus regular human insulin in combination with Basal insulin glargine**. *Endocr Pract* 2005, **11**(1):11-17.
148. Dailey G, Rosenstock J, Moses RG, Ways K: **Insulin glulisine provides improved glycemic control in patients with type 2 diabetes**. *Diabetes Care* 2004, **27**(10):2363-2368.
149. Heinemann L, Linkeschova R, Rave K, Hompesch B, Sedlak M, Heise T: **Time-action profile of the long-acting insulin analog insulin glargine (HOE901) in comparison with those of NPH insulin and placebo**. *Diabetes Care* 2000, **23**(5):644-649.
150. Berti L, Kellerer M, Bossenmaier B, Seffer E, Seipke G, Haring HU: **The long acting human insulin analog HOE 901: characteristics of insulin signalling in comparison to Asp(B10) and regular insulin**. *Horm Metab Res* 1998, **30**(3):123-129.
151. Porcellati F, Rossetti P, Ricci NB, Pampanelli S, Torlone E, Campos SH, Andreoli AM, Bolli GB, Fanelli CG: **Pharmacokinetics and pharmacodynamics of the long-acting insulin analog glargine after 1 week of use compared with its first**

administration in subjects with type 1 diabetes. *Diabetes Care* 2007, **30**(5):1261-1263.

152. Ashwell SG, Gebbie J, Home PD: **Twice-daily compared with once-daily insulin glargine in people with Type 1 diabetes using meal-time insulin aspart**. *Diabet Med* 2006, **23**(8):879-886.
153. Ratner RE, Hirsch IB, Neifing JL, Garg SK, Mecca TE, Wilson CA: **Less hypoglycemia with insulin glargine in intensive insulin therapy for type 1 diabetes. U.S. Study Group of Insulin Glargine in Type 1 Diabetes**. *Diabetes Care* 2000, **23**(5):639-643.
154. Pieber TR, Eugene-Jolchine I, Derobert E: **Efficacy and safety of HOE 901 versus NPH insulin in patients with type 1 diabetes. The European Study Group of HOE 901 in type 1 diabetes**. *Diabetes Care* 2000, **23**(2):157-162.
155. Rosenstock J, Park G, Zimmerman J, Group USIGTDI: **Basal insulin glargine (HOE 901) versus NPH insulin in patients with type 1 diabetes on multiple daily insulin regimens. U.S. Insulin Glargine (HOE 901) Type 1 Diabetes Investigator Group**. *Diabetes Care* 2000, **23**(8):1137-1142.
156. Becker RH, Dahmen R, Bergmann K, Lehmann A, Jax T, Heise T: **New insulin glargine 300 Units . mL-1 provides a more even activity profile and prolonged glycemic control at steady state compared with insulin glargine 100 Units . mL-1**. *Diabetes Care* 2015, **38**(4):637-643.
157. Ritzel R, Roussel R, Bolli GB, Vinet L, Brulle-Wohlhueter C, Glezer S, Yki-Jarvinen H: **Patient-level meta-analysis of the EDITION 1, 2 and 3 studies: glycaemic control and hypoglycaemia with new insulin glargine 300 U/ml versus glargine 100 U/ml in people with type 2 diabetes**. *Diabetes Obes Metab* 2015, **17**(9):859-867.
158. Owens DR: **Insulin preparations with prolonged effect**. *Diabetes Technol Ther* 2011, **13 Suppl 1**:S5-14.
159. Havelund S, Plum A, Ribel U, Jonassen I, Volund A, Markussen J, Kurtzhals P: **The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin**. *Pharm Res* 2004, **21**(8):1498-1504.
160. Kurtzhals P, Havelund S, Jonassen I, Markussen J: **Effect of fatty acids and selected drugs on the albumin binding of a long-acting, acylated insulin analogue**. *J Pharm Sci* 1997, **86**(12):1365-1368.
161. Plank J, Bodenlenz M, Sinner F, Magnes C, Gorzer E, Regittnig W, Endahl LA, Draeger E, Zdravkovic M, Pieber TR: **A double-blind, randomized, dose-response study investigating the pharmacodynamic and pharmacokinetic properties of the long-acting insulin analog detemir**. *Diabetes Care* 2005, **28**(5):1107-1112.
162. Brunner GA, Sendhofer G, Wutte A, Ellmerer M, Sogaard B, Siebenhofer A, Hirschberger S, Krejs GJ, Pieber TR: **Pharmacokinetic and pharmacodynamic properties of long-acting insulin analogue NN304 in comparison to NPH insulin in humans**. *Exp Clin Endocrinol Diabetes* 2000, **108**(2):100-105.
163. Klein O, Lynge J, Endahl L, Damholt B, Nosek L, Heise T: **Albumin-bound basal insulin analogues (insulin detemir and NN344): comparable time-action profiles but less variability than insulin glargine in type 2 diabetes**. *Diabetes Obes Metab* 2007, **9**(3):290-299.
164. Dornhorst A, Luddeke HJ, Sreenan S, Koenen C, Hansen JB, Tsur A, Landstedt-Hallin L: **Safety and efficacy of insulin detemir in clinical practice: 14-week follow-up data from type 1 and type 2 diabetes patients in the PREDICTIVE European cohort**. *Int J Clin Pract* 2007, **61**(3):523-528.
165. Heise T, Nosek L, Ronn BB, Endahl L, Heinemann L, Kapitza C, Draeger E: **Lower within-subject variability of insulin detemir in comparison to NPH insulin and insulin glargine in people with type 1 diabetes**. *Diabetes* 2004, **53**(6):1614-1620.

166. Hermansen K, Fontaine P, Kukolja KK, Peterkova V, Leth G, Gall MA: **Insulin analogues (insulin detemir and insulin aspart) versus traditional human insulins (NPH insulin and regular human insulin) in basal-bolus therapy for patients with type 1 diabetes**. *Diabetologia* 2004, **47**(4):622-629.
167. Home P, Bartley P, Russell-Jones D, Hanaire-Broutin H, Heeg JE, Abrams P, Landin-Olsson M, Hylleberg B, Lang H, Draeger E *et al*: **Insulin detemir offers improved glycemic control compared with NPH insulin in people with type 1 diabetes: a randomized clinical trial**. *Diabetes Care* 2004, **27**(5):1081-1087.
168. Mathieu C: **Can we reduce hypoglycaemia with insulin detemir?** *Int J Obes* 2004, **28 (Suppl. 2)**:S35-40.
169. Raslova K, Bogoev M, Raz I, Leth G, Gall MA, Hancu N: **Insulin detemir and insulin aspart: a promising basal-bolus regimen for type 2 diabetes**. *Diabetes Res Clin Pract* 2004, **66**(2):193-201.
170. Jonassen I, Havelund S, Hoeg-Jensen T, Steensgaard DB, Wahlund PO, Ribel U: **Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin**. *Pharm Res* 2012, **29**(8):2104-2114.
171. Seested T BA, Pyke C, Nishimura E: **Ultrastructural 3D visualization of insulin degludec multihexamers upon subcutaneous injection in Pig**. *Diabetes* 2016, **65 (Suppl. 1)**:A236.
172. Nishimura E SA, Hansen BF, Stidsen CE, Olsen GS, Schaäffer L, et al.: **Insulin degludec: a new ultra-long, basal insulin designes to maintain full metabolic effect while minimizing mitogenic potential**. *Diabetologia* 2010, **53 (Suppl. 1)**:S388-S389.
173. Heise T, Hovelmann U, Nosek L, Hermanski L, Bottcher SG, Haahr H: **Comparison of the pharmacokinetic and pharmacodynamic profiles of insulin degludec and insulin glargine**. *Expert Opin Drug Metab Toxicol* 2015, **11**(8):1193-1201.
174. Heise T, Hermanski L, Nosek L, Feldman A, Rasmussen S, Haahr H: **Insulin degludec: four times lower pharmacodynamic variability than insulin glargine under steady-state conditions in type 1 diabetes**. *Diabetes Obes Metab* 2012, **14**(9):859-864.
175. Ratner RE, Gough SC, Mathieu C, Del Prato S, Bode B, Mersebach H, Endahl L, Zinman B: **Hypoglycaemia risk with insulin degludec compared with insulin glargine in type 2 and type 1 diabetes: a pre-planned meta-analysis of phase 3 trials**. *Diabetes Obes Metab* 2013, **15**(2):175-184.
176. Mathieu C, Hollander P, Miranda-Palma B, Cooper J, Franek E, Russell-Jones D, Larsen J, Tamer SC, Bain SC, Investigators NNT: **Efficacy and safety of insulin degludec in a flexible dosing regimen vs insulin glargine in patients with type 1 diabetes (BEGIN: Flex T1): a 26-week randomized, treat-to-target trial with a 26-week extension**. *J Clin Endocrinol Metab* 2013, **98**(3):1154-1162.
177. Meneghini L, Atkin SL, Gough SC, Raz I, Blonde L, Shestakova M, Bain S, Johansen T, Begtrup K, Birkeland KI *et al*: **The efficacy and safety of insulin degludec given in variable once-daily dosing intervals compared with insulin glargine and insulin degludec dosed at the same time daily: a 26-week, randomized, open-label, parallel-group, treat-to-target trial in individuals with type 2 diabetes**. *Diabetes Care* 2013, **36**(4):858-864.
178. Kiselyov VV DL, Wu GY, Moyer J: **Analysis of basal insulin peglispro (BIL) binding kinetics with the use of a harmonic oscillator insulin receptor binding model**. *Diabetes* 2015, **64 (Suppl. 1)**:A264-A265.
179. Owens RA, Hansen RJ, Kahl SD, Zhang C, Ruan X, Koester A, Li S, Qian HR, Farmen MW, Michael MD *et al*: **In Vivo and In Vitro Characterization of Basal**

Insulin Peglispro: A Novel Insulin Analog. *J Pharmacol Exp Ther* 2016, **357**(3):459-465.

180. Sinha VP, Howey DC, Choi SL, Mace KF, Heise T: **Steady-state pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus**. *Diabetes Obes Metab* 2014, **16**(4):344-350.
181. Henry RR, Mudaliar S, Ciaraldi TP, Armstrong DA, Burke P, Pettus J, Garhyan P, Choi SL, Jacober SJ, Knadler MP *et al*: **Basal insulin peglispro demonstrates preferential hepatic versus peripheral action relative to insulin glargine in healthy subjects**. *Diabetes Care* 2014, **37**(9):2609-2615.
182. Bergenstal RM, Lunt H, Franek E, Travert F, Mou J, Qu Y, Antalis CJ, Hartman ML, Rosilio M, Jacober SJ *et al*: **Randomized, double-blind clinical trial comparing basal insulin peglispro and insulin glargine, in combination with prandial insulin lispro, in patients with type 1 diabetes: IMAGINE 3**. *Diabetes Obes Metab* 2016, **18**(11):1081-1088.
183. Buse JB, Rodbard HW, Trescoli Serrano C, Luo J, Ivanyi T, Bue-Valleskey J, Hartman ML, Carey MA, Chang AM, Investigators I: **Randomized Clinical Trial Comparing Basal Insulin Peglispro and Insulin Glargine in Patients With Type 2 Diabetes Previously Treated With Basal Insulin: IMAGINE 5**. *Diabetes Care* 2016, **39**(1):92-100.
184. Soos MA, Whittaker J, Lammers R, Ullrich A, Siddle K: **Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. Characterisation of hybrid receptors in transfected cells**. *Biochem J* 1990, **270**(2):383-390.
185. Slieker LJ BG, DiMarchi RD, Flora DB, Green LK, Hoffmann JA, Long HB, Fan L, Shields JE, Sundell KL, Surface PL, Chance RE: **Modifications in the B10 and B26-30 regions of the B chain of human insulin alter affinity for the human IGF-1 receptor more than for the insulin receptor**. *Diabetologia* 1997, **40 (Suppl. 2)**:S54-S61.
186. Schwartz GP, Burke GT, Katsoyannis PG: **A superactive insulin: [B10-aspartic acid]insulin(human)**. *Proc Natl Acad Sci U S A* 1987, **84**(18):6408-6411.
187. Nielsen FS, Jorgensen LN, Ipsen M, Voldsgaard AI, Parving HH: **Long-term comparison of human insulin analogue B10Asp and soluble human insulin in IDDM patients on a basal/bolus insulin regimen**. *Diabetologia* 1995, **38**(5):592-598.
188. Bornfeldt KE, Gidlof RA, Wasteson A, Lake M, Skottner A, Arnqvist HJ: **Binding and biological effects of insulin, insulin analogues and insulin-like growth factors in rat aortic smooth muscle cells. Comparison of maximal growth promoting activities**. *Diabetologia* 1991, **34**(5):307-313.
189. Drejer K, Kruse V, Larsen UD, Hougaard P, Bjorn S, Gammeltoft S: **Receptor binding and tyrosine kinase activation by insulin analogues with extreme affinities studied in human hepatoma HepG2 cells**. *Diabetes* 1991, **40**(11):1488-1495.
190. Hamel FG, Siford GL, Fawcett J, Chance RE, Frank BH, Duckworth WC: **Differences in the cellular processing of AspB10 human insulin compared with human insulin and LysB28ProB29 human insulin**. *Metabolism* 1999, **48**(5):611-617.
191. Chang CH, Toh S, Lin JW, Chen ST, Kuo CW, Chuang LM, Lai MS: **Cancer risk associated with insulin glargine among adult type 2 diabetes patients--a nationwide cohort study**. *PLoS One* 2011, **6**(6):e21368.

192. Colhoun HM, Group SE: **Use of insulin glargine and cancer incidence in Scotland: a study from the Scottish Diabetes Research Network Epidemiology Group**. *Diabetologia* 2009, **52**(9):1755-1765.
193. Jonasson JM, Ljung R, Talback M, Haglund B, Gudbjornsdottir S, Steineck G: **Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden**. *Diabetologia* 2009, **52**(9):1745-1754.
194. Eckardt K, May C, Koenen M, Eckel J: **IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts**. *Diabetologia* 2007, **50**(12):2534-2543.
195. Mayer D, Shukla A, Enzmann H: **Proliferative effects of insulin analogues on mammary epithelial cells**. *Arch Physiol Biochem* 2008, **114**(1):38-44.
196. Bronsveld HK, ter Braak B, Karlstad O, Vestergaard P, Starup-Linde J, Bazelier MT, De Bruin ML, de Boer A, Siezen CL, van de Water B *et al*: **Treatment with insulin (analogues) and breast cancer risk in diabetics; a systematic review and meta-analysis of in vitro, animal and human evidence**. *Breast Cancer Res* 2015, **17**:100.
197. Kuerzel GU, Shukla U, Scholtz HE, Pretorius SG, Wessels DH, Venter C, Potgieter MA, Lang AM, Koose T, Bernhardt E: **Biotransformation of insulin glargine after subcutaneous injection in healthy subjects**. *Curr Med Res Opin* 2003, **19**(1):34-40.
198. Sommerfeld MR, Muller G, Tschank G, Seipke G, Habermann P, Kurrle R, Tennagels N: **In vitro metabolic and mitogenic signaling of insulin glargine and its metabolites**. *PLoS One* 2010, **5**(3):e9540.
199. Tennagels N, Welte S, Hofmann M, Brenk P, Schmidt R, Werner U: **Differences in metabolic and mitogenic signallingof insulin glargine and AspB10 human insulin in rats [corrected]**. *Diabetologia* 2013, **56**(8):1826-1834.
200. Werner U, Korn M, Schmidt R, Wendrich TM, Tennagels N: **Metabolic effect and receptor signalling profile of a non-metabolisable insulin glargine analogue**. *Arch Physiol Biochem* 2014, **120**(4):158-165.
201. Gallagher EJ, Zelenko Z, Tobin-Hess A, Werner U, Tennagels N, LeRoith D: **Non-metabolisable insulin glargine does not promote breast cancer growth in a mouse model of type 2 diabetes**. *Diabetologia* 2016, **59**(9):2018-2025.
202. Investigators OT, Gerstein HC, Bosch J, Dagenais GR, Diaz R, Jung H, Maggioni AP, Pogue J, Probstfield J, Ramachandran A *et al*: **Basal insulin and cardiovascular and other outcomes in dysglycemia**. *N Engl J Med* 2012, **367**(4):319-328.
203. Investigators OT: **Cardiovascular and Other Outcomes Postintervention With Insulin Glargine and Omega-3 Fatty Acids (ORIGINALE)**. *Diabetes Care* 2016, **39**(5):709-716.
204. Rask-Madsen C, Kahn CR: **Tissue-specific insulin signaling, metabolic syndrome, and cardiovascular disease**. *Arterioscler Thromb Vasc Biol* 2012, **32**(9):2052-2059.
205. Administration USFaD: **Endocrinologic and Metabolic Drugs Advisory Committee Meeting**. In.; 2012.
206. Ginsberg H, Cariou B, Orchard T, Chen L, Luo J, Bastyr EJ, 3rd, Bue-Valleskey J, Chang AM, Ivanyi T, Jacober SJ *et al*: **Lipid changes during basal insulin peglispro, insulin glargine, or NPH treatment in six IMAGINE trials**. *Diabetes Obes Metab* 2016, **18**(11):1089-1092.
207. Freckmann G, Pleus S, Haug C, Bitton G, Nagar R: **Increasing local blood flow by warming the application site: beneficial effects on postprandial glycemic excursions**. *J Diabetes Sci Technol* 2012, **6**(4):780-785.
208. McVey E, Hirsch L, Sutter DE, Kapitza C, Dellweg S, Clair J, Rebrin K, Judge K, Pettis RJ: **Pharmacokinetics and postprandial glycemic excursions following insulin lispro delivered by intradermal microneedle or subcutaneous infusion**. *J Diabetes Sci Technol* 2012, **6**(4):743-754.

209. Boss AH, Petrucci R, Lorber D: **Coverage of prandial insulin requirements by means of an ultra-rapid-acting inhaled insulin**. *J Diabetes Sci Technol* 2012, **6**(4):773-779.
210. Pohl R, Hauser R, Li M, De Souza E, Feldstein R, Seibert R, Ozhan K, Kashyap N, Steiner S: **Ultra-rapid absorption of recombinant human insulin induced by zinc chelation and surface charge masking**. *J Diabetes Sci Technol* 2012, **6**(4):755-763.
211. Krasner A, Pohl R, Simms P, Pichotta P, Hauser R, De Souza E: **A review of a family of ultra-rapid-acting insulins: formulation development**. *J Diabetes Sci Technol* 2012, **6**(4):786-796.
212. Heise T, Hovelmann U, Brondsted L, Adrian CL, Nosek L, Haahr H: **Faster-acting insulin aspart: earlier onset of appearance and greater early pharmacokinetic and pharmacodynamic effects than insulin aspart**. *Diabetes Obes Metab* 2015, **17**(7):682-688.
213. Andersen G AB, Meiffren G, Ranson A, Seroussi C, Gaudier M, Soula O, Fischer A, Nosek L, Heise T: **Ultra-rapid BioChaperone insulin Lispro (BC-LIS): linear dose-response and faster absorption than insulin Lispro (LIS)**. *Diabetologia* 2015, **58 (Suppl. 1)**:S449.
214. Safavi-Hemami H, Gajewiak J, Karanth S, Robinson SD, Ueberheide B, Douglass AD, Schlegel A, Imperial JS, Watkins M, Bandyopadhyay PK *et al*: **Specialized insulin is used for chemical warfare by fish-hunting cone snails**. *Proc Natl Acad Sci U S A* 2015, **112**(6):1743-1748.
215. De Meyts P: **Structural basis for the poisonous activity of a predator's venom insulin**. *Nat Struct Mol Biol* 2016, **23**(10):872-874.
216. Jowett J, Woods C: **Therapeutic agents comprising insulin amino acid sequences**. In.: Google Patents; 2013.
217. **PE0139: A super-long-acting basal insulin for once-weekly administration** [http://www.phasebio.com/pages/pipeline/pe0139.php]
218. Roberts BK WX, Rosendahl MS, Mantripragada S: **The in vitro and in vivo pharmacology of AB101, a potential once-weekly basal subcutaneous insulin**. *Diabetes* 2015, **64 (Suppl. 1)**:A25.
219. Huh Y KJ, Yun N, Kil S, Son J, Chapel S: **Use of PKPD Model to Design and Analyze Restuls of a Euglycemic Clamp Study for a Very Long-acting Insulin Analogue HM12470**. In: *American Diabetes Association's 75th Scientific Sessions.* 2015.
220. Hwang SY CI, Kim JY, Jung SY, Kim DJ, Lee YM, Kim YH, Trautmann M, Hompesch M, Son JW, Kwon SC: **A Novel Very Long-Acting Insulin Analog (HM12470) with Potential for Once-Weekly Dosing, Has a Favorable PK, PD and Mitogenic Profile**. In: *American Diabetes Association's 74th Scientific Session.* 2014.
221. Chou DH, Webber MJ, Tang BC, Lin AB, Thapa LS, Deng D, Truong JV, Cortinas AB, Langer R, Anderson DG: **Glucose-responsive insulin activity by covalent modification with aliphatic phenylboronic acid conjugates**. *Proc Natl Acad Sci U S A* 2015, **112**(8):2401-2406.
222. Yu J, Zhang Y, Ye Y, DiSanto R, Sun W, Ranson D, Ligler FS, Buse JB, Gu Z: **Microneedle-array patches loaded with hypoxia-sensitive vesicles provide fast glucose-responsive insulin delivery**. *Proc Natl Acad Sci U S A* 2015, **112**(27):8260-8265.
223. Muniyappa R, Montagnani M, Koh KK, Quon MJ: **Cardiovascular actions of insulin**. *Endocr Rev* 2007, **28**(5):463-491.
224. Abraham MA, Filippi BM, Kang GM, Kim MS, Lam TK: **Insulin action in the hypothalamus and dorsal vagal complex**. *Exp Physiol* 2014, **99**(9):1104-1109.

225. Opie LH LG (ed.): **Fuels: aerobic and anaerobic metabolism**. Philadelphia: Williams & Wilkins; 2003.
226. Williams A (ed.): **Mitochondira in Cardiac Metabolism**: John Wiley & Sons; 1983.
227. Randle PJ, Garland PB, Hales CN, Newsholme EA: **The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus**. *Lancet* 1963, **1**(7285):785-789.
228. Sakai T, Ogawa S, Hosokawa M, Miyazaki T, Sakurai K, Nakamura Y: **Electrophysiological effects of flecainide in a canine 7 day old myocardial infarction model**. *Cardiovasc Res* 1989, **23**(3):177-183.
229. Shuralyova I, Tajmir P, Bilan PJ, Sweeney G, Coe IR: **Inhibition of glucose uptake in murine cardiomyocyte cell line HL-1 by cardioprotective drugs dilazep and dipyridamole**. *Am J Physiol Heart Circ Physiol* 2004, **286**(2):H627-632.
230. Davey KA, Garlick PB, Warley A, Southworth R: **Immunogold labeling study of the distribution of GLUT-1 and GLUT-4 in cardiac tissue following stimulation by insulin or ischemia**. *Am J Physiol Heart Circ Physiol* 2007, **292**(4):H2009-2019.
231. Asrih M, Lerch R, Papageorgiou I, Pellieux C, Montessuit C: **Differential regulation of stimulated glucose transport by free fatty acids and PPARalpha or -delta agonists in cardiac myocytes**. *Am J Physiol Endocrinol Metab* 2012, **302**(7):E872-884.
232. Ji L, Fu F, Zhang L, Liu W, Cai X, Zhang L, Zheng Q, Zhang H, Gao F: **Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress**. *Am J Physiol Endocrinol Metab* 2010, **298**(4):E871-880.
233. Shi YF, Liu N, Li YX, Song CL, Song XJ, Zhao Z, Liu B: **Insulin protects H9c2 rat cardiomyoblast cells against hydrogen peroxide-induced injury through upregulation of microRNA-210**. *Free Radic Res* 2015, **49**(9):1147-1155.
234. Wong VW, Mardini M, Cheung NW, Mihailidou AS: **High-dose insulin in experimental myocardial infarction in rabbits: protection against effects of hyperglycaemia**. *J Diabetes Complications* 2011, **25**(2):122-128.
235. Xing W, Yan W, Fu F, Jin Y, Ji L, Liu W, Wang L, Lv A, Duan Y, Zhang J *et al*: **Insulin inhibits myocardial ischemia-induced apoptosis and alleviates chronic adverse changes in post-ischemic cardiac structure and function**. *Apoptosis* 2009, **14**(9):1050-1060.
236. Braunersreuther V, Jaquet V: **Reactive oxygen species in myocardial reperfusion injury: from physiopathology to therapeutic approaches**. *Curr Pharm Biotechnol* 2012, **13**(1):97-114.
237. Reed JC, Paternostro G: **Postmitochondrial regulation of apoptosis during heart failure**. *Proc Natl Acad Sci U S A* 1999, **96**(14):7614-7616.
238. Regula KM, Ens K, Kirshenbaum LA: **Mitochondria-assisted cell suicide: a license to kill**. *J Mol Cell Cardiol* 2003, **35**(6):559-567.
239. Oltvai ZN, Milliman CL, Korsmeyer SJ: **Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death**. *Cell* 1993, **74**(4):609-619.
240. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ: **Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death**. *Cell* 1995, **80**(2):285-291.
241. Zou H, Li Y, Liu X, Wang X: **An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9**. *J Biol Chem* 1999, **274**(17):11549-11556.
242. Kelekar A, Thompson CB: **Bcl-2-family proteins: the role of the BH3 domain in apoptosis**. *Trends Cell Biol* 1998, **8**(8):324-330.

243. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: **Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery**. *Cell* 1997, **91**(2):231-241.
244. Cook SA, Sugden PH, Clerk A: **Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential**. *Circ Res* 1999, **85**(10):940-949.
245. Duan J, Zhang HY, Adkins SD, Ren BH, Norby FL, Zhang X, Benoit JN, Epstein PN, Ren J: **Impaired cardiac function and IGF-I response in myocytes from calmodulin-diabetic mice: role of Akt and RhoA**. *Am J Physiol Endocrinol Metab* 2003, **284**(2):E366-376.
246. Catalucci D, Latronico MV, Ceci M, Rusconi F, Young HS, Gallo P, Santonastasi M, Bellacosa A, Brown JH, Condorelli G: **Akt increases sarcoplasmic reticulum Ca2+ cycling by direct phosphorylation of phospholamban at Thr17**. *J Biol Chem* 2009, **284**(41):28180-28187.
247. Kim YK, Kim SJ, Yatani A, Huang Y, Castelli G, Vatner DE, Liu J, Zhang Q, Diaz G, Zieba R *et al*: **Mechanism of enhanced cardiac function in mice with hypertrophy induced by overexpressed Akt**. *J Biol Chem* 2003, **278**(48):47622-47628.
248. Reinartz M, Raupach A, Kaisers W, Godecke A: **AKT1 and AKT2 induce distinct phosphorylation patterns in HL-1 cardiac myocytes**. *J Proteome Res* 2014, **13**(10):4232-4245.
249. Gerich J, Becker RH, Zhu R, Bolli GB: **Fluctuation of serum basal insulin levels following single and multiple dosing of insulin glargine**. *Diabetes Technol Ther* 2006, **8**(2):237-243.
250. Lucidi P, Porcellati F, Rossetti P, Candeloro P, Andreoli AM, Cioli P, Hahn A, Schmidt R, Bolli GB, Fanelli CG: **Metabolism of insulin glargine after repeated daily subcutaneous injections in subjects with type 2 diabetes**. *Diabetes Care* 2012, **35**(12):2647-2649.
251. Shiramoto M, Eto T, Irie S, Fukuzaki A, Teichert L, Tillner J, Takahashi Y, Koyama M, Dahmen R, Heise T *et al*: **Single-dose new insulin glargine 300 U/ml provides prolonged, stable glycaemic control in Japanese and European people with type 1 diabetes**. *Diabetes Obes Metab* 2015, **17**(3):254-260.
252. Korsatko S, Deller S, Mader JK, Glettler K, Koehler G, Treiber G, Urschitz M, Wolf M, Hastrup H, Sondergaard F *et al*: **Ultra-long pharmacokinetic properties of insulin degludec are comparable in elderly subjects and younger adults with type 1 diabetes mellitus**. *Drugs Aging* 2014, **31**(1):47-53.
253. Kupcova V, Arold G, Roepstorff C, Hojbjerre M, Klim S, Haahr H: **Insulin degludec: pharmacokinetic properties in subjects with hepatic impairment**. *Clin Drug Investig* 2014, **34**(2):127-133.
254. Biester T, Blaesig S, Remus K, Aschemeier B, Kordonouri O, Granhall C, Sondergaard F, Kristensen NR, Haahr H, Danne T: **Insulin degludec's ultra-long pharmacokinetic properties observed in adults are retained in children and adolescents with type 1 diabetes**. *Pediatr Diabetes* 2014, **15**(1):27-33.
255. Uhlig M, Passlack W, Eckel J: **Functional role of Rab11 in GLUT4 trafficking in cardiomyocytes**. *Mol Cell Endocrinol* 2005, **235**(1-2):1-9.
256. Ciaraldi TP, Phillips SA, Carter L, Aroda V, Mudaliar S, Henry RR: **Effects of the rapid-acting insulin analog glulisine on cultured human skeletal muscle cells: comparisons with insulin and insulin-like growth factor I**. *J Clin Endocrinol Metab* 2005, **90**(10):5551-5558.
257. Ciaraldi TP, Carter L, Seipke G, Mudaliar S, Henry RR: **Effects of the long-acting insulin analog insulin glargine on cultured human skeletal muscle cells:**

comparisons to insulin and IGF-I. *J Clin Endocrinol Metab* 2001, **86**(12):5838-5847.
258. Hartmann T, Overhagen S, Ouwens DM, Raschke S, Wohlfart P, Tennagels N, Wronkowitz N, Eckel J: **Effect of the long-acting insulin analogues glargine and degludec on cardiomyocyte cell signalling and function**. *Cardiovasc Diabetol* 2016, **15**:96.
259. Duckworth WC, Gifford D, Kitabchi AE, Runyan K, Solomon SS: **Insulin binding and degradation by muscles from streptozotocin-diabetic rats**. *Diabetes* 1979, **28**(8):746-748.
260. Hue L, Taegtmeyer H: **The Randle cycle revisited: a new head for an old hat**. *Am J Physiol Endocrinol Metab* 2009, **297**(3):E578-591.
261. Stanley WC, Lopaschuk GD, McCormack JG: **Regulation of energy substrate metabolism in the diabetic heart**. *Cardiovasc Res* 1997, **34**(1):25-33.
262. Bielawska AE, Shapiro JP, Jiang L, Melkonyan HS, Piot C, Wolfe CL, Tomei LD, Hannun YA, Umansky SR: **Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion**. *Am J Pathol* 1997, **151**(5):1257-1263.
263. Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, Orci L, Unger RH: **Lipotoxic heart disease in obese rats: implications for human obesity**. *Proc Natl Acad Sci U S A* 2000, **97**(4):1784-1789.
264. Rosen P, Du X, Tschope D: **Role of oxygen derived radicals for vascular dysfunction in the diabetic heart: prevention by alpha-tocopherol?** *Mol Cell Biochem* 1998, **188**(1-2):103-111.
265. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, Utsumi H, Hamasaki N, Takeshita A: **Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction**. *Circ Res* 2001, **88**(5):529-535.
266. Morad M, Suzuki YJ: **Redox regulation of cardiac muscle calcium signaling**. *Antioxid Redox Signal* 2000, **2**(1):65-71.
267. Simon HU, Haj-Yehia A, Levi-Schaffer F: **Role of reactive oxygen species (ROS) in apoptosis induction**. *Apoptosis* 2000, **5**(5):415-418.
268. **FDA approves two new drug treatments for diabetes mellitus** [http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm464321.htm]
269. Song DH, Shin JH, Park YJ, Im DS, Bae SM, Kwon SC: **An insulin conjugate using an immunoglobulin fragment**. In.: Google Patents; 2011.
270. Strohl WR: **Fusion Proteins for Half-Life Extension of Biologics as a Strategy to Make Biobetters**. *BioDrugs* 2015, **29**(4):215-239.
271. Kang JK, SE; Choi, IY; Bae, S; Yoon, YK; Lee, KH; Park, KM; Kwon, SC; Son, J: **Pharmacokinetic and pharmacodynamic effects of a single dose of the long-acting GLP-1R agonist HM11260C in subjects with type 2 diabetes mellitus.** *Diabetes* 2012, **61 (Supplement 1)**.
272. **What is the Quantum Project?** [http://www.hanmipharm.com/ehanmi/handler/Rnd-ProjectQuantum]
273. **What is LAPSCOVERY?** [http://www.hanmipharm.com/ehanmi/handler/Rnd-ProjectBio]
274. Song DH, Shin JH, Lee MJ, Hong SH, Kwon SC, Lee GS: **A polypeptide complex comprising non-peptidyl polymer having three functional ends**. In.: Google Patents; 2010.
275. Roopenian DC, Akilesh S: **FcRn: the neonatal Fc receptor comes of age**. *Nat Rev Immunol* 2007, **7**(9):715-725.

276. Kontermann RE: **Strategies for extended serum half-life of protein therapeutics**. *Curr Opin Biotechnol* 2011, **22**(6):868-876.
277. **Guidance for Industrie - Diabetes Mellitus: Developing Drugs and Therapeutic Biologics for Treatment and Prevention** [http://www.fda.gov/downloads/Drugs/.../Guidances/ucm071624.pdf]
278. Enzmann HG, Weise M: **Current european regulatory perspectives on insulin analogues**. *Diabetol Metab Syndr* 2011, **3**:14.
279. Lepore M, Pampanelli S, Fanelli C, Porcellati F, Bartocci L, Di Vincenzo A, Cordoni C, Costa E, Brunetti P, Bolli GB: **Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting human insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro**. *Diabetes* 2000, **49**(12):2142-2148.
280. Gough SC, Harris S, Woo V, Davies M: **Insulin degludec: overview of a novel ultra long-acting basal insulin**. *Diabetes Obes Metab* 2013, **15**(4):301-309.
281. Choi IH, S; Kim, S; Jung, D; Kim, YM; Lee, Y; Kim, M; Trautmann, M; Hompesch, M; Son, J; Kwon, S: **Long-acting basal insulin (HM12470) offers once-weekly dosing potential with a favourable PK, PD and mitogenic profile.** *Diabetologia* 2014, **57 (Supplement 1)**.
282. Peyrot M, Barnett AH, Meneghini LF, Schumm-Draeger PM: **Insulin adherence behaviours and barriers in the multinational Global Attitudes of Patients and Physicians in Insulin Therapy study**. *Diabet Med* 2012, **29**(5):682-689.
283. Peyrot M, Barnett AH, Meneghini LF, Schumm-Draeger PM: **Factors associated with injection omission/non-adherence in the Global Attitudes of Patients and Physicians in Insulin Therapy study**. *Diabetes Obes Metab* 2012, **14**(12):1081-1087.
284. Hwang SY KJ, Kim JY, Choi IY, Hompesch M, Kwon SC: **Mechanisms for Long-Acting Properties of the Novel Weekly Insulin HM12470**. *Diabetes* 2016, **65 (Supplement 1)**.
285. Tennagels N, Werner U: **The metabolic and mitogenic properties of basal insulin analogues**. *Arch Physiol Biochem* 2013, **119**(1):1-14.
286. Aronson D, Rayfield EJ: **How hyperglycemia promotes atherosclerosis: molecular mechanisms**. *Cardiovasc Diabetol* 2002, **1**:1.
287. Herring R, Jones RH, Russell-Jones DL: **Hepatoselectivity and the evolution of insulin**. *Diabetes Obes Metab* 2014, **16**(1):1-8.
288. Waldhausl W, Bratusch-Marrain P, Gasic S, Korn A, Nowotny P: **Insulin production rate following glucose ingestion estimated by splanchnic C-peptide output in normal man**. *Diabetologia* 1979, **17**(4):221-227.
289. Hordern SV, Wright JE, Umpleby AM, Shojaee-Moradie F, Amiss J, Russell-Jones DL: **Comparison of the effects on glucose and lipid metabolism of equipotent doses of insulin detemir and NPH insulin with a 16-h euglycaemic clamp**. *Diabetologia* 2005, **48**(3):420-426.
290. Logtenberg SJ, Kleefstra N, Houweling ST, Groenier KH, Gans RO, van Ballegooie E, Bilo HJ: **Improved glycemic control with intraperitoneal versus subcutaneous insulin in type 1 diabetes: a randomized controlled trial**. *Diabetes Care* 2009, **32**(8):1372-1377.
291. Wang H, Wang AX, Liu Z, Barrett EJ: **Insulin signaling stimulates insulin transport by bovine aortic endothelial cells**. *Diabetes* 2008, **57**(3):540-547.
292. Dernovsek KD, Bar RS, Ginsberg BH, Lioubin MN: **Rapid transport of biologically intact insulin through cultured endothelial cells**. *J Clin Endocrinol Metab* 1984, **58**(4):761-763.
293. King GL, Johnson SM: **Receptor-mediated transport of insulin across endothelial cells**. *Science* 1985, **227**(4694):1583-1586.

294. Bar RS, Harrison LC, Muggeo M, Gorden P, Kahn CR, Roth J: **Regulation of insulin receptors in normal and abnormal physiology in humans**. *Adv Intern Med* 1979, **24**:23-52.
295. Bar RS, Gorden P, Roth J, Kahn CR, De Meyts P: **Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients: effects of starvation, refeeding, and dieting**. *J Clin Invest* 1976, **58**(5):1123-1135.
296. Archer JA, Gorden P, Roth J: **Defect in insulin binding to receptors in obese man. Amelioration with calorie restriction**. *J Clin Invest* 1975, **55**(1):166-174.
297. Archer JA, Gorden P, Gavin JR, 3rd, Lesniak MA, Roth J: **Insulin receptors in human circulating lymphocytes: application to the study of insulin resistance in man**. *J Clin Endocrinol Metab* 1973, **36**(4):627-633.
298. Choi IY PS, Hwang SY, Lee GH, Kim YH, Kwon SC: **Super-Agonistic Activation of GLP-1 Receptor by CA Exendin-4 (exendin-4 analog) with Fast Dissociation Rate Constant**. *Diabetes* 2013, **62 (Supplement 1)**.
299. Choi IY PS, Trautmann M, Moon MJ, Kim JY, Lee YM, Hompesch M, Kwon SC: **Underlying Superagonistic Mechanisms of Efpeglenatide in Glycaemic Control and Weight Loss Potency**. *Diabetes* 2016, **65 (Supplement 1)**.
300. Burmeister WP, Gastinel LN, Simister NE, Blum ML, Bjorkman PJ: **Crystal structure at 2.2 A resolution of the MHC-related neonatal Fc receptor**. *Nature* 1994, **372**(6504):336-343.
301. Burmeister WP, Huber AH, Bjorkman PJ: **Crystal structure of the complex of rat neonatal Fc receptor with Fc**. *Nature* 1994, **372**(6504):379-383.
302. Martin WL, West AP, Jr., Gan L, Bjorkman PJ: **Crystal structure at 2.8 A of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding**. *Mol Cell* 2001, **7**(4):867-877.
303. Rankin DJ: **Therapy supervision: the phenomena and the need**. *Clin Nurse Spec* 1989, **3**(4):204-208.
304. Roopenian DC, Christianson GJ, Sproule TJ, Brown AC, Akilesh S, Jung N, Petkova S, Avanessian L, Choi EY, Shaffer DJ *et al*: **The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs**. *J Immunol* 2003, **170**(7):3528-3533.
305. Wani MA, Haynes LD, Kim J, Bronson CL, Chaudhury C, Mohanty S, Waldmann TA, Robinson JM, Anderson CL: **Familial hypercatabolic hypoproteinemia caused by deficiency of the neonatal Fc receptor, FcRn, due to a mutant beta2-microglobulin gene**. *Proc Natl Acad Sci U S A* 2006, **103**(13):5084-5089.
306. Waldmann TA, Terry WD: **Familial hypercatabolic hypoproteinemia. A disorder of endogenous catabolism of albumin and immunoglobulin**. *J Clin Invest* 1990, **86**(6):2093-2098.
307. Simister NE, Rees AR: **Isolation and characterization of an Fc receptor from neonatal rat small intestine**. *Eur J Immunol* 1985, **15**(7):733-738.
308. Jones EA, Waldmann TA: **The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat**. *J Clin Invest* 1972, **51**(11):2916-2927.
309. Rodewald R: **pH-dependent binding of immunoglobulins to intestinal cells of the neonatal rat**. *J Cell Biol* 1976, **71**(2):666-669.
310. Raghavan M, Bonagura VR, Morrison SL, Bjorkman PJ: **Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants**. *Biochemistry* 1995, **34**(45):14649-14657.
311. Ward ES, Zhou J, Ghetie V, Ober RJ: **Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans**. *Int Immunol* 2003, **15**(2):187-195.

312. Antohe F, Radulescu L, Gafencu A, Ghetie V, Simionescu M: **Expression of functionally active FcRn and the differentiated bidirectional transport of IgG in human placental endothelial cells**. *Hum Immunol* 2001, **62**(2):93-105.
313. Artunc F, Schleicher E, Weigert C, Fritsche A, Stefan N, Haring HU: **The impact of insulin resistance on the kidney and vasculature**. *Nat Rev Nephrol* 2016, **12**(12):721-737.
314. Spiekermann GM, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, Lencer WI: **Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung**. *J Exp Med* 2002, **196**(3):303-310.
315. Bitonti AJ, Dumont JA, Low SC, Peters RT, Kropp KE, Palombella VJ, Stattel JM, Lu Y, Tan CA, Song JJ *et al*: **Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway**. *Proc Natl Acad Sci U S A* 2004, **101**(26):9763-9768.
316. Dumont JA, Bitonti AJ, Clark D, Evans S, Pickford M, Newman SP: **Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway**. *J Aerosol Med* 2005, **18**(3):294-303.
317. Heise T, Meneghini LF: **Insulin stacking versus therapeutic accumulation: understanding the differences**. *Endocr Pract* 2014, **20**(1):75-83.
318. Bansal V, Davis GM, Ismail-Beigi F: **Risk of insulin accumulation (stacking) with use of novel ultralong-acting insulin formulations**. *Endocr Pract* 2014, **20**(9):990-991.
319. Horvath K, Jeitler K, Berghold A, Ebrahim SH, Gratzer TW, Plank J, Kaiser T, Pieber TR, Siebenhofer A: **Long-acting insulin analogues versus NPH insulin (human isophane insulin) for type 2 diabetes mellitus**. *Cochrane Database Syst Rev* 2007(2):CD005613.
320. Devries JH, Nattrass M, Pieber TR: **Refining basal insulin therapy: what have we learned in the age of analogues?** *Diabetes Metab Res Rev* 2007, **23**(6):441-454.
321. Heller S, Buse J, Fisher M, Garg S, Marre M, Merker L, Renard E, Russell-Jones D, Philotheou A, Francisco AM *et al*: **Insulin degludec, an ultra-longacting basal insulin, versus insulin glargine in basal-bolus treatment with mealtime insulin aspart in type 1 diabetes (BEGIN Basal-Bolus Type 1): a phase 3, randomised, open-label, treat-to-target non-inferiority trial**. *Lancet* 2012, **379**(9825):1489-1497.
322. Garber AJ, King AB, Del Prato S, Sreenan S, Balci MK, Munoz-Torres M, Rosenstock J, Endahl LA, Francisco AM, Hollander P *et al*: **Insulin degludec, an ultra-longacting basal insulin, versus insulin glargine in basal-bolus treatment with mealtime insulin aspart in type 2 diabetes (BEGIN Basal-Bolus Type 2): a phase 3, randomised, open-label, treat-to-target non-inferiority trial**. *Lancet* 2012, **379**(9825):1498-1507.
323. Grunberger G, Chen L, Rodriguez A, Tinahones FJ, Jacober SJ, Bue-Valleskey J, Investigators I: **A randomized clinical trial of basal insulin peglispro vs NPH in insulin-naive patients with type 2 diabetes: the IMAGINE 6 trial**. *Diabetes Obes Metab* 2016, **18 Suppl 2**:34-42.
324. Lu M, Inboriboon PC: **Lantus insulin overdose: a case report**. *J Emerg Med* 2011, **41**(4):374-377.
325. Thornton S, Gutovitz S: **Intravenous overdose of insulin glargine without prolonged hypoglycemic effects**. *J Emerg Med* 2012, **43**(3):435-437.
326. Ashawesh K, Padinjakara RN, Murthy NP, Nizar H, Anwar A: **Intentional overdose with insulin glargine**. *Am J Health Syst Pharm* 2009, **66**(6):534.
327. Oduru M, Ahmad M: **Massive levemir (long-acting) insulin overdose: case report**. *Case Rep Med* 2012, **2012**:904841.

328. Kumar A, Hayes CE, Iwashyna SJ, Boustani R, Duncan L, Mickell JJ, Francis G: **Management of intentional overdose of insulin glargine**. *Endocrinol Nutr* 2012, **59**(9):570-572.
329. Eldred AE, Mustafa OG, Hunt KF, Whitelaw BC: **Problem based review: the patient who has taken an overdose of long-acting insulin analogue**. *Acute Med* 2013, **12**(3):167-172.
330. Campbell IW, Ratcliffe JG: **Suicidal insulin overdose managed by excision of insulin injection site**. *Br Med J (Clin Res Ed)* 1982, **285**(6339):408-409.
331. Brambell FW, Hemmings WA, Morris IG: **A Theoretical Model of Gamma-Globulin Catabolism**. *Nature* 1964, **203**:1352-1354.
332. Humphrey JH, Fahey JL: **The Metabolism of Normal Plasma Proteins and Gamma-Myeloma Protein in Mice Bearing Plasma-Cell Tumors**. *J Clin Invest* 1961, **40**(9):1696-1705.
333. Yu Z, Lennon VA: **Mechanism of intravenous immune globulin therapy in antibody-mediated autoimmune diseases**. *N Engl J Med* 1999, **340**(3):227-228.
334. Raghavan M, Chen MY, Gastinel LN, Bjorkman PJ: **Investigation of the interaction between the class I MHC-related Fc receptor and its immunoglobulin G ligand**. *Immunity* 1994, **1**(4):303-315.
335. Getman KE, Balthasar JP: **Pharmacokinetic effects of 4C9, an anti-FcRn antibody, in rats: implications for the use of FcRn inhibitors for the treatment of humoral autoimmune and alloimmune conditions**. *J Pharm Sci* 2005, **94**(4):718-729.
336. Liu L, Garcia AM, Santoro H, Zhang Y, McDonnell K, Dumont J, Bitonti A: **Amelioration of experimental autoimmune myasthenia gravis in rats by neonatal FcR blockade**. *J Immunol* 2007, **178**(8):5390-5398.
337. Vaccaro C, Zhou J, Ober RJ, Ward ES: **Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels**. *Nat Biotechnol* 2005, **23**(10):1283-1288.
338. Ober RJ, Radu CG, Ghetie V, Ward ES: **Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies**. *Int Immunol* 2001, **13**(12):1551-1559.

Danksagung

Mein Dank gilt Herrn Prof. Eckel für die Bereitstellung des Themas, das fortwährende Interesse an dieser Arbeit und für viele konstruktive Diskussionen, welche maßgeblich zum Gelingen dieser Arbeit beigetragen haben. Darüber hinaus bedanke ich mich für das Ermöglichen der Teilnahme an internationalen Kongressen.

Herrn Prof. Lammert danke ich für die Übernahme des Korreferats und das Interesse an meiner Arbeit.

Darüber hinaus bedanke ich mich bei den (ehemaligen) Mitgliedern der Paul-Langerhans-Gruppe und anderen Arbeitsgruppen für die gemeinsame Zeit. Besonderer Dank gilt hierbei Nina, die mich als Betreuerin stets zu einer selbstständigen Arbeitsweise motiviert hat. Zusätzlicher Dank gilt Silja, die mich vor ihrem Mutterschutz als Betreuerin übernommen und mich super am DDZ eingearbeitet hat. Dieser Dank gilt ebenso Sabrina, die mich anfangs bei den Kontraktionsversuchen an die Hand genommen hat. Bei Ira, Tina und Tanya möchte ich mich auch noch sehr herzlich bedanken, da sie stets ein offenes Ohr für Fragen, Probleme oder sonstige Anliegen bei einem Kaffee oder privat hatten. Manuela gilt auch noch besonderer Dank, da sie immer viel von mir gehalten hat und mir stets Mut zugeredet hat. Nicht vergessen sind natürlich Andrea und Birgit, die stets die Stimmung aufgelockert haben und mir mit Rat und Tat zur Seite standen. Ein riesiger Dank geht zusätzlich an meine erste und einzige Studentin, Carolyn, die im Labor wunderbar produktiv war und wo aus der Betreuung im Labor eine richtige Freundschaft gewachsen ist.

Großer Dank ist natürlich auch meiner Freundin Alisa geschuldet, die alle meine Launen besonders in der Endphase der Doktorarbeit mit einem mehr oder minder großen Lächeln ertragen hat und mich stets in allen Lebensbereichen unterstützt wo immer sie kann! Danke!

Weiterer Dank gilt allen meinen Freunden und allen Menschen, die sich von mir immer wieder Geschichten aus dem Labor anhören mussten. Danke Sebastian, Ingo, Michael, Andreas, Constancia, Martin und alle die ich vergessen habe zu erwähnen!

Großer Dank gilt Mama, Bruder, Oma, Opa und allen anderen aus der Familie, die stets an mich geglaubt haben und die mir diese Ausbildung ermöglicht haben. Ich denke mein Vater wäre stolz auf mich.

HOO-RAH!

Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

(Thorsten Hartmann)

Düsseldorf, den 22. Februar 2017