Vasodilator effect of glucagon, glucagon-like peptide-1 and GLP-1 receptor agonists, and role of oxidative stress in their vasoactivity

Ph.D. thesis

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Abbreviations

ApoE: Apolipoprotein E

Akt/PKB: Protein Kinase B

ANP**:** Atrial Natriuretic Peptide

ATP: Adenosine triphosphate

AUC: Area Under the Curve

BK_{Ca}: Large-conductance calcium activated potassium channel

CaMK: Ca^{2+}/calmodulin-regulated protein kinase

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CO: Carbon monoxide

COX-1: Cyclo-oxygenase-1

CSE: Cystathionine gamma-lyase

DPI: Diphenyleneiodonium chloride

DPP-4: Dipeptidyl peptidase-4

ELISA: Enzyme-linked immunosorbent assay

eNOS: Endothelial Nitric Oxide Synthase

ERK 1/2: extracellular signal–regulated kinase 1/2

GIP: Glucose-dependent insulinotropic peptide

GLP-1: Glucagon-like-peptide-1

GLP-1R: Glucagon-like-peptide-1 receptor

NADPH: Nicotinamide adenine dinucleotide phosphate

NO: Nitric oxide

H_2O_2: Hydrogen peroxide

H_2S: Hydrogen sulphide

H89:N-(2-(p-Bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide dihydrochloride

HFD: High-Fat Diet

HPLC: High Performance Liquid Chromatography

HDR: High Responders To High-Fat Diet

HO: Heme oxygenase

ICAM-1: Intercellular Adhesion Molecule 1

IRS-1: Insulin-Receptor-Substrate-1

IRS-2: Insulin-Receptor-Substrate-2

LDR: Low responders to high-fat diet

K_{ATP}: ATP-sensitive potassium channel

KCNQ: Voltage-gated potassium channel subfamily

K_v: Voltage-gated potassium channel

L-NAME: N_ω-Nitro-L-arginine methyl ester hydrochloride

MAPK: Mitogen-activated protein kinase

3MST: 3-mercaptopyruvate-sulfurtransferase

m-Tyr: Meta-tyrosine

NASH: Nonalcoholic steatohepatitis

NCX: Sodium-calcium exchanger

NFκB: Nuclear Factor κB

$O_2^{-\bullet}$: superoxide anion

ODQ: 1H-1,2,4-oxadiazolo[4,3-a]quinoxalin-1-one

OGTT: Oral glucose tolerance test

o-Tyr: Ortho-tyrosine

p-Tyr: Para-tyrosine

PI3K: Phosphatidylinositol 3-Kinase

PKA: Protein kinase A

PKG: Protein kinase G

PLC: Phospholipase C

PPG: DL-propargylglycine

ROS: Reactive oxygen species

sGC: Soluble guanylyl cyclase

SOD: Superoxide dismutase

TEA: Tetraethylammonium chloride

T2DM: Type 2 diabetes mellitus

1. Abstract

Background

Glucagon, a pancreatic peptide hormone is widely known for its role as an antagonist of the effects of insulin in the maintenance of blood glucose homeostasis, while it also reduces vascular resistance in several organs. Although the vasodilator potential of glucagon was formerly suggested, no direct evidence of the glucagon induced vasodilatation was available so far.

Apart from its effects on blood glucose levels, the incretine hormone, glucagon-like peptide-1 (GLP-1) also relaxes certain arteries, which might explain the blood pressure lowering effect of GLP-1 mimetics, which are used in the everyday-care of type 2 diabetic (T2DM) patients. Native GLP-1 causes dose-dependent relaxation of the thoracic aorta of rats and other arteries via nitric oxide (NO), cyclic adenosine monophosphate (cAMP) and ATP-sensitive potassium channels (K_{ATP}), however, through a mechanism not thoroughly described.

The dose-dependent vasodilator effect of exendin-4 (exenatide) in the rat aorta has previously been demonstrated, although the precise mechanism is not completely described. Another GLP-1 mimetic, liraglutide induces nitric oxide production in vascular endothelial cells, however, there was no evidence for its direct vasodilator effect before.

Previous data of our workgroup indicated that the accumulation of oxidized amino acids (meta- and ortho-tyrosine) due to oxidative stress may play an important role in the impaired insulin-induced vasoactive properties of different arterial segments. There is evidence that incorporation of these amino acids into cellular proteins may lead to certain hormonal resistances, which might be restored by supplementation with the physiologic isoform, para-tyrosine (p-Tyr).

Aims

Hereby we aimed to provide direct evidence that glucagon causes vasodilatation of the rat thoracic aorta *ex vivo*, and since the mechanism of the vasoactive effect of glucagon has not been studied before, we also intended to investigate the mediators involved in the vasodilatation induced by glucagon.We also aimed to compare the vasodilator potential of glucagon, GLP-1 and insulin.

We have aimed to provide *ex vivo* evidence for the hypothesis that exenatide and liraglutide may cause relaxation of central blood vessels, for example the thoracic aorta, involving three gasotransmitters, namely nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide (H_2S) and to investigate further mediators involved in the vasodilatation induced by exenatide and liraglutide.

In our third study, we wished to prove that the physiological amino acid para-tyrosine is capable of restoring hypercholesterolemia-induced increased pathological tyrosine content of the vascular wall, thus attenuating oxidative stress-induced vascular damage.

Methods

The vasoactive effect of glucagon, insulin, GLP-1, exenatide and liraglutide was studied on isolated thoracic aortic rings of adult rats. The vasoactive effect of liraglutide was also studied on rat femoral artery. Two millimetre-long vessel segments were mounted in a wire myograph and preincubated with potential inhibitors of vasodilatation. To investigate the mechanism of the vasodilatation caused by glucagon, we determined the role of the receptor for glucagon and the receptor for GLP-1. To investigate mediators involved in the vasodilatation evoked by glucagon, exenatide and liraglutide, we studied the effect of various inhibitors of the enzymes producing the three gasotransmitters, inhibitors of reactive oxygen species formation, NADPH oxidase, prostaglandin synthesis, inhibitors of protein kinases, potassium channels and an inhibitor of the Na^+/Ca^{2+}-exchanger.

In the other study, four week-old rats were divided into three groups. Rats in the control group were kept on a regular diet, rats in the cholesterol-fed group received high-fat diet, while the third group of rats received high-fat diet with para-tyrosine supplementation for 16 weeks. Vascular response of the thoracic aorta to insulin and liraglutide was assessed by a DMT multi-myograph. Vascular para-, meta- and ortho-tyrosine (p-, m-, o-Tyr) content was measured with HPLC. Plasma insulin level was evaluated with ELISA technique, and cholesterol level was measured via an enzymatic reaction using spectrophotometry.

Results

Glucagon causes dose-dependent relaxation in the rat thoracic aorta, which is as potent as that of insulin, but greater than that of GLP-1(7-36) amide. Vasodilatation by GLP-1 is partially mediated by the glucagon receptor. The vasodilatation due to glucagon is evoked via the glucagon-receptor, but also via the receptor for GLP-1 (GLP-1R), and it is mainly endothelium-independent. The contribution of gasotransmitters, prostaglandins, the NADPH oxidase enzyme, free radicals, potassium channels and the Na^+/Ca^{2+}-exchanger is also significant.

Exenatide caused dose-dependent relaxation of rat thoracic aorta, which was evoked via the GLP-1 receptor and was mediated mainly by H_2S but also by NO and CO. Prostaglandins and superoxide free radical ($O_2^{-\bullet}$) also play a part in the relaxation. Inhibition of soluble guanylyl cyclase (sGC) significantly diminished vasorelaxation. We found that ATP-sensitive-, voltage-gated- (K_v) and calcium-activated large-conductance potassium channels (BK_{Ca}) are also involved in the vasodilation, but that seemingly the inhibition of the KCNQ-type voltage-gated potassium channels resulted in the most remarkable decrease in the rate of vasorelaxation. Inhibition of the Na^+/Ca^{2+}-exchanger abolished most of the vasodilation.

According to our findings, liraglutide activates endothelial cells and vascular smooth muscle cells leading to the production of NO, CO, H_2S,

superoxide anion, and hydrogen peroxide. Increased production of such relaxing factors promotes the activation of protein kinase- A and -G, resulting in the activation of potassium channels (ATP-sensitive-, voltage-gated-, large-conductance-calcium activated), which profoundly contributes to the activation of the Na^{+}/Ca^{2+}-exchanger, thereby leading to calcium efflux and smooth muscle relaxation and vasorelaxation.

In the oxidative stress study, plasma cholesterol level was significantly higher in the cholesterol-fed group, while the level of cholesterol in the cholesterol+para-tyrosine group did not differ significantly from that of the controls. Plasma level of insulin after glucose stimulation was decreased in the cholesterol-fed group, while that in the para-tyrosine supplemented group did not differ significantly from the controls. Elevated vascular meta-tyrosine/para-tyrosine ratio, as a sign of oxidative-stress in cholesterol-fed rats, could be avoided by para-tyrosine supplementation. Cholesterol feeding resulted in vascular insulin-and liraglutide resistance, which was restored by para-tyrosine.

Conclusions

Glucagon causes dose-dependent relaxation of rat thoracic aorta *in vitro*, which is mediated by the receptor for glucagon and the receptor for GLP-1, while the vasodilatation evoked by GLP-1 also evolves partially via the receptor for glucagon, thereby, a possible crosstalk between the two hormones at receptorial level could occur.

Exenatide induces vasodilation in rat thoracic aorta with the contribution of all three gasotransmitters. We provide *in vitro* evidence for the potential ability of exenatide to lower central (aortic) blood pressure, which could have relevant clinical importance.

We reveal the contribution of all gasotransmitters in the vasorelaxation induced by liraglutide. We provide *ex vivo* evidence that liraglutide is capable of causing vasodilatation in the central and peripheral vessels, thereby supporting the clinical observation that it lowers blood pressure.

Decreased glucose-stimulated insulin secretion due to hypercholesterolemia, and elevated vascular level of meta-tyrosine, leading to vascular liraglutide-and insulin-resistance, can both be restored with the supplementation of the physiological amino acid, para-tyrosine. The importance of a physiologic compound with the ability of restoring oxidative stress-induced functional vascular damage, could be of valuable clinical relevance.

Keywords

Aortic rings, Exenatide, Glucagon, Glucagon-like peptide-1, Hormon-resistance, Hypercholesterolemia, Insulin, Liraglutide, Meta-tyrosine, Oxidative-stress, Para-tyrosine, Vasodilatation

2. Összefoglalás

Háttér

A glukagon inzulint ellensúlyozó szerepe a vércukor fenntartásában széleskörben ismert, ugyanakkor számos szervben csökkenti az érellenállást. Habár korábban is felvetették a glukagon vazodilatátor hatását, ezidáig nem létezett közvetlen bizonyíték a glukagon által kiváltott vazodilatációra.

A vércukor - szintre gyakorolt hatásán kívül az inkretin hormon, a glucagon-szerű peptid-1 (GLP-1) szintén relaxál bizonyos artériákat, mely jelenség magyarázhatja a 2-es típusú cukorbetegség mindennapi kezelésében alkalmazott GLP-1 mimetikumok vérnyomás csökkentő hatását.

A natív GLP-1 dózisfüggő vazodilatációt vált ki a patkányok mellkasi aortájában illetve más artériákban is, melyben nitrogén monoxid, cAMP, és ATP-szenzitív kálium csatornák vesznek részt, ugyanakkor a pontos mechanizmus nem tisztázott.

Az exendin-4-ről (exenatid) már korábban kimutatták, hogy a patkány aortában dózisfüggő vazodilatációt okoz, mindazonáltal a pontos mechanizmus nem került leírásra.

Egy másik GLP-1 mimetikum, a liraglutid, nitrogén-monoxid képződést indukál vaszkuláris endotélsejtekben, de a vazodilatátor képességéről korábban nem volt adat.

Munkacsoportunk korábbi eredményei arra utaltak, hogy az oxidatív stressz hatására felhalmozódó oxidtált aminosavak (meta-és orto-tirozin) fontos szerepet játszhatnak a különböző artériaszakaszok károsodott inzulin-indukálta vazoaktív képességben. Bizonyított, hogy ezen aminosavak sejtfehérjékbe képesek beépülni, ezáltal hormonrezisztenciákat okozhatnak, mely kivédhető lehet a fiziológiás izoforma, a para-tirozin szupplementációjával.

Célkitűzések

Célul tűztük ki, hogy közvetlen bizonyítékot szolgáltassunk arra, hogy a glukagon vazodilatációt vált ki a patkány aortában *in vitro*, és mivel a glukagon vazoaktív hatását korábban nem vizsgálták, meg szerettük volna határozni a glukagon-által indukált vazodilatáció mediátorait. Ezen felül össze kívántuk hasonlítani a glukagon, a GLP-1 és az inzulin vazodilatációs potenciálját.

In vitro kívántuk igazolni azt a hipotézisünket, hogy az exenatid és a liraglutid csökkenthetik a centrális vérnyomást, melyben szerepe van három gazotranszmitternek, név szerint a nitrogén-monoxidnak (NO), a szén-monoxidnak (CO) és a kén-hidrogénnek (H_2S), valamint tisztázni kívántuk az exenatid és a liraglutid által kiváltott vazodilatáció további mediátorait.

Második vizsgálatunk során bizonyítani kívántuk, hogy a fiziológiás aminosav para-tirozin képes az érfal hiperkoleszerinémia és oxidatív stessz hatására megnövekedett patológiás tirozin tartalmát helyreállítani, ezáltal csökkentve a funkcionális vaszkuláris károsodást.

Módszerek

A glukagon, az inzulin, a glukagon-szerű peptid-1, az exenatid és a liraglutid vazoaktív hatását felnőtt patkányok izolált mellkasi aortáján vizsgáltuk. A liraglutid vazoaktív hatását patkány femorális artérián is vizsgáltuk. Miográf celláiba fűztünk fel két milliméteres érszakaszokat, és előinkubáltuk azokat a vazodilatáció lehetséges gátlóival. A glukagon által kiváltott vazodilatáció mechanizmusának megállapítása céljából vizsgáltuk a glukagon-receptor és a GLP-1-receptor szerepét a glukagon indukálta vazodilatációban. A glukagon, az exenatid és a liraglutid által kiváltott vazodilatáció mechanizmusának tisztázása céljából előinkubáltuk az érszakaszokat különböző gáztranszmitterek képződését gátló anyagokkal, reaktív oxigéntermékek képződésének gátlóival, NADPH-oxidázgátlóval, prosztaglandinszintézis-gátlóval, proteinkinázok gátlóival, káliumcsatornák valamint a Na^+/Ca^{2+}-cseretranszporter gátlójával.

Oxidatív stressz vizsgálatunk során négyhetes patkányokat osztottunk három csoportba. A kontrol csoportban hagyományos tápot kaptak az állatok, a

koleszterin-táplált csoportban magas zsírtartalmú diétában részesültek, míg a harmadik csoportban az állatok a magas zsírtartalmú diéta mellett para-tirozin szupplementációban részesültek 16 héten keresztül. Az inzulin-és a liraglutid kiváltotta érválaszt DMT multi-miográf segítségével vizsgáltuk. Az ér para-, meta- és orto-tirozin tartalmát HPLC-vel vizsgáltuk. A plazmainzulin-szintet ELISA módszerrel, a koleszterin koncentrációját pedig enzimatikus módszerrel, spektrofotometriásan határoztuk meg.

Eredmények

A glukagon dózisfüggő vazodilatációt okoz a patkány mellkasi aortában, mely hasonló nagyságú az inzulin által kiváltott vazodilatációhoz, ugyanakkor nagyobb, mint a GLP-1(7-36) amid által okozott. A GLP-1 által okozott vazodilatáció részben a glukagon receptorán keresztül jön létre. A glukagonindukálta vazodilatáció főleg a glukagon-receptoron, de kisebb részben a GLP-1 receptorán keresztül alakul ki, az ér endotéliumtól függetlenül. Jelentős szerepet játszanak még ezen túlmenően a vazodilatációban a gátranszmitterek, a prosztaglandinok, a NADPH-oxidáz enzim, szabadgyökök, káliumcsatornák és a Na^{+}/Ca^{2+}-cseretranszporter.

Az exenatid szintén dózisfüggő vazodilatációt okoz a patkány mellkasi aortában, mely a GLP-1-receptoron keresztül, főképp H_2S, kisebb részt NO és CO közvetítésével valósul meg. Az exenatid által kiváltott vazodilatációban szerepe van még a prosztaglandinoknak és a szuperoxid szabad gyöknek is. A szolubilis guanilát cikláz enzim gátlása kivédte a vazodilatációt. Azt találtuk, hogy az ATP-szenzitív, a feszültség-függő, valamint a nagy vezetőképességű kálcium aktiválta káliumcsatornáknak is van szerepe, de láthatólag a KCNQ-típusú feszültség-függő kálium csatornák gátlása vezetett a legnagyobb arányú csökkenéshez az exenatid-által kiváltott vazodilatációban. ANa^{+}/Ca^{2+}-cseretranszporter gátlása szinte teljesen kivédte a vazodilatációt.

Eredményeink alapján a liraglutid aktiválja az endotélsejteket és a vaszkuláris simaizom-sejteket, ezáltal NO, CO,H_2S, szuperoxid szabad gyök és hidrogén-peroxid termelődést okoz. Ezen mediátorok megnövekedett

képződése hozzájárul a protein kináz-A és –G aktivációjához, mely káliumcsatornák (ATP-szenzitív-, feszültségfüggő-, és nagy vezetőképességű kálcium-aktiválta) aktivációját eredményezi, ami pedig végül aktiválja aNa^+/Ca^{2+}-cseretranszportert, ezáltal kálcium kiáramláshoz, simaizom relaxációhoz illetve vazodilatációhoz vezetve.

Oxidatív stressz vizsgálatunk során a koleszterinnel etetett csoportban szignifikánsan magasabb plazma-koleszterin szintet mértünk, ugyanakkor a plazma-koleszterin szint nem különbözött szignifikánsan a koleszterinnel etetett és koleszterinnel etetett és para-tirozin szupplementációban részesülő csoportok között. A glukóz-stimmulációt követő inzulin-szekréció csökkent volt a koleszterinnel etetett csoportban, míg a para-tirozin szupplementációban is részesülő csoportban nem különbözött a kontrolokétól.

A koleszterinnel etetett állatoknál tapasztalt emelkedett érfali meta-tirozin/para-tirozin arány kivédhető volt para-tirozin szupplementációjával. A koleszterin etetés hatására létrejött vaszkuláris inzulin- és liraglutid-rezisztenica szintén kivédhető volt para-tirozinnal.

Következtetések

A glukagon dózisfüggő vazodilatációt okoz a patkány mellkasi aortában *in vitro*, mely a glukagon-receptoron és a GLP-1 receptoron keresztül valósul meg, míg a GLP-1 által okozott vazodilatáció szintén részben a glukagon-receptoron keresztül jön létre. Igy tehát, lehetséges, hogy a két hormon és a receptoraik között átfedés van.

Az exenatid és a liraglutid mindhárom gazotranszmitter közvetítésével vazodilatációt okoz a patkány mellkasi aortában. *In vitro* bizonyítottuk, hogy az exenatid és a liraglutid csökkentheti a centrális vérnyomást, melynek klinikai jelentősége nagy. Ezen felül bizonyítottuk, hogy a liraglutid a perifériás ereket is dózisfüggő módon relaxálja.

A hiperkoleszterinémia által kiváltott csökkent inzulinszekréció, valamint a vaszkuláris liraglutid- és inzulinrezisztenciához vezető emelkedett érfali meta-tirozin tartalom is helyreállítható a fiziológiás aminosav, para-tirozin

szupplementációjával. A klinikai jelentősége ennek az lehet, hogy egy fizológiás anyag alkalmas lehet az oxidatív stressz indukálta funkcionális vaszkuláris károsodás kivédésre.

Kulcsszavak

aortagyűrűk, exenatid, glukagon, glukagon-szerű peptid-1, hormonrezisztencia, hiperkoleszterinémia, inzulin, liraglutid, meta-tirozin, oxidatív-stressz, para-tirozin, vazodilatáció

3. Introduction

3.1. Gastrointestinal peptide hormones: glucagon and glucagon-like peptides

3.1.1. Glucagon

Glucagon, a 29 amino acid peptide hormone, was first isolated from a pancreatic extract in the 1950s (1). It is produced by the alphacells of the Langerhans islets of the pancreas, from its precursor, proglucagon, a 160-amino acid polypeptide (1). Proglucagon is also the precursor molecule for two other important peptide hormones, glucagon-like peptide-1 and 2 (GLP-1 and GLP-2), which also have relevant biological activity (1).Glucagon is also found in some cells of the stomach and intestine; moreover, it is also present in the central nervous system (1).

3.1.1.1. Biological actions of glucagon

Glucagon is mainly known for its role in the maintenance of blood glucose homeostasis, as it opposes the effects of insulin on glucose metabolism (1). In the liver, glucagon stimulates both glycogenolysis and glyconeogenesis from pyruvate, lactate, glycerol and some amino acids, thereby increasing blood glucose levels (1). Moreover, it stimulates fatty acid oxidation and reduces the level of cholesterol, triglycerides, and attenuates the release of very-low density lipoprotein (2).

Various extra-hepatic effects of glucagon have been described, such as positive inotropic and chronotropic effects in the heart, while in the gastrointestinal tract it acts as a smooth muscle relaxant (1, 2). It might even attenuate food intake and body fat mass, as well as it promotes weight loss and increases energy expenditure via the activation of brown adipose tissue (2).

Glucagon might stimulate its own secretion in the alpha cells of the pancreas and also secretion of insulin via its own receptor on the pancreatic β-cells, as it stimulates glucose-dependent insulin release (1,2). It is also essential for β-cell

differentiation during the fetal life (2). It was also suggested that glucagon might have a role in retinal function and in the perception of sweet or umami taste (2).

3.1.1.1.1. Vasodilatation induced by glucagon

Glucagon decreases vascular resistance in several organs, indirectly suggesting its vasodilator effect, while the mechanism of the vasodilator effect of glucagon is still unknown (3).

In strips of rabbit renal artery, the glucagon-induced vasodilatation was dose-dependently inhibited by Ca^{2+}-antagonists, suggesting that its vasodilator effect evolves via the increase of intracellular calcium levels (3). In rat renal arteries *in vivo*, the vasodilator response to glucagon was shown to be evoked with contribution of nitric oxide (4).

Glucagon induces dose-dependent vasodilatation in sympathetically-innervated arterial vascular bed of dog liver *in vivo*, however, the vasodilator potential of glucagon was shown to be less remarkable, compared to that of other hormones (secretin, prostaglandin E_2), and the onset of action was slow (5).

Glucagon decreases coronary vascular pressure in isolated dog heart (6), while in isolated rat heart it potentiates coronary reperfusion following ischemia, and increases NO production (7).

Despite the number of studies investigating the mechanism of the glucagon-induced decrease in vascular resistance, the precise mechanism remains unclear.

3.1.1.2. Signal transduction of the effects of glucagon

Metabolic effects of glucagon are commonly known to evolve via the G-protein coupled glucagon-receptor, through the activation adenylyl cyclase increasing cyclyc adenosine monophosphate (cAMP) levels as well as activating the phospholipase C (PLC)/protein kinase C (PKC) pathway (1, 2). However, besides the activation of the cAMP-dependent protein kinase A

(PKA), glucagon has also been shown to activate the extracellular signal-regulated protein kinase 1/2 (ERK1/2) in a clonal cell line of human embryonic kidney cells (8). The glucagon-induced activation of ERK 1/2 is known to be dependent of PKA activation (8).

In traumatic brain injury, glucagon was shown to be protective against impaired cerebral vasodilation via the activation of the cAMP-PKA pathway (9), while besides the upregulation of cAMP, another study demonstrated that the inhibition of the ERK and mitogen activated protein kinase (MAPK) pathway by glucagon also contributes to its protective effect (10).

3.1.1.3. Medical uses of glucagon

Glucagon in the acute treatment of hypoglycaemia

The most widespread therapeutical use of glucagon is in the acute treatment of severe hypoglycaemia in diabetic patients, administered either intravenously, intramuscular or as a subcutaneous injection (11). Prefilled glucagon pens, Glucagon Emergency Kit® (Eli Lilly and Co, Indianapolis,IN) and the GlucaGen® Hypokit® (Novo Nordisk A/S, Bagsværd, Denmark) can be administered rapidly in acute hypoglyacaemia for the unconscious patient by a second person. Its effect should be observed immediately (11).

Glucagon might also be used to prevent hypoglycaemia in type 1 diabetic patients, given simultaneously with insulin in a closed-loop insulin pump system, the so-called 'bionic pancreas' (12).

Glucagon in beta-blocker overdose

Based on its positive chrono-and inotropic effects, glucagon antagonises the effects of beta-blockers in the heart, therefore it might alternatively be used in beta-blocker overdose, through an intravenous infusion (13). However, this is an expensive way of treatment, while patients must be monitored for severe gastrointestinal side effects (13).

Glucagon in calcium channel blocker overdose

Although it is not common in clinical practice, glucagon can be safely used in the management of hemodynamic instability associated with calcium channel blocker poisonings (14).

Glucagon in oesophageal soft food bolus obstruction

Soft oesophageal bolus impaction of steakhouse syndrome might be treated by glucagon with caution, but only transiently prior to endoscopic treatment (15).

3.1.2. The incretin hormones

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 and 2 (GLP-1,2) are peptide hormones secreted shortly within the ingestion of nutrients, potentiating the secretion of insulin (1). The level of incretins in fasted state is low, while it raises rapidly following food intake (1).

GIP, first named as gastric inhibitory polypeptide, inhibits gastric acid secretion and stimulates insulin secretion in animals and humans (1). GIP is secreted mainly in the duodenum, although it is also present in the central nervous system (1). It is released in the small intestine in response to glucose or fat ingestion, enhancing glucose-stimulated insulin secretion(1). Since it's inhibitory effect occurs only in pharmacological doses, while the incretin effect develops also in physiological levels, it was renamed, referring to the true nature of its action (1). The new name is glucose-dependent insulinotropic peptide (1).

3.1.2.1. GLP-1

GLP-1(7-36) amiderepresents the majority of GLP-1 in the circulation; the representation of the other active form, GLP-1 (7-37) amide is smaller (16). GLP-1 is released from the intestinal L-cells in response to food intake, and degraded rapidly by the enzyme dipeptidyl peptidase-4 (DPP-4) (16).The gene sequence of GLP-1and GLP-2 are both homologous to glucagon's andboth peptide hormones are derived from proglucagon via posttranslational processing (1). Based on its role in the therapy of type 2 diabetes mellitus, we focused on the effects of GLP-1.

The receptor for GLP (GLP-1R) is present on the pancreatic islets, kidney, lung, heart, and multiple regions of the peripheral and central nervous system, as well ason endothelial and vascular smooth muscle cells (16). A broad expression of GLP-1R mRNA in the thoracic aorta of rats has been identified (17).

3.1.2.1.1. Biological effects of GLP-1

GLP-1 potentiates glucose-stimulated insulin secretion, and promotes the transcription of the proinsulin gene (1, 16). The blood glucose lowering effect of GLP-1 develops only at elevated glucose levels (16). It promotes the proliferation of β-cells, and inhibits their apoptosis (16).

It is well known, that GLP-1 attenuates gastric emptying and secretion, hepatic glucose production, as well as decreases the appetite directly in the central nervous system (16).

On the other hand,it directly suppresses the secretion of glucagon from the pancreatic α-cells, although this effect develops also indirectly via the stimulation of insulin and somatostatin secretion (16).

GLP-1 is known to be cardioprotective, and also increases the cardiac output (16). Cardioprotective and vasodilator effects of GLP-1 were mediated mainly via the GLP-1R in human studies; however, this effect was also suggested to be partially independent of GLP-1R (18).

3.1.2.1.2. Vasodilatation induced by GLP-1

Numerous publications pointed out that native GLP-1 causes concentration-dependent relaxation of different arteries; however, controversial data have been published about the mechanism of the vasodilator effect of GLP-1 (17-22). In the rat thoracic aorta, native GLP-1 was shown to cause vasorelaxation with the involvement of cyclic adenosine monophosphate (cAMP) and the ATP-sensitive potassium channels (K_{ATP}), independent of the receptor for GLP-1 (17). The same study reported that NO, hydrogen peroxide and prostaglandins are not mediating the vasodilator effect of GLP-1 (17). GLP-1 exerted dose-dependent vasorelaxation in the pulmonary arteries of rats in an endothelium-dependent manner (19, 20). In contrast, the effect of GLP-1 causing vasodilatation of the rat femoral artery was found to be endothelium-independent but it evoked via the GLP-1R (21). However, GLP-1 is known to increase the plasma level of the potent vasodilator NO, and also increases microvascular blood flow in an NO-dependent manner (22).

These findings may indicate that GLP-1 induces vasodilatation via different pathways in the different parts of the arterial system.

3.1.2.1.3. Signal transduction of the GLP-1 effects

Metabolic effects of GLP-1 develop via the 463 amino acid-containing heptahelical G-protein-coupled GLP-1R, which, as mentioned above, is widely expressed in the body (pancreatic islets, heart, lung, kidney, stomach, central and peripheral nervous system, endothelial cells, vascular smooth muscle cells, but not liver, skeletal muscle or adipose tissue) (16).

Binding of GLP-1 to its receptor increases intracellular cAMP formation and activates cAMP-dependent protein kinase A, which leads to insulin secretion (16). In smooth muscle cells the elevation of cAMP and cGMP (cyclic guanosine monophosphate) levels causes vasodilatation (16). Recent studies demonstrated that the Ca^{2+}/calmodulin-regulated protein kinase (CaMK), protein kinase B (PKB, Akt), phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinases (MAPK, ERK1/2) are also involved in the signalling of

GLP-1 in β-cells (1, 16). Moreover, GLP-1R activation leads to a closure of the ATP-sensitive potassium channels (K_{ATP}) in β-cells, which proves the diversity of the GLP-1 signalling pathways (16). There is evidence, that PKA is not the only protein by which cAMP acts (16). Besides cAMP, GLP-1 activates cAMP-binding proteins known as cAMP-regulated guanine nucleotide exchange factors (GEFs or Epac), although cAMP may show higher affinity for PKA than for Epac (16). Although these mechanisms are proven to regulate GLP-1 effects in β-cells, it is not clarified whether the same signalling pathways are involved in the relaxation of vascular smooth muscle cells.Besides the activation of K_{ATP} channels, GLP-1 is also involved in modulating the activity of the voltage-gated potassium channels (K_v) (16,17).

3.1.2.2. The incretin-based therapies

Type 2 diabetes is characterized by reduced glucose sensitivity of the β–cells, delayed, as well as a reduced meal-induced insulin secretion, loss of first phase insulin response to glucose stimulation and loss of regular oscillatory insulin secretion (23).

Besides the insulin resistance of the β-cells, muscles, and the liver; incretin deficiency/resistance, hyperglucagonemia, increased glucose reabsorption and accelerated lipolysis are all important in the development of glucose intolerance in type 2 diabetic individuals (24).

GLP-1administration lowers the blood glucose level and reduces food intake in type 2 diabetic patients via the stimulation of insulin secretion,inhibition of gastric emptying andglucagon secretion (16, 23).

Since GLP-1 itself is degraded rapidly by the DPP-4 enzyme, in the therapy of T2DM, genetically, structurally modified, enzyme-resistant peptides, inhibitors of the dipeptidyl peptidase-4 enzyme have been developed (23).

Beneficial effects of incretin-based therapies include improved glycaemic control with a low risk of hypoglycaemia and no weight gain or even weight loss, improvement of serum lipid profiles, blood pressure lowering effect and

cardioprotection following ischaemia via GLP-1R-dependent and -independent mechanisms (25).

In our research, we focused on the vasodilator effect of GLP-1 mimetics, therefore we give a more detailed description about these.

3.1.2.2.1. GLP-1 mimetics

3.1.2.2.1.1. Exenatide

Exenatide (exendin-4) is a 39 amino acid GLP-1 receptor agonist, which shows 53% homology to native human GLP-1 (16, 25). It was originally isolated from the venom of the *Heloderma suspectum* lizard (16). The *in vivo* potency of exendin-4 is 5-10 times higher than that of GLP-1 (23). It mimics the actions of GLP-1, reduces blood glucose levels via binding to the pancreatic GLP-1R (23). Exenatide stimulates glucose-dependent insulin secretion and enhances first-phase insulin secretion and also delays gastric emptying, while it also suppresses the inappropriately high glucagon secretion (23).

Exenatide, given either twice daily or once weekly as a subcutaneous injection, reduces food intake, facilitates weight loss, and also has an insulin-sensitizing effect, like GLP-1 (26).

Both endogenous GLP-1 and exendin-4 induced vasorelaxation of the rat thoracic aorta via the ATP-sensitive potassium channels and cAMP (17). Although number of studies demonstrated that both native GLP-1 and its mimetics induce vasodilatation, furthermore, some studies focused on their effect in the thoracic aorta, the precise mechanism of vasodilatation still remains unclear (17-22).

Central (aortic) blood pressure, the pressure measured in the aorta, is a major determinant of cardiovascular outcomes (27), therefore, the possible effect of exenatide to lower the pressure in the aorta as well as in other arteries could be beneficial in clinical practice.

3.1.2.2.1.2. Liraglutide

Liraglutide is a long acting GLP-1 analogue, with 97% homology to native human GLP-1, but it is also resistant to the DDP-4 enzyme (23, 25). It also mimics all of the GLP-1 actions (23). Liraglutide is administered once daily as a subcutaneous injection (25).

Liraglutide induces NO-production in vascular endothelial cells (28), however so far there has been no evidence, that any of the GLP-1 analogues would be able to induce the synthesis of other gasotransmitters.

In an $ApoE^{-/-}$ deficient mouse model, liraglutide was shown to have non-PKA-dependent, GLP-1R-dependent effects in the regulation of endothelial nitric oxide synthase (eNOS) enzyme expression and attenuated intracellular adhesion molecule-I (ICAM-1) expression in aortic endothelial cells, referring to the role of liraglutide in the inhibition of endothelial cell dysfunction (29).

A previous study, which demonstrated the GLP-1R-dependent, NO-independent systolic blood pressure lowering effect of liraglutide, also reported that the antihypertensive effect of liraglutide evoked due to the increased secretion of the atrial natriuretic peptide (ANP) (30).

3.1.3. Potential mediators of the vasodilatation induced by the GLP-1 mimetics

NO, CO, and H_2S are gaseous signalling molecules, mediating vasodilatory effects in the arterial tree (31). They are all known to act via the formation of cyclic nucleotides (cAMP/cGMP) and activation of intracellular protein kinases (PKA/PKG) and to cause vasodilation via modulating the activity of potassium channels (32). The vasorelaxation in response to H_2S often evolves via the activation of K_{ATP} channels and the KCNQ-type voltage-gated potassium channels (31, 32). In mice models of hypertension, KCNQ channel openers are proven to reduce arterial blood pressure (33). Both CO and superoxide ($O_2^{-\bullet}$) are known activators of K_v channels (34). The vasodilator effect of CO partially lays on the activation of these channels; moreover, the

activation of PKG or the activation of PKA by NO also results in the opening of these channels (31, 34).

3.2. Role of oxidative stress in the development of chronic diseases

Oxidative stress significantly contributes to development of diabetes mellitus and its complications, chronic kidney disease and inflammatory diseases,as well as the development of the complications associated with these diseases (35).

Atherosclerotic vascular disease, developing among others due to high levels of cholesterol in the blood, is a chronic inflammatory disease, which induces the hardening of the arterial wall, caused by the accumulation of white blood cells, lipoproteins and cholesterol crystals (36).

Insulin resistance is characterized by decreased glucose uptake by insulin-dependent parenchymal cells and decreased glucose-induced insulin secretion (36). There may also be a concomitant impaired GLP-1-induced insulin secretion, known as GLP-1 resistance in patients with type 2 diabetes mellitus (37).

3.2.1. The development of insulin resistance

Permanent hypercholesterolaemia leads to oxidative stress (38) and increases the activity of nuclear factor κB (NFκB) through the initiation of it's translocationinto the nucleus, leading to changes in gene expression (38). This results in the transcription of proinflammatory cytokines, thereby increasing oxidative stress (38). Activation of NFκB and the accompanying inflammatory changes are critical determinants of complications (39). The key step in the development of insulin resistance, in association with the increased oxidative stress, is the inhibitory serine phosphorylation of two important molecules in the insulin-signalling, namely insulin-receptor-substrate-1 and 2 (IRS-1, IRS-2),

instead of the activating tyrosine-phospohrylation (40). This leads to the attenuation of insulin-signalling via IRS (the Akt/PKB way) (40).

3.2.2. Effect of oxidative stress on the formation of tyrosine isoforms

Export para-tyrosine is synthesized mainly in the kidneys via an enzymatic reaction from its precursor amino acid, phenylalanine (41). While physiologically phenylalanine is transformed only to para-tyrosine by the enzyme phenylalanine-hydroxylase, under pathological circumstances, as a result of oxidative stress, due to hydroxyl free radical, phenylalanine can also be transformed into two other tyrosine isoforms, meta-tyrosine, and ortho-tyrosine (41, 42). While para-tyrosine is formed enzymatically as well as via the oxidative processes, meta- and ortho-tyrosine are only formed in the presence of hydroxyl free radical in humans (41). These isoforms are markers of hydroxyl free radical formation thus markers of oxidative stress (41).

3.2.3. Possible effect of tyrosine isoforms

There is evidence that these pathological isoforms may be incorporated into cellular proteins leading to altered cell function, hence leading to for example hormone-resistances and alterations of vascular function (43). The imbalance between physiological and non-physiological tyrosine isoforms has also been suggested to be involved in insulin-, acetylcholine- and erythropoietin-resistance (44, 45). Former data of our workgroup proved in an erythroid cell line that incorporation of ortho- and meta-tyrosine into cellular proteins leads to erythropoietin-resistance that could be overcome by increasing concentrations of added para-tyrosine (46).

Insulin-induced relaxation of arteries is known to be impaired in diabetes mellitus, while the level of ortho–tyrosine is increased (47,48). Our workgroup previously demonstrated that elevated vascular level of ortho-tyrosine significantly contributes to impaired insulin-induced relaxation of arteries (49).

4. Aims

Our aims were to demonstrate that glucagon, glucagon-like peptide-1 and GLP-1 agonists exenatide and liraglutide exert vasodilatation in the rat thoracic aorta.

We wished to determine the receptors, mediators, second messengers and downstream mediatorsinvolved in the vasodilator effect of glucagon, GLP-1 mimetics exenatide and liraglutide *in vitro* in the rat thoracic aorta.

We also intended to prove the preventive effect of para-tyrosine on the target organs and to demonstrate its effect in hormone resistant states in a model of hypercholesterolaemia and metabolic syndrome in rats. Based on our former studies, we hypothesized, that with oral supplementation of the physiological amino acid, para-tyrosine, the damage due to oxidative stress, characterized by elevated meta-tyrosine levels, may be prevented.

5. Methods

5.1. Investigation of vasodilator mechanisms

5.1.1. Animals

We performed our experiments with the permission of the Hungarian Local Animal Experiment Committee in accordance with the'Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985). Adult, 10-12 week old (280-340 g) male CFY Sprague-Dawly rats were kept on ad libitum standard chow and continuous water supply. Animals were originally purchased from Charles River Laboratories GmbH (Sulzfeld, Germany). On the day of the experiment, after anesthetization with ether, rats were killed by decapitation using a guillotine.

5.1.2. Vasoreactivity experiments

The thoracic aorta and the femoral artery were gently excised from rats and were placed in oxygenated (95% O_2/5% CO_2), ice-cold Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM glucose, 1.6 mM $CaCl_2$*$2H_2O$, pH 7.4). As described earlier, the perivascular fat and connective tissue were gently removed (50), and 2 mm long segments of the vessel were mounted on two stainless steel wires (40 μm in diameter), and placed in 5 ml organ baths of a wire myograph (Danish Multimyograph Model 610M, DMT- USA Inc., Atlanta, GA, USA). After 30 minutes of equilibration, the aortic rings were placed under tension of 1 g, while in the case of femoral artery segments the modified method of Fésüs et al. was used (17, 51). Rings were allowed to rest for 30 minutes, isometric tension was continuously recorded. After a 30 minutes resting period, vessel rings were preconstricted with 100 nM epinephrine as described earlier (49), which in our previously performed experiments had shown 60% contraction force of the 60 mM KCl contraction (52). After all vessel segments had reached a stable contraction plateau, increasing doses of either glucagon (Sigma-Aldrich, St. Louis, MO, USA), GLP-1 (Sigma-Aldrich, St. Louis, MO, USA), exenatide (Byetta® injection, Bristol-Myers Squibb–AstraZeneca, Budapest, Hungary), or liraglutide (Victoza®

injection, Novo Nordisk Hungary, Budapest, Hungary) were administered to the organ baths, and relaxant responses were assessed. The applied doses of glucagon were 0.1, 1, 2.5, 10, 25 μM. The dose of agonists that was applied to relax the aorta correlated with the dose of epinephrine we used to preconstrict the vessels. Plasma epinephrine level is approximately 30 pM at rest, while in our experiments we used 100 nM, which is a 3000 times higher concentration (53). Since the dose of epinephrine needed to be increased, to achieve sufficient preconstriction, also the applied doses of glucagon needed to be increased accordingly. The plasma exenatide level was found to be 70 pM, while in our experiments we used an approximately341-45.300 times higher concentration (23.9, 71.7, 310, 788, 1980, 3170 nM) (54). Similarly, the doses of and liraglutide correlated well with the above mentioned.The plasma level of liraglutide is 10 nM and in our experiments we used an up to 3000 times higher concentration (2.6, 5.06, 15.6, 29.2 μM) (55).

In one set of experiments, the vasodilator efficacy of glucagon, insulin and glucagon-like peptide-1 amide fragment (7-36) was compared.

In order to identify the extracellular and intracellular mediators of the vasodilator effect of glucagon, exenatide and liraglutide, we performed a series of experiments. Prior to contracting the vessels with epinephrine, we preincubated the vessels (n=5 of each experiment) with different materials.

To determine whether the vasodilatation due to glucagon evoked via the glucagon receptor, in one set of experiments vessels were preincubated with a specific glucagon receptor antagonist (hGCGR-antagonist) (25 μM, 30 min). To investigate whether the glucagon-like peptide-1-receptor (GLP-1R) is involved in the vasodilatation evoked by glucagon, vessels were preincubated with GLP-1R antagonist exendin(9-39) (25 μM, 30 min) (17). To test the hypothesis, that GLP-1 might induce vasodilatation via the glucagon-receptor, some vessels were incubated with hGCGR-antagonist (75 μM, 30 min), prior to performing the experiment with GLP-1(7-36).

To determine whether the vasodilation due to exenatide evoked via the GLP-1R, we preincubated vessels with the GLP-1R antagonist exendin(9-39) (32 μM,

30 min). Because the affinity of exendin(9-39) to bind GLP-1R is smaller than that of exenatide, we applied a ten times higher concentration of the receptor antagonist than the highest dose of exenatide (56).

In one set of experiments we mechanically removed the endothelium of the vessels by gently rubbing a hair through it. The effect of denudation was verified by the loss of response to 3 μM acetylcholine. We incubated one group of vessels with the eNOS inhibitor L-NAME (N_{ω}-Nitro-L-arginine methyl ester hydrochloride) (300 μM, 30 min). Other vessels were incubated with the potent heme oxygenase inhibitor Tin-protoporphyrin IX dichloride (10 μM, 30min), others with DL-Propargylglycine, inhibitor of cystathionine-γ-lyase (10 mmol/l, 30 min), or with the relatively selective COX-1 inhibitor indomethacin (3 μM, 30 min). We tested the effects of superoxide dismutase (SOD; 200 U/ml, 30 min) and catalase (1000 U/ml,30 min). H89 hydrochloride (5 μM, 30 min) was used to block protein kinase A (PKA), and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ, 3 μM, 30 min) was used to inhibit the effect of soluble guanylyl cyclase (sGC).

To block the large-conductance calcium-activated potassium channels (BK_{Ca} channels) some vessels were incubated with tetraethylammonium (TEA, 2 mM) for 30 minutes (57). To block the ATP-sensitive potassium (K_{ATP}) channels we used glibenclamide (10 μM, 30 min) (57). KCNQ-type voltage-dependent potassium channels were blocked by incubation with XE991 (30 μM, 15 min) (50). The Na^{+}/Ca^{2+}-exchanger was blocked by incubation with its specific inhibitor SEA0400 (4 μM, 30 min) (58).

The contribution of the NADPH oxidase in the vasodilatation induced by glucagon was demonstrated by inhibiting it with diphenyleneiodonium chloride (DPI) (10 μM, 30 min).

To exclude the effect of spontaneous vessel relaxation we performed untreated time-control experiments; however, the spontaneous vessel relaxation of untreated aortic rings was not significant. To test the effect of the specific inhibitors on the permanence of the epinephrine-induced plateau, we performed

a row of control experiments, and found that most of the chemicals had a slight vasodilatory effect which could not have a significant influence on the results.

5.1.3. Chemicals

Chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA, except for Tin-protoporphyrin IX dichloride, which was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA); XE991 was purchased from Ascent Scientific Ltd. (Avonmouth, Bristol, UK), while epinephrine was purchased from Richter-Gedeon Hungary (Budapest, Hungary). SEA0400 was synthesized in the Institute of Pharmaceutical Chemistry, University of Szeged, Hungary, and was a generous gift of Professor Ferenc Fülöp.

5.1.4. Data acquisition and statisticalanalysis

Myodaq 2.01 M610+ software was used for data acquisition and display. We expressed the rate of relaxation caused by insulin, GLP-1, glucagon, exenatide, and liraglutide as the percentage of the contraction evoked by epinephrine.

Statistical analysis was performed by using SPSS Version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined using repeated measures ANOVA with Bonferroni post hoc test. Values are shown as mean ±SE. A value of *P* less than 0.05 was considered to be significant.

5.2. Metabolic animal model

5.2.1. Animals

Experiments were performed with the permission of the Hungarian Local Animal Experiment Committee in accordance with the‘Principles of laboratory animal care’ (NIH publication no. 85–23, revised 1985). Rats were purchased from Charles River Laboratories GmbH (Sulzfeld, Germany). We divided 4

weeks old male Sprague-Dawly rats into three groups. Rats in the first group (Contr; n=10) were kept on standard chow (19 % crude protein; 1% lysine; 4% crude fat; 1% calcium; 7% crude ash; 35000 IU Vitamin A; 1000 IU Vitamin D3; 150 mg Vitamin E; 0.7% phosphorus; 12 mg copper) for four months and recieved vehiculum after one month via an oral tube for six days per week. Rats in the second group (Chol; n=10) were kept on high fat diet (70 cal%fat) for four months and after one month recieved vehiculum via an oral tube six days per week. Rats in the third group (Chol+p-Tyr; n=10) were kept on high fat diet for four months and recieved para-tyrosine supplementation (1.76 mg/die) via an oral tube after the first month, six days per week.

Body weight and food intake was recorded in every three days. Oral glucose tolerance tests (OGTT) were performed after the first, second, third and fourth month of the study.

On the day of the experiment, cardiac puncture was performed in ether narcosis for blood sampling, and finally rats were killed by decapitation using a guillotine. Blood was obtained for HPLC and enzymatic analysis, while the thoracic aorta was prepared for HPLC analysis and myography experiments.

5.2.2. Vasoreactivity experiments

As the thoracic aorta was carefully removed from the rat, vasoreactivity experiments were carried out as described above.

When all vessel segments had reached a stable contraction plateau, increasing doses of insulin (Actrapid® injection) (10; 50;100; 500; 1000 mU/ml) or liraglutide (Victosa® injection) (2.6, 5.06, 15.6, 29.2 μM) were applied to the organ baths, and relaxant responses were assessed.

5.2.3. Investigation of metabolic parameters

Plasma total-cholesterol level was determined using a one-step enzymatic method (Diagnosticum Zrt, Budapest, Hungary) with fluorescence spectrophotometry (Benchmark Plus microplate spectrophotometer).

Plasma concentrations of insulin were measured using a Rat Ultrasensitive Insulin ELISA kit (Alpco, Salem, NH, USA), detected with a microplate spectrophotometer.

5.2.4. Measurement of tyrosine isoforms in the rat thoracic aorta

An overnight hydrolysis in hydrochloric acid was performed at 120°C on equal sized thoracic aortic segments. Subsequently precipitate was separated by centrifugation. The supernatant was filtered through a syringe filter (0.2 μm) before analysis. Finally, levels of p- and m-Tyr were determined using reverse phase-HPLC (C_{18} silica column, 250x4 mm) with fluorescence detection (λ_{EX}=275 nm; λ_{EM}=305 nm) as described earlier (59). Concentrations were calculated using an external standard. Ratios of m- and p-Tyr were then calculated.

5.2.5. Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Version 22.0 (SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used as test of normality. Intergroup analyses were performed using Student's t-test or ANOVA with Bonferroni *post hoc* test as appropriate, in case of normal distribution. Kruskal-Wallis test followed by Mann-Whitney U test was used when distribution was not normal. Jonckheere-Terpstra test was used to assess tendency. Values are shown as mean±SE or median(interquarter range). Values of *P* less than 0.05 were considered significant.

6. Results

6.1. Vasoactive effect of glucagon and glucagon-like peptide-1 analogues

6.1.1. Glucagon

Glucagon induces endothelium-independent vasodilatation of the rat aorta, comparable to the vasodilatation caused by insulin and GLP-1

Glucagon caused dose-dependent vasodilatation of the rat thoracic aorta, which was as effective, as the vasodilatation evoked by insulin. GLP-1(7-36) also caused dose-dependent vasodilatation of the rat thoracic aorta *in vitro*. Glucagon and insulin proved to be more potent vasodilators in the rat thoracic aorta than native GLP-1(7-36) (Fig 1A). LogEC50 values for the glucagon induced vasodilatation were not significantly different from that of insulin, (median(IQR) logEC50 values -5.336(0.27); -5.313(0.21); respectively, p=0.958); while it was significantly lower than that of GLP-1, (-5.336(0.27) vs. -4.385(0.27), p=0.003).

Vessels with mechanically damaged endothelium showed no decrease in the vasodilator response to glucagon, moreover, the vasodilatation in endothelium-denuded vessels was more pronounced, than that in endothelium-intact vessels (logEC50 values-5.336(0.27) vs -4.78(0.21) p=0.013) (Fig 1B).

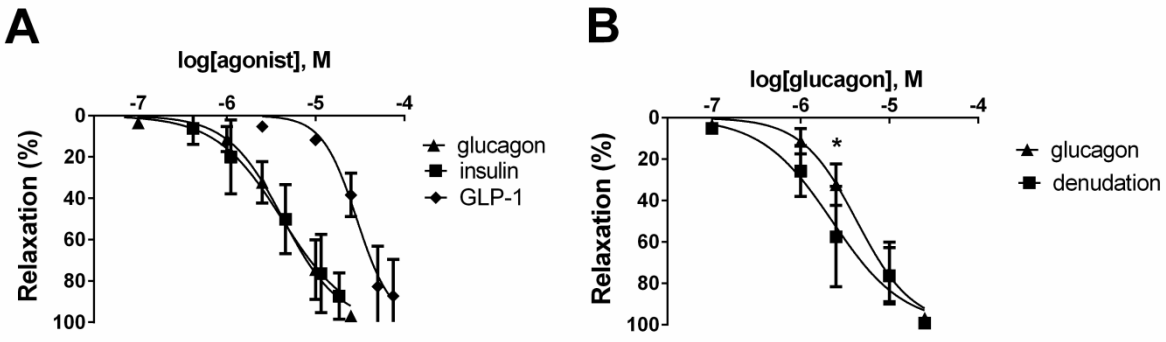

Figure 1. Concentration-relaxation curves representing a comparison of the vasodilator potential of glucagon, insulin and GLP-1(7-36) amide (A). Role of the endothelium in the vasodilatation evoked by glucagon (B). (n=6) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

Glucagon causes vasodilatation via the receptor for glucagon and GLP-1

Inhibition of the glucagon-receptor with its antagonist significantly decreased the vasodilator response to glucagon (Fig 2A). On the other hand, GLP-1R inhibition with its specific antagonist, exendin (9-39) also caused a significant, albeit seemingly less expressed reduction in the vasodilatation caused by glucagon (Fig 2B).

Glucagon-like peptide-1 causes vasodilatation via the glucagon-receptor

Concentration-dependent vasorelaxation caused by GLP-1(7-36) amide was significantly reduced in vessels preincubated with a glucagon-receptor antagonist (hGCGR-antagonist),although the glucagon-receptor blocker inhibited the GLP-1-induced vasodilatation only at smaller concentrations, but it did not inhibit the vasodilatation when the highest dosage of GLP-1 was applied (Fig 2C).

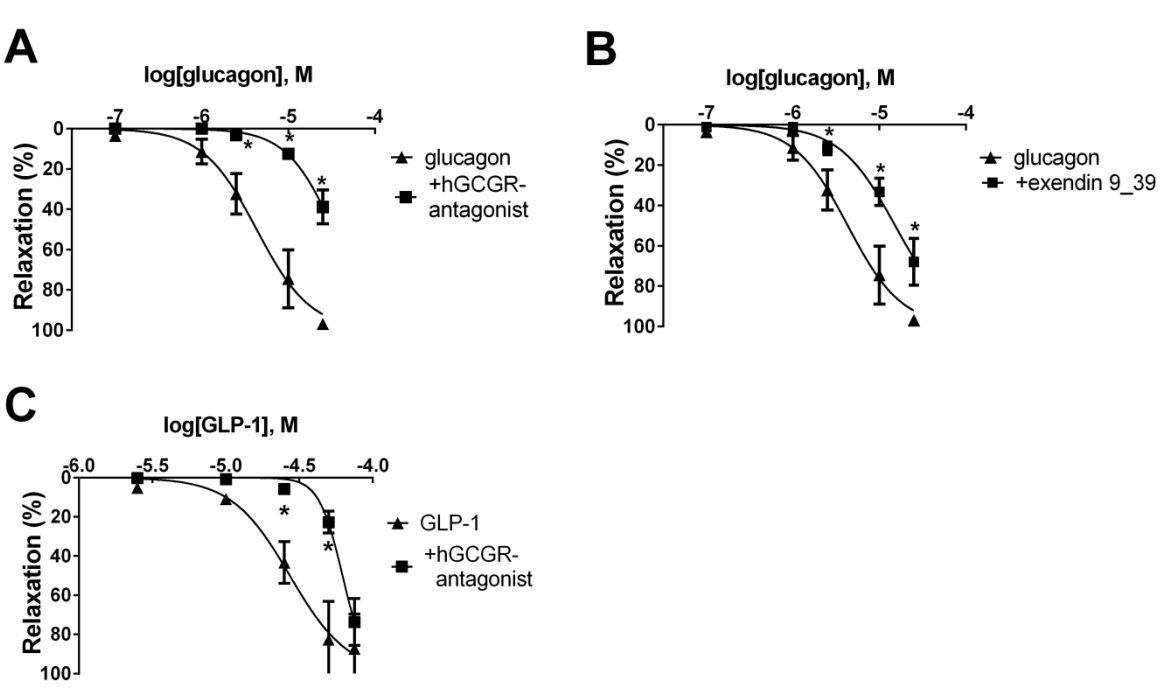

Figure 2. Receptors in the vasodilator effect of glucagon and GLP-1: effect of a glucagon-receptor antagonist (hGCGR-antagonist) (A) and GLP-1R inhibition by the receptor antagonist exendin(9-39) (B). Inhibition of the GLP-1 induced vasodilatation by glucagon-receptor blockade (C). (n=4) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

Involvement of NADPH oxidase enzyme in the vasodilatation induced by glucagon

Glucagon-induced vasodilatation was significantly decreased when vessels were preincubated with superoxide dismutase (Fig 3A),or catalase (Fig 3B), or the NADPH oxidase inhibitor DPI (Fig 3C).

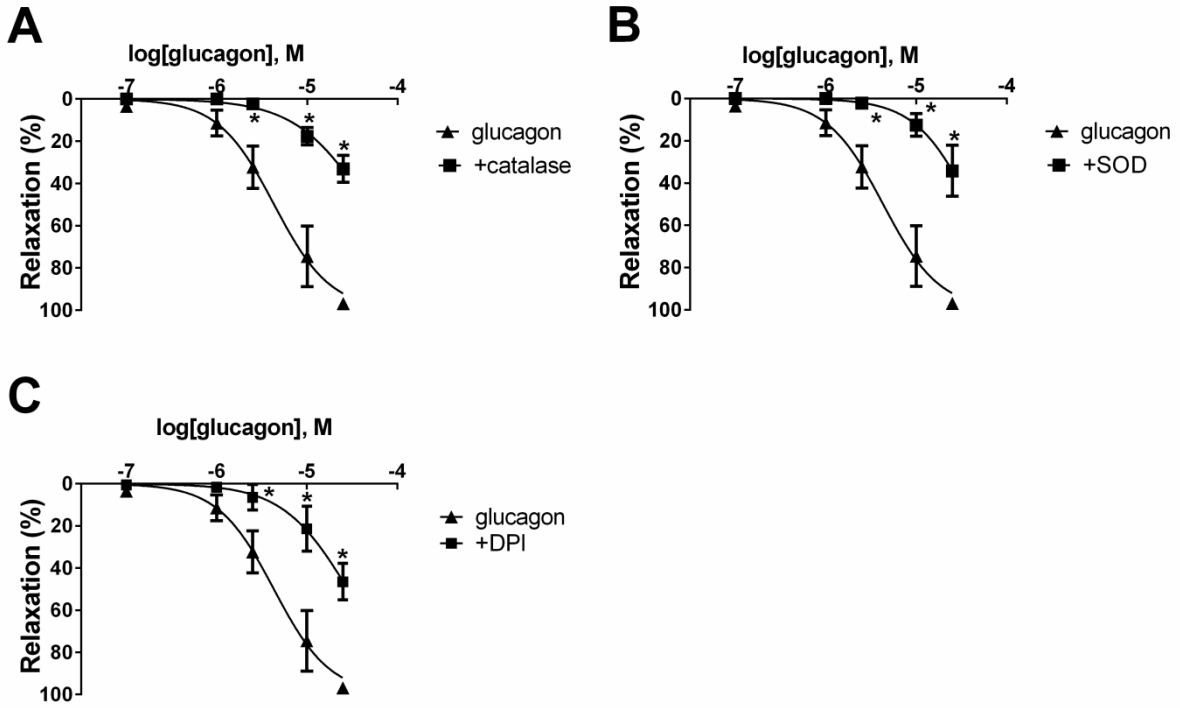

Figure 3. Concentration-relaxation curves of glucagon alone and with the addition of hydrogen peroxide formation blocker catalase (A), superoxide dismutase (SOD) (B) or NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) (C). (n=4) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

Contribution of gasotransmitters and the effect of COX-1 inhibition in the vasodilatation evoked by glucagon

Inhibition of NO production with the eNOS inhibitor L-NAME significantly inhibited vasodilatation when lower dosages of glucagon were applied, however, it had no effect when higher concentrations of glucagon were used (Fig 4A). The blockade of CO formation with the heme oxigenase inhibitor Tin-protoporphyrin (Fig 4B) and the inhibition of the H_2S generating cystathionine-γ-lyase with DL-propargylglycine (PPG) (Fig 4C) both significantly inhibited the vasodilator effect of glucagon.

Prostaglandin synthesis inhibition with indomethacin resulted in a significantly reduced vasodilatation to glucagon (Fig 4D).

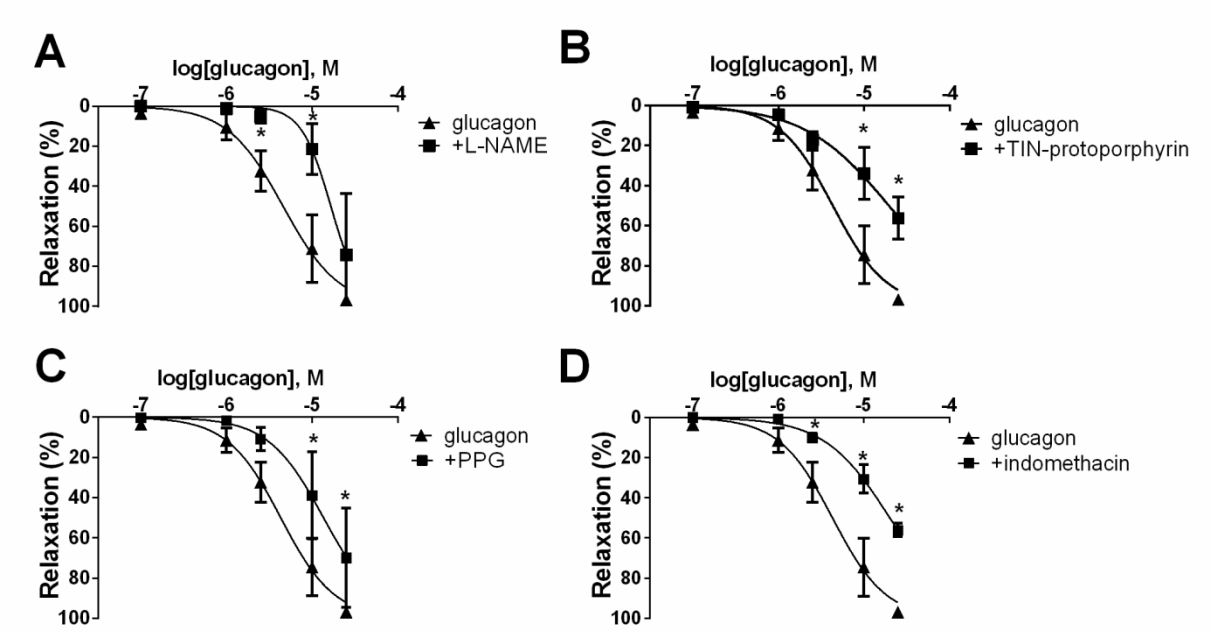

Figure 4. Concentration-relaxation curves showing the possible role of gasotransmitters and prostaglandins in the vasodilator effect of glucagon: eNOS inhibition with L-NAME (A). Blocking CO production via the inhibition of the enzyme heme oxygenase with Tin-protoporphyrin IX dichloride (B). Inhibition of H_2S production by inhibiting cystathionine-γ-lyase with DL-Propargylglycine (PPG) (C). Inhibition of prostaglandin synthesis with indomethacin (D). (n=4) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

Role of protein kinase G and protein kinase A in the vasodilation caused by glucagon

Soluble guanylyl cyclase inhibitor ODQ almost completely abolished the vasodilator effect of glucagon (Fig 5A).

Using H89, an inhibitor of PKA, the vasodilator response to glucagon was significantly decreased (Fig 5B).

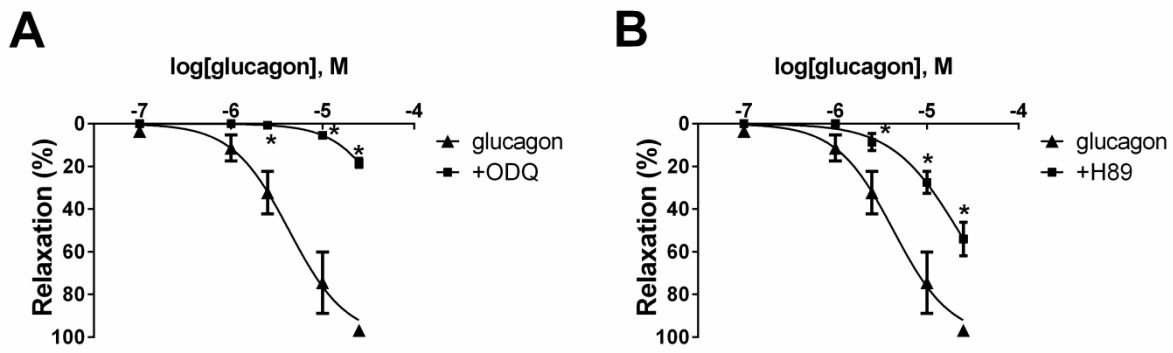

Figure 5. Effector molecules in the vasodilatation induced by glucagon: Inhibition of soluble guanylyl cyclase with 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) (A). cAMP-dependent protein kinase A (PKA) blockade with H89 hydrochloride (B). (n=4) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

Role of ion channels and transporters in the vasodilator effect of glucagon

Blockade of the large-conductance-calcium activated potassium channels by TEA (Fig 6A) and ATP-sensitive potassium channel inhibition with glibenclamide both significantly inhibited the vasodilatation in response to glucagon (Fig 6B).

Although to a lower extent, KCNQ-type K_v channel inhibition using XE991 also reduced the vasodilator effect of glucagon (Fig 6C).

Inhibition of the sodium-calcium exchanger (NCX) with SEA0400 significantly decreased the vasodilatation evoked by glucagon (Fig 6D).

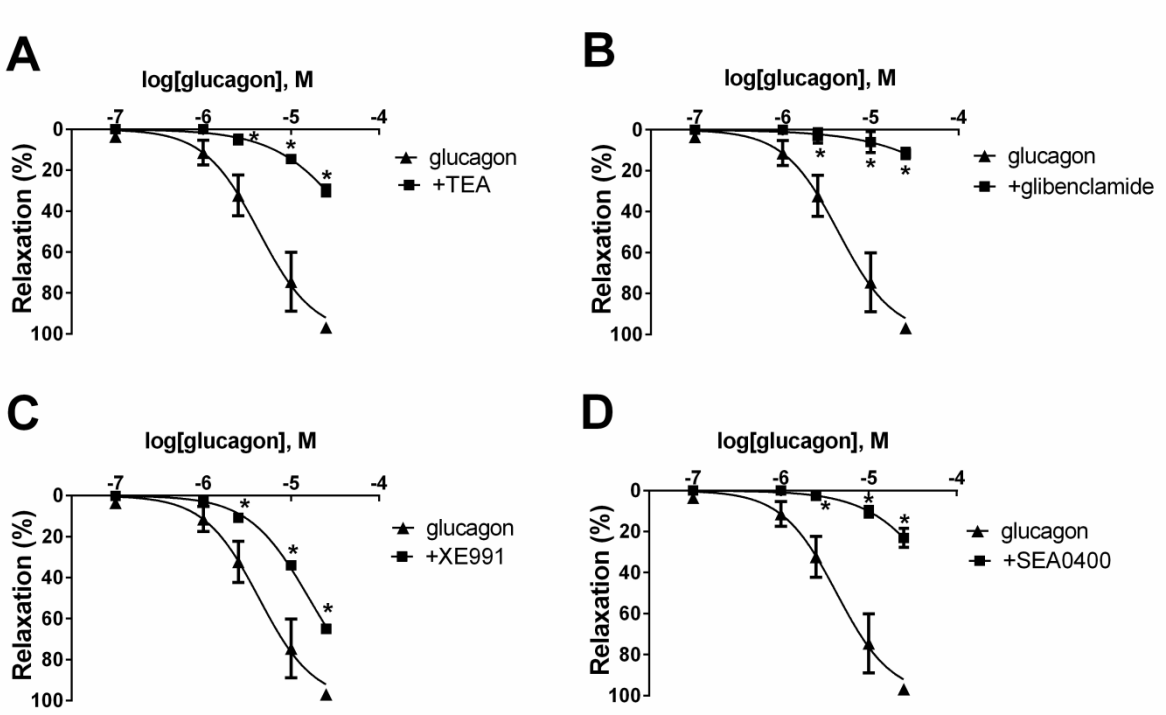

Figure 6. Involvement of potassium channels and the Na^+/Ca^{2+}-exchanger in the vasodilator effect of glucagon: Inhibition of the large-conductance calcium-activated potassium channels with tetraethylammonium (TEA) (A). Blockade of the ATP-sensitive potassium channels with glibenclamide (B). KCNQ-type Kv channels were blocked by XE991 (C). Selective inhibition of the Na^+/Ca^{2+}-exchanger with SEA0400 (D). (n=4 each) *P<0.01 compared to the relaxation evoked by glucagon only (at respective concentration of glucagon).

6.1.2. Exenatide

Exenatide relaxes rat thoracic aorta in a dose-dependent manner

After precontracting the vessels with epinephrine, time-control experiments showed that spontaneous vessel relaxation was not significant (Figure 7A). Following the epinephrine-induced contraction, in another set of experiments we administered increasing doses of exenatide to the organ baths to assess the vasoactive effect of this GLP-1R agonist. We found a dose-dependent relaxation of the rat thoracic aorta due to exenatide (Figure 7B).

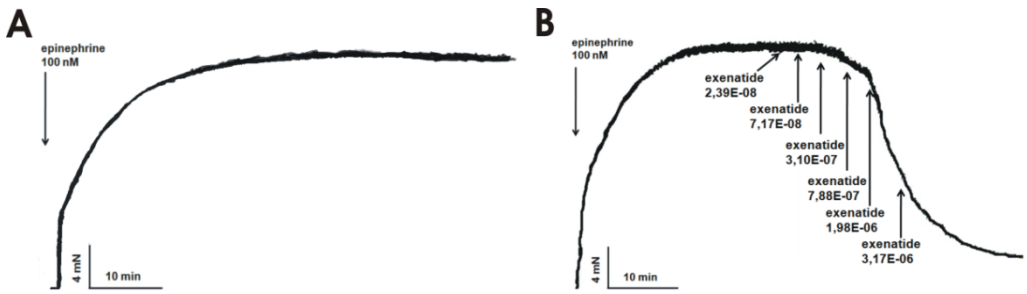

Figure 7. Effect of exenatide on the vasoactivity of rat thoracic aorta. Original records of myography experiments. Time-control of an epinephrine contracted aortic segment (A). Dose-dependent vasodilatory effect of exenatide on rat thoracic aorta following epinephrine contraction. 23.9, 71.7, 310, 788, 1980, 3170 nanomoles of exenatide were used to relax the vessels (B) (n=5 of each experiment).

Role of GLP-1 receptor in the vasodilation of exenatide

In our experiments exenatide induced vasodilation in a GLP-1R dependent manner, since preincubation with the specfic GLP-1R antagonist exendin(9-39) almost entirely blocked the vasodilation when the maximal dose of exenatide was applied, and totaly inhibited relaxation when smaller concentrations of the GLP-1 agonist were administered to the chambers (Figure 8A).

Effects of exenatide after endothelial denudation

When the endothelium of the thoracic aorta was mechanically removed, the relaxation due to exenatide was significantly decreased (Figure 8B).

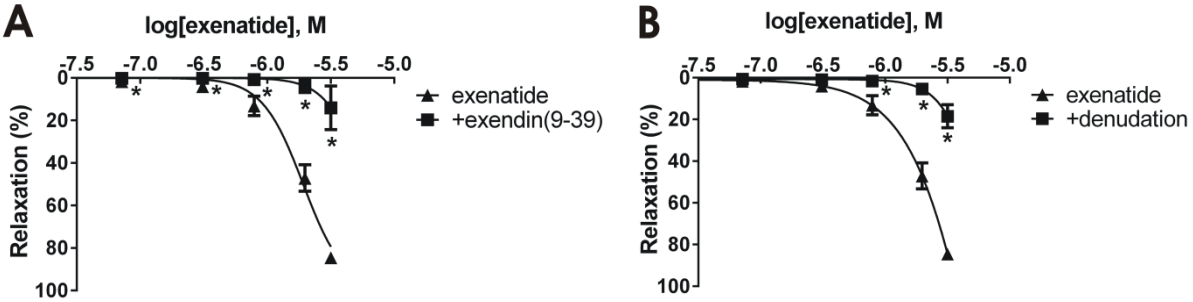

Figure 8. Role of GLP-1 receptor and endothelial denudation in the vasodilatation due to exenatide. Exenatide concentration-relaxation curves of vessels treated with exenatide only (▲) and vessels preincubated with GLP-1R antagonist exendin(9-39) (■) (A). Vasodilation evoked by exenatide in endothelium-intact and endothelium-denuded vessels (B). (n=5 of each experiment) *P<0.01 compared to exenatide only (at respective concentration of exenatide).

Effect of exenatide on the production of gasotransmitters

Incubation of vessels with the eNOS inhibitor L-NAME led to a mild but significant decrease in the relaxation of the rat thoracic aorta (Figure 9A).To determine further mediators of the vasodilator effect of exenatide, we examined the role of CO and H_2S. When we preincubated vessels with Tin-protoporphyrin, a potent heme oxygenase inhibitor, the vasorelaxation to exenatide was significantly reduced (Figure 9B). The inhibition of NO-synthesis and the inhibiton CO-production only partially decreased the rate of vasodilation: we therefore wished to prove that the third gasotransmitter, H_2S also plays a part in the vasoactive effect of exenatide. The inhibition of cystathionine-γ-lyase by preincubating vessels with PPG resulted in a significant decrease in the rate of relaxation (Figure 9C). Comparing the effects of these three gasotransmitters leading to vasodilation in response to exenatide, H_2S seemed to have the most remarkable effect.

Effect of the inhibition of prostaglandin biosynthesis

Incubation of vessels with the COX inhibitor indomethacin for 30 minutes significantly decreased vasodilation to exenatide (Figure 9D).

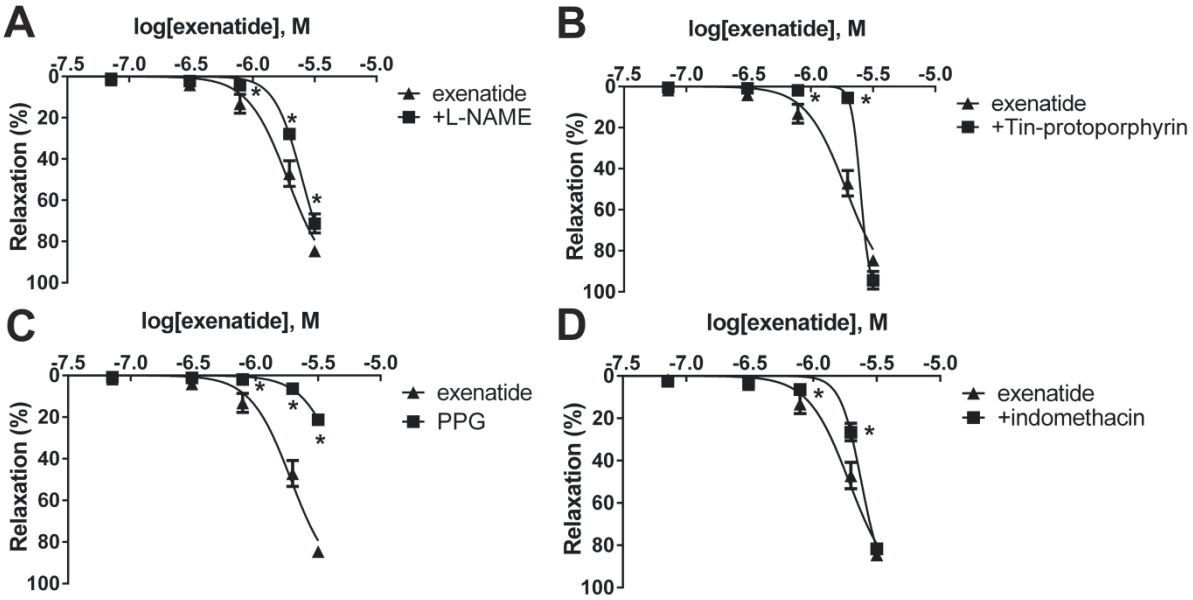

Figure 9. Role of gasotransmitters and prostaglandins in the vasodilatory effect of exenatide. Inhibition of eNOS with L-NAME (A). Inhibition of CO production Tin-protoporphyrin IX dichloride (B). Blocking H_2S production with PPG (C). Inhibition of prostaglandin production with indomethacin (D). (n=5 of each experiment) *P<0.01 compared to the relaxation caused by exenatide only (at respective concentration of exenatide).

Free radicals play a part in the vasoactive effect of exenatide

To determine the role of ROS in the vasorelaxation caused by exenatide, we preincubated vessels with superoxide dismutase or with catalase. The rate of relaxation was significantly decreased in both experiments; however, SOD proved to be a much more potent inhibitor of vasodilation to exenatide (Figure 10).

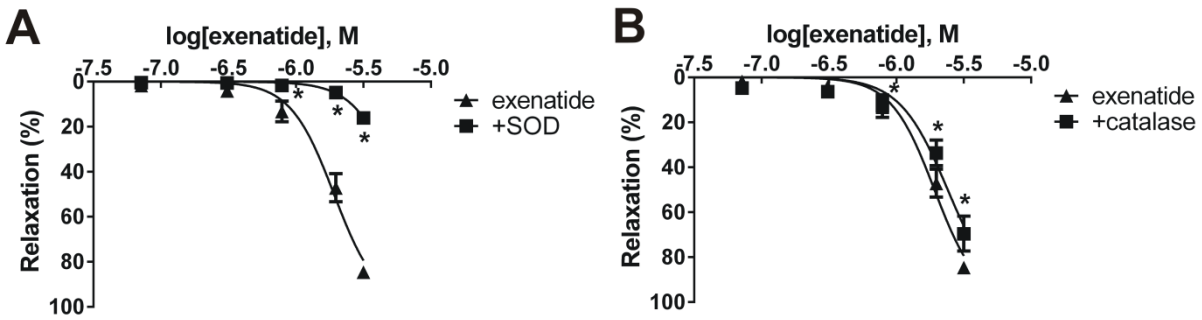

Figure 10. Effect of free radicals on the vasodilation due to exenatide. Concentration-relaxation curve of exenatide with/without the addition of 200 U/ml of the free radical scavanger superoxide dismutase (SOD) (A). Concentration-relaxation curve analyzing the possible role of hydrogen peroxide by blocking its formation with 1000 U/ml catalase (B). (n=5 of each experiment) *P<0.01 compared to the relaxation caused by exenatide only (at respective concentration of exenatide).

Effects of the inhibition of the cAMP-dependent protein kinase A and cGMP-dependent protein kinase G

In order to determine the second messenger of the dilatation caused by exenatide, we incubated vessels with H89, an inhibitor of PKA. This caused only a mild decrease in the vasorelaxation at a low concentration (Figure 11A). In turn, inhibition of soluble guanylyl cyclase by ODQ significantly inhibited the vasorelaxation at higher concentrations as well (Figure 11B).

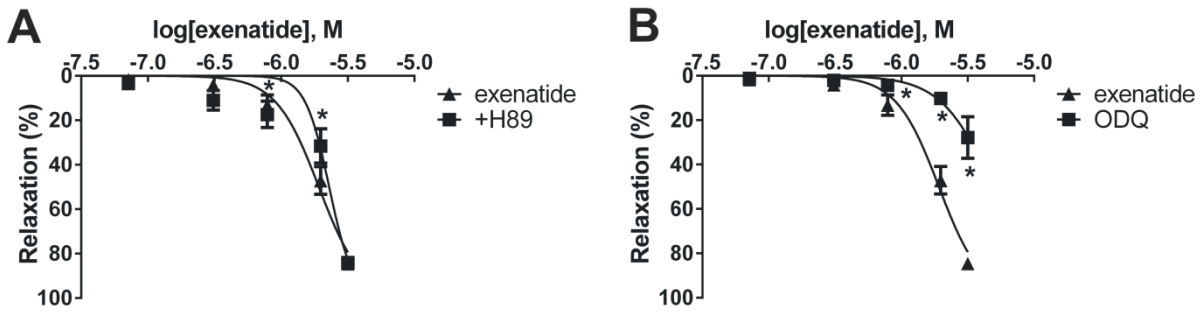

Figure 11. Concentration-relaxation curves showing the possible effector molecules of the exenatide induced vasodilation. Blocking cAMP-dependent protein kinase A (PKA) with 5 μM H89 hydrochloride (A). Inhibiton of soluble guanylyl cyclase with 3 μM ODQ (B). (n=5 of each experiment) *P<0.01 compared to the relaxation caused by exenatide only (at respective concentration of exenatide).

Role of potassium channels in the vasodilator effect of exenatide

Preincubating vessels with three different potassium channel blockers before adding increasing concentrations of exenatide to the myograph chambers resulted in a signifcant decrease in the relaxation in all cases. Incubating one group of vessels with TEA, an inhibitor of the BK_{Ca} channels, demonstrated inhibition of vasodilation (Figure 12A). Relaxation was also inhibited by a blockade of the K_{ATP} channels by preincubation with glibenclamide (Figure 12B*)*. In the group of vessels preincubated with XE991, a KCNQ (a type of K_v channels) channel inhibitor, most of the vasodilation was abolished (Figure 12C). However, there was a less pronounced decrease of vasodilation with the inhibition of BK_{Ca} and K_{ATP} channels compared to the inhibition of K_v channels.

Effects of inhibiting the Na^+/Ca^{2+}-exchanger with SEA0400

We found that SEA0400, an inhibitor of the Na^+/Ca^{2+}-exchanger markedly inhibited vasorelaxation. Preincubation of vessels with SEA0400 almost completely abolished the whole of the vasodilation caused by exenatide (Figure 12D).

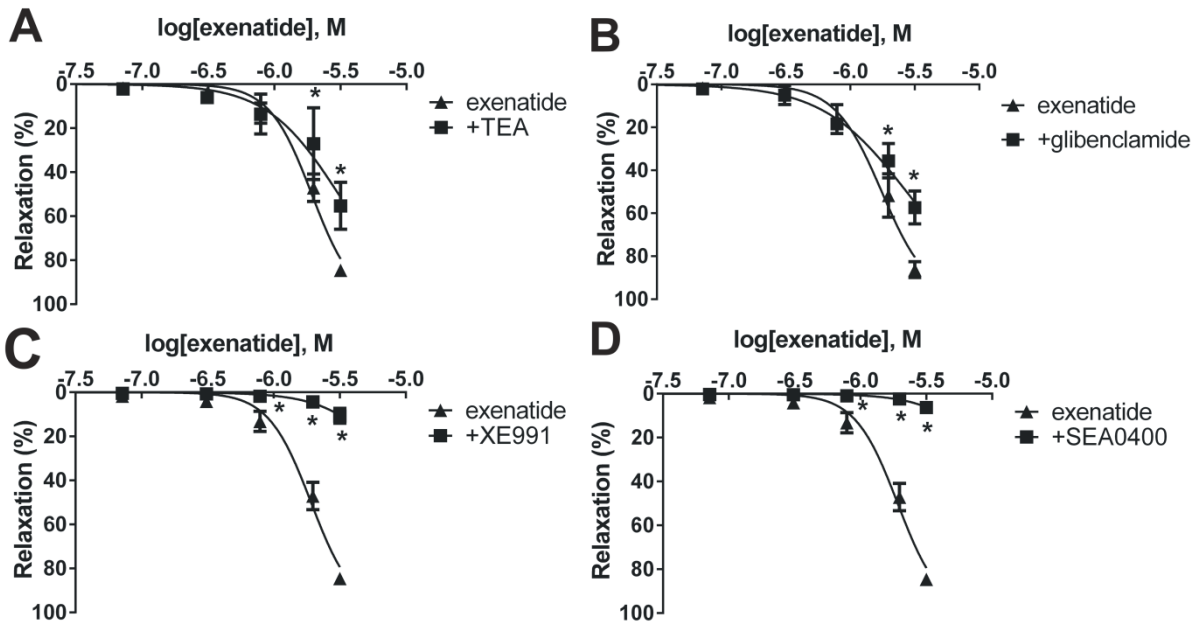

Figure 12. Role of potassium channels and the Na^+/Ca^{2+}-exchanger in the vasodilator effect of exenatide. Blockade of large-conductance calcium-activated potassium channels with 2 mM tetraethylammonium (TEA) (A). Inhibition of ATP-sensitive potassium channels with 10 μM glibenclamide (B). KCNQ-type Kv channels blocked by 30 μM XE991 (C). Selective inhibition of the Na+/Ca2+-exchanger with 4 μM SEA0400 (D). (n=5 of each experiment) *P<0.01 compared to the relaxation caused by exenatide only (at respective concentration of exenatide).

6.1.3. Liraglutide

Liraglutide causes dose-dependent vasorelaxation

To start with, we evaluated the spontaneous relaxation of the vessel precontracted with epinephrine, however untreated vessels showed no significant spontaneous relaxation (Fig. 13A,B). When assessing the vasoactive effect of this GLP-1 analogue, we administered increasing doses of liraglutide to the vessel chambers after the precontraction with epinephrine. In our experiment we showed that liraglutide dose-dependently relaxed the rat thoracic aorta and the femoral artery, however, respective concentrations of liraglutide elicited greater vasodilation in the femoral artery than in the thoracic aorta (Table 1).

Effect of endothelial denudation

Mechanical removal of the endothelium of the thoracic aorta caused a mild inhibition of the relaxation caused by liraglutide. However, this difference in the relaxation between endothelium-intact and endothelium-denuded vessels proved to be significant except for the lowest dosage of liraglutide (Fig. 13*C*). In the femoral artery, endothelial denudation caused no change in the vasodilator response to liraglutide (Table 1).

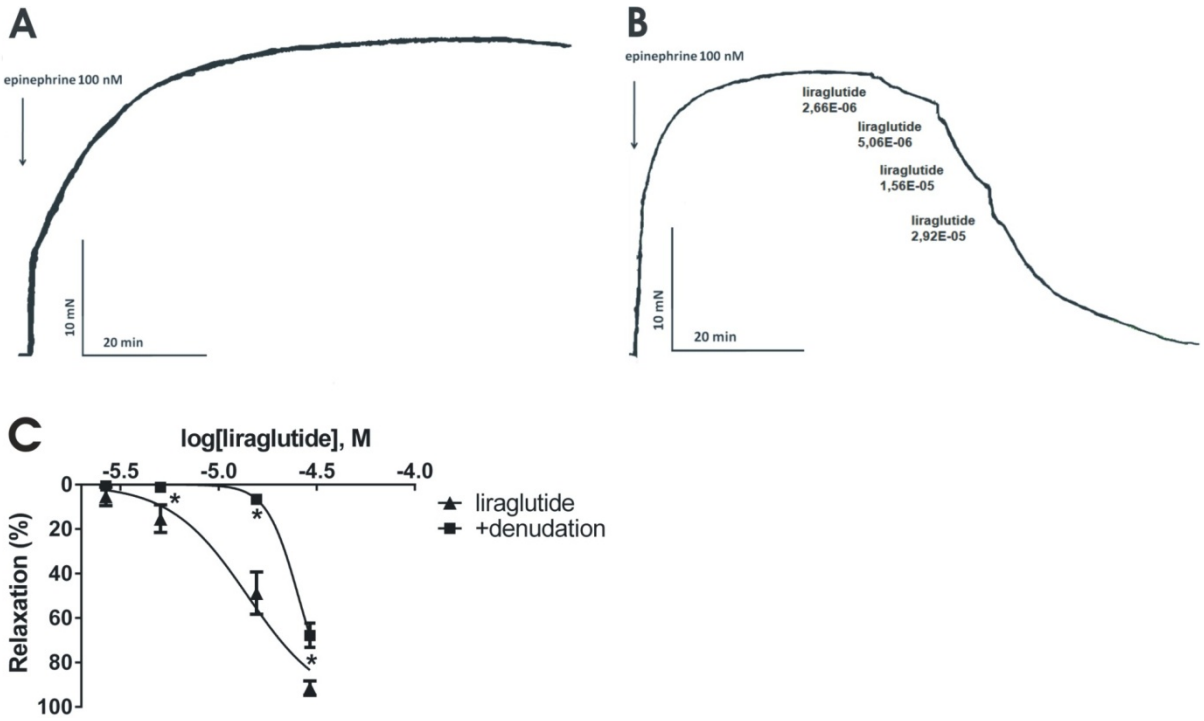

Figure 13. Original record, a time-control of an epinephrine precontracted aortic ring (A). Original record of the response of an isolated thoracic aorta segment to 100 nM epinephrine followed by increasing doses of liraglutide (B) (n=5). Liraglutide concentration-relaxation curves in endothelium-intact (▲) and endothelium-denuded (■) vessels (n=5) (C). *P<0.001 compared to the relaxation caused by liraglutide only (at respective concentration of liraglutide).

Role of gasotransmitters in vasodilatation caused by liraglutide

We incubated a group of vessels with L-NAME, and found that inhibition of eNOS partially avoids vasodilatation caused by liraglutide in both arteries. Decrease in the magnitude of relaxation proved to be significant (Fig. 14A, Table 1).

To determine further mediators of the vasodilator effect of liraglutide, we tested the role of CO. We preincubated vessels with Tin-protoporphyrin, which resulted in a significantly milder relaxation in the thoracic aorta, but it had no significant effect in the femoral artery (Fig. 14B, Table 1).

Because the inhibition of NO-synthesis and the inhibition of CO-production only partially decreased the vasodilatation, we wanted to determine whether H_2S also plays a part in the vasoactive effect of liraglutide. We inhibited cystathionine-γ-lyase by preincubating vessels with PPG, which significantly decreased the relaxation in both vessels (Fig 14C, Table 1).

Effect of indomethacin, a prostaglandin synthesis inhibitor

Incubation of vessels with the COX inhibitor indomethacin for 30 minutes caused significantly smaller vasodilatation in response to liraglutide in the thoracic aorta, but it had no effect in the femoral artery (Fig. 14D, Table 1).

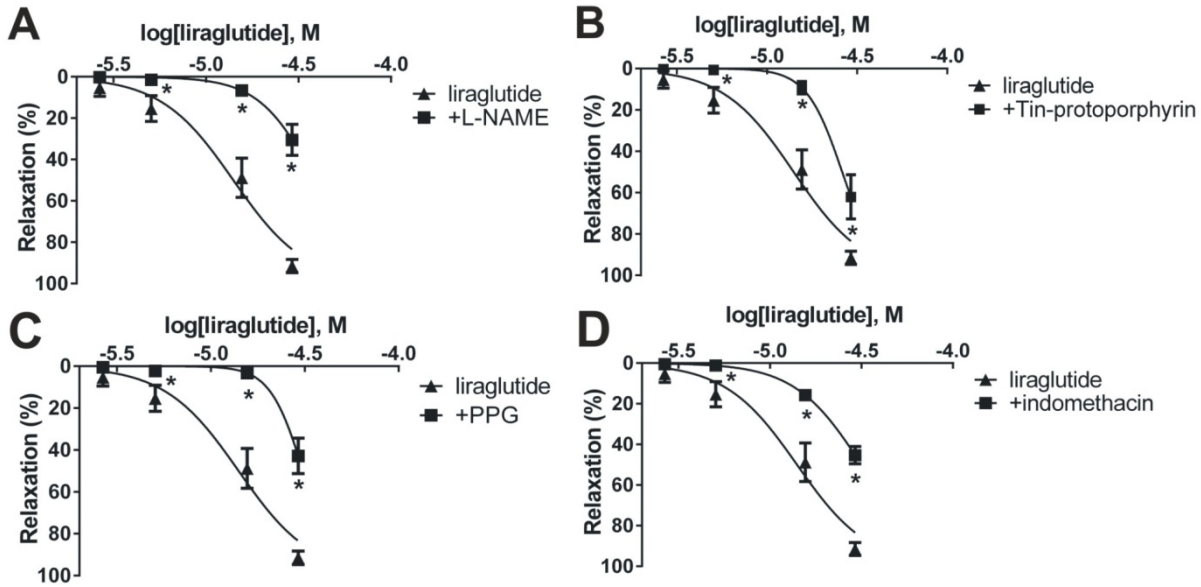

Figure 14. Concentration-relaxation curves of liraglutide (▲) and liraglutide+inhibitors of the potential mediators of the vasodilation caused by liraglutide (■).Inhibition of NOS (Nitric oxide synthase) with 300 μM L-NAME (A) (n=5). Inhibition of CO production by blocking heme oxygenase with 10 μM Tin-protoporphyrin IX dichloride (B) (n=5). Blocking H_2S production by inhibiting cystathionine-γ-lyase with 10 mM DL-Propargylglycine (PPG) (C) (n=5). Inhibiting prostaglandin production with 3 μM indomethacin (D) (n=5). *P<0.001 compared to the relaxation caused by liraglutide only (at respective concentration of liraglutide).

Free radicals contribute to the effect of liraglutide

In order to evaluate the role of ROS in the vasorelaxation caused by liraglutide, we preincubated vessels with superoxide dismutase or with catalase. We found significant inhibition of relaxation in both experiments in case of the aorta, however, we proved that in the femoral artery only H_2O_2 is involved in the liraglutide induced vasorelaxation (Fig. 15, Table 1).

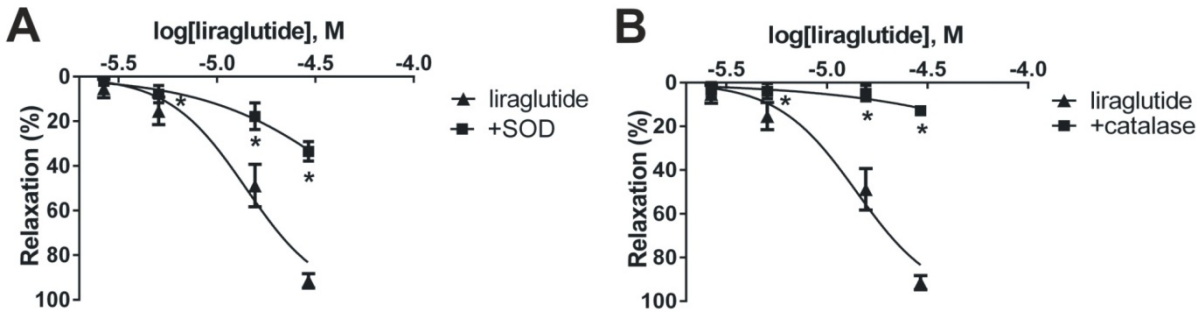

Figure 15. Role of free radicals in the effect of liraglutide. Concentration-relaxation curve of liraglutide + 200 U/ml of the free radical scavanger superoxid dismutase (SOD) (A) (n=5). Concentration-relaxation curve showing the possible role of hydrogen peroxide by blocking its formation with 1000 U/ml catalase (B) (n=5). *P<0.001 compared to the relaxation caused by liraglutide only (at respective concentration of liraglutide).

The role of cAMP-dependent protein kinase A and cGMP-dependent protein kinase G

To determine the second mediator of the dilatation caused by liraglutide, we incubated vessels with H89, the inhibitor of PKA. This almost completely abolished the relaxation induced by liraglutide in both arteries (Fig. 16A, Table 1). We inhibited soluble guanylyl cyclase by incubating vessels with ODQ. This led to a significant inhibition of vasorelaxation in both cases (Fig. 16, Table 1).

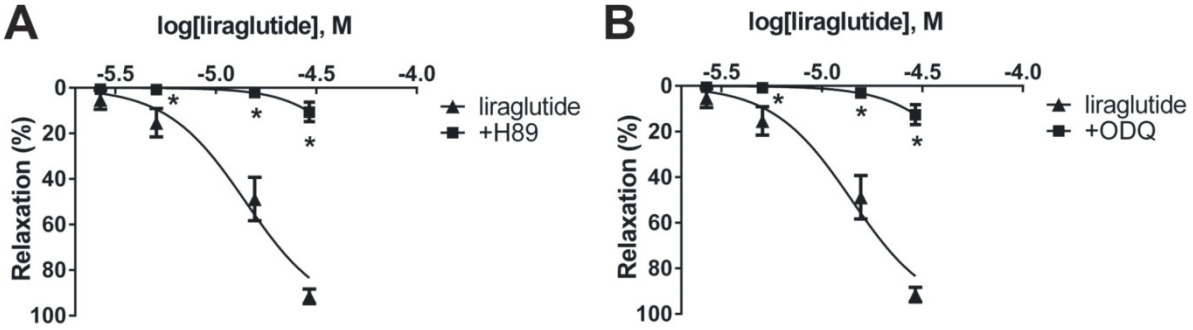

Figure 16. Concentration-relaxation curves showing the possible effector molecules of the liraglutide induced vasodilation. Blocking cAMP-dependent protein kinase A (PKA) with 5 μM H89 hydrochloride (A) (n=5). Inhibiton of soluble guanylyl cyclase with 3 μM ODQ (B) (n=5). *P<0.001 compared to the relaxation caused by liraglutide only (at respective concentration of liraglutide).

Inhibition of potassium channels with TEA, glibenclamide and XE991

Incubating one group of vessels with TEA, inhibitor of the BK_{Ca} channels proved to be a very potent inhibitor of vasodilatation (Fig. 17A, Table 1). Blockade of the K_{ATP} channels by preincubation with glibenclamide also inhibited most of the aortic vasodilatation (Fig. 17*B*). To block the KCNQ type K_v channels, we preincubated one group of vessels with XE991, a specific inhibitor of the channel, which also inhibited the vasodilatation in both arteries (Fig. 17C, Table 1).

Inhibition of the Na^+/Ca^{2+}-exchanger with SEA0400

Another potent inhibitor of vasorelaxation was SEA0400, inhibitor of the Na^+/Ca^{2+}-exchanger. Preincubation of one group of vessels with SEA0400 abolished almost the whole of the vasodilatation caused by liraglutide (Fig. 17D, Table 1).

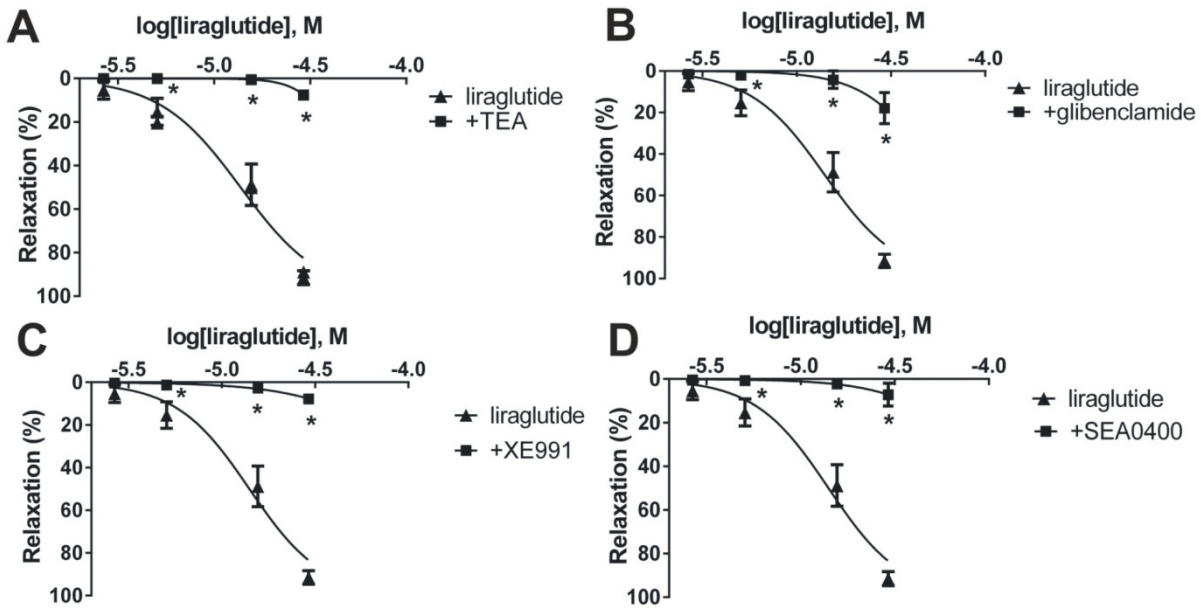

Figure 17. Liraglutide concentration-relaxation curves with (■) and without (▲) an inhibitor of a possible way of the vasodilatory effect. Blockade of large-conductance calcium-activated potassium channels with 2 mM tetraethylammonium (TEA) (A) (n=5). Inhibition of ATP-sensitive potassium channels with 10 μM glibenclamide (B) (n=5). KCNQ-type K_v channels were blocked by 30 μM XE991 (C) (n=5). Selective inhibition of the Na^+/Ca^{2+}-exchanger with 4 μM SEA0400 (D) (n=5). *P<0.001 compared to the relaxation caused by liraglutide only (at respective concentration of liraglutide).

Table 1. Log EC_{50} values of liraglutide induced vasodilation in the persence of different inhibitors in rat thoracic aorta and femoral artery

	log EC_{50} of liraglutide		p
	Thoracic aorta	Femoral artery	
liraglutide alone	-4.89(0.04)	-5.11(0.10)	0.004
liraglutide + denudation	-4.58(0.02)*	-4.90(0.17)	0.004
L-NAME	-4.40(0.05)*	-5.36(0.18)*	0.001
Tin-PP	-4.59(0.01)*	-5.05(0.15)	0.016
PPG	-4.48(0.16)*	-4.71(0.21)**	0.200
indomethacin	-4.49(0.09)*	-5.10(0.21)	0.029
SOD	-4.30(0.01)*	-4.99(0.16)	0.029
catalase	-3.67(0.96)*	-5.21(0.15)*	0.010
H89	-4.18(0.19)*	-4.65(0.08)**	0.029
ODQ	-4.27(0.29)*	-4.80(0.13)**	0.029
TEA	-4.27(0.08)*	-4.48(0.20)**	0.343
glibenclamide	-4.37(0.08)*	-5.08(0.34)	0.016
XE991	-3.88(0.15)*	-4.72(0.18)*	0.100
SEA0400	-3.52(0.75)*	-4.63(0.02)**	0.029

Abbreviations: L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; Tin-PP, Tin-protoporphyrin IX dichloride; PPG, DL-Propargylglycine; SOD, superoxide dismutase; H89, N[2-(p Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; TEA, tetraethylammonium chloride

Data expressed as median(IQR)

*: $p<0.05$ vs. log EC50 of liraglutide alone in the same vessel segment

**: $p<0.01$ vs. log EC50 of liraglutide alone in the same vessel segment

6.2. Effect of oxidative stress in cholesterol-fed rats

Baseline characteristics of the animals

Body weight

Body weight of the rats in the control group was significantly higher than that of the rats fed with cholesterol or cholesterol+para-tyrosine. However, no significant difference was observed in the body weight of the two cholesterol fed groups throughout the study (Table 2).

Table 2. Baseline characteristics of animals.

		Week 4	Week 8	Week 12	Week 16	Week 20
Body weight [g]	Contr	64 (37)	225 (58)	348 (75)	420 (62)	437 (55)
	Chol	95 (24)*	183 (17)*	268 (18)*	345 (42)**	377 (80)**
	Chol+para	67 (32)$^{\#}$	145 (56)**	232 (67)**	317 (41)**	349 (36)**
Blood glucose OGTT 0 min	Contr	-	3.94 ±0.39	4.64±0.37	4.65±0.28	4.71±0.32
	Chol		4.29±1.04	5.23±0.28	4.90±0.67	4.91±0.49
	Chol+para		4.79 ±0.70*	5.21±0.82	4.45±0.66	4.75±0.36
Blood glucose OGTT 15	Contr	-	10.06±3.21	10.17±2.20	10.6±2.06	8.43±1.58
	Chol		9.18±2.26	9.90±1.73	9.66±1.36	9.08±1.24
	Chol+para		9.37±1.47	10.61±1.11	9.27±1.42	9.12±1.18
Blood glucose OGTT 30	Contr	-	9.10±1.94	10.07±2.27	9.00±1.33	9.38±1.56
	Chol		8.13±1.54	8.34±0.85	8.66±0.77	9.41±1.67
	Chol+para		9.22±1.54	11.01±3.94	9.50±1.92	10.05±1.23
Blood glucose OGTT 60	Contr	-	8.62±1.91	8.49±1.70	8.63±1.08	8.44±0.92
	Chol		8.74±2.20	8.79±0.88	9.60±0.63	8.94±1.47
	Chol+para		8.15±1.36	8.91±0.94	9.28±2.12	9.53±0.82
Blood glucose OGTT 120	Contr	-	6.53±0.99	6.09±0.98	7.66±1.21	7.77±0.60
	Chol		6.95±1.19	7.20±1.34	8.18±1.10	8.43±1.02
	Chol+para		7.46±1.37	7.97±1.22**	8.03±1.04	8.82±0.79*
Liver weight [g]	Contr	-	-	-	-	14 (4)
	Chol					26 (3)***
	Chol+para					24 (3)***
Liver weight/body weight [g/kg]	Contr	-	-	-	-	28.74 (3.14)
	Chol					67.16 (14.88)***
	Chol+para					67.73 (6.40)***

*: p<0.05 vs. Contr; **: p<0.01 vs. Contr; ***: p<0.001 vs. Contr; $^{\#}$: p<0.05 vs. Chol
Data presented as mean±SD or median (interquarter range).

Blood glucose levels during oral glucose tolerance test

The oral glucose tolerance tests (OGTT) (age 8, 12, 16, 20 weeks) showed no significant differences between the blood glucose profiles of the three groups (Table 1, Fig 18A). No remarkable difference was shown in the area under the curve (AUC) analysis of the blood glucose profile of the three groups during OGTT at week 20 (Fig 18B).

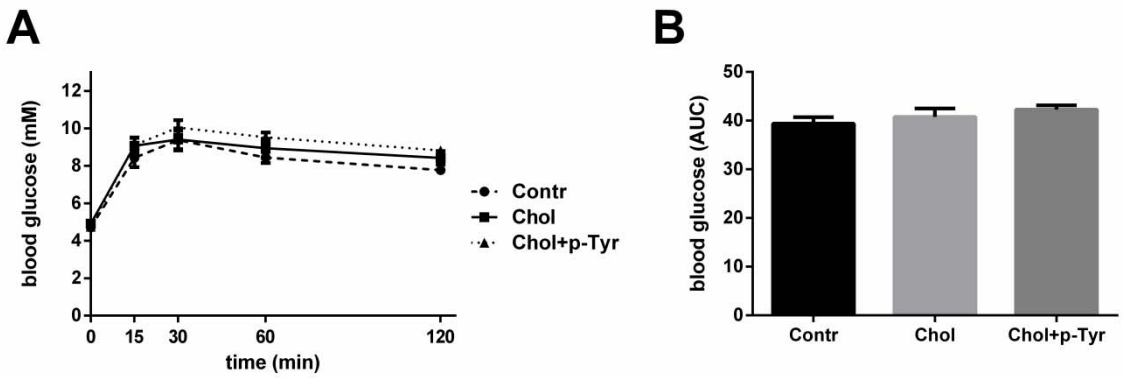

Figure 18. Blood glucose levels during OGTT (week 20) at 0, 15, 30, 60 and 120 min (A) and area under the curve (AUC) of blood glucose during OGTT (B) in control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr).

Liver weight

Liver weight of rats after execution was significantly higher in both the cholesterol fed and the cholesterol+para-tyrosine fed group than that of control groups, nevertheless, para-tyrosine supplementation had no significant effect on the cholesterol-induced hepatomegaly (Table 1).

Plasma level of insulin during OGTT at week 20

Although no significant difference was seen in the plasma insulin level of the rats in the different groups during the glucose tolerance test at week 20 (Fig 19A), the plasma insulin AUC was significantly decreased in the cholesterol fed group compared to the controls. The plasma insulin AUC of the cholesterol+para-tyrosine group was not significantly different neither from the control, nor from cholesterol fed group (Fig 19B).

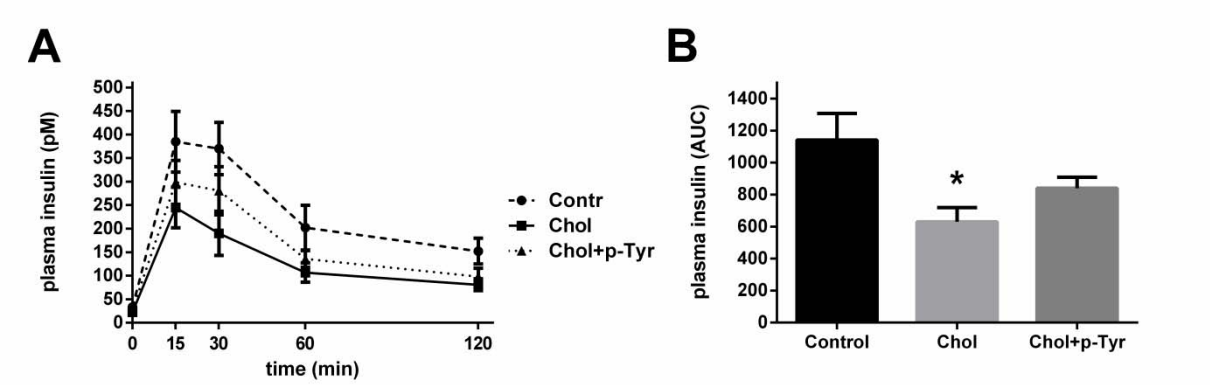

Figure 19. Plasma levels of insulin during OGTT (week 20) at 0, 15, 30, 60 and 120 min (A) and area under the curve (AUC) of plasma insulin during OGTT (B) in control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr). *: $p<0.05$ vs. Contr.

Serum cholesterol levels

Serum cholesterol levels were higher in cholesterol fed and also in cholesterol fed and para-tyrosine supplemented groups compared to the control group, however, no significant difference was observed as a result of para-tyrosine supplementation (Fig 20).

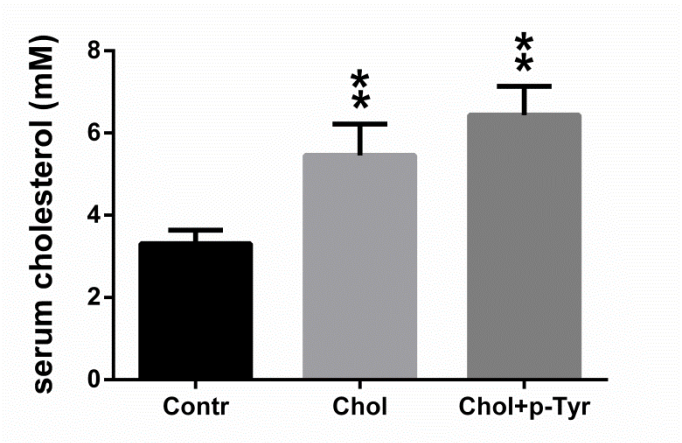

Figure20. Serum level of cholesterol in control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr). **: $p<0.01$ vs. Contr.

Elevated m-Tyr/p-Tyr ratio in the thoracic aorta of cholesterol-fed rats

The m-Tyr/p-Tyr ratio in the thoracic aorta of cholesterol fed rats was significantly higher than that in the control group, while in the group receiving

para-tyrosine supplementation the m-Tyr/p-Tyr ratio did not differ significantly from the control group (Fig 21).

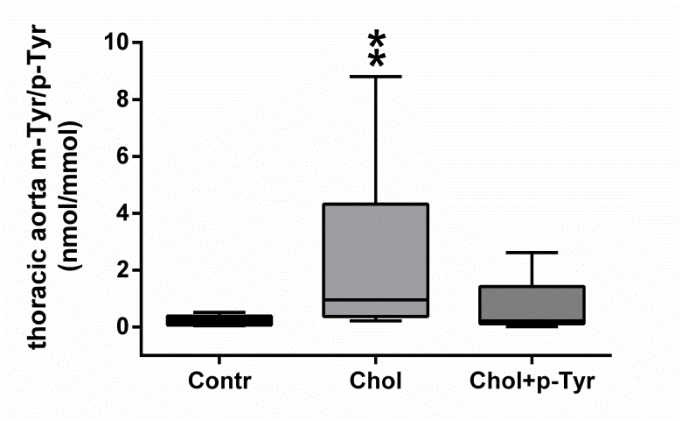

Figure 21. Ratios of protein-bound m-Tyr and p-Tyr in thoracic aorta of control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr). **: $p<0.01$ vs. Contr.

Para-tyrosine supplementation restores cholesterol-induced vascular liraglutide and insulin resistance

Four months cholesterol-feeding resulted in a significantly decreased vasodilator response of the thoracic aorta to liraglutide, compared to the vasodilation evoked by liraglutide in the thoracic aorta of rats on normal diet. The cholesterol-induced vascular liraglutide-resistance was restored by para-tyrosine supplementation (Fig 22).

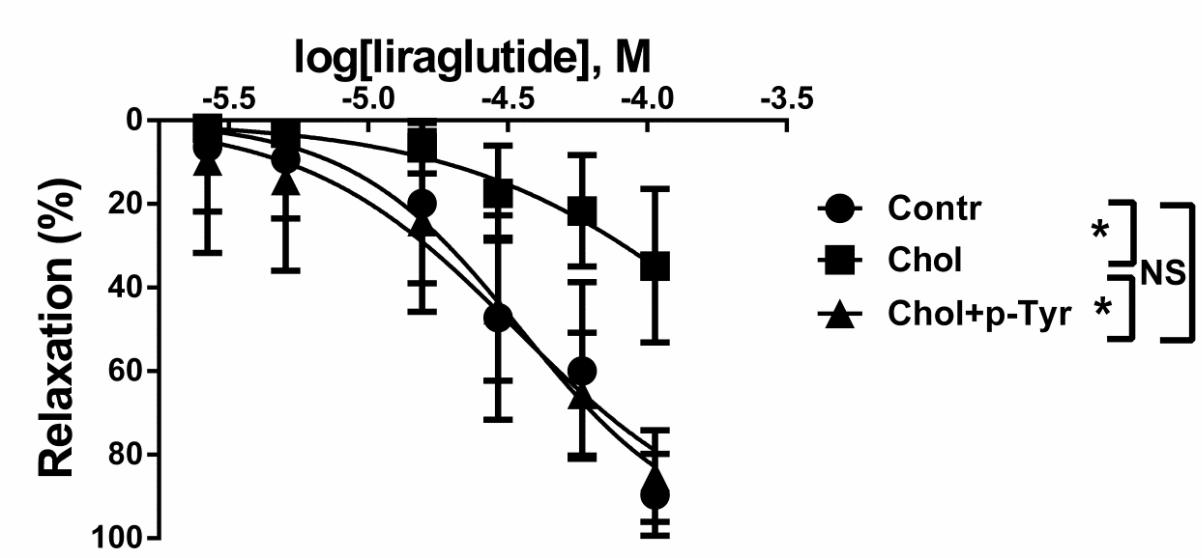

Figure 22. Liraglutide induced relaxation of the thoracic aorta of control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr). *: $p<0.05$.

Similarly, cholesterol-feeding led to decreased vascular insulin-sensitivity, which could be prevented by para-tyrosine supplementation (Fig 23).

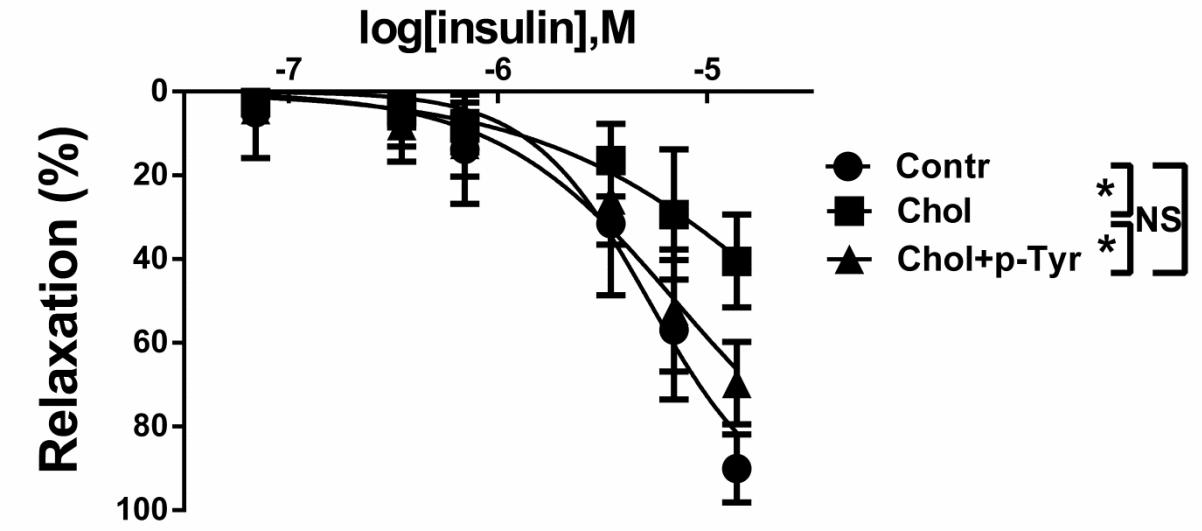

Figure 23. Insulin induced relaxation of the thoracic aorta of control rats (Contr), cholesterol fed rats without p-Tyr supplementation (Chol) and cholesterol fed rats with p-Tyr supplementation (Chol+p-Tyr). *: $p<0.05$.

7. Discussion

7.1. Hypothetical mechanism of the vasodilatation induced by glucagon and glucagon-like peptide agonists

The major novel findings of our studies are as follows: glucagon, exanatide and liraglutide dose-dependently relax the rat thoracic aorta *in vitro*. The vasodilator potential of glucagon is the same as that of insulin and it is greater than that of GLP-1(7-36) amide. The vasodilatation in response to glucagon is mainly endothelium-independent, and it evokes mostly via the glucagon-receptor, but it is also mediated by the GLP-1R. GLP-1(7-36) amide also dilates the rat thoracic aorta, which is partially mediated by the glucagon receptor. According to our findings further mediators of the vasodilatation evoked by glucagon are gasotransmitters, prostaglandins and NADPH oxidase producing free radicals, the soluble guanylyl cyclase and PKA, resulting in the activation of potassium channels and finally the NCX, which leads to smooth muscle relaxation, hence vasodilatation (Figure 24).

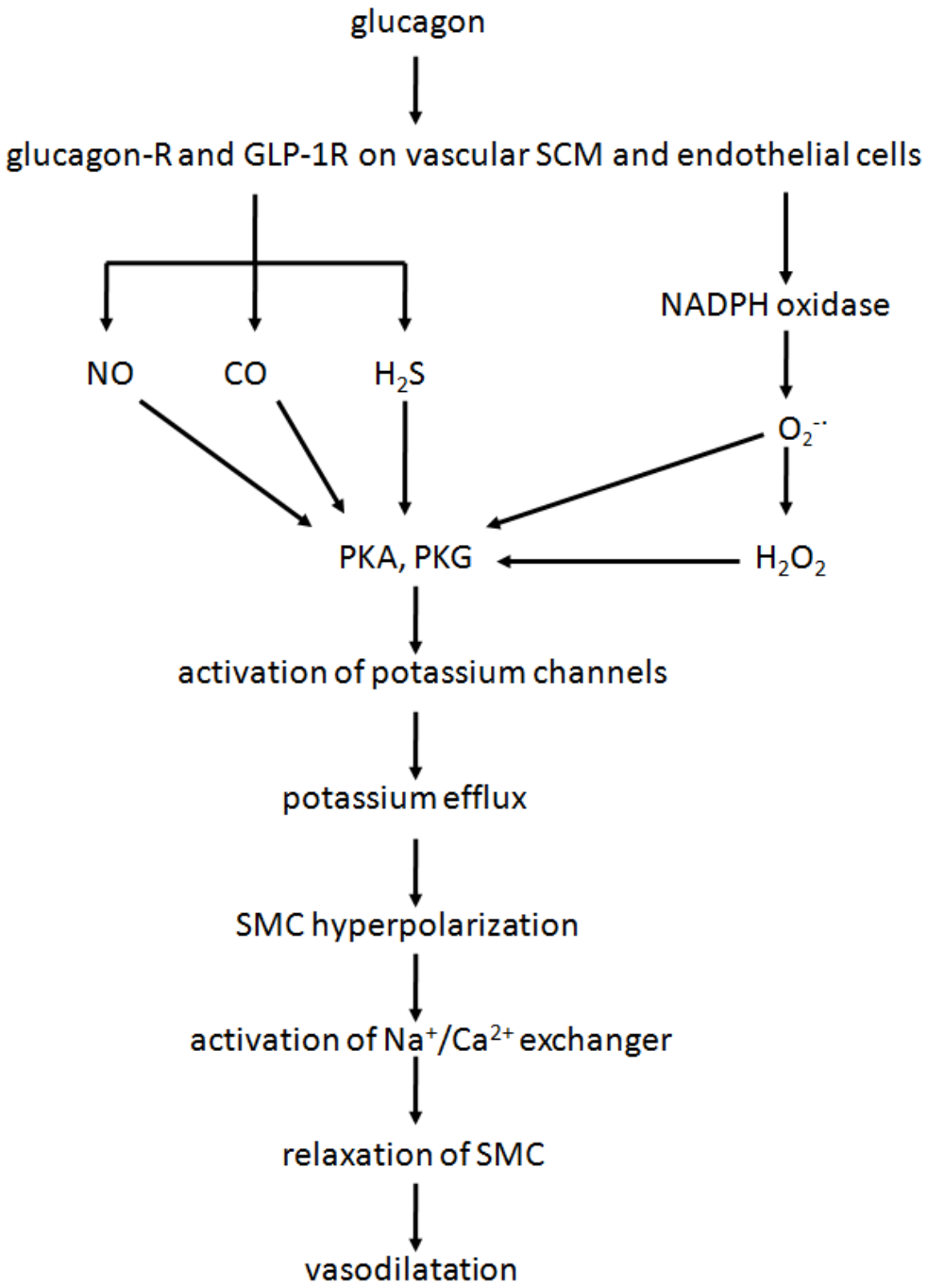

Figure 24. Hypothetical mechanism of the vasodilation induced by glucagon. NO: nitric oxide, H_2S: hydrogen sulfide, CO: carbon monoxide, $O_2^{-\bullet}$: superoxide anion, H_2O_2: hydrogen peroxide, PKA: cAMP-dependent protein kinase, PKG: cGMP-dependent protein kinase, SMC: smooth muscle cell.

Based on our findings, the hypothetical mechanism of the vasorelaxation caused by exenatide is as follows: exenatide binds to GLP-1R and activates both endothelial and vascular smooth muscle cells, leading to the production of H_2S, NO, CO, $O_2^{-\bullet}$, H_2O_2 and prostaglandins.Formation of these relaxing factors contributes to the activation of potassium channels either directly or by activating PKG or - to a lesser extent - PKA. Subsequent activativation of the Na^+/Ca^{2+}-exchanger resulting in calcium efflux leads to smooth muscle relaxation, and thus vasorelaxation (Figure 25).

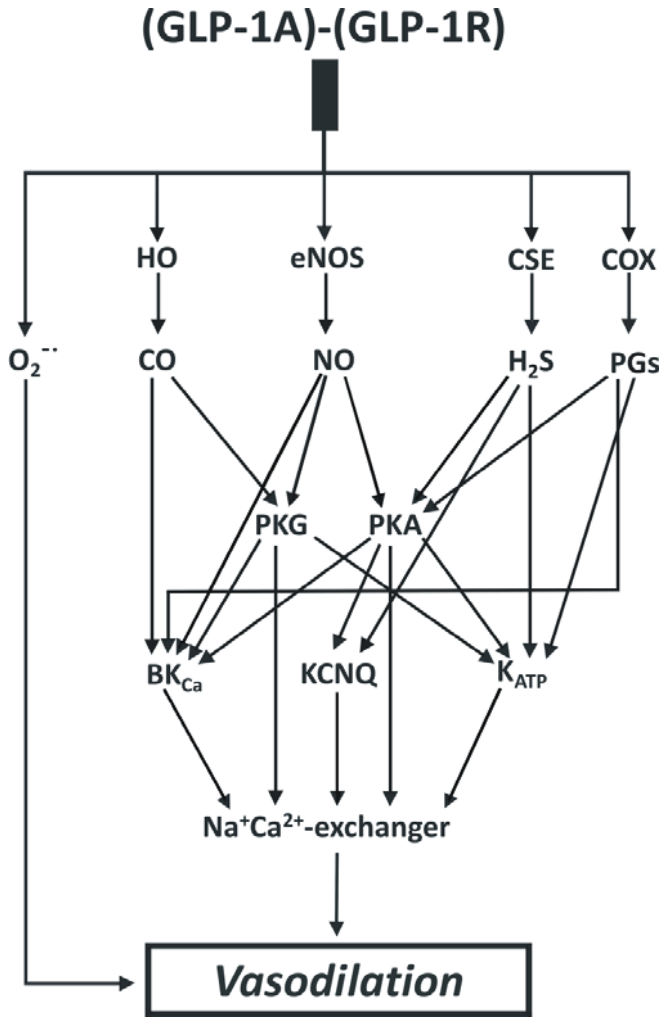

Figure 25. Hypothetical mechanism of the exenatide induced vasodilation according to our data and according to previous examinations [3-7, 10-14, 19, 22, 24-26, 28-29, 35-37, 44-46,] HO: heme oxygenase, eNOS endothelial nitric oxide synthase, CSE: cystathionine-γ-lyase, COX: cyclooxygenase, PG: prostaglandin, H_2S: hydrogen sulphide NO: nitric oxide, CO: carbon monoxide, $O_2^{-\bullet}$: superoxide anion, PKA: cAMP-dependent protein kinase, PKG: cGMP-dependent protein kinase, BKCa:large-conductance calcium activated potassium channel, KCNQ: a type of voltage-gated potassium channel, K_{ATP}: ATP-sensitive potassium channel.

In the rat thoracic aorta liraglutide activates endothelial cells and vascular smooth muscle cells resulting in an increased production of NO, CO, H_2S, $O_2^{-\bullet}$, and H_2O_2. These relaxing factors activate PKA and PKG, resulting in the activation of potassium channels, which in turn activates the Na^+/Ca^{2+}-exchanger, thereby leading to calcium efflux and smooth muscle relaxation and vasorelaxation (Figure 26). In contrast to the aorta, in the femoral artery, the vasodilatation induced by liraglutide was found to be independent of the endothelium, however partially mediated by NO, H_2S and H_2O_2, while PKA and sGC were both involved, activating the calcium-activated and the KCNQ-type voltage-gated potassium channels, resulting in vasodilation via the activation of the Na^+/Ca^{2+}-exchanger.

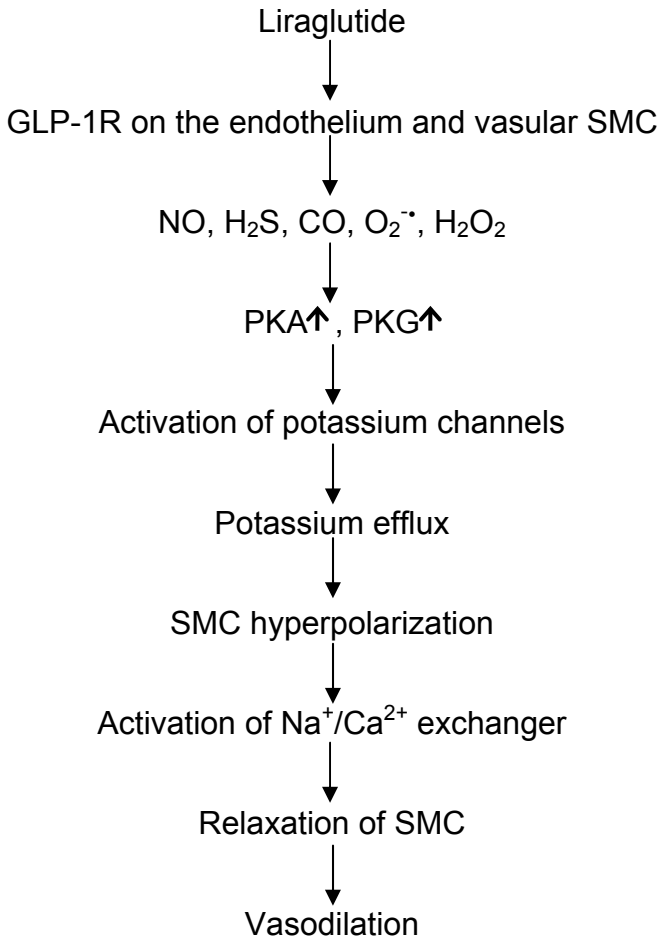

Figure 26. Hypothetical mechanism of liraglutide induced vasodilation. NO: nitric oxide, H_2S: hydrogen sulfide, CO: carbon monoxide, $O_2^{-\bullet}$: superoxide anion, H_2O_2: hydrogen peroxide, PKA: cAMP-dependent protein kinase, PKG: cGMP-dependent protein kinase, SMC: smooth muscle cell.

7.1.1. Mechanism of the glucagon-induced vasodilatation

Decreased vascular resistance and relaxation of hepatic and other, peripheral arteries has formerly been attributed to glucagon (3-7), however, the precise description of the vasodilator mechanism of glucagon has so far not been given.

7.1.1.1. Role of the glucagon-and the GLP-1 receptor in the vasodilatation evoked by glucagon and GLP-1

Metabolic actions of glucagon evolve via the glucagon receptor (1), however, it has not been investigated whether its vasodilator effect is transmitted via the glucagon receptor. Here we demonstrate that glucagon induces vasodilatation via the activation of both the glucagon- and the glucagon-like peptide-1-receptor. Moreover, we reveal that the glucagon receptor is also responsible, at least partially, for the GLP-1-induced vasodilatation. These two receptors are homologous G-protein coupled receptors (60). A study of chimeric glucagon/GLP-1 peptides proved that the major determinant of the glucagon/GLP-1 selectivity of the receptor is the amino-terminal of the extracellular domain of the GLP-1R (60). The homology of these receptors might be the reason for the cross-talk of the glucagon-and GLP-1-induced vasodilatation.

7.1.1.1.1. Significance of the receptorial crosstalk among glucagon, GLP-1 and receptor for glucagon and GLP-1

As mentioned above, type 2 diabetes is commonly treated by analogues of native GLP-1 and agonists of the GLP-1R, as well as dipeptidyl peptidase-4 inhibitors, inhibitors of the enzyme degrading incretin hormones (GLP-1 and GIP), thereby elevating the level of GLP-1 (16). These drugs are also known to decrease glucagon level (16). Like GLP-1, its analogues also cause vasodilatation (17). Speculatively, based on our novel findings, the drugs that

increase the level of GLP-1, they might also induce vasodilatation via the glucagon receptor. Moreover, glucagon and its receptors have been suggested to be potential targets for the treatment of type 2 diabetes and its complications (61).

Although the presence of glucagon receptors on hepatocytes is well known, moreover, their density is increased following exercise in fasting in rats (62), the presence of GLP-1 receptors on hepatocytes is not so evident (1,63). Despite this fact, increasing number of evidencesindicate, that among their pleotropic effects, GLP-1 analogues have a beneficial effect on liver function (64,65,66). Liraglutide, a long acting GLP-1 analogue, decreases lipotoxicity as well as increases hepatic insulin sensitivity in non-alcoholic steatohepatitis (NASH) (63). Another study also demonstrated, that liraglutide significantly improved liver function and histological features in NASH patients with glucose intolerance (65). Liraglutide and exenatide, another GLP-1 agonist, were shown to improve transaminase levels as well as histology in patients with NASH (66). Sitagliptin, an inhibitor of the DPP-4 enzyme, also showed improvement in transaminases (66). The possibility that GLP-1 receptor agonists may cross-activate glucagon receptors e.g. on the hepatocytes, could explain this beneficial effect of the GLP-1 agonist drugs.

Our novel finding might be one of the underlying mechanisms explaining the pleotropic effects of GLP-1, as we demonstrated, that GLP-1 activates the glucagon-receptors as well, moreover, glucagon also acts on GLP-1 receptors. For instance, a dissociation of endocrine and metabolic effects of the Roux-en-Y gastric bypass operation was found in a mildly obese patients with type 2 diabetes, namely, when compared to the preoperative meal tolerance test, after the operation, no increase of GLP-1 and insulin secretion, but improved hepatic and peripheral insulin sensitivity were found (67).

Another pleotropic effect of GLP-1 may be the recently described stimulation of the expression of a novel insulin-mimetic adipocytokine, visfatin, via the PKA pathway, which might also influence glucose metabolism (68).

7.1.1.2. Gaseous and non-gaseous mediators of the glucagon induced vasodilatation

The role of NO and prostaglandins in the glucagon-induced vasodilatation have previously been presented (4), but we went further and reveal that all the three gasotransmitters, nitric oxide, hydrogen sulphide and carbon monoxide, reactive oxygen species (ROS)-superoxide anion ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) are parts of the vasodilatation induced by glucagon. Production of ROS is mediated by the NADPH oxidase, while the products may activate the sGC-cGMP-PKG pathway, leading to vasodilatation (69).

Insulin was also previously shown to induce vasodilatation via increasing NO production through the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway (70,71). However, insulin was also shown to cause vasoconstriction via the MAPK pathway via inducing the production of endothelin-1 (72). A pathway-specific impairment in phosphatidylinositol 3-kinase-dependent signalling is present in insulin resistance, thereby contributing to endothelial dysfunction (72).

Similarly to the signal transduction of insulin, PI3K/Akt is also involved in the effect of glucagon together with the cAMP/PKA, PLC/PKC and ERK, MAPK pathways (8). We demonstrated the role of PKA and the sGC-cGMP-protein kinase G pathway in the vasodilatation induced by glucagon.

7.1.1.3. Involvement of ion transporters in the vasoactive effect of glucagon

Potassium channels are frequently targets of gasotransmitters, thereby leading to vasodilatation (34). Glucagon activates the ATP-sensitiv- the large-conductance calcium-activated and the KCNQ-type voltage-gated potassium channels and causes vasorelaxation.

The terminal effector of the glucagon-induced vasodilatation according to our experiments is the sodium-calcium exchanger. The Na^+/Ca^{2+}-exchanger or NCX is a transmembrane protein found almost in all cell types (73). It is

responsible for calcium efflux and sodium influx in hyperpolarization, whereas when the cell membrane is depolarized, its activity is reversed and it induces calcium influx (73).

7.1.2. Vasodilator mechanism of GLP-1 and its agonists

7.1.2.1. GLP-1 receptor-dependent and independent effects of GLP-1 and its agonists

Also GLP-1 and other related peptides are known to induce vasodilatation in central as well as peripheral vessels, however, both GLP-1R-dependent and –independent vasodilator mechanisms of GLP-1 mimetics have been described (18). In Glp1R-/- mice, native GLP-1 reduced the ischemic damage after ischemia-reperfusion and also increased the production of cGMP, thereby leading to vasodilatation, and increased coronary flow (18). However, the same study reported GLP-1R dependent cardioprotective and glycaemic effects of native GLP-1 amide (18). It has also been suggested that GLP-1 peptides induce vascular relaxation in a GLP-1R-independent manner, at least in the rat aorta, independently of its well-know metabolic actions (17).

In our experiments, GLP-1 mimetic liraglutide caused dose-dependent relaxation of the rat thoracic aorta and the femoral artery, and it proved to be a more potent vasodilator in the femoral artery. The receptor of the vasodilatation induced by liraglutide was not investigated.

According to our results, GLP-1 mimetic exenatide causes relaxation of the rat thoracic aorta in a GLP-1Rdependent manner. This finding is congruent with a study that demonstrated that GLP-1R agonists reduce systolic blood pressure via the GLP-1R, however the same study reported that the antihypertensive effect was NO-independent and that vasodilatation is evoked by the increased secretion of the atrial natriuretic peptide due to GLP-1R activation (30). Non-PKA GLP-1R dependent effects have been shown in the regulation of eNOS expression in aortic endothelial cells in an $ApoE^{-/-}$ mouse

model, suggesting the role of GLP-1 agonists in the inhibition of endothelial cell dysfunction (29).

7.1.2.2. Role of the endothelium in the vasorelaxation caused by exenatide and liraglutide

A previous study stated that the relaxation caused by physiological isoform GLP-1(7-36)amide was not altered by endothelial denudation (17). However, in our experiments, endothelial denudation partilally inhibited both the exenatide and the liraglutide-induced vasodilatation. In contrast to the aorta, in the femoral artery the liraglutide induced vasodilatation wasendothelium-independent.These findings indicate the role of the endothelium in vasorelaxation due to the GLP-1 mimetics, but not the endogenous GLP-1-amides (17).

7.1.2.3. Gasotransmitters mediating the vasoactivity of liraglutide and exenatide

7.1.2.3.1. Role of nitric oxide

Endogenous GLP-1 causes endothelium-dependent vasorelaxation via NO; however, data regarding GLP-1-induced endothelium-independent vasorelaxation have also been published (19-22). NO, formed by the endothelial nitric oxide synthase (eNOS) enzyme, acts via activating soluble guanylyl cyclase (sGC) leading to an increased production of cyclic-guanosine monophosphate (cGMP) which activates the cGMP-dependent protein kinase (PKG) (31). Alternatively, NO was shown to increase cAMP levels in cardiac myocytes by activating adenylyl cyclase in a cGMP-independent manner, resulting in the activation of the cAMP-dependent protein kinase (PKA) (74). On the other hand, in vascular smooth muscle cells NO may have vasodilatory effect via the direct activation of the large-conductance calcium activated potassium channels (BK_{Ca} channels) (34).

Previously, endothelial denudation and preincubation with L-NAME was shown to have no significant effect on the vasodilatation caused by endogenous GLP-1 in rat thoracic aorta, which is in contrast with our findings (17).

GLP-1 was also shown to cause significant vasorelaxation of rat saphenous artery and recruitment of muscle microvasculature via the NO/PKA pathway(75), however, we found that these mechanisms are only partially responsible for the vasodilatation evoked by exenatide and liraglutide in the thoracic aorta of rats, and in case of the liraglutide also in the femoral artery. The possible explanation for the discrepancies between former studies and ours may be that we studied the effects of the GLP-1 agonist exenatide while in previous studies the endogenous, physiological isoform GLP-1(7-36) amide was used.

Our results suggest that other gaseous mediators (CO and H_2S) also take part in the vasodilatation induced by the GLP-1 mimetics.

7.1.2.3.2. Involvement of carbon monoxide

Although previously only NO was found to be involved in the GLP-1-induced vasodilatation (19-22), we found that CO and H_2S also contribute. CO, an important regulator of vascular tone, is a vasodilator gaseous molecule formed from heme by heme oxygenase (HO) in vascular smooth muscle cells (31). Similar to NOS, HO has three isoforms, of which HO-2 is constitutively expressed in endothelial cells and vascular smooth muscle cells (31, 76). CO acts through the activation of sGC, but also increases the calcium sensitivity of BK_{Ca} channels, which in turn leads to smooth muscle hyperpolarization (76). Another vasodilator pathway of CO is the activation of the voltage-dependent potassium channels (K_v) (76). A relationship between GLP-1-effects and CO has not been described earlier, however, in our study we demonstrated that CO significantly contributes to the vasorelaxation caused by exenatide and liraglutide in the thoracic aorta.

7.1.2.3.3. Role of hydrogen sulphide

We also demonstrated the involvement of the third influential gasotransmitter, H_2S in the vasorelaxation evoked by GLP-1 mimetics.H_2S is

also known to relax blood vessels (32). It is produced in endothelial cells and vascular smooth muscle cells from homocystein, cysthationine or L-cysteine by cystathionine-γ-lyase, cystathionine β-synthase and 3-mercaptopyruvate sulfurtransferase (3MST) (77). H_2S and NO are both fundametal in the maintenance of vascular tone (78). It has also been proven that H_2S regulates the availability of NO in the vasculature (78). H_2S may act through the sGC-cGMP-PKG pathway and also via the activation of K_{ATP} and KCNQ-type voltage-gated potassium channels, which causes hyperpolarization of smooth muscle cells (32, 79). H_2S was also shown to activate adenylyl cyclase, which generates cAMP, thereby activates PKA leading to vasodilation (32). It causes vasodilatation via K_{ATP} channel activation, similar to endogenous GLP-1 (17, 32). In our experiments, inhibition of H_2S synthesis resulted in a significantly decreased vasorelaxation in the rat thoracic aorta and also in the femoral artery.

7.1.3. Prostaglandins and reactive oxygen species, as mediators of the vasoactive effect of exenatide and liraglutide

In contrast with previous findings, our experiments showed that prostaglandins and reactive oxygen species are also involved in the vasorelaxation due to exenatide and liraglutide, although to a variable extent (17). In certain arteries, reactive oxygen species play a role in the mediation of vasodilatation (80-82). ROS were also found to relax both pulmonary and mesenteric arteries (83, 33). Hydrogen peroxide activates K_v channels, thereby causing dilation of the arteries (80, 81). H_2O_2 has previously been found to induce both endothelium-dependent and endothelium-independent relaxation in the rabbit's aorta (80). In superoxide-dismutase (SOD) knockout mice eNOS-dependent endothelium-mediated vasorelaxation is impaired, suggesting the role of superoxide anion ($O_2^{-\bullet}$) in vasodilatation (83).

Our results suggest, that H_2O_2 contributes to the exenatide-induced vasorelaxation inthe thoracic aorta, and to the liraglutide induced vasorelaxation in the femoral artery and thoracic aorta.

7.1.4. Protein kinases in the vasodilatation caused by the GLP-1 agonists

Activation of PKA by liraglutide, and the exenatide induced formation cAMP has been described formerly, but these studies did not mention the involvement of PKG (16, 17). However, we found that both PKA and PKG are involved in the vasoactive effect of exenatide and liraglutide.

7.1.5. Role of ion transporters in the liraglutide- and exenatide-induced vasodilatation

The role of potassium channels in the effects of GLP-1 on β-cells has thoroughly been described (1, 11), moreover, GLP-1 was formerly shown to cause vasodilation of rat thoracic aorta via the activation of K_{ATP} channels (17). In a human ischaemia-reperfusion injury model subcutaneously administered exenatide was found to be protective against endothelialdysfunction via the opening of K_{ATP} channels (84). K_{ATP} and K_v channels are inhibited by GLP-1 in ß-cells (16, 17), but we found that both channels are activated by exenatide and liraglutide in the vasculature, leading to vasodilatation of the isolated rat thoracic aorta or femoral artery. Unlike GLP-1, sulphonylurea drugs are known inhibitors of vasodilatation via K_{ATP} channels, thereby, when liraglutide and a sulphonylurea are applied simultaneously for the treatment of type 2 diabetes, the sulphonylurea component might inhibit the vasodilator effect of liraglutide (85).

Moreover, we found that KCNQ type voltage-gated potassium channels are activated by exenatide and liraglutide in the vasculature, which leads to vasodilatation. Both carbon monoxide and superoxide are known activators of these channels (31). The vasorelaxation caused by H_2S, which, as we demonstrated, is involved in the vasorelaxation evoked by liraglutide, often arises via the activation of K_{ATP} and KCNQ-type K_v channels (50).

Activation of the large conductance calcium activated potassium channels (BK_{Ca} channels) by GLP-1 mimetics has not been described previously, although they are constantly expressed in vascular smooth muscle cells (34).Nevertheless, we found thattheblockade of these channels with TEA also inhibited vasorelaxation, but that this occurred only in higher concentrations of the GLP-1 mimetics. It is well-established that the vasodilator effect of CO partially depends on the activation of these channels, moreover, the activation of PKG by NO or the activation of PKA by its mediators result in the activation of these channels (31, 34).

We demonstrated that the inhibition of the transmembrane protein sodium-calcium exchanger markedly inhibited vasodilatation evoked by exenatide and liraglutide, which has not been shown before. NO was also observed to decrease the vasoconstriction mediated by the NCX in rat aorta, which is congruent with our findings, namely, that the GLP-1 mimetics induces the production of NO (86).

7.1.6. Potential clinical significane of our findings

In this study we provide *ex vivo* evidence for the possibility that GLP-1 receptor agonists exenatide and liraglutide cause relaxation of the thoracic aorta *in vitro*, thereby might possibly decrease central (aortic) blood pressure.

Central blood pressure indicates the load affecting the left ventricle, the coronary and cerebral vessels, and it correlates closely with the risk of cardiovascular (CV) events, thereby demonstrating the value of our findings in the clinical context (87). The CAFE study (The Conduit Artery Functional Endpoint Study) established that central aortic pressure is a strong predictor of clinical outcomes (27). There is also *in vivo* evidence that exenatide reduces both systolic and diastolic blood pressure (88). Moreover, GLP-1 receptor agonists are associated with outstanding improvements of other CV risk factors such as body weight and lipid profiles, while they have only a small effect on heart rate and QTc. (25). Exenatide was found to be more beneficial than biguanides, dipeptidyl peptidase-4 inhibitors, thiazolidindiones, or basal insulin,

in reaching the therapeutic goals recommended by the American Diabetes Association (ADA) in the treatment of type 2 diabetes, which is also promising in the reduction of cardivascular risk (89). Treatment with exenatide has shown to have similarly beneficial effects on microvascular endothelial function, oxidative stress, vascular activation and markers of inflammation as metformin in patients with pre-diabetes and obesity (90). Taking the above mentioned into consideration, the ability of a drug used for the treatment of diabetes to further lower central (aortic) blood pressure may be highly beneficial.

We provide *ex vivo* evidence for the mechanism of the liraglutide induced vasorelaxation in the thoracic aorta and in the femoral artery. We have also proved that liraglutide is a more potent vasodilator in the femoral artery, speculating that liraglutide may reduce the arterial stiffness gradient, which could be highly beneficial in clinical practice.

7.2. Role of oxidative stress in the vasorelaxation caused by liraglutide and insulin

The treatment of type 2 diabetes has improved in the past decades as the underlying pathomechanisms were recognised (24). The ominous octet is well known to describe the factors contributing to the development of glucose intolerance: insulin resistance of the pancreatic β-cells, muscle, liver and the brain; incretin resistance or deficiency; hyperglucagonaemia; increased glucose reabsorption and accelerated lipolysis (24). The identification of these factors resulted in a paradigm shift in the treatment, however, importance of the prevention of the disease still cannot be enough emphasized. With better understanding of the underlying molecular mechanisms, a more effective prevention would be available.

7.2.1. Effect of para-tyrosine supplementation on the growth and body weight of cholesterol-fed rats

Although, high-fat diet (HFD) induced obesity is the most widely studied model of type 2 diabetes (T2DM) (91), in our study the rats did not develop

obesity, but they had hypercholesterolemia, and exhibited an important feature observed in T2DM, namely decreased insulin secretion. Although in our experiments metabolic changes also developed when cholesterol-feeding was supplemented with para-tyrosine, despite the presence of risk factors, the physiologic amino acid supplementation prevented some of the target organ damages.

A recent study described that mice on high-fat diet could be categorised into two types based on their weight gain, low (LDR)-or a high responder (HDR) type (92). Low-responder mice were less obese, less hyperglycaemic and showed only a mild change in plasma lipid profile, however, both types of mice developed impaired insulin secretion in response to glucose stimulation (92). Nevertheless, there are studies, in which it is described, that HFD did not induce extra weight gain in rats compared to those on a regular diet, and it did not cause an alteration of basal glycaemia (93).

Insulin effect is essential for normal growth, thus if it is impaired in early stages of the development, it leads to a decrease in growth (94). According to the literature, concerning the age of rats when HFD was started, the earlier HFD was initiated, the less likely the animals were to develop obesity (92, 93). When HFD was started at the age of 8 weeks, rats either developed obesity (HDR) or did not (LDR) (92), but when HFD was started earlier, rats did not show extra weight gain, compared to the controls (93). This suggests, that when cholesterol feeding is started early, the decreased insulin effect results in impaired insulin-like growth factor (IGF) effect, thus hindering the growth of rats.

7.2.2. Role of oxidative stress in glucose-stimulated insulin secretion

It was shown, that when the level of cholesterol in pancreatic β-cells is elevated, it attenuates glucose-stimulated insulin secretion (95), which is congruent with our results, that following glucose stimulation, decreased insulin response was to be seen in the cholesterol-fed group. This phenomenon was not seen in rats treated with para-tyrosine, supporting our hypothesis, that para-

tyrosine might be useful in preventing oxidative stress-induced impaired insulin response. This raises the question, whether even the metabolic-resistance to insulin could be prevented by para-tyrosine. It is well established, that other amino acids, but not para-tyrosine increase insulin secretion (96). Particularly, arginine, leucine, isoleucine, valine, lysine, and threonine are likely to act as insulin secretagogues (96).

7.2.3. Role of tyrosine isoforms in oxidative stress-related diseases

Permanent hypercholesterolemia leads to oxidative stress(97); while diseases, associated with increased oxidative stress are known to be associated with elevated plasma and urinary levels of the non-physiological amino acid isoforms meta-and ortho-tyrosine (47, 48, 59). Our former studies proved that plasma level of para-tyrosine is decreased in patients with chronic kidney disease, while the excretion of ortho-tyrosine in the urine is higher in patients with chronic kidney disease and type 2 diabetes mellitus (59).

The physiological and pathological isoforms and their possible pathogenetical role can also be regarded in a broader concept, as some data indicated their potential role in resistance to specific hormones (35). Regarding erythropoietin efficacy/resistance, our workgroup found that the level of para-tyrosine is decreased in dialyzed patients, while ortho-tyrosine and ortho-tyrosine/para-tyrosine ratio was shown to be significantly higher (45). Ortho-tyrosine/para-tyrosine ratio proved to be an independent predictor of erythropoietin resistance index, indicating that ortho-tyrosine/para-tyrosine ratio may be responsible for the resistance to erythropoiesis-stimulating agents in dialyzed patients (45).

Regarding insulin sensitivity/resistance our workgroup found that short-term (4 weeks) administration of the antioxidant resveratrol resulted in a reduction of urinary ortho-tyrosine excretion in patients with type 2 diabetes mellitus (98). A parallel improvement in insulin signaling and in insulin sensitivity was observed; the former was estimated by the phosphorylation of Akt (protein kinase B) and the latter was expressed with the calculated marker of insulin

resistance, the homeostasis model assessment-insulin resistance (HOMA-IR) (98).

Former studies indicated that the level of ortho-tyrosine is elevated in type 2 diabetes mellitus, and proved that ortho-tyrosine concentration is elevated in the aortic proteins of diabetic monkeys (48, 49). The role of reactive oxygen species in diabetes-induced endothelial dysfunction was also indicated formerly (99).

The present study is a part of a series of experiments regarding the possible role of the pathological and physiological tyrosine isoforms. First, in a cross-sectional study, our workgroup investigated the role of vascular oxidative state by the assessment of vascular (thoracic and abdominal aorta, femoral artery) ortho-tyrosine concentration (100). Higher ortho-tyrosine content was found in the central blood vessels, while with the decrease of ortho-tyrosine concentration toward the peripheral vessels insulin-induced vasorelaxation increased (100).

In a longitudinal, interventional study, oral ortho-tyrosine treatment of rats for 4 weeks significantly increased vascular ortho-tyrosine content, and at the same time, impaired insulin-induced relaxation was demonstrated in isolated femoral arteries (49). The same study proved that accumulation of ortho-tyrosine in the vascular wall could contribute to impaired insulin-induced relaxation of arteries, interfering with the PI3K/Akt/eNOS/NO signalling pathway (49). It was also demonstrated in this study, that ortho-tyrosine could be incorporated into the proteins of endothelial cells (49). Insulin-induced eNOS phosphorylation was also decreased in endothelial cells cultured in the presence of ortho-tyrosine (49).

7.2.3.1. Impaired liraglutide-and insulin-induced vasorelaxation due to oxidative stress

Finally, in our present, longitudinal, interventional experiments, the level of meta-tyrosine was elevated in the wall of the thoracic aorta of cholesterol-fed rats, which was associated with decreased insulin-and liraglutide-induced

vasodilatation. Since the consequences of altered blood flow and endothelial dysfunction is a major problem in diabetes mellitus (72), the importance of a physiologic compound with the ability of either preventing or restoring vasodilatation would be remarkable. According to our results, para-tyrosine is capable of restoring some of the vascular function damaged by oxidative stress, thus raising the possibility of a protective effect of para-tyrosine.

8. Conclusions

Glucagon may activate both the glucagon- and the GLP-1-receptor, thereby leading to dose-dependent, endothelium-independent vasodilatation with the contribution of NADPH oxidase, free radicals, gasotrasmitters, prostaglandins, PKA, sGC, potassium channels and finally the NCX.

We demonstrated that exenatide causes vasorelaxation in the rat thoracic aorta in a GLP-1R dependent manner mainly via H_2S but also via NO, CO, $O_2^{-\bullet}$and prostaglandins, and that this effect can be mediated via the activation of PKA and PKG. Through the induction of these mediators, exenatide also influences the activity of potassium channels and the Na^+/Ca^{2+} -exchanger.

Liraglutide induces vasodilation in both central and peripherial vessels. According to our findings the vasodilator effect of liraglutide was evoked by three gasotransmitters (NO, CO, H_2S) and also by reactive oxygen species and prostaglandins via activation of PKA and PKG, which involves the activation of several potassium channels and the Na^+/Ca^{2+}-exchanger. This mechanism is far more diverse than it was assumed earlier.

Incorporation of the oxidative stress-induced pathological tyrosine isoforms leads to hormone-resistances. We show that the physiological amino acid para-tyrosine is capable of restoring hypercholesterolemia-induced increased meta-tyrosine content of the vascular wall, thus attenuating functional vascular damage.

9. List of the Ph.D. theses

1. Glucagon induces dose-dependent vasodilatation via the glucagon- and the GLP-1-receptor *in vitro.*

2. Further mediators of the glucagon induced vasodilatation are the NADPH oxidase, free radicals, gasotransmitters, prostaglandins, PKA, sGC, potassium channels and the Na^+/Ca^{2+}-exchanger.

3. The vasodilatation induced by native GLP-1 is partially evoked via the glucagon-receptor.

4. GLP-1 mimetics exenatide and liraglutide induce dose-dependent vasorelaxation of the rat thoracic aorta *in vitro*, via H_2S but also via NO, CO, $O_2^{-\bullet}$ and prostaglandins, and this effect can be mediated via the activation of PKA and PKG. Through the induction of these mediators, exenatide and liraglutide also influence the activity of potassium channels and the Na^+/Ca^{2+} -exchanger.

5. The relaxation evoked by liraglutide in the rat femoral artery is greater than that in the thoracic aorta.

6. Cholesterol feeding results in increased meta-tyrosine concentration in the wall of the rat thoracic aorta associated with decreased vascular response to liraglutide and insulin.

7. Supplementation with the physiological amino acid para-tyrosine restores hypercholesterolemia induced increased meta-tyrosine content of the vascular wall, thus attenuates functional vascular damage.

10. List of publications used for the thesis

Sélley E, Kun S, Szijártó IA, Laczy B, Kovács T, Fülöp F, Wittmann I, Molnár GA: Exenatide induces aortic vasodilation increasing hydrogen sulphide, carbon monoxide and nitric oxide production. *Cardiovasc Diabetol* 2014, 13:69. doi: 10.1186/1475-2840-13-69. IF:3.67

Sélley E, Molnár GA,Kun S, Szijártó IA, Laczy B, Kovács T, Fülöp F, Wittmann I:Complex vasoactivity of liraglutide. Contribution of three gasotransmitters. *Art Res* 2015, doi:10.1016/j.artres.2015.04.001. IF:-

Selley E, Kun S, Kürthy M, Kovacs T, Wittmann I, Molnar GA: Para-Tyrosine Supplementation improves insulin- and liraglutide- induced vasorelaxation in cholesterol-fed rats.*Protein Pept Lett* 2015, 22:736-742.IF: 1.06

Selley E, Kun S, Szijártó IA, Kertész M, Wittmann I, Molnar GA:Vasodilator effect of glucagon. Receptorial crosstalk among glucagon, GLP-1 and receptor for glucagon and GLP-1. *Horm Metab Res* 2016;48: 1–8. IF: 2.029

10.1. List of publications not related to the thesis

Kun S, Mikolás E, Molnár GA, **Sélley E**, Laczy B, Csiky B, Kovács T, Wittmann I. Association of plasma ortho-tyrosine/para-tyrosine ratio with responsiveness of erythropoiesis-stimulating agent in dialyzed patients. *Redox Rep* 2014 Apr 3.IF: 1.52

Mikolás E, Kun S, Laczy B, Molnár GA, **Sélley E**, Kőszegi T, Wittmann I. Incorporation of Ortho- and Meta-Tyrosine Into Cellular Proteins Leads to

Erythropoietin-Resistance in an Erythroid Cell Line. *Kidney Blood Press Res* 2014 Apr 9;38(2-3):217-225.IF: 1.82

Molnár GA, Laczy B, **Sélley E**, Kun S, Wittmann I: Az antidiabeticumok és a cardiovascularis kockázat. *Diabetol Hungarica* 22, 103-110, 2014. 03. IF:-

Gergő A Molnár, Esztella Zsóka Mikolás, István András Szijártó, Szilárd Kun, **Eszter Sélley** and István Wittmann. Tyrosine isomers and hormonal signaling. Possible role of hydroxyl free radical in insulin resistance. *World J Diab* 2015. IF:-

Kun S, Molnár GA, **Sélley E**, Szélig L, Bogár L, Csontos C, Miseta A., Wittmann I. Insulin therapy of non-diabetic septic patients is predicted by para-tyrosine/phenylalanine ratio and by hydroxyl radical-derived products of phenylalanine. *Oxidative Med* 2015. IF: 3.51

Molnár GA, Kun S, **Sélley E**, Kertész M, Szélig L, Csontos C, Böddi K, Bogár L,Miseta A, Wittmann I: Role of Tyrosine Isomers in Acute and Chronic Diseases Leading to Oxidative Stress - a review. Curr Med Chem. 2016 Jan 18. IF: 3.85

Brasnyó P, Kovács T, Molnár GA, **Sélley E**, Kun Sz, Vas T, Laczy B, Fekete K, Kovács K, Meszáros GL, Winkler G, Sümegi B and Wittmann I.: Resveratrol Causes Gender-dependent and Bardoxolone Methyl-like Effects in Patients with IgA Nephropathy: Pilot Study. J Nutr Food Sci 2016, 6: 442. DOI: 10.4172/2155-9600.1000442. IF: 1.2

Cumulative impact factor: 18.66

Number of independent citations: 14

10.2. List of presentations and abstracts

Kun Szilárd, Mikolás E., **Sélley E**., Wittmann I.:Az oxidált aminosav, orto-tirozin szerepe az erythropoetin-rezisztencia kialakulásában. *I. MATHINÉ Budapest, 21.03.2013.*

Sélley E, Kun Sz., Molnár G., Degrell P., Kürthy M., Kovács T., Wittmann I.: Para-tirozin szupplementáció javítja az inzulin- és a liraglutide-kiváltotta vazorelaxációt koleszterinnel etetett patkányokban. *II. MATHINÉ Budapest, 14.03.2014.*

Mikolás Esztella Zsóka,Kun Sz**.**, Laczy B., Molnár G.A., **Sélley E**., Kőszegi T., Wittmann I.: A cukorbetegekben tapasztalt korai anaemia lehetséges oka. *II. MATHINÉ Budapest, 14.03.2014.*

Sélley E, Molnár G.A., Kun Sz**.**, Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I. A liraglutid komplex vazoaktív hatása. Három gázmolekula jelátvivő szerepe. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) p. &. (2014)*

Kun Szilárd, **Sélley E**., Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I., Molnár G.A. Az exenatid a kén-hidrogén, a szén-monoxid és a nitrogén-monoxid termelődésének fokozása révén kelthet centrális vasodilatatiót. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) p. &. (2014)*

Mikolás Esztella**,** Kun Sz., Laczy B., Molnár G.A., **Sélley E**., Kőszegi T., Wittmann I. A cukorbetegekben tapasztalt korai anaemia lehetséges oka. *MDT*

XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) p. &. (2014)

Molnár Gergő Attila, **Sélley E.,** Kun Sz**.**, Degrell P., Kürthy M., Kovács T., Wittmann I. Para-tirozin szupplementáció javítja az inzulin- és a liraglutid kiváltotta vasorelaxatiót koleszterinnel etetett patkányokban. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) pp. 105-107. (2014)*

Mikolás Esztella,Kun Sz., Laczy B., Molnár G.A., **Sélley E.**, Kőszegi T., Wittmann I.: Az orto- és meta-tirozin beépülés in vitro erythropoetin-rezisztenciához vezet. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

Sélley E., Molnár G.A., Kun Sz**.**, Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I.: A GLP-1 analógok vese artériára gyakorolt hatása. Gázmolekulák jelátvivő szerepe. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

Molnár Gergő Attila,Kun Sz., Mikolás E., **Sélley E.**, Csiky B., Wittmann I.: Az erythropoetin-rezisztencia és az oxidált aminosav, az orto-tirozin összefüggése. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

E. Sélley, Sz. Kun, I.A. Szijártó, B. Laczy, T. Kovács, F. Fülöp, I. Wittmann, G.A. Molnár: Vasodilatation by lixisenatide in the rat thoracic aorta, mediated by an unknown receptor. Third International Symposium on Hypertension, Translational Medicine in Hypertension. *Osijek*, 28.11.2014.

Kun Szilárd**,** Molnár G.A., **Sélley E.**, Szélig L., Bogár L., Csontos Cs., Miseta A., Wittmann I.: Nem-diabéteszes szeptikus betegek inzulinterápiájának független prediktora a para-tirozin/fenilalanin hányados és a fenilalanin oxidált

származékai. *MDT XXIII. Kongresszusa Budapest, 15.05.2015.DIABETOLOGIA HUNGARICA 2015/2*

Sélley E., Kun Sz., Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I., Molnár G.A.: A lixiszenatid vazodilatációt okoz cGMP közvetítésével ismeretlen receptoron keresztül.*MDT XXIII. Kongresszusa Budapest, 15.05.2015.DIABETOLOGIA HUNGARICA 2015/2*

Resveratrol hatásának vizsgálata IgA nephropathiában. (Pilot vizsgálat)

Kovács Tibor**,** Brasnyó P., Molnár G.A., **Sélley E**., Kun Sz., Vas T., Laczy B., Fekete K., Kovács K., Mészáros G.L., Winkler G., Sümegi B., Wittmann I.*MANET XXXII. Nagygyűlése és Továbbképző Tanfolyama Siófok*, 12.11.2015.

Sélley Eszter, Kun Szilárd, Szijártó István András, Kertész Melinda, Wittmann István, Molnár Gergő Attila: A glukagon vazodilatációs hatása. Receptoriális szintű kölcsönhatás a glukagon és a GLP-1 között. *MDT XXIV. Kongresszusa Debrecen*, 28.04.2016.

10. References

1. Mayo KE, Miller LJ, Bataille D et al. International Union of Pharmacology. XXXV. The Glucagon Receptor Family. *Pharmacol Rev* 2003; 55:167–194.
2. Charron MJ, Vuguin PM: Lack of glucagon receptor signaling and its implications beyond glucose homeostasis. *J Endocrinol* 2015; 224: R123-R130.
3. Gagnon G, Regoli D, Rioux F: Studies on the mechanism of action of glucagon in strips of rabbit renal artery. *Br J Pharmacol* 1980; 69: 389–396.
4. Tolins JP: Mechanisms of glucagon-induced renal vasodilation: role of prostaglandins and endothelium-derived relaxing factor. *J Lab Clin Med* 1992; 120:941-948.
5. Richardson PD, Withrington PG: The vasodilator actions of isoprenaline, histamine, prostaglandin E2, glucagon and secretin on the hepatic arterial vascular bed of the dog. *Br J Pharmacol* 1976; 57: 581–588.
6. Moir TW, Nayler WG: Coronary Vascular Effects of Glucagon in the Isolated Dog Heart. *Circ Res* 1970; Vol. XXVI.
7. Rosic M, Pantovic S, Rosic G, Tomic-Lucic A, Labudovic T, Zivkovic V, Jakovljevic V: Glucagon effects on ischemic vasodilatation in the isolated rat heart. J Biomed Biotechnol 2010; 2010:231832. doi: 10.1155/2010/231832.
8. Jiang Y1, Cypess AM, Muse ED, Wu CR, Unson CG, Merrifield RB et al.: Glucagon receptor activates extracellular signal-regulated protein kinase 1/2 via cAMP-dependent protein kinase. Proc Natl Acad Sci U S A 2001; 98:10102-10107.
9. Armstead WM, Kiessling JW, Cines DB, Higazi AA: Glucagon protects against impaired NMDA-mediated cerebrovasodilation and cerebral autoregulation during hypotension after brain injury by activating cAMP protein kinase A and inhibiting upregulation of tPA. *J Neurotrauma* 2011; 28:451-457.
10. Armstead WM, Riley J, Cines DB, Higazi AA: Combination therapy with glucagon and a novel plasminogen activator inhibitor-1-derived peptide enhances protection against impaired cerebrovasodilation during hypotension after traumatic brain injury through inhibition of ERK and JNK MAPK. *Neurol Res* 2012; 34:530-537.
11. Kedia N.: Treatment of severe diabetic hypoglycemia with glucagon: an underutilized therapeutic approach. *Diabetes Metab Syndr Obes* 2011; 4: 337–346.

12. Castle JR, Engle JM, El Youssef J, Massoud RG, Yuen KC, Kagan R, Ward WK. Novel use of glucagon in a closed-loop system for prevention of hypoglycemia in type 1 diabetes. *Diabetes Care* 2010; 33:1282-1287.
13. Peterson CD, Leeder JS, Sterner S.: Glucagon therapy for beta-blocker overdose. *Drug Intell Clin Pharm* 1984; 18:394-398.
14. Doyon S, Roberts JR: The use of glucagon in a case of calcium channel blocker overdose. *Ann Emerg Med* 1993; 22:1229-33.
15. Khayyat YM: Pharmacological Management of Esophageal Food Bolus Impaction. *Emerg Med Internat* 2013, ID 924015 (http://dx.doi.org/10.1155/2013/924015).
16. Drucker DJ: The biology of incretin hormones. 2006; 3:153–165.
17. Green BD, Hand KV, Dougan JE, McDonnell BM, Cassidy RS, Grieve DJ: GLP-1 and related peptides cause concentration-dependent relaxation of rat aorta through a pathway involving KATP and cAMP. *Arch Biochem Biophys* 2008, 478:136–142.
18. Ban K, Noyan-Ashraf M.H, Hoefer J, Bolz S-S, Drucker DJ, Husain M: Cardioprotective and Vasodilatory Actions of Glucagon-Like Peptide 1 Receptor Are Mediated Through Both Glucagon-Like Peptide 1 Receptor–Dependent and –Independent Pathways. Circulation 2008; 117:2340-2350.
19. Golpon HA, Puechner A, Welte T, Wichert PV, Feddersen CO: Vasorelaxant effect of glucagon-like peptide-(7-36)amide and amylin on the pulmonary circulation of the rat. *Regul Pept* 2001; 102:81-86.
20. Richter G, Feddersen O, Wagner U, Barth P, Göke B: GLP-1 stimulates secretion of macromolecules from airways and relaxes pulmonary artery. *Am J Physiol* 1993; 265: L374-381.
21. Nyström T, Gonon AT, Sjöholm A, Pernow J.: Glucagon-like peptide-1 relaxes rat conduit arteries via an endothelium-independent mechanism. *Regul Pept* 2005; 125:173-177.
22. Chai W, Dong Z, Wang N, Wang W, Tao L, Cao W, Liu Z: Glucagon-Like Peptide 1 Recruits Microvasulature and Increases Glucose Use in Microvasculature and Increases Glucose Use in Muscle via Nitric Oxide-Dependent Mechanism. *Diabetes* 2012; 61: 888-896.
23. Verspohl EJ: Novel therapeutics for type 2 diabetes: incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors. *Pharmacol Ther* 2009; 124:113-138.
24. DeFronzo RA: From the Triumvirate to the Ominous Octet: A New Paradigm for

the Treatment of Type 2 Diabetes Mellitus. *Diabetes* 2009; 58: 773–795.

25. Petrie JR: The cardiovascular safety of incretin-based therapies: a review of the evidence. *Cardiovasc Diabetol* 2013; 12:130.
26. DeFronzo RA, Ratner RE, Han J, Kim DD, Fineman MS, Baron AD: Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. *Diab Care* 2005; 28:1092-1100.
27. Williams B, Lacy PS, Thom SM, Cruickshank K, Stanton A, Collier D, Hughes AD, Thurston H, O'Rourke M; CAFE Investigators; Anglo-Scandinavian Cardiac Outcomes Trial Investigators; CAFE Steering Committee and Writing Committee: Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: principal results of the Conduit Artery Function Evaluation (CAFE) study. *Circulation* 2006, 7; 113.
28. Hattori Y, Jojima T, Tomizawa A, Satoh H, Hattori S, Kasai K, Hayashi T: A glocagon-like peptide-1 (GLP-1) analogue, liraglitide, upregulates nitric oxide production and exerts anti-inflammatory action in endothelial cells. *Diabetologia* 2010; 53:2256-2263.
29. Gaspari T, Liu H, Welungoda I, Hu Y, Widdop RE, Knudsen LB, Simpson RW, Dear AE.: A GLP-1 receptor agonist liraglutide inhibits endothelial cell dysfunction and vascular cell adhesion molecule expression in ApoE-/- mouse model. *Diab Vasc Dis Res* 2011; 8:117-124.
30. Kim M, Platt MJ, Shibasaki T, Quaggin SE, Backx PH, Seino S, Simpson JA, Drucker DJ: GLP-1 receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure.*Nat Med* 2013; 19:567-575.
31. Mustafa AK, Gadalla MM, Snyder SH: Signaling by Gasotransmitters. *Sci Signal* 2009, 2(68).
32. Martelli A, Testai L, Breschi MC, Lawson K, McKay NG,Miceli F, Taglialatela M, Calderone V: Vasorelaxation by hydrogen sulphide involves activation of Kv7 potassium channels.*Pharmacol Res*2013, 70: 27-34.
33. Zavaritskaya O, Zhuravleva N, Schleifenbaum J, Gloe T, Devermann L,Kluge R, Mladenov M, Frey M, Gagov H, Fésüs G,Gollasch M, Schubert R: Role of KCNQ channels in skeletal muscle arteries and periadventitial vascular dysfunction.*Hypertension* 2013, 61:151-159.
34. Ko EA, Han J, Jung ID, Park WS: Physiological roles of K^+ channels in vascular smooth muscle cells. *J Smooth Muscle Res* 2008, 44: 65-81.
35. Molnár GA, Mikolás E, Szijártó IA,Kun Sz, Sélley E, WittmannI.: Tyrosine isomers and hormonal signaling: A possible role for the hydroxyl free radical in

insulin resistance. *World J Diabetes* 2015; 6: 500–507.

36. Aso Y,Wakabayashi S,Yamamoto R,Matsutomo R,Takebayashi K,I.: Metabolic syndrome accompanied by hypercholesterolemia is strongly associated with proinflammatory state and impairment of fibrinolysis in patients with type 2 diabetes: synergistic effects of plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor.*Diabetes Care* 2005; 28:2211-2216.
37. Herzberg-Schäfer S, Heni M, Stefan N, Häring HU, Fritsche A.: Impairment of GLP1-induced insulin secretion: role of genetic background, insulin resistance and hyperglycaemia. *Diabetes Obes Metab* 2012; Suppl 3:85-90. doi: 10.1111/j.1463-1326.2012.01648.x.
38. Wilson SH, Caplice NM, Simari RD et al. :Activated nuclear factor-kappaB is present in the coronary vasculature in experimental hypercholesterolemia. *Atherosclerosis* 2000; 148:23–30
39. Ramana KV, Friedrich B, Srivastava S, Bhatnagar A, Srivastava SK.: Activation of nuclear factor-kappaB by hyperglycemia in vascular smooth muscle cells is regulated by aldose reductase*. Diabetes* 2004; 53:2910-2920.
40. Boura-Halfon S, Zick Y: Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 2009; 296: E581-591
41. Galano A, Cruz-Torres A. OH radical reactions with phenylalanine in free and peptide forms. *Org Biomol Chem* 2008;6:732–738.
42. Stadtman ER, Berlett BS. Fenton chemistry–amino acid oxidation. *J Biol Chem* 1991; 266:17201–17211.
43. Klipcan L, Moor N, Kessler N, Safro MG. Eukaryotic cytosolic and mitochondrial phenylalanyl-tRNA synthetases catalyze the charging of tRNA with the meta-tyrosine. *Proc Natl Acad Sci USA* 2009; 106 :11045–11048.
44. Rudich A, Kozlovsky N, Potashnik R, Bashan N. Oxidant stress reduces insulin responsiveness in 3T3-L1 adipocytes. *Am J Physiol* 1997; 272:935–940.
45. Kun S, Mikolás E, Molnár GA, Sélley E, Laczy B, Csiky B, Kovács T, Wittmann I. Association of plasma ortho-tyrosine/para-tyrosine ratio with responsiveness of erythropoiesis-stimulating agent in dialyzed patients. *Redox Rep*. 2014.
46. Mikolás E, Kun S, Laczy B, Molnár GA, Sélley E, Kőszegi T, Wittmann I. Incorporation of Ortho- and Meta-Tyrosine Into Cellular Proteins Leads to Erythropoietin-Resistance in an Erythroid Cell Line. *Kidney Blood Press Res* 2014; 38: 217-225.
47. Vivekanadan-Giri A, Wang J H ,Byun J , Pennathur S . Mass spectrometric quantifi cation of amino acid oxidation products identifies oxidative mechanisms

of diabetic end-organ damage. *Rev Endocr Metab Disord* 2008; 9: 275–287

48. Pennathur S , Wagner J D , Leeuwenburgh C , Litwak K N , Heinecke J W .A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease . *J Clin Invest* 2001; 107 : 853–860
49. Szijártó IA, Molnár GA, Mikolás E, Fisi V, Cseh J, Laczy B, Kovács T, Böddi K, Takátsy A, Gollasch M, Koller A, Wittmann I.:Elevated vascular level of ortho-tyrosine contributes to the impairment of insulin-induced arterial relaxation. *Horm Metab Res* 2014; 46:749-752.
50. Köhn C, Schleifenbaum J, Szijártó IA, Markó L, Dubrovska G,Huang Y, Gollasch M: Differential effects of cystathionine-γ-lyase-dependent vasodilatory H_2S in periadventitial vasoregulation of rat and mouse aortas. *PLoS One* 2012, 7:e41951
51. Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft FC, Gollasch M: Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* 2007; 75:719-727.
52. Halmai R, Szijártó IA, Fehér E, Fésüs G, Molnár GA, Brasnyó P, Fülöp F, Gollasch M, Koller A, Wittmann I.: Cigarette smoke elicits relaxation of renal arteries. *Eur J Clin Invest* 2011; 41: 195-202.
53. Goldstein DS, Eisenhofer G, Kopin IJ: Sources and significance of plasma levels of catechols and their metabolites in humans. *J Pharmacol Exp Ther* 2003, 305:800-811.
54. DeYoung MB, MacConell L, Sarin V, Trautmann M, Herbert P: Encapsulation of exenatide in poly-(D,L-lactide-co-glycolide) microspheres produced an investigational long-acting once-weekly formulation for type 2 diabetes. *Diabetes Technol Ther* 2011; 13: 1145-1154.
55. Malm-Erjefält M, Bjørnsdottir I, Vanggaard J, Helleberg H, Larsen U, Oosterhuis B, van Lier JJ, Zdravkovic M, Olsen AK: Metabolism and excretion of the once-daily human glucagon-like peptide-1 analog liraglutide in healthy male subjects and its in vitro degradation by dipeptidyl peptidase IV and neutral endopeptidase. *Drug Metab Dispos* 2010; 38:1944-1953.
56. Xiao YF, Nikolskaya A, Jaye DA, Sigg DC: Glucagon-like peptide-1 enhances cardiac L-type Ca^{2+} currents via activation of the cAMP-dependent protein kinase A pathway. *Cardiovasc Diabetol* 2011, 10:6.
57. Ko EA, Han J, Jung ID, Park WS: Physiological roles of K+ channels in vascular smooth muscle cells. *J Smooth Muscle Res* 2008, 44: 65-81.
58. Iwamoto T, Kita S: Hypertension, Na^+/Ca^{2+} exchanger, and Na^+, K^+-ATPase.

*Kidney Int*206; 69:2148-2154.

59. Molnár GA, Wagner Z, Markó L, Kőszegi T, Mohás M, Kocsis B, Matus Z, Wagner L, Tamaskó M, Mazák I, Laczy B, Nagy J, Wittmann I: Urinary ortho-tyrosine excretion in diabetes mellitus and renal failure: Evidence for hydroxyl radical production. *Kidney Int* 2005; 68:2281-2287.
60. Runge S, Wulff BS, Madsen K, Bräuner-Osborne H, Knudsen LB: Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. *Br J Pharmacol* 2003; 138:787-794.
61. XC. Li, JL. Zhuo: Targeting glucagon receptor signalling in treating metabolic syndrome and renal injury in Type 2 diabetes: theory versus promise. *Clin Sci* 2007 113: 183–193.
62. Melançon A, Gagnon V, Milot M, Charest É, Foucher D,Péronnet F, UnsonO'Brien CG, Asselin E,Lavoie C: Liver glucagon receptors(GluR): effect of exercise and fasting on binding characteristics, GluR-mRNA, and GluR protein content in rats.*Horm Metab Res* 2013;45:716-721.
63. Pyke C, Heller RS, Kirk RK, Ørskov C, Reedtz-Runge S, Kaastrup P, HvelplundA, Bardram L, Calatayud D, Knudsen LB: GLP-1 receptor localization in monkey and human tissue: novel distribution revealed with extensively validated monoclonal antibody. *Endocrinology* 2014; 155:1280-1290.
64. Armstrong MJ, Hull D, Guo K, Barton D, Hazlehurst JM, Gathercole LL, Nasiri M, Yu J, Gough SC, Newsome PN, Tomlinson JW: Glucagon-Like Peptide 1 Decreases Lipotoxicity in Non-Alcoholic Steatophepatitis*. J Hepatol*. 2015. pii: S0168-8278(15)00624-8. doi: 10.1016/j.jhep.2015.08.038.
65. Eguchi Y, Kitajima Y, Hyogo H, Takahashi H, Kojima M, Ono M, Araki N, Tanaka K, Yamaguchi M, Matsuda Y, Ide Y, Otsuka T, Ozaki I, Ono N, Eguchi T, Anzai K; Japan Study Group for NAFLD (JSG-NAFLD): Pilot study of liraglutide effects in non-alcoholic steatohepatitis and non-alcoholic fatty liver disease with glucose intolerance in Japanese patients (LEAN-J). *Hepatol Res* 2015; 45:269-278. doi: 10.1111/hepr.12351.
66. Olaywi M, Bhatia T, Anand S, Singhal S: Novel anti-diabetic agents in non-alcoholic fatty liver disease: a mini-review. *Hepatobiliary Pancreat Dis Int* 2013; 12:584-588.
67. Yamashita K, Sato Y1, Oki K2, Kishimoto H2, Yamauchi K1, Aizawa T1: Marked improvement of insulin sensitivity without enhancement of GLP-1 and insulin secretion after Roux-en-Y gastric bypass surgery in a mildly obese patient with diabetes. *Horm Metab Res* 201; 46:424-426.

68. Liu R, Ding X, Wang Y, Wang M, Peng Y: Glucagon-like peptide-1 upregulates visfatin expression in 3T3-L1 adipocytes. *Horm Metab Res* 2013; 45:646-651.

69. Paravicini TM, Touyz RM: NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 2008; 31 Suppl 2:S170-80

70. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD .Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* 1994; 94 : 1172–1179.

71. Lee JH, Ragolia L.: AKT phosphorylation is essential for insulin-induced relaxation of rat vascular smooth muscle cells. *Am J Physiol Cell Physiol* 2006; 291: C1355–C1365.

72. Muniyappa R, Montagnani M, Koh KK, Quon MJ: Cardiovascular actions of insulin .*Endocr Rev* 2007; 28 : 463–491

73. Blaustein MP, Lederer WJ: Sodium/calcium exchange: its physiological implications.*Physiol Rev* 1999, 79:763–854.

74. Vila-Petroff MG, Younes A, Egan J, Lakatta EG, Sollott SJ: Activation of distinct cAMP-dependent and cGMP-dependent pathways by nitric oxide in cardiac myocytes. *Circ Res* 1999, 14(84):1020–1031.

75. Dong Z, Chai W, Wang W, Zhao L, Fu Z, Cao W, Liu Z: Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitmen. *Am J Physiol Endocrinol Metab* 2013, 15(304):E222–228.

76. Wilkinson WJ, Kemp PJ: Carbon monoxide: emerging regulator of ion channels. *J Phisiol* 2011, 589:3055–3062.

77. Kimura H: Hydrogen sulfide: its production and functions.*Exp Physiol* 2011, 96(9)**:**833–835.

78. Coletta C, Papapetropouls A, Erdelyi K, Olah G, Módis K, Panopoulos P, Asimakopoulou A, Gerő D, Sharina I, Martin E, Szabo C: Hydrogen sulfide and nitric oxide mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci U S A* 2012, 5(109):9161–9166.

79. Bucci M, Papapetropoulus A, Vellecco V, Zhou Z, Zaid Z, Giannogonas P, Cantalupo A, Dhayade S, Karalis KP, Wang R, Feil R, Cirino G: CGMP-dependent protein kinase contributes to hydrogen sulfide-stimulated vasorelaxation.*PLoS One* 2012, 7**:**e53319.

80. Zembowicz A, Hatchett RJ, Jakubowski AM, Gryglewski RJ: Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta.*Br J Pharmacol* 1993, 110:151–158.
81. Gao YJ, Hirota S, Zhang DW, Janssen LJ, Lee RMK: Mechanisms of hydrogen-peroxide-induced biphasic response in rat mesenteric artery.*Br J Pharmacol* 2003, 138**:**1085–1092.
82. Snetkov VA, Smirnov SV, Kua J, Aaronson PI, Ward JPT, Knock GA: Superoxide differentially controls pulmonary and systemic vascular tone through multiple signalling pathways.*Cardiovasc Res* 2011, 89:214–224.
83. Cooke CL, Davidge ST: Endothelial-dependent vasodilation is reduced in mesenteric arteries from superoxide dismutase knockout mice.*Cardiovasc Res* 2003, 60:635–662
84. Ha SJ, Kim W, Woo JS, Kim JB, Kim SJ, Kim WS, Kim MK, Cheng XW, Kim KS: Preventive effects of exenatide on endothelial dysfunction induced by ischemia-reperfusion injury via KATP channels. *Arterioscler Thromb Vasc Biol* 2012, 32:474–480.
85. Ravel D, Levens N, Félétou M, Néliat G, Auclair J, Bouskela E: Differential effects of sulphonylureas on the vasodilatory response evoked by K(ATP) channel openers. *Fundam Clin Pharmacol* 2003; 17:61-69.
86. Zhao J, Majewski H: Endothelial nitric oxide attenuates Na^{+}/Ca^{2+}exchanger-mediated vasoconstriction in rat aorta.*Br J Pharmacol* 2008, 154:982–990.
87. Roman MJ, Devereux RB, Kizer JR, Okin PM, Lee ET, Wang W, Umans JG, Calhoun D, Howard BV: High central pulse pressure is independently associated with adverse cardiovascular outcome: the strong heart study.*J Am Coll Cardiol* 2009, 54:1730–1734.
88. Robinson LE, Holt TA, Rees K, Randeva HS, O'Hare JP: Effects of exenatide and liraglutide on heart rate, blood pressure and body weight: systematic review and meta-analysis.*BMJ Open* 2013,24:3.
89. Meloni AR, DeYoung MB, Han J, Best JH, Grimm M: Treatment of patients with type 2 diabetes with exenatide once weekly versus oral glucose-lowering medications or insulin glargine: achievement of glycemic and cardiovascular goals**.***Cardiovasc Diabetol* 2013, 23(12)**:**48.
90. Kelly AS, Bergenstal RM, Gonzalez-Campoy JM, Katz H, Bank AJ: Effects of exenatide vs. metformin on endothelial function in obese patients with pre-diabetes: a randomized trial.*Cardiovasc Diabetol* 2012, 11:64.
91. Peyot ML, Pepin E, Lamontagne J, Latour MG, Zarrouki B, Lussier R et al.

Beta-cell failure in diet-induced obese mice stratified according to body weight gain: secretory dysfunction and altered islet lipid metabolism without steatosis or reduced beta-cell mass. *Diabetes* 59:2178-87; 2010.

92. Zaman MQ, Leray V, Le Bloc'h J, Thorin C, Ouguerram K, Nguyen P.:Lipid profile and insulin sensitivity in rats fed with high-fat or high-fructose diets *Br J Nutr* 2011;106 Suppl 1:S206-210.
93. Ishii Y, Ohta T, Sasase T, Morinaga H, Hata T, Miyajima K, Katusda Y, Masuyama T, Shinohara M, Kakutani M, Matsushita M.: A high-fat diet inhibits the progression of diabetes mellitus in type 2 diabetic rats. *Nutr Res* 2010; 30:483-491.
94. Laron Z.: Insulin-a growth hormone. *Arch Physiol Biochem* 2008 114:11-16.
95. Lee AK, Yeung-Yam-Wah V, Tse FW, Tse A. Cholesterol elevation impairs glucose-stimulated Ca[2+] signaling in mouse pancreatic β-cells. *Endocrinology* 52:3351-61; 2011.
96. Nilsson M, Holst JJ, Björck IM. : Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose-equivalent drinks. *AmJ Clin Nutr* 2007 85:996-1004.
97. Wilson SH, Caplice NM, Simari RD et al.: Activated nuclear factor-kappaB is present in the coronary vasculature in experimental hypercholesterolemia. *Atherosclerosis* 2000; 148:23–30.
98. Brasnyó P, Molnár GA, Mohás M, Markó L, Laczy B, Cseh J, Mikolás E, Szijártó IA, Mérei A, Halmai R, Mészáros LG, Sümegi B, Wittmann I.: Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients. *Br J Nutr* 2011;106: 383-389.
99. Pieper GM, Langenstroer P , Siebeneich W . Diabetic-induced endothelial dysfunction in rat aorta: role of hydroxyl radicals. *Cardiovasc Res* 1997; 34: 145–156.
100. Szijártó IA, Molnár GA, Mikolás E, Fisi V, Laczy B, Gollasch M, Koller A, Wittmann I.: Increase in insulin-induced relaxation of consecutive arterial segments toward the periphery: Role of vascular oxidative state. *Free Radic Res* 2014; 48:749-757.

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