Experimental Diabetes Research

Glucagon-Like Peptide-1 and Diabetes

Guest Editors: Matteo Monami, Giovanni Di Pasquale, Edoardo Mannucci, and Carlo Maria Rotella

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Editorial

Glucagon-Like Peptide-1 and Diabetes

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This special issue was focused on the role and the effects of glucagon-like peptide-1 (GLP-1) in type 2 diabetes. This gastrointestinal hormone, which is mainly secreted after meals, enhances glucose-stimulated insulin release and inhibits food intake. The response of circulating active GLP-1 concentrations after a standard meal, as well as after an oral glucose load, has been reported to be reduced in type 2 diabetic patients in comparison with healthy subjects. Available data suggest that GLP-1 plays a relevant role in the regulation of postprandial glucose metabolism in physiologic conditions. Furthermore, the impairment of GLP-1 secretion after meals could contribute to the pathogenesis of hyperglycemia in type 2 diabetes. Several new drugs act through the GLP-1 signaling system to stimulate insulin release and regulate blood glucose levels in patients with diabetes.

This special issue includes 8 articles: three mechanistic studies and five reviews and meta-analyses, exploring the putative favourable effects of the incretins on beta-cell function and mass and on gastrointestinal motor function. The reviews and meta-analyses are focused on the promising beneficial extraglycaemic effects of the incretin-based therapy, including those on central nervous system (cognitive impairment) and cardiovascular risk. Last but not least, an interesting and intriguing review on gene therapy using expression vectors of GLP-1 and other incretin mimetics in the salivary gland for the treatment of type 2 diabetes mellitus (T2 DM) is presented.

Matteo Monami

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Research Article

Subthreshold α_2-Adrenergic Activation Counteracts Glucagon-Like Peptide-1 Potentiation of Glucose-Stimulated Insulin Secretion

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The pancreatic β cell harbors α_2-adrenergic and glucagon-like peptide-1 (GLP-1) receptors on its plasma membrane to sense the corresponding ligands adrenaline/noradrenaline and GLP-1 to govern glucose-stimulated insulin secretion. However, it is not known whether these two signaling systems interact to gain the adequate and timely control of insulin release in response to glucose. The present work shows that the α_2-adrenergic agonist clonidine concentration-dependently depresses glucose-stimulated insulin secretion from INS-1 cells. On the contrary, GLP-1 concentration-dependently potentiates insulin secretory response to glucose. Importantly, the present work reveals that subthreshold α_2-adrenergic activation with clonidine counteracts GLP-1 potentiation of glucose-induced insulin secretion. This counteractory process relies on pertussis toxin- (PTX-) sensitive Gi proteins since it no longer occurs following PTX-mediated inactivation of Gi proteins. The counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by subthreshold α_2-adrenergic activation is likely to serve as a molecular mechanism for the delicate regulation of insulin release.

1. Introduction

Glucose-stimulated insulin secretion plays an irreplaceable role in the control of glucose homeostasis since insulin is the only hormone capable of lowering blood glucose in the body [1–3]. This pancreatic endocrine hormone is packed in β cell secretory granules. These granules undergo exocytosis to release their insulin cargo into the bloodstream in response to elevated blood glucose levels [1–3]. Upon elevation of the plasma glucose level, the β cell efficiently takes up glucose through glucose transporters. Thereafter, subsequent glucose metabolism drastically raises the intracellular ATP level. The resultant rise in the ATP/ADP ratio closes ATP-sensitive K^+ (K_{ATP}) channels, causing depolarization of the plasma membrane. The membrane depolarization in turn opens voltage-gated Ca^{2+} (Ca_V) channels, mediating Ca^{2+} influx. The consequent increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers direct interactions between exocytotic proteins situated in the insulin-containing granule membrane and those localized in the plasma membrane. Eventually, the interaction between exocytotic proteins initiates the fusion of insulin-containing granules with the plasma membrane, that is, insulin exocytosis [1–3].

On top of the aforementioned consensus paradigm, glucose-stimulated insulin secretion is, in fact, regulated by complex neural mechanisms [4, 5]. It is well known that the autonomic nervous system innervates pancreatic islet cells where parasympathetic endings release a bunch of substances, for example, acetylcholine and vasoactive intestinal polypeptide, to potentiate glucose-stimulated insulin secretion [5, 6]. On the contrary, sympathetic terminals exocytose adrenergic and peptidergic transmitters to inhibit the insulin secretory process [4, 5]. Treatment with the main sympathetic transmitter noradrenaline fully shuts down insulin secretion from either islets or β cell aggregates perifused with high glucose [7, 8]. Mechanistically noradrenaline acts on α_2 receptors coupled to pertussis toxin- (PTX-) sensitive Gi proteins in β cells, reducing glucose-stimulated

insulin secretion through inhibition of intracellular cAMP formation, Ca_V channels, glucose metabolism, and the exocytotic machinery as well as elevation of K_{ATP} channel activity [4, 5].

Glucose-stimulated insulin secretion is subjected not only to the complex neural regulation, but also to various different types of hormonal regulation [9–16]. The islet β cell is able to sense its own released molecules, such as zinc and ATP, and hormones released from its neighboring cells to autocrinally and paracrinally regulate insulin secretion in response to glucose stimulation [13–18]. A number of systemic hormones impinge on islet β cells to coordinate insulin secretory response to glucose [9–12, 19]. A group of gastrointestinal hormones has long attracted a great deal of attention and categorized as incretins due to their stimulatory action on glucose-stimulated insulin secretion [9–12]. One of the most important incretin hormones is glucagon-like peptide-1 (GLP-1), which is secreted from intestinal L-cells into the bloodstream after a meal [9–12]. Upon encounter with β cells, this incretin binds to Gs protein-coupled receptors on these cells, resulting in activation of adenylyl cyclases, Ca_V channels, glucose metabolism, and the exocytotic machinery as well as inhibition of K_{ATP} channels [9–12, 20–22]. As consequence of these events, potentiation of glucose-stimulated insulin secretion occurs [9–12, 20–22].

Although either noradrenergic or GLP-1 signaling system in the regulation of glucose-stimulated insulin secretion has been clarified, it is not known whether these two signaling systems interact to gain adequate and timely insulin release in response to glucose stimulation [4, 5, 9–12, 20–22]. In the present work, we describe that subthreshold α_2-adrenergic activation counteracts glucagon-like peptide-1 potentiation of glucose-stimulated insulin secretion in a PTX-sensitive Gi protein-dependent manner.

2. Materials and Methods

2.1. Cell Culture. INS-1 cells were cultivated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with the following additives: 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/100 μg/ml penicillin/streptomycin, 10 mM N-[2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (Invitrogen). Briefly, the cells at about 70% confluency were trypsinized. The resultant cell suspension was seeded into 24-well cell culture plates. The cells were maintained at 37°C in a humidified 5% CO_2 incubator. They were grown to approximately 70% confluence and then subjected to analysis of insulin secretion.

2.2. Static Insulin Secretion. Approximately 70% confluent INS-1 cells in 24-well plates were used for insulin secretion experiments. The cells were kept at 37°C in a humidified 5% CO_2 incubator during the course of an experiment except when their bath solutions need to be changed. The experiments were carried out in Krebs-Ringer bicarbonate HEPES buffer (KRBH) consisting of (in mM) 140NaCl, 3.6KCl, 1.5$CaCl_2$, 0.5$MgSO_4$, 0.5NaH_2PO_4, 2$NaHCO_3$, 10HEPES, 0.1% bovine serum albumin (BSA), pH 7.4. First, the cells were rinsed with glucose-free KRBH and then maintained in the same buffer for 2 h. Thereafter, they were rinsed and preincubated with glucose-free KRBH for 30 min. To characterize the concentration-response relationships of the α_2-adrenergic agonist clonidine and the incretin GLP-1 as well as interactions between noradrenergic and GLP-1 signaling systems, the cells were rinsed and incubated with 3 or 11 mM glucose KRBH containing different concentrations of clonidine and/or GLP-1 for 30 min. Clonidine and/or GLP-1 were applied simultaneously with glucose. To determine a possible dependence of α_2-adrenoceptor regulation of GLP-1 receptors on PTX-sensitive Gi proteins, the cells were pretreated with 100 ng/ml PTX in RPMI 1640 medium for 18 h. Subsequently, the toxin medium was removed. The cells were rinsed and incubated with glucose-free KRBH as described above and subjected to incubations with 3 or 11 mM glucose KRBH containing different concentrations of clonidine and/or GLP-1 for 30 min. Finally, the treatments with the different reagents were stopped by putting the culture plates on ice. Supernatants were carefully aspirated from each well to prepare samples for insulin quantification. The samples were centrifuged at $1000 \times$ g for 3 min to remove detached cells and stored at -20°C until insulin immunoassay was performed.

2.3. Radioimmunoassay. A standard insulin immunoassay was used to evaluate static insulin secretion from INS-1 cells subjected to different treatments [23, 24]. Briefly, duplicate samples were measured. The calibration curve was constructed from insulin standard at 5, 10, 20, 40, 80, and 160 mIU/L. Radioactivity was counted by a γ-counter.

2.4. Statistical Analysis. All data are presented as mean ± SEM. Statistical significance was evaluated by one-way ANOVA, followed by least significant difference (LSD) test. The significance level was determined at both the 0.05 and 0.01 levels.

3. Results

3.1. Clonidine Concentration-Dependently Inhibits Glucose-Stimulated Insulin Secretion. To determine the concentration response relationship of clonidine inhibition of glucose-stimulated insulin secretion, we examined the effect of 30 min incubation with clonidine at concentrations ranging from 0.003 to 10 μM on insulin release from INS-1 cells challenged with 11 mM glucose. As shown in Figure 1, incubation with 11 mM glucose for 30 min resulted in a significant insulin secretion as compared with that with 3 mM glucose ($n = 6$, $P < .01$). This confirms that the cells used in this set of experiments reliably responded to such stimulation to secrete an appreciable amount of insulin. We therefore adopted this sufficient and reliable stimulation to test for the effect of clonidine on glucose-stimulated insulin secretion. Figure 1 shows that in the concentration range of 0.003–10 μM, clonidine concentration-dependently inhibited insulin release from INS-1 cells exposed to 11 mM

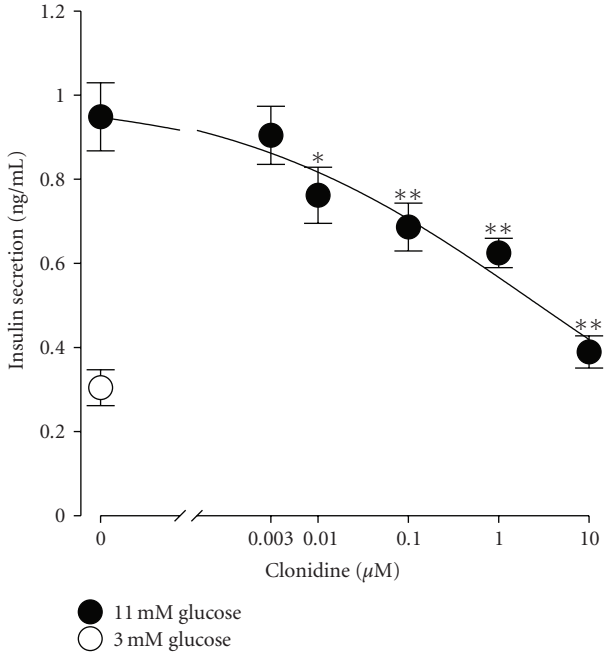

Figure 1: The α_2-adrenergic agonist clonidine concentration-dependently depresses glucose-stimulated insulin secretion from INS-1 cells. Static insulin secretion was performed with cells subjected to stepwise elevation of glucose concentration from 3 to 11 mM for 30 min in the absence or presence of clonidine and determined by a standard insulin radioimmunoassay. Cells exposed to 11 mM glucose (closed circle at the far left) released significantly more insulin than those to 3 mM glucose (open circle) ($n = 6$, $P < .01$). In the concentration range of 0.003–10 μM, clonidine produced a concentration-dependent inhibition of insulin release induced by 11 mM glucose. The inhibition became statistically significant at 0.01 μM clonidine ($n = 6, P < .05$) and was statistically significant at higher clonidine concentrations ($n = 6, P < .01$). The subthreshold and ED_{50} concentration of clonidine were calculated to be 0.003 and 4 μM, respectively. In this and all other figures, data are presented as means ± SEM. Statistical significance was evaluated by one-way ANOVA, followed by least significant difference (LSD) test. $^*P < .05$ and $^{**}P < .01$ versus 11 mM glucose-treated group.

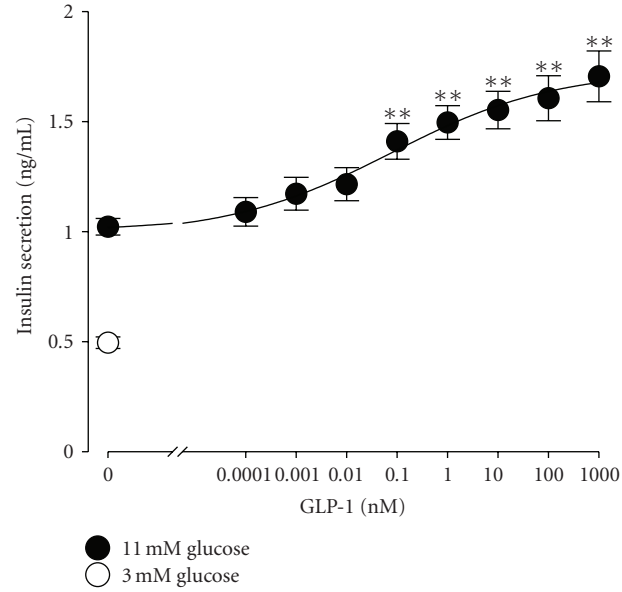

Figure 2: The incretin GLP-1 concentration-dependently potentiates glucose-stimulated insulin secretion from INS-1 cells. A stepwise increase in glucose concentration from 3 to 11 mM for 30 min was used to induce static insulin secretion from cells exposed to different concentrations of GLP-1. Released insulin was measured by a standard insulin radioimmunoassay. 11 mM glucose incubation (closed circle at the far left) caused a significant insulin secretion from cells preincubated with 3 mM glucose ($n = 6$, $P < .01$ versus 3 mM glucose-treated group represented by an open circle). GLP-1 at concentrations ranging from 0.0001 to 1000 nM potentiated glucose-stimulated insulin secretion in a concentration-dependent manner. GLP-1 at concentrations of 0.1 nM and higher significantly enhanced insulin release from cells stimulated with 11 mM glucose ($n = 6$, $P < .01$ versus 11 mM glucose-treated group). The subthreshold and ED_{50} concentration of GLP-1 were estimated to be 0.01 and 0.1 nM, respectively. $^{**}P < .01$ versus 11 mM glucose-treated group.

glucose. The effect became statistically significant when clonidine concentration reached 0.01 μM and higher ($n = 6$, $P < .05$ at 0.01 μM, $P < .01$ at 0.1, 1 and 10 μM). The subthreshold and ED_{50} concentration of clonidine were estimated to be 0.003 and 4 μM, respectively.

3.2. Glucagon-Like Peptide-1 Concentration-Dependently Stimulates Glucose-Stimulated Insulin Secretion. To reveal the concentration-response relationship of GLP-1 potentiation of glucose-stimulated insulin secretion, we evaluated the insulin secretory response of INS-1 cells stimulated with 11 mM glucose for 30 min in the presence of GLP-1 in the concentration range 0.0001 to 1000 nM. Figure 2 shows that 11 mM glucose treatment for 30 min produced a significant increase in insulin secretion in comparison with 3 mM glucose treatment ($n = 6$, $P < .01$). This validates that the glucose responsiveness of the cells employed in this set of experiments. As illustrated in Figure 2, GLP-1 in the concentration range 0.0001 to 1000 nM significantly potentiated insulin release induced by 11 mM glucose in a concentration-dependent manner. The statistically significant potentiation occurred when GLP-1 concentration was raised to 0.1 nM and higher ($n = 6$, $P < .01$). A concentration of 0.01 nM was considered as the subthreshold concentration of GLP-1 on its potentiation of glucose-stimulated insulin secretion. The ED_{50} concentration of GLP-1 for potentiating the insulin secretory response to glucose was calculated to be 0.1 nM.

3.3. Subthreshold Clonidine Suppresses the Stimulatory Effect of Glucagon-Like Peptide-1 on Glucose-Stimulated Insulin Secretion. The pancreatic β cell is equipped with both the α_2-adrenergic receptor and the GLP-1 receptor which are impinged by the sympathetic transmitter adrenaline/noradrenaline and the incretin hormone GLP-1, respectively [4, 5, 9–12, 20–22]. Both of these systems critically regulate glucose-stimulated insulin secretion [4, 5, 9–12, 20–22]. This inevitably raises the question whether they are insulated from each other or one cross-talks with

the other in pancreatic β cells. To tackle this issue, we examined how subthreshold α_2-adrenergic activation affects GLP-1 potentiation of glucose-stimulated insulin secretion.

Validation of the capacity of the cells applied in this set of experiments to release insulin in response to glucose was likewise performed. As illustrated in Figure 3, treatment with 11 mM glucose for 30 min gave rise to a significant insulin release as compared with that with 3 mM glucose ($n = 10$, $P < .01$). As expected, cells exposed to clonidine at the subthreshold concentration 3 nM did not alter their insulin secretory response to 11 mM glucose ($n = 10$, $P > .05$ versus group subjected to only 11 mM glucose stimulation) (Figure 3). In contrast, cells treated with GLP-1 at the ED_{50} concentration 0.1 nM following 11 mM glucose stimulation released significantly more insulin than cells subjected to only 11 mM glucose stimulation ($n = 10$, $P < .05$). Importantly, cells incubated with the ED_{50} concentration of GLP-1 plus the subthreshold concentration of clonidine secreted significantly less insulin than cells treated with the ED_{50} concentration of GLP-1 alone following 11 mM glucose stimulation ($n = 10$, $P < .01$) (Figure 3). The insulin secretory response to 11 mM glucose was very similar among group treated with the ED_{50} concentration of GLP-1 plus the subthreshold concentration of clonidine, group treated with the subthreshold concentration of clonidine alone, and untreated group ($n = 10$, $P > .05$) (Figure 3). The data demonstrate that the subthreshold concentration of clonidine completely counteracted the potentiation of glucose-stimulated insulin secretion by the ED_{50} concentration of GLP-1.

3.4. Counteraction of Glucagon-Like Peptide-1 Potentiation of Glucose-Stimulated Insulin Secretion by Clonidine Relies on Pertussis Toxin-Sensitive Gi Proteins. Multiple intracellular signaling events, such as decreases in cAMP production, Ca_V channel activity, glucose metabolism, and exocytotic capacity as well as an increase in K_{ATP} conductance occur upon activation of α_2-adrenergic receptors on the β cell to depress glucose-stimulated insulin secretion [4, 5]. All these events are dependent on the PTX-sensitive Gi protein that is an immediate mediator for α_2-adrenergic activation [4, 5]. This made us wonder if counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by clonidine relies on PTX-sensitive Gi proteins. To circumvent this issue, we evaluated if PTX-mediated inactivation of Gi proteins could prevent counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by subthreshold α_2-adrenergic activation.

The cells used in this set of experiments were proved to be quite sensitive to glucose with regard to their insulin secretory responsiveness. Figure 4 shows that both control and PTX-pretreated cells released a significant amount of insulin when glucose concentration was raised from 3 to 11 mM ($n = 11$, $P < .01$). Cells pretreated with 100 ng/ml PTX in for 18 h secreted significantly more insulin than cells without PTX pretreatment following 11 mM glucose stimulation ($n = 11$, $P < .01$) (Figure 4). Clonidine at both the subthreshold concentration 3 nM and the ED_{50} concentration 4 μM had no effect on insulin secretory response

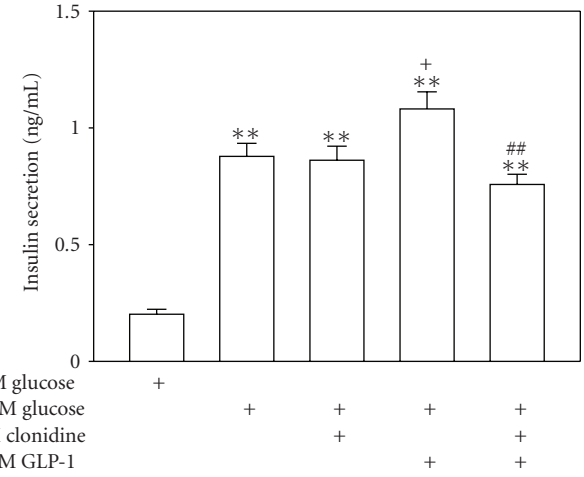

Figure 3: Subthreshold α_2-adrenergic activation with clonidine counteracts GLP-1 potentiation of glucose-stimulated insulin secretion. A standard insulin radioimmunoassay was employed to examine the insulin secretory response of cells treated with the subthreshold concentration of clonidine, the ED_{50} concentration of GLP-1 and their combinations following a stepwise stimulation with glucose from 3 to 11 mM for 30 min. Treatment with 11 mM glucose caused a significant insulin secretion as compared with that with 3 mM glucose ($n = 10$, $P < .01$). Cells incubated with clonidine at the subthreshold concentration 3 nM and control cells displayed similar insulin secretory responses to 11 mM glucose ($n = 10$, $P > .05$). However, cells treated with GLP-1 at the ED_{50} concentration 0.1 nM displayed significantly enhanced insulin secretion in comparison with untreated cells following 11 mM glucose stimulation ($n = 10, P < .05$). Furthermore, cells exposed to the ED_{50} concentration of GLP-1 plus the subthreshold concentration of clonidine exhibited significantly less insulin secretion than cells treated with the ED_{50} concentration of GLP-1 alone following 11 mM glucose stimulation ($n = 10$, $P < .01$). Control cells and cells treated either with the ED_{50} concentration of GLP-1 plus the subthreshold concentration of clonidine or the subthreshold concentration of clonidine alone released similar amounts of insulin in response to 11 mM glucose ($n = 10$, $P > .05$). $^{**}P < .01$ versus 3 mM glucose-treated group, $^{+}P < .05$ versus 11 mM glucose-treated group. $^{\#\#}P < .01$ versus the ED50 concentration of GLP-1 plus the subthreshold concentration of clonidine group subjected to 11 mM glucose incubation.

to 11 mM glucose in PTX-pretreated cells ($n = 11$, $P > .05$ versus PTX-pretreated group subjected to only 11 mM glucose stimulation) (Figure 4). However, GLP-1 at the ED_{50} concentration 0.1 nM significantly enhanced insulin secretion from PTX-pretreated cells following 11 mM glucose stimulation ($n = 11$, $P < .01$ versus PTX-pretreated group subjected to only 11 mM glucose stimulation) (Figure 4). Most importantly, the subthreshold concentration of clonidine was unable to counteract potentiation of glucose-stimulated insulin secretion by the ED_{50} concentration of GLP-1 in PTX-pretreated cells ($n = 11$, $P > .05$ versus PTX-pretreated group treated with 11 mM glucose plus 0.1 nM GLP-1) (Figure 4). The data reveal that counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by clonidine relies on PTX-sensitive Gi proteins.

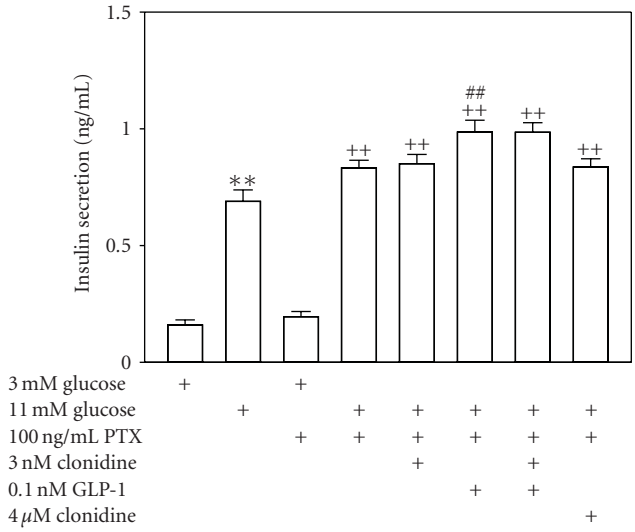

Figure 4: Uncoupling of Gi proteins with PTX prevents counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by clonidine. Cells pretreated with 100 ng/ml PTX for 18 h and control cells were subjected to analysis of static insulin secretion induced by 11 mM glucose for 30 min in the absence or presence of the subthreshold concentration and ED_{50} concentration of clonidine, the ED_{50} concentration of GLP-1, and their combinations. Insulin secretion was evaluated by a standard insulin radioimmunoassay. Both control and PTX-pretreated cells well responded to 30 min stimulation with 11 mM glucose ($n = 11$, $P < .01$ versus corresponding 3 mM glucose-treated groups). PTX-pretreated cells released significantly more insulin than control cells following 11 mM glucose stimulation ($n = 11$, $P < .01$). Neither the subthreshold concentration (3 nM) nor the ED_{50} concentration (4 μM) of clonidine altered glucose-stimulated insulin secretion in PTX-pretreated cells ($n = 11$, $P > .05$ versus PTX-pretreated group subjected to only 11 mM glucose stimulation). In contrast, GLP-1 at the ED_{50} concentration of 0.1 nM induced a significant potentiation of glucose-induced insulin release from PTX-pretreated cells ($n = 11$, $P < .01$ versus PTX-pretreated group subjected to only 11 mM glucose stimulation). Intriguingly, glucose-stimulated insulin secretion from PTX-pretreated cells incubated with the ED_{50} concentration of GLP-1 alone did not significantly differ from that from those subjected to incubation with the ED_{50} concentration of GLP-1 plus the subthreshold concentration of clonidine ($n = 11$, $P > .05$). Clonidine at the subthreshold concentration could no longer influence potentiation of glucose-stimulated insulin secretion by the ED_{50} concentration of GLP-1 in PTX-pretreated cells. $^{**}P < .01$ versus 3 mM glucose-treated group without PTX pretreatment, $^{++}P < .01$ versus 11 mM glucose-treated group without PTX pretreatment or PTX-pretreated group subjected to 3 mM glucose incubation, $^{\#\#}P < .01$ versus PTX-pretreated group subjected to 11 mM glucose stimulation.

4. Discussion

Glucose homeostasis critically relies on the complex regulation of glucose-stimulated insulin secretion by autonomic impulses and humoral inputs to the pancreatic β cell [4, 5, 9–16]. α_2-Adrenergic and GLP-1 receptors on the pancreatic β cell transduce signals from their corresponding ligands adrenaline/noradrenaline and GLP-1 to control glucose-induced insulin release [4, 5, 9–12]. The present work confirms that the α_2-adrenergic agonist clonidine and the incretin GLP-1 concentration-dependently inhibits glucose-induced insulin release at concentration ranges similar to those employed in previous studies [8, 25, 26]. Furthermore, it also estimates the subthreshold and ED_{50} concentration of theses two agonists. These parameters are critical for examination of the counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by subthreshold α_2-adrenergic activation.

Most importantly, the present study shows for the first time that insulin-secreting INS-1 cells exposed to the ED_{50} concentration of GLP-1 together with the subthreshold concentration of clonidine release significantly less insulin than cells treated with the ED_{50} concentration of GLP-1 alone following glucose stimulation. Furthermore, it also uncovers that the antagonistic interaction of the α_2-adrenergic signaling system with the GLP-1 signaling system critically depends on PTX-sensitive Gi proteins. These findings provide evidence that α_2-adrenergic or GLP-1 signaling system does not operate independently, but instead the former effectively antagonizes the latter to enable the pancreatic β cell to appropriately execute its unique function glucose-stimulated insulin secretion. In fact, interactions between G protein-coupled receptor signaling pathways have been intensively investigated in other cell types and neurons in particular [27–33]. Such interactions rely on multilevel mechanisms [27–33]. They occur at the receptor level due to receptor heterodimerization, which is either G protein-dependent or -independent [27–30]. The heterodimerization is able to alter the ligand binding affinity and/or signal transduction efficacy of dimerized receptors [27–30, 32, 33]. Interactions between G protein-coupled receptor signaling pathways can also bypass the receptor level and come about downstream of receptors as a result of crosstalk between receptor signaling cascades [31]. In general, these well-characterized mechanisms are applicable to the counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by subthreshold α_2-adrenergic activation in the pancreatic β cell. The in-depth mechanisms whereby the α_2-adrenergic signaling system antagonizes the GLP-1 signaling system in the pancreatic β cell remain to be characterized.

There is no doubt that the healthy body requires the efficient amount of insulin to remove extra glucose from the blood stream into body cells most of the time. However, the healthy body needs less insulin to boost blood glucose levels in some circumstances, such as stress, exercise, low blood glucose, and other environmental challenges. The counteraction of GLP-1 potentiation of glucose-stimulated insulin secretion by subthreshold α_2-adrenergic activation definitely fits in with these circumstances where sympathetic activity is elevated [34]. It adds a new level of complexity to the classical paradigm for the regulation of glucose-evoked insulin release. Under certain pathological conditions, for example, diabetes, hypertension, obesity, and aging, sympathetic activity and/or expression of α_2-adrenergic receptors in the β cell significantly increase [35–39]. Increases in sympathetic activity and/or expression of α_2-adrenergic receptors in the β cell likely exaggerate the antagonistic

interaction of the α_2-adrenergic signaling system with the GLP-1 signaling system in the pancreatic β cell to provoke and aggravate diabetes [35–39].

5. Conclusions

α_2-Adrenergic receptors and GLP-1 receptors on insulin-secreting INS-1 cells transduce signals from their corresponding ligands clonidine and GLP-1 to govern glucose-induced insulin release. Importantly, the former also interacts with the latter to brake potentiation of glucose-induced insulin release by the latter. In fact, subthreshold α_2-adrenergic activation is enough to counteract GLP-1 potentiation of glucose-induced insulin secretion in a PTX-sensitive Gi protein-dependent fashion. Such a counteraction is able to serve as a molecular mechanism for the delicate control of insulin release in the healthy body. Most likely, this counteractory process is exaggerated to provoke and aggravate diabetes since obesity, aging, and diabetes are highly associated with elevated sympathetic activity [35–39].

Acknowledgments

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Erratum

Erratum to "Subthreshold α_2-Adrenergic Activation Counteracts Glucagon-Like Peptide-1 Potentiation of Glucose-Stimulated Insulin Secretion"

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There are three errors in the original paper. First, the last sentence of the legend for Figure 3 should have read "$^{\#\#}P < .01$ versus group subjected to treatment with the ED_{50} concentration of GLP-1 and stimulation with 11 mM glucose." Second, the word "inhibits" in the third line of the right column on page 5 should have read "regulate." Third, the typo "LD" in Reference 23 on page 6 should have read "L_D". We apologize to readers for these errors.

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Review Article

Activation of the GLP-1 Receptor Signalling Pathway: A Relevant Strategy to Repair a Deficient Beta-Cell Mass

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Recent preclinical studies in rodent models of diabetes suggest that exogenous GLP-1R agonists and DPP-4 inhibitors have the ability to increase islet mass and preserve beta-cell function, by immediate reactivation of beta-cell glucose competence, as well as enhanced beta-cell proliferation and neogenesis and promotion of beta-cell survival. These effects have tremendous implication in the treatment of T2D because they directly address one of the basic defects in T2D, that is, beta-cell failure. In human diabetes, however, evidence that the GLP-1-based drugs alter the course of beta-cell function remains to be found. Several questions surrounding the risks and benefits of GLP-1-based therapy for the diabetic beta-cell mass are discussed in this review and require further investigation.

1. The Rationale for Restoration of Beta-Cell Mass in Diabetic Patients

Both type 1 and type 2 diabetes are characterised by deficits in beta-cell mass (~99% deficit in long-standing type 1 diabetes, ~65% deficit in long-standing type 2 diabetes [1]). There is little doubt regarding the importance of increased autoimmune-mediated beta-cell death in type 1 diabetes, and recent studies in type 2 diabetes suggest that the frequency of beta-cell apoptosis is also significantly increased, although other factors cannot be excluded, such as the failure of beta-cell mass to expand adequately in response to rising secretory demands by adapting beta-cell replication and neogenesis. Loss of beta-cells in both types of diabetes implies that restoration of endogenous insulin secretion and normalisation of hyperglycemia in such patients might be accomplished through the supplementation of islet cells. Indeed, hyperglycemia in both types of diabetes is reversed by pancreas transplantation, and intraportal transplantation of isolated islets temporarily restores glucose control. Unfortunately, replacement of beta-cell mass by islet or pancreas transplantation is associated with both surgical morbidity and the adverse effects of chronic immunosuppression. Some of the risks and side effects, including ischemic and enzymatic damage caused by the islet isolation and purification protocol as well as the concerns of thrombosis and portal hypertension induced by transplanting islets into the liver portal vein, are intrinsic to the islet transplantation procedure itself [2]. Moreover, there is an insufficient supply of pancreases available for the increasing number of people with diabetes, thus preventing the widespread implementation of this intervention. There is, therefore, a need for alternative approaches for restoring functional beta-cell mass in patients with diabetes.

Conceivable approaches to achieve beta-cell supplementation consist of restoring an endogenous source and/or implanting an autologous- or nonautologous-derived source. At present, there are different strategies under investigation: (1) transplantation of beta cells generated in vitro from nonautologous embryonic stem cells, (2) transplantation of beta-cells generated in vitro from patient's own adult stem cells, and (3) stimulation of beta-cell regeneration in vivo from patient's own endogenous cell sources.

An alternative strategy for the restoration of beta-cell mass in patients with diabetes is to foster in vivo beta-cell regeneration from patient's endogenous cell sources. There is

now evidence that beta-cell mass is dynamic and capable of undergoing adaptive changes in response to different secretory demands. In humans, beta-cell mass increases by ~50% in obesity, and both insulin secretion and beta-cell mass have been shown to increase in pregnant women [3]. Likewise, beta-cell mass in rodents increases by ~2.5-fold towards the end of pregnancy and is rapidly decreased through increased apoptosis and reduced replication postpartum. In humans, the overall capacity for beta-cell replication is much lower than in rodents, and very few replicating beta cells (one cell in ~50 islets of ~100 beta-cells each per cross-section) can be found in adult human pancreas [1]. There is, however, a capacity for increased beta-cell replication in humans: beta-cell replication has been reported to be more than ten times higher in human pancreas adjacent to gastrin-producing tumours [4] and in the pancreas of an old patient with recent-onset type 1 diabetes [5]. Indeed, the emerging understanding of beta-cell growth in the adult, either from precursor cells found in the pancreatic ducts or/and from residual beta cells, holds the promise of developing new strategies for stimulating beta-cell regeneration. Such approach necessitates the delivery of appropriate growth factors to these cells to obtain a full beta-cell phenotype. GLP-1 could be one of the most promising candidates for doing so. The following sections review our current understanding of the therapeutic potential of the GLP-1 receptor (GLP-1R) agonists for the diabetic beta-cell population.

2. Activation of the GLP-1R Signalling Pathway and Beta-Cell Functions

GLP-1 replenishes beta-cell insulin stores via increased insulin mRNA stability, gene transcription, and biosynthesis. It stabilizes mRNA encoding preproinsulin, thereby stabilizing and upregulating its expression [6, 7]. GLP-1 increases *insulin* gene transcription and biosynthesis via activation of both PKA-dependent and -independent signalling pathways. PDX-1, the most extensively studied insulin transcription factor, is a key effector for the GLP-1R signaling pathway on *insulin* gene transcription and biosynthesis, as well as differentiation, proliferation, and survival of the beta cell. GLP-1 has been shown, both in vitro and in vivo, to be involved in regulation of PDX-1 by increasing its total protein levels, and its translocation to the nucleus, followed by its binding to the A-box element and the GG2 element of the rat and human insulin promoters and resultant increase in activity of the *insulin* gene promoter in beta cells [8–13]. The regulation of PDX-1 by GLP-1 mainly occurrs via cAMP/PKA-dependent signaling pathway [10]. Nevertheless, GLP-1 triggers expression and nuclear localization of PDX-1 involves the phosphorylation of FoxO1 via transactivation of the EGFR and PI-3K/PKB pathway, resulting in deactivation and nuclear exclusion of FoxO1 and consequent disinhibition of Foxa2-dependent pdx-1 gene promoter activity [14, 15]. In addition, FoxO1 and PDX-1 mutually exclude each other from the nucleus of the beta cell [14]. The GLP-1R signaling pathway also mediates *insulin* gene transcription via basic region-leucine zipper transcription factors that are related structurally to the transcription factor CREB, and these directly bind to CRE sites on the insulin gene promoter. This effect is independent of Gsα, cAMP/PKA, and PKC and may be mediated by the 90-kDa ribosomal S6 kinase and mitogen- and stress-activated protein kinase family of CREB kinases [16, 17].

GLP-1 is one of the most potent substances known to stimulate glucose-induced insulin secretion (GIIS), and its stimulatory activity is exerted via binding to its receptor on beta cells. This binding results in activation of adenylyl cyclase with consequent production of cAMP and subsequent activation of PKA and the Epac family. GLP-1-mediated activation of PKA results in phosphorylation of the SUR1 KATP channel subunit via an ADP-dependent mechanism, facilitating its closure [18]. This is followed by membrane depolarization and triggering of the insulin secretory pathway. Treatment with the PKA inhibitor 8-bromoadenosine-3′,5′-cyclic mono-phosphorothioate, Rp-isomer [19], or H89 [18] abolishes GLP-1-induced inhibition of the KATP channels. SUR1(−/−) islets lack an insulin secretory response but exhibit a normal rise in cAMP to GLP-1, implicating cAMP-dependent PKA-independent signal transduction pathway [20, 21]. It is now clear that the action of cAMP produced by GLP-1 signaling is mediated not just by PKA, but also by Epac2 [22, 23], and Epac2 also inhibits the function of KATP channels in rodent and human beta-cells via interaction with SUR1 [24, 25]. A recent study has also demonstrated that the scaffold protein, β-arrestin-1, facilitates GLP-1-stimulated cAMP production via interaction with GLP-1R [26]. GLP-1 signaling also antagonizes voltage-dependent K^+ (Kv) channels via cAMP/PKA-dependent pathway in beta cells, which prevents beta-cell repolarization by reducing Kv currents [27]. However, MacDonald et al., [27] identified a role of PI-3K with subsequent activation of PKCζ in the antagonism of the Kv current by GLP-1. This occurred via epidermal growth factor receptor (EGFR) transactivation, not via the G protein-regulated isoform p110γ [27]. L-type Ca^{2+} channels are also phosphorylated by PKA, leading to increase of their open probability and enhancement of Ca^{2+} influx [28–30]. Activation of GLP-1R also increases intracellular Ca^{2+} through Ca^{2+}-release from the endoplasmic reticulum via the inositol 1,4,5 triphosphate receptors activated by PKA and the ryanodine receptors activated by Epac2 [31, 32]. A recent study has indicated that GLP-1 elevates intracellular Ca^{2+} concentration via stimulation of the nicotinic acid adenine dinucleotide phosphate and cyclic ADP-ribose production [33], catalyzed by cyclic ADP-ribose cyclases, that stimulates glucose-induced Ca^{2+} mobilization [34]. Direct effects of GLP-1 signaling on insulin-containing vesicles have also been described. Lester et al. [35] proposed a mechanism by which changes in insulin secretion are associated with phosphorylation of the vesicle-associated protein synapsin-1 by PKA followed by dephosphorylation by calcineurin [35]. PKA may also regulate the vesicle priming through the phosphorylation of RIM proteins [36]. It is known that RIM proteins bind Rab3a, which serves to tether the vesicle to the plasma membrane [37] and also bind Munc13-1 to create a link between synaptic vesicle tethering and priming [38]. RIM proteins also bind Epac2, and this binding participates

in the regulation of docking and fusion of insulin-containing vesicles to the plasma membrane [39, 40]. In addition, Epac2 interacts with Picollo, a RIM2-interacting protein on insulin-containing vesicles, in a Ca^{2+}-dependent manner [41].

Intracellular cAMP levels have long been recognized to be critical for normal GIIS (glucose-competence) [42]. Thus, receptors linked to cAMP production, such as the GLP-1R play an important role to control cellular cAMP levels. It has been very recently proposed that the biological process regulated by GLP-1 to control the beta-cell glucose competence was dependent on the level of IGF-1R expression and on IGF-2 secretion [43]. Finally, since GLP-1 also stimulates the expression of GLUT2 transporters and glucokinase, which determine the rate of glycolysis, it helps to confer glucose competence to beta cells and thereby increase the efficacy (maximal effect) and potency (threshold concentration) of glucose as a stimulus for insulin secretion [44]. However, this interpretation is questionable in the light of recent extensive data indicating that GLP-1 barely affects beta-cell intermediary metabolism and that metabolic signalling does not significantly contribute to GLP-1 potentiation of GIIS [45].

3. Pharmacological Activation of the GLP-1R Signalling Pathway in Glucose-Insensitive Diabetic Beta Cells

Enhancement of GIIS from the beta cell is one of principal goals for treatment of patients with T2D. Because the mechanism underlying the insulinotropic action of GLP-1 in an in vitro model of glucose-unresponsive beta cells (dispersed rat beta cells) has been shown to involve activation of adenylate cyclase and cAMP production [42], we investigated the effect of GLP-1 stimulation on cAMP production and GIIS in GK/Par rats with spontaneous T2D [46]. Diabetic GK/Par islets were able to amplify their cAMP content in response to GLP-1 in the presence of high glucose, and this was associated with a strong insulin release with restitution of their insulin secretory competence to glucose [47]. GLP-1-stimulated cAMP generation was instrumental in the GLP-1-triggered insulin release at high glucose since insulin release became no longer reactive to GLP-1 when AC isoforms were acutely blocked by the AC blocker dd-Ado. Since we also demonstrated that GK/Par beta cells suffer from some degree of cAMP-resistance, one may conclude that GLP-1 at pharmacological dosage, is able to generate within the GK/Par beta cell, cAMP levels high enough to cope for the reduced effectiveness of cAMP [47]. Furthermore, we report that GLP-1 also normalizes GIIS in islets from n-STZ rats (another recognized model of rat diabetes with glucose-unresponsive beta cells) [48]. This suggests that restoration by GLP-1 of glucose responsiveness in the diabetic beta cell is not restricted to the GK model, but is probably a more generalized mechanism.

4. Activation of the GLP-1R Signalling Pathway and Beta-Cell Growth/Survival

Repair or expansion of the beta-cell population can be achieved through stimulation of beta-cell proliferation and/or neogenesis, and slowing the rate of beta-cell apoptosis. Abundant in vitro and in vivo studies have shown that GLP-1, and its analogs such as Exendin-4 (Ex-4) are capable of inducing beta-cell proliferation in normal rodent islets and insulinoma cell lines [49–52]. This proliferative seems to involve the activation of immediate early genes such as c-jun, junD, nur77, and c-fos [53], and the implication of different intermediary signaling molecules such as PI3K, PKB/Akt, and PKCzeta [53, 54]. Studies in INS-1 cells have also indicated that betacellulin- (BTC-) mediated transactivation of the epidermal growth factor receptor/erb-B1 is a prerequisite for GLP-1-induced proliferative effects in these cells [49]. However, BTC failed to induce proliferation in a different insulinoma cell line, RINm5F and also in fetal human beta cells [55]. The importance of the Pdx1 transcription factor in mediating the proliferative effects of GLP-1 in beta cells was demonstrated using mice with beta cell-specific inactivation of the Pdx1 gene. Ex-4-mediated proliferation was blocked in isolated islets from these mice, suggesting that Pdx1expression is essential for Ex-4-induced proliferative effects [51]. Interestingly, Ex-4 induces Pdx1 expression in human fetal islet cell cultures and promotes functional maturation and proliferation of human islet cell cultures transplanted under the rat kidney capsule [56]. It has become clear that GLP-1 acts by means of Gsα and PI-3K/PKB to stimulate beta cell proliferation and survival. A beta-cell-specific Gsα deficiency in mice results in diabetes characterized by reduced insulin secretion and beta-cell mass with the primary defect being in decreased beta-cell proliferative capacity [57]. It has been also shown that GLP-1 inhibits FoxO1 transcriptional activity through phosphorylation-dependent nuclear exclusion in beta cells [15], and the ability of Ex-4 to increase beta-cell mass was blunted in transgenic mice expressing constitutively nuclear FoxO1 in beta cells [15]. FoxO1 inactivation plays an important role in the effect of GLP-1 on the expression of the two important transcription factors PDX-1 and Foxa2 [16]. GLP-1 activation of PI-3K/PKB facilitates acute nuclear translocation of existing PDX-1. Indeed, mice with a beta-cell-specific inactivation of PDX-1 do not display a proliferative response to Ex-4 treatment [51]. GLP-1 activation of PI-3K is mediated by transactivation of EGFRs via GLP-1R-mediated activation of c-Src that in turn activates a membrane-bound metalloproteinase, with concomitant release of the soluble ligand BTC which is an agonist of EGFRs [49]. This is also followed by activation and translocation to the nucleus of PKCζ, resulting in enhancement of the stimulatory effect of GLP-1 on beta-cell proliferation [53]. GLP-1 also exerts its stimulatory effects on beta-cell proliferation through CREB-mediated Irs2 gene expression, leading to activation of PI-3K/PKB [58]. GLP-1R activation has been shown to upregulate the expression of cyclin D1 [59, 60], and this effect is likely to be mediated by PKA-dependent activation of CREB [60, 61]. A recent study showed that GLP-1R signaling via cAMP/PKA activates β-catenin/T-cell factor-like 2- (TCF7L2-) dependent Wnt signaling in the proliferation through upregulation of cyclin D1 [62]. A prominent role for β-cateninTCF7L2-dependent Wnt signaling is now acknowledged after the reports that GSK-3β overexpression in mice

induces beta cell mass restriction and the development of diabetes [63], that genetic disruption of GSK-3β in beta-cells results in increased beta-cell mass and that beta-cell regeneration can be promoted by systemic administration of GSK-3β inhibitors to streptozotocin-induced neonatal diabetic rats [64]. Unexpectedly, the proliferative effect of GLP-1 was recently related to IGF-1R expression and autocrine secretion of IGF-2 by the beta-cell, since this effect was suppressed by Igf-1r gene inactivation and by IGF-2 immunoneutralization or knockdown [43].

GLP-1R activation reduces beta-cell apoptosis in purified rodent and human islets as well as beta-cell lines after exposure to many cytotoxic agents, including reactive oxygen species, glucose, free fatty acid, palmitate, cytokines, tumor necrosis factor-α (TNF-α), immunosuppressive reagents, and dexamethasone [66]. A role for endogenous GLP-1 in prevention of beta-cell death was demonstrated by the increased susceptibility to streptozotocin-induced apoptosis in GLP-1R knockout mice; conversely, streptozotocin-induced apoptosis was significantly reduced by coadministration of Ex-4 [67]. Ex-4 also reduces biochemical markers of islet ER stress in vivo and ER stress-associated beta-cell death in a PKA-dependent manner [68, 69]. Similar to GLP-1-induced beta-cell proliferation, antiapoptotic effects of GLP-1 in beta cells are mediated by promotion of FoxO1 nuclear exclusion and consequent upregulation of PDX-1 and Foxa2 expression via EGFR- and PI3K-dependent activation of PKB and cAMP/PKA-dependent activation of CREB, leading to upregulation of IRS2 protein expression and activation of PKB. Also similar to the induction of proliferation, activation of IGF-1 receptor expression and IGF-2 secretion participate to the GLP-1-induced the protection of the beta cells against cytokine-induced apoptosis, through [43]. The protective effect of GLP-1 on beta-cell glucolipotoxicity is also mediated by PKB activation and possibly its downstream target nuclear factor-κB [70]. In addition, recent studies suggest that GLP-1R agonists protect beta cells from proinflammatory cytokine-induced apoptosis by inhibiting the c-Jun NH2-terminal kinase pathway via upregulation of islet-brain 1, a potent blocker of the c-Jun NH2-terminal kinase pathway [71], and activation of the extracellular signal-regulated kinase 1/2-dependent pathway [72].

GLP-1R was confirmed to be present in pancreatic ducts in mouse, rat, and human [73, 74]. GLP-1R exists in the AR42J acinar cell line derived from a rat pancreatic tumor and treatment with GLP-1 or Ex-4 causes increases in both intracellular cAMP and Ca^{2+} levels [75]. Activation of GLP-1R signaling either in ductal or acinar cell lines or in vivo in rodents has resulted in differentiation of a fraction of these cells toward an islet-like phenotype, in association with activation of PKC and MAPK, and transcription factors necessary for an endocrine phenotype such as PDX-1, as well as the glucose-sensing factors glucokinase and GLUT2 [76]. GLP-1R activation of those cells also affects transforming growth factor-β signaling pathways, resulting in reduced Smad activity [77, 78]. AR42J cells, even without GLP-1R activation, have the potential to be converted into endocrine [79] but are negative for islet hormones and their transcripts under usual culture conditions [76]. When these cells were exposed to GLP-1 or Ex-4, approximately 20% of the cells contained insulin protein and were capable of releasing insulin in a glucose-mediated mode [76]. Such GLP-1 effect was also observed in Capan-1 cell line [80] and rat ARIP and human PANC-1 cell lines. Similar to the AR42J cells, GLUT2 and glucokinase transcripts were induced in these cell lines [81]. In particular, the differentiation-promoting activity of GLP-1 requires the expression of PDX-1, because PANC-1 cells, which lack endogenous PDX-1, differentiate only when transfected with PDX-1, whereas rat ARIP cells that express PDX-1 are susceptible to undergoing differentiation into insulin-secreting cells [81]. In the Capan-1 cell line, differentiation to insulin-producing cells was also seen when they were transfected with PDX-1, and PDX-1 antisense totally inhibited such conversion [80]. In human pancreatic ducts also where GLP-1 receptor is abundantly expressed, Ex-4 treatment in vitro increases the number of insulin-producing cells. This suggests that GLP-1/Ex-4 is useful to facilitate beta-cell neogenesis in adult pancreatic ducts [74].

5. Pharmacological Activation of the GLP-1R Signalling Pathway in Models of Deficient Beta-Cell Mass

Acute or chronic treatment of diabetic rodents with GLP-1R agonists stimulates beta-cell proliferation and neogenesis and slows the rate of beta-cell apoptosis, leading to an expansion of beta-cell mass. In vivo administration of GLP-1, Ex-4 or other degradation-resistant analogs has been shown to increase beta-cell mass in different prediabetic and diabetic rodent models [11, 65, 82–86]. Administration of GLP-1 or Ex-4 for several days resulted in stimulated expansion of beta-cell mass and increased beta-cell proliferation in old glucose-intolerant rats [84], adult db/db mice, Zucker rats, pancreatectomized rats and mice, or intrauterine growth-retarded rats [87]. A transient treatment of GLP-1 or Ex-4 in STZ-treated newborn rats resulted in a sustained improvement of beta-cell mass through increased beta-cell neogenesis and replication [83] (Figure 1). We obtained similar conclusion in the nSTZ model after in vivo administration of a DPPIV inhibitor (Figure 2). Moreover, diabetic Lepdb/Lepdb mice treated with Ex-4 for 2 weeks showed enhanced expression of PDX-1 in the ducts (favoring the presence of GLP-1R in ductal cells, as referenced above) and the exocrine tissue [11], which means that GLP-1R agonists aid in islet neogenesis, because ductal cells have been thought to be the main source for endocrine neogenesis [88].

Taking advantage of the GK/Par rat model of spontaneous T2D, we have raised the question of what is the impact of GLP-1 or Ex-4 treatment, in terms of beta-cell mass enlargement and long-term improvement of glucose homeostasis. To address this issue, we investigated the ability of GLP-1 or Ex-4 treatment to promote beta-cell proliferation in young GK/Par rats during the prediabetic stage and thereby to prevent the pathological progression of the T2D when animals become adults. GK/Par rats were submitted to GLP-1 or Ex-4 injection from postnatal day 2 to day 6 only [84]. Both treatments enhanced, on day 7, pancreatic

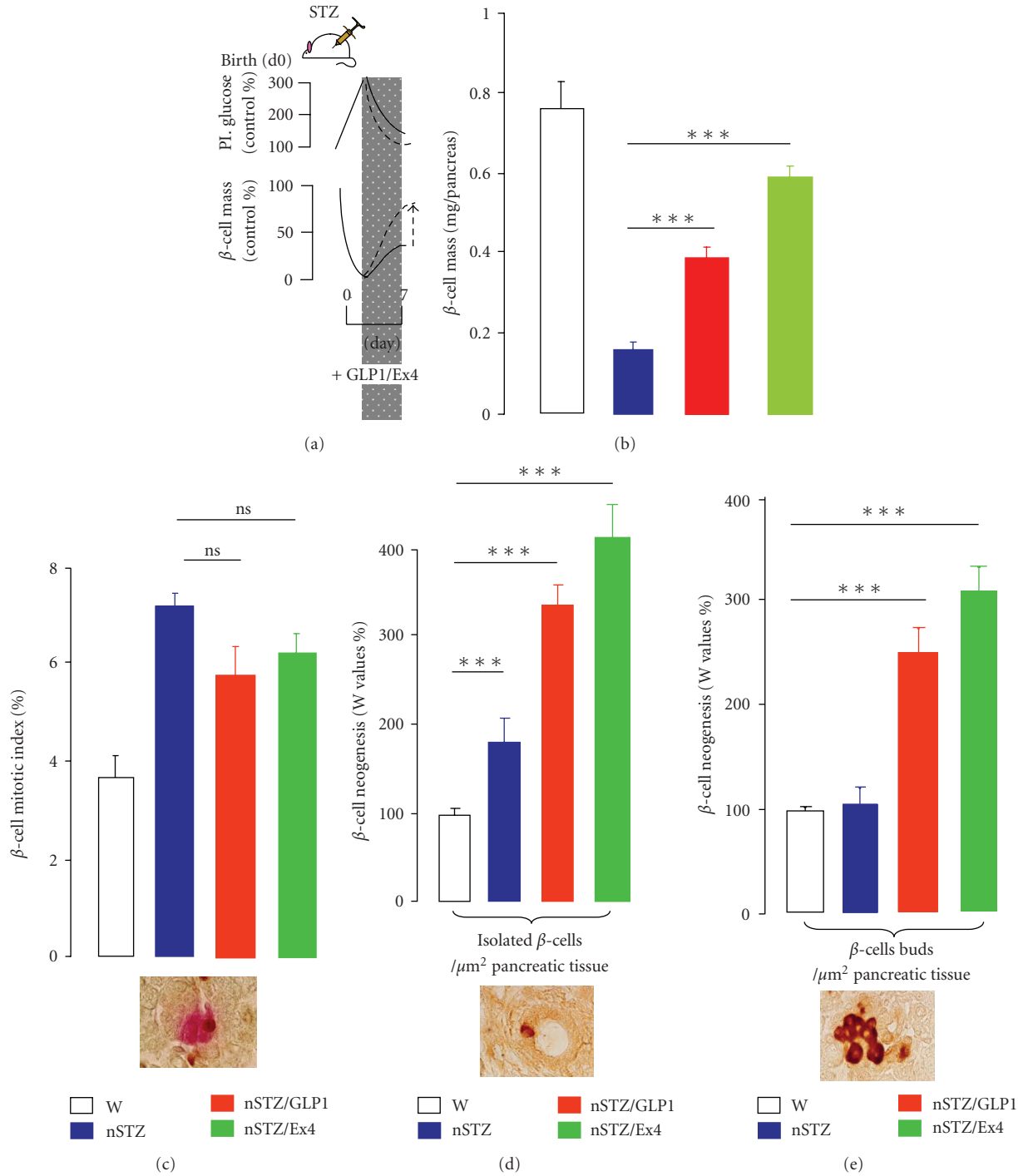

Figure 1: GLP-1 or Exendin-4 (Ex-4) activates beta-cells regeneration in vivo. The aim was to investigate in the rat model of neonatal beta-cell regeneration (nSTZ model), the capacity of in vivo treatments with GLP-1 or Ex-4 to promote beta-cell regeneration. To this end, nSTZ rats from the Wistar strain (W) were submitted to GLP-1 or Ex-4 administration from postnatal day 2 to day 6 only, and their beta-cell masses were tested on day 7 (a) and (b). In the nSTZ/GLP-1 and nSTZ/Ex-4 groups, total beta-cell masses per pancreas were both significantly increased (***$P < .001$) as compared with values in untreated nSTZ rats, representing, respectively, 51% and 71% of the control Wistar beta-cell mass, while nSTZ beta-cell mass represented only 21% of the control Wistar value. Beta-cell BrdU labeling index (c) in the untreated nSTZ rats was found to be significantly increased ($P < .001$) as compared with Wistar group. In the nSTZ/GLP-1 and nSTZ/Ex-4 groups, it was similarly increased. A representative figure is given with double immunostaining for BrdU and insulin in 7-day-old nSTZ rats (magnification ×1000). To estimate activation of neogenesis (d) and (e), the number of single beta-cells incorporated into the duct epithelium and the number of beta-cell clusters budding from ducts were quantified. The number of isolated beta-cell within pancreatic tissue of nSTZ rats represented 185% of Wistar value and the number of beta-cell buds in pancreatic tissue in nSTZ rats represented 106% of Wistar value. These two parameters were strongly increased in nSTZ/GLP-1 and nSTZ/Ex-4 rats as compared to untreated nSTZ rats (***$P <$ .001). A representative figure is given with indirect immunoperoxydase staining for insulin in 7 day-old nSTZ/GLP-1 rats (magnification ×1000). Adapted from Tourrel et al. [83].

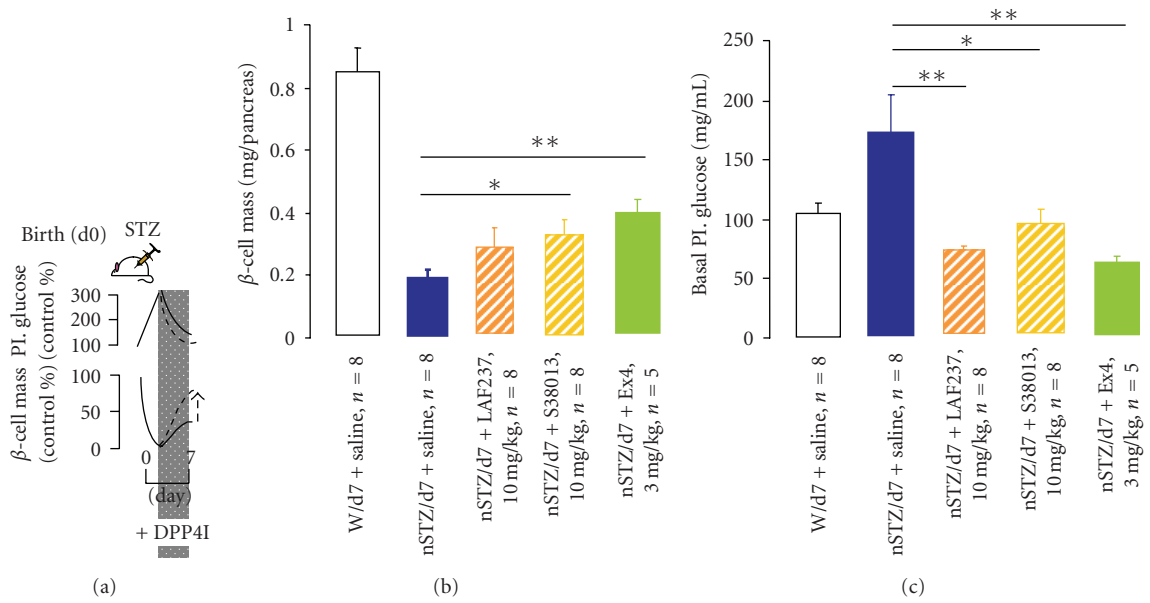

Figure 2: DPPIV inhibitors activate beta-cells regeneration in vivo. The aim was to investigate in the rat model of neonatal beta-cell regeneration (nSTZ model), the capacity of in vivo treatment by DPPIV inhibitors (LAF237 or S38013) to promote beta-cell regeneration. To this end, nSTZ rats were submitted to DPPIV administration from postnatal day 2 to day 6 only, and their beta-cell masses were tested on day 7. Ex-4 was taken as a beta-cell growth stimulator comparator. In the 7-day-old untreated nSTZ group, total beta-cell mass per pancreas was only 22% of the value in the untreated normal group ($P < .001$). In the nSTZ/LAF237 group, the total beta-cell mass increase (by 46%) did not reach statistical significance. In the nSTZ/S38013 group, the total beta-cell mass increase (by 68%) reached statistical significance ($^*P < .05$). Beta-cell mass in the nSTZ/Ex-4 group was twice increased ($^{**}P < .01$). In the 7-day-old untreated nSTZ group, basal plasma glucose value was significantly increased by 70% ($P < .05$) as compared to that of untreated normal group. By contrast, in the nSTZ/LAF237, nSTZ/S38013, and nSTZ/Ex-4 groups, basal plasma glucose levels were significantly decreased ($P < .05$ or $P < .01$) at the end of treatment as compared with those in the untreated nSTZ group and reached values no longer significantly different from those in untreated normal pups.

insulin contents and total beta-cell mass by stimulating both beta-cell neogenesis and beta-cell regeneration. Followup of biological characteristics from day 7 to adult age (2 months) showed that both treatment exerted long-term favorable influence on beta-cell mass and glycaemic control at adult age. As compared to untreated GK/Par rats, 2-month-old GLP-1 or Ex-4-treated GK rats exhibited improved glucose-stimulated insulin secretion, in vivo after intravenous glucose load or in vitro using isolated perfused pancreas. Moreover, plasma glucose disappearance rate was increased in both treated GK/Par groups compared to untreated GK/Par group [84]. These findings model indicate that a GLP-1 or Ex-4 treatment limited to the prediabetic period, delays the installation, and limits the severity of T2D in the GK/Par model.

GLP-1R activation also promotes preservation and expansion of beta-cell mass in type 2 diabetic rodent models through protecting beta cells against the deleterious effects of the diabetic milieu (i.e., increased cytokine toxicity, glucose toxicity, and lipotoxicity). Ex-4 treatment of Lepdb/Lepdb mice decreases activation of caspase-3 and prevents beta-cell apoptosis through PKB and MAPK [85] and infusion with GLP-1 drastically reduced the number of apoptotic beta cells in islets of Zucker diabetic rats [65].

Collectively, the studies above mentioned so far indicate that GLP-1R agonists may prove useful for expansion of human beta cells either cultured in vitro, after transplantation, or after sustained treatment of diabetic subjects in vivo.

6. Current Issues That Challenge the Beneficial Effects of GLP-1R Agonists for Beta-Cell Therapy

6.1. Risk of Pancreatic Tumor Formation in Patients Receiving GLP-1R Agonist Supplementation. If GLP-1R agonists were to be used continuously to treat diabetes, then uncontrolled beta-cell proliferation would become an issue unless there were brakes on the system. Klinger et al. [89] shed light on cellular mechanisms that may indeed limit the proliferative effect of GLP-1 in beta-cells: GLP-1 provides its own brakes because it leads to the rapid and strong expression of four negative regulators of intracellular signalling: RGS2 (regulator of G protein signalling 2), Dusp14 (dual-specificity phosphatase 14, also called MAP kinase phosphatase 6, a negative feedback regulator of the mitogen-activated protein kinase signaling cascade), Icer (inducible cAMP early repressor), and Crem-α (cAMP responsive element modulator alpha). However, an obvious question to be further studied is whether unrestrained beta-cell proliferation may result from loosening of the GLP-1 effect upon these negative regulators of beta-cell growth, during long-term treatment.

6.2. Risk of Pancreatitis in Patients Receiving GLP-1R Agonist Supplementation. Another concern with respect to clinical use of GLP-1 relates to reports of pancreatitis in some patients long-term treated with long-acting GLP-1 receptor agonists [90]. However, amylase levels and pancreatic markers of inflammation were found reduced in Ex-4-treated mice, and Ex-4 did not increase the severity of pancreatitis in a murine model of this condition [91]. Furthermore, no evidence was found for an increased incidence of pancreatitis in a large cohort of patients treated with either exenatide or sitagliptin, compared with those treated with metformin or glyburide [92]. Notwithstanding, a recent paper in the HIP diabetic rat model treated with sitagliptin has reported ductal cell hyperplasia in all sitagliptin-treated animals, acinar to ductal metaplasia in some, and haemorrhagic pancreatitis in one isolated case [93]. Histologic evidence of pancreatic acinar inflammation has also been found in rats treated with Ex-4 and their acinar cells were abnormal in appearance and had a greater frequency of cell death [94]. Pancreatic ductal replication is increased in humans with obesity and/or type 2 diabetes [95], providing a possible link between the increased risk of pancreatitis in individuals with obesity and/or T2D. In common with the HIP rat model of diabetes, pancreatic ductal replication was also increased in humans with obesity and T2D [95]. The mechanisms that induce increased pancreatic ductal replication in patients with obesity and/or type 2 diabetes are unknown. Excessive fat accumulation in pancreas could induce local inflammation [95]. Increased beta cell apoptosis in type 2 diabetes is also associated with inflammation and increased local cytokines [96]. Such combination might activate islet regeneration via duct-related progenitors, comparable to the process proposed for acinar tissue in chronic pancreatitis. GLP-1 therapy may potentially amplify ductal hyperplasia since it has been reported to activate pancreatic regenerative efforts with increased duct cells positive for PDX-1 [74]. Given the clinical gravity of pancreatitis, a better understanding of this issue is important.

6.3. Relevance of GLP-1R Agonist Supplementation for T1D. Although regenerative and antiapoptotic actions of GLP-1 or Ex-4 have been demonstrated in both normoglycemic and diabetic animal models, the majority of these studies were conducted in animal models of T2D [83–87]. In contrast, much less is known about whether the actions of GLP-1R agonists are maintained in the setting of an ongoing autoimmune attack, as is the case in the NOD mouse, the BB rat, and in human subjects with type 1 diabetes (T1D). Zhang et al. [97] have shown that continuous delivery of GLP-1 via an osmotic minipump in prediabetic NOD mice results in significant increases in beta-cell mass and replication rate, together with a significant reduction in the rate of beta-cell apoptosis. Hence, it seems possible that GLP-1R activation may be able to enhance beta-cell mass even in the presence of an autoimmune attack, if therapy is initiated before the onset of hyperglycemia. Ex-4 has also been administered to NOD mice alone or in combination with different immune modulators, lisofylline [98], antilymphocyte serum [99], or anti-CD3 immunotherapy [100]: the highest frequency of diabetes remission was observed in animals that received the combination treatments, suggesting a beneficial synergistic effect between immunomodulators and the Ex-4 regenerative agent. Furthermore, recent studies have reported that increasing the levels of circulating GLP-1 by inhibiting dipeptidyl peptidase-4 results in prolonged islet graft survival and decreased insulitis in diabetic NOD mice [101], that Ex-4 in vitro decreased IFN-γ-induced expression of several inflammatory mediators in human islets and MIN6 cells [102] and that Ex-4 in vivo induced a recovery of beta-cell proliferation during the initial stages of insulitis in the BB/Worcester rat [103]. The finding of an anti-inflammatory action of Ex-4 may have implications for the treatment of both types of diabetes, since the presence of immune cells in islets from human T2D diabetic patients and from animal models of T2D has been reported [96].

6.4. No Reliable Method to Assess Beta-Cell Mass in Patients Receiving GLP-1R Agonist Supplementation. Among the demonstrated biological actions of GLP-1R agonists, none has generated more interest than the findings of enhanced beta-cell growth and survival in rodent diabetic models [104]. The possibility that incretin therapy may not only improve beta-cell function but also increase beta-cell mass in patients with T2D has, therefore, created much excitement. Determination of the success of therapeutic strategies designed to enhance beta-cell regeneration requires reliable methods for the assessment of beta-cell mass. In animal models, beta-cell mass can be easily calculated as the product of pancreatic weight and the fractional beta-cell area in cross-sections from different regions of the pancreas. In human, there are no direct measures currently available to determine whether an antidiabetes drug has the ability to alter the course of T2D by increasing beta-cell mass (replication, neogenesis) or attenuating beta-cell apoptosis. Noninvasive imaging techniques that can assess islet mass are currently being explored, but have not yet reached the sensitivity that is required for use in humans, and pancreatic tissue for histological examination cannot be ethically procured for research purposes only. This leaves no direct means for testing direct effects of GLP-1-based drugs on beta-cell mass in diabetic patients.

6.5. Significance of the Islet-Derived GLP-1 Source and Its Modulation by GLP-1R Agonist Supplementation. Beside production and secretion of GLP-1 by the enteroendocrine L cells throughout the intestinal epithelium, there is now growing evidence that under certain conditions, islet alpha-cells are an extraintestinal site for GLP-1 production, perhaps to support the function and/or survival of neighboring beta-cells. While proglucagon is expressed in islet alpha-cells, PC2 is the predominant processing enzyme in these cells, cleaving proglucagon to yield glucagon rather than GLP-1. However, under certain conditions, alpha cells do express PC1/3 and liberate GLP-1 from proglucagon instead. Several models of pancreatic injury have been associated with islet GLP-1 production. Treatment of neonatal rats with STZ increases pancreatic GLP-1 content [105]. STZ treatment of adult rats increases PC1/3 expression in glucagon-immunoreactive cells in islets and increases GLP-1 levels in islets and

plasma [106]. It has been shown recently that treatment of isolated mouse islets with a PC1/3-expressing adenovirus induces GLP-1 release from alpha cells, increases GIIS, and promotes islet survival [107]. In addition, transplantation of PC1/3-expressing alpha-cells increases plasma GLP-1 levels and improves glucose homeostasis in rodent models of type 1 and type 2 diabetes [108]. Thus, manipulation of proglucagon processing in the alpha cell to yield GLP-1 can be wiewed as a startegy for enhancing islet function and survival. Since GLP-1 seem to be expressed in islets under certain conditions, it might be necessary to revise our understanding of how this hormone modulates beta-cell secretion and growth. Our current view relies on the network of neural and endocrine signals originating in the gut after food intake that stimulate insulin secretion. We should now also consider the possibility that intraislet GLP-1 signals might modulate insulin secretion and/or influence beta-cell survival. Determining the physiological importance of the islet-derived GLP-1 source during diabetes and its modulation during administration of exogenous GLP-1R agonists is a clinically relevant issue.

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Research Article

Assessment of Glucagon-Like Peptide-1 Analogue and Renin Inhibitor on the Binding and Regulation of GLP-1 Receptor in Type 1 Diabetic Rat Hearts

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This study focuses on the effects of long-term renin-angiotensin system suppression and/or incretin mimetic therapies on the regulation and binding affinity of GLP-1 to its receptor in the coronary endothelium (CE) and cardiomyocytes (CMs) of type 1 diabetic male Sprague-Dawley rats. The groups assessed are normal (N), streptozotocin-induced diabetic (D), Insulin treated (DI), Exendin-4 treated (DE), Aliskiren treated (DA), cotreated with Insulin and Aliskiren (DIA) and cotreated with exendin-4 and Aliskiren (DEA). Heart perfusion with ^{125}I-GLP-1 was performed to estimate GLP-1 binding affinity ($\tau = 1/k - n$) to its receptor in the heart. Western Blotting was assessed to determine the expression variation of GLP-1 receptor in the heart. Plasma GLP-1 levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Diabetes decreased the τ value on CE and increased it on CMs compared to normal. The combination of Exendin-4 with Aliskiren showed a normalizing effect on the binding affinity of GLP-1 at the coronary endothelium, while at the cardiomyocyte level Exendin-4 treatment alone was the most effective.

1. Introduction

Diabetes mellitus currently affects more than 170 million individuals worldwide [1]. Other than hyperglycemia, diabetes mellitus can cause a 2-3-fold increase in the occurrence of cardiovascular disease (CVD) [2]. Both manifestations are easily triggered by oxidative stress, glucose intolerance, and inflammation; hence, they probably exhibit similar underlying processes that lead to their pathogenesis [1]. The incretin hormone, glucagon-like peptide-1 (GLP-1), plays an important role in maintaining glucose homeostasis. Receptor signaling on the pancreas leads to enhanced insulin biosynthesis, secretion, and β-cell proliferation [3, 4]. GLP-1 has also been suggested to ameliorate left ventricular function, because of its antiapoptotic and insulin-like properties [5]. The incretin effect, described as the enhanced response of insulin release after an oral glucose load, has been shown to be reduced in diabetes mellitus [6]. This defect in GLP-1 secretion has been reported in both Type 1 and Type 2 diabetes mellitus [7]. On the other hand, a recent study reported that GLP-1 levels are not decreased in type 2 diabetic patients [8]. Therefore, the effect of diabetes mellitus on the secretion of GLP-1 is a controversial issue. The renin angiotensin system (RAS) controls and regulates the electrolyte-fluid homeostasis and blood pressure by acting on organs in the cardiovascular, renal, and adrenal systems [9]. Clinically, RAS blockage has been proposed to alleviate diabetic complications. In 2007, Aliskiren, a potent renin inhibitor, was approved for clinical use. It is unique due to its low molecular weight, its orally active property, and its nonpeptide nature that makes it resistant to enzymatic degradation [10, 11]. Aliskiren binds to renin, hence blocking the attachment of angiotensinogen to it and its consequent conversion to angiotensin I [12]. It was also shown to reduce

left ventricular hypertrophy as efficiently as angiotensin receptor blockers (ARBs) [13]. This study aims to assess the effect of the GLP-1 analogue, Exendin-4, and the renin inhibitor, Aliskiren, and their cotreatment on the binding kinetics of GLP-1 to its receptor at both the coronary endothelial and cardiomyocyte levels in type 1 diabetic rats.

2. Materials and Methods

The experiments were conducted with prior approval of the Institutional Review Board and Animal Care Committee of the American University of Beirut (AUB). All animals were handled, treated, and sacrificed in accordance with the guidelines of the American Association for Laboratory Animal Sciences (AALAS) on *Humane Care and Use of Laboratory Animals*. For all the parameters, per animal per group, mentioned below, the experimental data are presented as mean value ± standard error of mean (SEM). Student's *t*-test was employed to estimate the significance among the different experimental groups. *P* values of less than .05 were considered significant.

2.1. Animals. Male Sprague-Dawley rats (6 weeks old, 175–250 g body weight) were purchased from Harland, The Netherlands, and bred at the Animal House Unit, American University of Beirut. They were housed at four rats per cage (24 animals per group), fed Purina pellets and tap water ad libitum, and kept for a period of one month at a constant temperature with a daily 12 h light : 12 h dark cycle.

2.2. Treatment and Monitor Plan. Rats were divided into seven groups as follows: Group N ($n = 24$): normal control, received a placebo by oral gavage (tap water, 4 mL/kg body weight), once daily (qd); Group D ($n = 24$): rats with diabetes type 1 were injected intraperitoneally (ip) with 3 cc/kg body weight normal saline solution (NSS), twice daily (bid), and were given placebo (water) by oral gavage (4 cc/kg body weight, qd); Group DI ($n = 24$): rats with diabetes type 1 were injected ip with bovine insulin (Sigma Chemical Company, St. Louis, MI, USA), 0.28 unit/cc, 1 unit/kg body weight, once in the morning (qAM), and subcutaneous insulin glargine (Lantus) injections (1.25 unit/cc, 1 unit/kg body weight) (Sanofi-Aventis, USA), once in the afternoon (qPM); Group DE ($n = 24$): rats with diabetes type 1 were injected intraperitoneally (ip) with Exendin-4 (0.03 μg/kg body weight, bid) (Sigma Chemical Company, St. Louis, Mich, USA); Group DA ($n = 24$): rats with diabetes type 1 were administered Aliskiren (50 mg/kg body weight, qd) (Novartis Pharma Stein AG, Switzerland) by oral gavage. Group DIA ($n = 24$): rats with diabetes type 1 were injected ip with bovine insulin qAM, injected subcutaneously insulin glargine qPM, and were administered Aliskiren (50 mg/kg body weight, qd) by oral gavage. Group DEA ($n = 24$): rats with diabetes type 1 were injected intraperitoneally (ip) with Exendin-4 and were administered Aliskiren (50 mg/kg body weight, qd) by oral gavage.

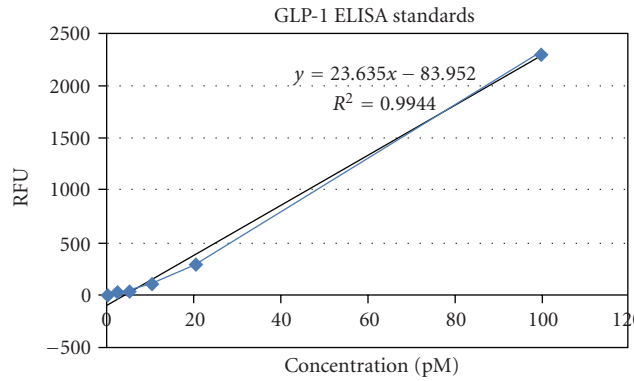

Figure 1: Standard curve for GLP-1.

2.3. Induction of Diabetes. Groups D, DI, DE, DA, DIA and DEA were induced to type 1 diabetes mellitus by a single intravenous injection of streptozotocin (STZ; 85 mg/kg bw) (Sigma Chemical Co., Saint Louis, Mo, USA) in saline acidified to pH 4.5 with 0.1 M citrate buffer [14]. Three days later, nonfasting blood glucose level was measured using Accu-Chek (Accu-Chek Instant Test; Roche Diagnostics GmbH, Mannheim, Germany); a level of ≥250 mg/dL confirmed type 1 diabetes mellitus.

2.4. Body Weight and Blood Glucose. All the animals were weighed weekly, and blood glucose levels were determined [15] using Accu-Chek (Accu-Chek instant test, Roche Diagnostics GmbH, Mannheim, Germany) weekly during one month of treatment.

2.5. Cardiac Hypertrophy Was Assessed Macroscopically. After one month of treatment, wet heart weight was recorded ($N = 16$). Heart weight (H.W.) to body weight (B.W.) ratio (H.W./B.W.) was determined and averaged that served as an index for comparison among different groups.

2.6. Enzyme-Linked Immunosorbent Assay of GLP-1. Rats were anesthetized, and blood was collected from the sublingual vein on days 1, 7, 14, 21, and 28 of the treatment period, at a constant time range of 9 to 10 AM. For each 1 mL blood, 10 μL of DPP-IV enzyme inhibitor was added within 30 seconds of the collection of blood to prevent GLP-1 degradation. Enzyme-Linked Immunosorbent Assay (ELISA) was performed to detect the levels of active GLP-1 (7–36 and 7–37) in the plasma of the rats using the Glucagon-Like Peptide-1 (Active) ELISA kit (ELISA KIT LINCO Research, Mish, USA). Standards of known concentrations of active GLP-1 and the samples were added to the 96-well plate. GLP-1 detection conjugate and substrate were added according to the kit manual to generate a reaction, and the plate was read on the Fluorescent Plate reader at wavelengths of 355/460 nm. A relative fluorescent unit (RFU) curve was plotted using these standards (Figure 1). Since the amount of fluorescence generated is directly proportional to the concentration of GLP-1, the concentration of GLP-1 in the plasma samples can be derived.

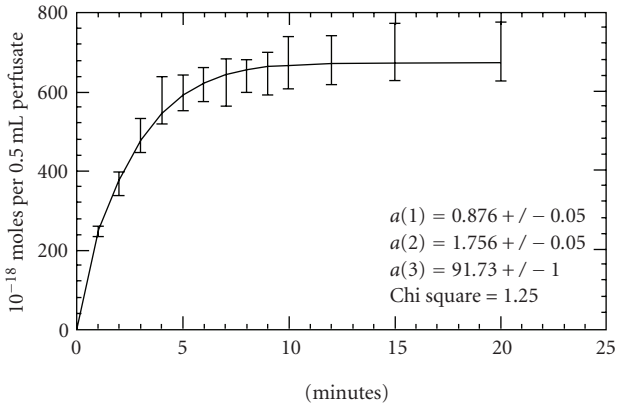

Figure 2: A representative time dependent [^{125}I]-GLP-1 concentration curve in the effluent collected during heart perfusion in Normal group (N) at the level of endothelium. Data points were curve fitted using equation 2 as described by Haddad et al. [17]. The values $a(1)$, $a(2)$, and $a(3)$ were employed to calculate k_n and k_{-n}.

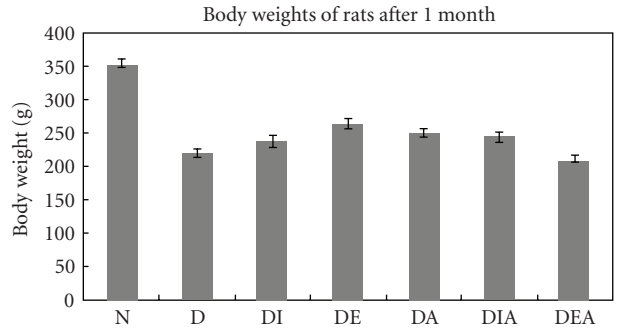

Figure 3: Body weights of rats in the seven experimental groups after 1 month of treatment (N = 24).

2.7. Surgical Procedures. After one month of treatment, the rats were weighed and anesthetized by intramuscular injection of Ketamine (100 mg/kg body weight) (AUB-MC, Lebanon) and Xylazine (10 mg/kg body weight) (Interchemie, Casternary, Holland) and then fixed to a heating pad to prevent rapid cooling. The anterior chest wall was excised longitudinally up to the xyphoid, thus exposing the entire thoracic cavity and the heart for perfusion [16].

2.8. Perfusion of Rat Heart with ^{125}I-GLP-1. The major veins and arteries including the inferior, superior vena cava, pulmonary artery, and pulmonary vein were ligated. In addition, the left and right lungs were tied in order to block the blood flow to them and to other organs. The aorta was slightly cut, and a polyethylene catheter was inserted (from cephalad to caudad) into its lumen to reach the aortic valve. The right atrium was punctured, and another catheter was inserted to carry out the perfusate. Both catheters were secured with a suture [17]. A 50 mL syringe filled with heparinized Ringer-Lock buffer containing 20 meq/L K^+, oxygenated with 95% O_2, 5% CO_2 at 37°C linked to an infusion pump was attached to the inlet. This solution cleared the heart from blood and clots for 15 minutes; infusion of a Ringer-Lock buffer solution for another 15 minutes cleared the heart from heparin. These steps were followed by the perfusion of a buffer containing 8.25×10^{-4} nM/L [^{125}I] GLP-1 (specific activity, 2200 Ci/mmol; Santa Cruz Biotech., Calif, USA) at a rate of 1 mL/min. Heart perfusion was performed in 16 rats from each set divided into one subgroup ($n = 8$) perfused with buffer alone; and the other ($n = 8$) perfused with 20 mmol/L 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Sigma), which has a mild detergent action, to slough off the capillary endothelial lining. The perfusate was collected for 20 minutes at specific time intervals (between 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10, 10–12, 12–15, 15–20 minutes); after each collection, 500 μL of the sample was put in a liquid scintillation vial containing 4 mL of Ecolume scintillation cocktail, which was later assayed for radioactivity by a liquid scintillation analyzer. The surgical procedure and perfusion model as described by Bikhazi et al. [16] and Haddad et al. [17] were followed to determine the binding kinetics of ^{125}I-GLP-1 (specific activity, 2200 Ci/mmol; Santa Cruz Biotech., Calif, USA) to its receptor at the level of endothelial cells and cardiac myocytes (Figure 2).

2.9. Western Blot. After the thoracic cage was excised and the inferior vena cava was cut, a fine needle was inserted in the beating heart washing it several times with saline water. The heart was then removed and immediately put in a beaker containing isopentane and dry ice. When the heart was snap frozen, it was cut transversely into 4 sections: apex, S1, S2, and base. The S1 heart sections were later homogenized, and proteins were extracted using Sucrose Hepes Tris-Buffer and a serine protease inhibitor, PMSF. 150 μg of the sample protein was loaded and separated by 10% polyacrylamide gel electrophoresis for 1 hour and 30 minutes. The bands were then transferred on a nitrocellulose membrane via the Transblot unit, PowerPac HC, (Bio-Rad Laboratories, Calif, USA) for 2 hours. The membrane was incubated with primary polyclonal antibody rabbit anti-GLP-1 receptor α IgG (Santa Cruz Biotech., Calif, USA) (diluted 1/200) for 1 hour. Three consecutive washes with Tris/Tween solution, after which the membrane was incubated with the secondary antibody, mouse antirabbit IgG-HRP 2° (Santa Cruz Biotech.) (1/500) for 2 hours, then washed 4 times with the same Tris/Tween solution. Equal volumes of reagents A and B of chemiluminescence solution were mixed and poured onto the membrane. The immunoblotted bands were later developed on a Fuji Medical X-ray film (Agfa-Gevaert N.V., Mortsel, Belgium).

3. Results

3.1. Body Weights. The mean body weights for the seven rat groups after one month of treatment are shown in Figure 3. One can observe the significant decrease ($P < .001$) in the body weight of all diabetic groups compared to the normal.

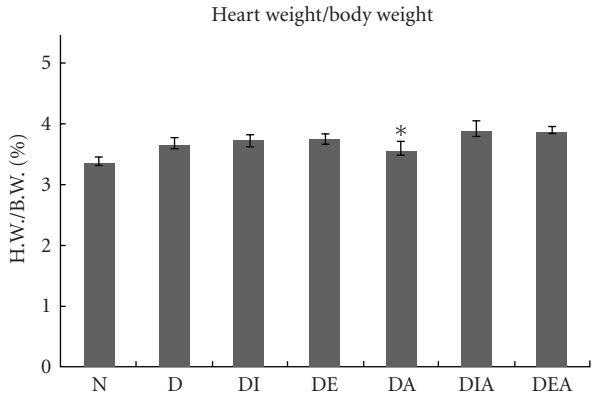

Figure 4: Mean ratios of heart weight per body weight of the seven rat groups after 1 month. *indicates no significance compared with normal group (N = 16).

3.2. Cardiac Hypertrophy Was Assessed Macroscopically. The mean ratios of heart weight to body weights are represented in Figure 4. The data show a significantly increased heart weight to body weight ratio in all the diabetic groups compared to the normal (D, DI, DE, DIA, DEA) except the group treated with Aliskiren (DA).

3.3. Plasma Glucose and GLP-1 Levels. There was a significant increase in plasma glucose levels in all the diabetic groups compared to the normal. It is notable that 12.5% of the Aliskiren-treated group exhibited normal blood glucose levels after one month of treatment. GLP-1 levels in the plasma of normal rats were assessed by ELISA, and a range of 7–9 pM was observed with no significant variation throughout a month. Upon the induction of diabetes mellitus, however, GLP-1 levels increased with a maximum peak of 28 pM at day 28, compared to that of the normal. GLP-1 levels of diabetic rats treated with Insulin (DI), Exendin (DE), or Aliskiren (DA) were normalized. The combination of Aliskiren with Insulin (DIA) was shown to be better than the combination of Exendin-4 and Aliskiren (DEA), because it corrected and nearly normalized GLP-1 levels in the diabetic rats (Figure 5).

3.4. Binding Kinetics of GLP-1 to Its Receptor on the Coronary Endothelium and Cardiomyocytes after One Month of Treatment. Time-dependent radioactive GLP-1 ([^{125}I]-GLP-1) concentration curves of all the animal models were mathematically curve-fitted using a first-order Bessel function physical model describing a 1 : 1 stoichiometry for reversible binding of GLP-1 with its receptor [16]. These curves were used to determine the GLP-1 forward binding constant (k_n), reversal constant (k_{-n}), dissociation constant ($K_d = k_{-n}/k_n$), and the affinity time constant ($\tau = 1/k_{-n}$) with its receptor on the coronary endothelium cells and cardiomyocytes [16, 17]. The derived K_d and τ values at the coronary endothelium are represented in Table 1, and those values at the cardiomyocyte level are represented in Table 2. One can notice the extremely significant increase in K_d constant in the diabetic untreated (D), treated with

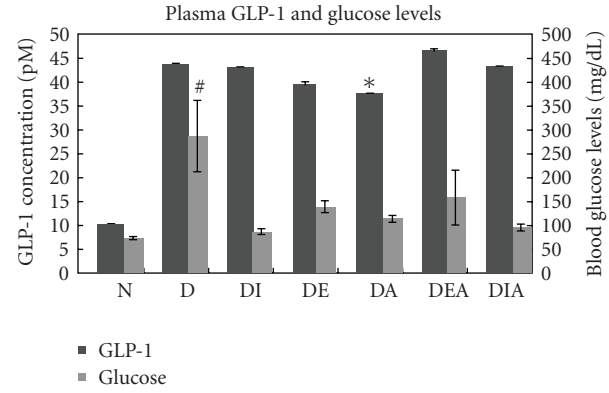

Figure 5: Plasma glucose (N = 24) and glucagon-like peptide-1 levels (N = 6) in all the animal groups after one month of treatment. *indicates significance with $P < .05$ compared with Glucose level of diabetic (D); #indicates extreme significance with $P < .001$ compared with GLP-1 level of normal (N).

Table 1: The calculated dissociation constants (K_d) and binding affinity constants (τ) of glucagon-like peptide-1 (GLP-1) with its receptor at the coronary endothelium (N = 8).

Rat Group	K_d (moles $\times 10^{-15}$)	τ (min)
Normal (N)	0.069 ± 0.005^{a}	$1.14 \pm 0.065^{a'}$
Diabetic (D)	0.700 ± 0.029^{b}	$0.37 \pm 0.007^{b'}$
Diabetic + Insulin (DI)	0.110 ± 0.025^{c}	$2.17 \pm 0.23^{c'}$
Diabetic + Exendin-4 (DE)	0.580 ± 0.029^{d}	$0.45 \pm 0.01^{d'}$
Diabetic +Aliskiren (DA)	0.857 ± 0.043^{e}	$0.38 \pm 0.007^{e'}$
Diabetic + Exendin-4 + Aliskiren (DEA)	0.230 ± 0.039^{f}	$1.35 \pm 0.0913^{f'}$
Diabetic + Insulin + Aliskiren (DIA)	0.650 ± 0.068^{g}	$0.657 \pm 0.021^{g'}$

Coronary endothelium: dissociation constants significant at $P < .05$ for (a, f), (b, d), (b, e), (c, f) and $P < .001$ for (a, b), (a, d), (a, e),(a, g), (b, c), (b, f), (c, d), (c, e), (c, g), (d, e), (d, f), (e, f), (f, g). The other comparative values are not significant ($P > .05$). Binding affinities significant at $P < .05$ for (c′, f′) and $P < .001$ for (a′, b′), (a′, c′), (a′, d′), (a′, e′), (a′, g′), (b′, c′), (b′, d′), (b′, f′), (b′, g′), (c′, d′), (c′, e′), (c′, g′), (d′, e′), (d′, f′), (d′, g′), (e′, f′), (e′, g′), (f′, g′). The other comparative values are not significant ($P > .05$).

Exendin-4 (DE), treated with Aliskiren (DA), cotreated with Exendin-4 and Aliskiren (DEA), and cotreated with Insulin and Aliskiren (DIA) compared to the normal. However, diabetic treated with Insulin (DI) showed no significance compared to the normal. Diabetes decreased the τ value on CE and increased it on CM compared to normal. Exendin-4 treatment partially corrected τ value in both CE and CM. Aliskiren treatment did not alter τ from diabetics in the CE, but its combination with Exendin-4 normalized it.

3.5. Western Blot Analysis. Western blotting was assessed to determine the expression variation of GLP-1 receptor in the heart. The GLP-1 receptor band densities were measured using ImageJ program. The housekeeping gene, β-actin, was also blotted, and a mean ratio was done to correct any differences in loading of the proteins. Our data showed no

Table 2: The calculated dissociation constants (K_d) and binding affinity constants (τ) of glucagon-like peptide-1 (GLP-1) with its receptor at the cardiomyocytes (N = 8).

Rat group	K_d (moles $\times 10^{-15}$)	τ (min)
Normal (N)	0.64 ± 0.021^{a}	$0.34 \pm 0.005^{a'}$
Diabetic (D)	0.33 ± 0.018^{b}	$0.6 \pm 0.018^{b'}$
Diabetic + Insulin (DI)	0.47 ± 0.019^{c}	$0.43 \pm 0.009^{c'}$
Diabetic + Exendin-4 (DE)	0.59 ± 0.025^{d}	$0.41 \pm 0.0084^{d'}$
Diabetic + Aliskiren (DA)	0.12 ± 0.014^{e}	$1.37 \pm 0.0938^{e'}$
Diabetic + Exendin-4 + Aliskiren (DEA)	0.077 ± 0.01^{f}	$1.63 \pm 0.13^{f'}$
Diabetic + Insulin + Aliskiren (DIA)	0.19 ± 0.027^{g}	$1.33 \pm 0.08^{g'}$

Cardiomyocytes: dissociation constants significant at $P < .05$ for (b, c), (b, g), (c, d), (e, f), (f, g) and $P < .001$ for (a, b), (a, c), (a, e), (a, f), (a, g), (b, d), (b, e), (b, f), (c, e), (c, f), (c, g), (d, e), (d, f), (d, g), (f, g). The other comparative values are not significant ($P > .05$). Binding affinities significant at $P < .001$ for (a′, b′), (a′, c′), (a′, d′), (a′, e′), (a′, f′), (a′, g′), (b′, c′), (b′, d′), (b′, e′), (b′, f′), (b′, g′), (c′, e′), (c′, f′), (c′, g′), (d′, e′), (d′, f′), (d′, g′). The other comparative values are not significant ($P > .05$).

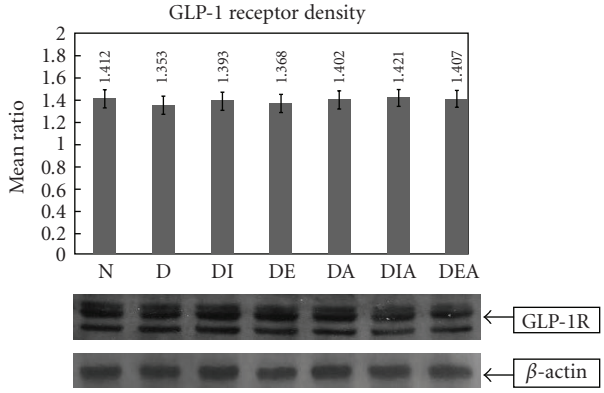

Figure 6: A representative Western blot GLP-1 receptor bands, β-actin bands and their mean ratio (N = 6).

significant change in the densities of GLP-1 receptor bands between the normal and the treated groups (Figure 6).

4. Discussion

The GLP-1 receptor has been localized in many tissues, including the heart [4]. Recently, it was shown that this (GLP-1) receptor is absent in the mouse cardiac fibroblasts, but, abundant in the endocardium. Moreover, endothelium and coronary smooth muscle cells of the heart were also shown to bear GLP-1 receptors [18]. Upon binding to its receptor, GLP-1 produces numerous effects, but because of its very short physiologic half-life, its use as a therapeutic agent is not practical [19]. Incretin mimetics have been introduced in the market to be used as therapies for diabetes mellitus [20]. Our study aimed at assessing the modulation of GLP-1 receptor upon the usage of different treatment modalities including insulin, Exendin-4 (GLP-1 receptor agonist), and Aliskiren (potent renin inhibitor).

After one month of treatment, body weights of the rats in each group were recorded and our results showed a significant decrease in body weights of all diabetic rats compared to the normal. This can be explained by the process of muscle wasting that occurs in diabetic patients [21]. Interestingly, diabetic rats treated with Exendin-4 showed a significant increase in their body weight compared to the diabetic untreated (Figure 3). Moreover, Cardiac hypertrophy was assessed macroscopically (Figure 4); the ratio of heart weight to body weight revealed that there is a significant increase in most diabetic rats compared to that of the normal. This indicated the presence of cardiac hypertrophy in the diabetic rats. However, heart weight over body weight ratio of diabetic rats treated with Aliskiren showed no significance when compared to the ratio of normal. One can conclude that the beneficial effect of Aliskiren as a cardioprotective agent is manifested [13].

Blood glucose levels were extremely increased in all diabetic groups treated or untreated (Figure 5). The intravenous injection of streptozotocin (STZ)—a glucosamine-nitrosourea antibiotic with structural similarity to glucose that is readily taken up by the pancreatic β cells—causes β cells toxicity and ultimately necrosis, leading to deficiency in insulin secretion. The use of Streptozotocin to induce type 1 diabetes in rats is advantageous, because it is easy, fast, and the effect of diabetes on the heart can be evaluated [22]. Our aim was to assess the long-term effects of treatments on blood glucose level in type 1 diabetic rats. It is not surprising that after one month of insulin treatment blood glucose levels of the diabetic rats were not lowered/corrected, since insulin directly treats diabetes on a daily basis—as a short-term and not a long-term treatment. Insulin glargine may be expected to lower the blood glucose levels since it is a long-acting insulin; however, the duration of its action does not reach 24 hours with some recipients, which may be reflected by hyperglycemia [23]. Although not significant, Exendin-4 treatment showed slight improvement in the blood glucose levels. On the other hand, Aliskiren treatment significantly decreased blood glucose levels compared to the untreated. In fact, administration of Aliskiren alone normalized blood glucose levels of 12.5% of the treated rats. Recent studies have shown that Aliskiren improves insulin sensitivity in type 2 diabetic mice [24]; however, there are no reports on Aliskiren's effect on blood glucose in type 1 diabetes mellitus. The different combinations of Aliskiren with insulin or Exendin-4, however, exhibited no beneficial effects on the blood glucose levels compared to the normal.

GLP-1 levels in the plasma of normal rats showed a range of 7–9 pM with no significant variation throughout a month. Upon the induction of diabetes mellitus, however, GLP-1 levels increased with a maximum peak of 28 pM at day 28, compared to that of the normal. GLP-1 levels in diabetic rats treated with Insulin (DI), Exendin-4 (DE), or Aliskiren (DA) were normalized. The combination of Aliskiren with Insulin (DIA) was shown to be better than the combination of Exendin-4 and Aliskiren (DEA), because it corrected and nearly normalized GLP-1 levels in the diabetic rats. It is possible that the DPP-IV enzyme is upregulated in the diabetic state, thereby, inactivating GLP-1 molecules

and reducing their affinity to the receptors. Hence, GLP-1 secretion is enhanced in the diabetic state to overcome this inactivation. Studies have reported that circulating DPPIV enzyme activity and mRNA are both enhanced in STZ-treated rats [25].

Rat heart perfusion technique showed an extremely significant decrease of GLP-1 affinity to its receptor in the diabetic state (D) compared to the normal (N) (1.14 ± 0.065 min versus 0.37 ± 0.007 min) at the level of the coronary endothelium. In diabetic rats treated with insulin (DI), GLP-1 affinity to its receptor increased beyond the normal (1.14 ± 0.065 min versus 2.17 ± 0.23 min), suggesting a direct effect of insulin on the GLP-1 receptor. Treatment with Exendin-4 (DE) (0.45 ± 0.01 min) slightly enhanced, while Aliskiren (DA) (0.38 ± 0.007) showed no significant change in its affinity compared to the diabetic (D) (0.37 ± 0.007 min). Interestingly, however, their combined therapy (DEA) (1.35 ± 0.0913 min) normalized the affinity constant. The cotreatment with Insulin and Aliskiren (DIA) showed some enhancement (0.657 ± 0.021 min) but could not be compared to the effect executed by (DEA) (1.35 ± 0.0913 min) (Table 1).

In parallel, it is interesting to note that the densities of GLP-1 receptors in the heart obtained by the Western blotting showed no difference between the normal or any of the diabetic-treated groups (Figure 6), while heart perfusion results showed differences in GLP-1 binding affinity between CM and CE in both the normal and the diabetic rats (Tables 1 and 2). At the level of the cardiomyocyte, our results showed a significant increase in the affinity of GLP-1 to its receptor in the diabetic compared to the normal and compared to the CHAPS-untreated diabetic. It is possible that GLP-1 receptors located on endothelial cells are different from those present on the cardiomyocytes, suggesting the existence of multiple subtypes of GLP-1 receptor, thereby explaining the difference in affinity constants in CHAPS-treated and -untreated diabetic rats. Another possible explanation could be that GLP-1 receptor expression is higher in the coronary endothelium than in cardiomyocytes. Insulin and Exendin-4 treatments showed slight improvement in the affinity (0.43 ± 0.009 min and 0.41 ± 0.0084 min, resp.), but Aliskiren and its combination treatments (DEA) and (DIA) did not lower the affinity (Table 2).

These two findings could hint the possibility of different GLP-1 receptors existing in the coronary endothelium and cardiomyocytes. In fact, there have been discussions about the existence of a putative second GLP-1 receptor during a European GLP-1 Club Meeting in Marseille, where scientists have concluded that "although no molecular evidence has yet been presented, there are circumstantial data to suggest that such receptors do exist" [26].

According to Nystrom et al. [27], GLP-1 receptors are located on the human coronary endothelial cells. Binding of GLP-1 to those receptors was reported to induce vasodilation, probably mediated through Nitric Oxide production. Furthermore, this effect was eliminated upon the removal of the endothelial lining [26]. Our results showed a significant reduction of GLP-1 affinity to its receptor in the diabetic rat coronary endothelium compared to the normal. It is also probable that in the diabetic state, GLP-1 receptors on the coronary endothelial cells are modified in such a way that the binding affinity of GLP-1 to them is reduced, hence, resulting in reduced NO production and subsequent vasoconstriction. Moreover, this reduction in affinity was corrected beyond the normal by the treatment with insulin, suggesting a direct role of insulin on GLP-1 receptor modulation. The affinity constant in the diabetic rats was improved with Exendin-4 treatment, however Aliskiren did not ameliorate it. Interestingly, the combination of the two treatment modalities normalized the affinity suggesting synergy between the Exendin-4 and Aliskiren treatments. Recently, Dong et al. reported that, upon the administration of Aliskiren, the potent renin inhibitor, nitric oxide synthase production by endothelial cells was significantly restored [28]. In addition, GLP-1 infusion has been reported to induce vasodilation [26]. Combining Aliskiren and Exendin-4 probably causes an interaction, resulting in an exaggerated effect on the binding affinity of GLP-1 to its receptor on the coronary endothelium.

5. Conclusions

Although many questions remain unanswered regarding the GLP-1 analogue, Exendin-4 and the renin-inhibitor, Aliskiren, it is evident that treatment with both Exendin-4, and Aliskiren greatly improves the GLP-1 binding affinity at the coronary endothelium level of the type 1 diabetic rat. It is therefore crucial to further investigate and to try and find a common ground between GLP-1 signaling pathway and the renin that could indicate a crosstalk between the two. Moreover, future investigations are crucial to unmask the possible existence of another GLP-1 receptor, hence explaining the difference in their affinities at the CE and CM levels. In conclusion, clinical studies on Aliskiren and/or its combination with Exendin-4 in type 1 diabetes mellitus could verify their long-term beneficiary effects on blood glucose levels and cardiomyopathy and further shed light on their ability to prevent the progression of diabetic complications especially in the heart.

Acknowledgments

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Review Article

Effects of GLP-1 and Incretin-Based Therapies on Gastrointestinal Motor Function

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Glucagon-like peptide 1 (GLP-1) is a hormone secreted predominantly by the distal small intestine and colon and released in response to enteral nutrient exposure. GLP-1-based therapies are now used widely in the management of type 2 diabetes and have the potential to be effective antiobesity agents. Although widely known as an incretin hormone, there is a growing body of evidence that GLP-1 also acts as an enterogastrone, with profound effects on the gastrointestinal motor system. Moreover, the effects of GLP-1 on gastrointestinal motility appear to be pivotal to its effect of reducing postprandial glycaemic excursions and may, potentially, represent the dominant mechanism. This review summarizes current knowledge of the enterogastrone properties of GLP-1, focusing on its effects on gut motility at physiological and pharmacological concentrations, and the motor actions of incretin-based therapies. While of potential importance, the inhibitory action of GLP-1 on gastric acid secretion is beyond the scope of this paper.

1. Introduction

A role for gastrointestinal peptides (or factors) in the maintenance of mammalian glucose homeostasis had been speculated for more than 100 years. The search for these gut peptides was stimulated initially by the discovery of secretin by Bayliss and Starling in 1902 [1] and advanced by the work of others [2–5] before it fell out of favour. It was revived subsequently by the observations, by Elrick et al. [6] and McIntyre et al. [7] in 1964, that an oral glucose load resulted in a much greater insulin response than an intravenous glucose load despite resulting in comparable blood glucose concentrations—the so-called "incretin effect" [8]. Glucagon-like peptide-1 (GLP-1) was discovered in the 1980s following sequencing of the proglucagon gene and was shown soon after that time to have fulfilled the physiological criteria for an "incretin" as specified by Creutzfeldt [9], that is, a hormone released from intestinal cells following a nutrient load, which leads to a glucose dependent insulin response. GLP-1 was the second incretin to be characterized after glucose-dependent insulinotropic polypeptide (GIP), which had been discovered a decade earlier.

GLP-1, however, does not fit as well as GIP within Creutzfeldt's incretin definition [10]. For example, in healthy subjects [11] and type 2 diabetic patients [12], postprandial (as opposed to fasting) levels of insulin and C peptide are *decreased* by exogenous GLP-1, rather than stimulated (Figure 1), and when the slowing of gastric emptying induced by GLP-1 is reversed by the prokinetic drug erythromycin, the glucose lowering effect is attenuated [13]. The properties of GLP-1 as an enterogastrone (i.e., a factor that slows gastric emptying and inhibits gastric acid secretion) have also been appreciated [10, 14, 15]. In fact, it has been suggested that the actions of GLP-1 to slow gastric emptying, and thereby the entry of nutrients into the small intestine to delay their absorption, may outweigh its insulinotropic and glucagonostatic effects [16]. In contrast to GLP-1, GIP has little effect on gastric emptying [17]. If anything, there is some evidence that GIP may modestly accelerate emptying from the stomach [18].

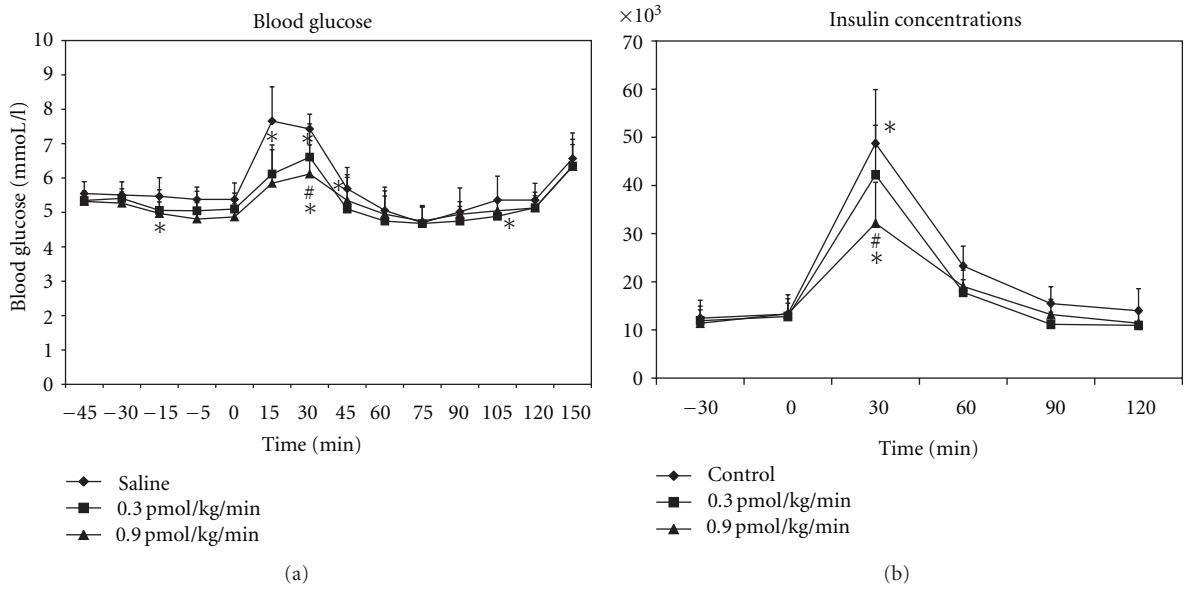

Figure 1: Blood glucose (a) and plasma insulin (b) concentrations during IV infusion of GLP-1, at 0.3 and 0.9 pmol/kg/minutes, or 0.9% saline, in 10 healthy humans. Data are means ± SEM. *GLP-1 0.3 and 0.9 versus saline: $P < .05$; #GLP-1 0.9 versus GLP-1 0.3: $P < .05$ (adapted from Little et al. 2006 [19], and used with permission from the publisher).

Some studies have reported reduced GLP-1 levels in type 2 diabetic patients [18, 20–24], although this has not been found uniformly to be the case [25, 26]. It should be noted, however, that the efficacy of GLP-1-based therapy does not depend on a deficiency of endogenous peptide. Pharmacological "replacement" of GLP-1 is not straightforward owing to its very short half-life. GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4), making it impractical for most clinical applications. This has provided the rationale for the development, and subsequent widespread use, of synthetic longer-acting analogues, such as exenatide and liraglutide (the two approved GLP-1 analogues), and DPP-4 inhibitors, like sitagliptin and vildagliptin, to improve glycaemic control in type 2 diabetic patients [27].

2. The Physiology of Gastrointestinal Motility

An overview of the physiology of gastrointestinal motility is useful in understanding the role and importance of GLP-1 in gut function. Gastric emptying, a highly regulated process of delivering chyme from the stomach to the small intestine, involves a complex interplay between the gastrointestinal smooth muscle, gastric pacemaker cell networks, the so-called interstitial cells of Cajal, and neurohormonal systems, particularly inhibitory feedback arising from the interaction of nutrients with the small intestine. Gastric and small intestinal motility is of predominutesantly two types: (a) peristaltic, in the interdigestive or fasted phase, and (b) segmented contractions in the fed, or postprandial state. Interdigestive motility is comprised of three sequential phases (phase I or quiescent, lasting ~40 minutesutes, phase II or intermittent, lasting ~50 minutesutes, and phase III or regular contractions, at about 3/minutesute in the stomach and 10–12/minutesute in the small intestine, and lasting ~5–10 minutesutes) and is called the "migrating motor complex" (MMC) [28]. Transit of indigestible solid occurs mainly in late phase II and phase III of the MMC [29].

Once food (solid, liquid, or mixed) arrives in the stomach, the MMC is replaced by the postprandial motor pattern. For a solid or mixed meal, the proximal and distal parts of the stomach have different functions. The proximal stomach is concerned with the storage of food and accommodates the ingested meal by decreasing its tone [30, 31], enabling its volume to increase without a substantial increase in intragastric pressure [31, 32]. In contrast, the distal portion of the stomach is concerned with the mixing and trituration of the meal. Antral contractions pulverise the digestible solid contents against the closed pylorus until they have achieved a size of 1-2 mm [33]. Phasic and tonic contractions localised to the pylorus play a major role in the regulation of gastric emptying so food particles are delivered to the duodenum following the opening of the pylorus, predominutesantly in a pulsatile manner [34]. The rate and pattern of gastric emptying are dependent on the composition (solid, semisolid, or liquid), osmolarity, caloric content, and size of the particles in the meal ingested. Liquids are preferentially transferred to the small intestine before solids. Nonnutrient liquids empty in an overall exponential pattern, while a more linear rate is observed as the nutrient and caloric content of the liquid meal increase. The presence of food in the stomach reduces appetite, and distension of the antrum, rather than the proximal stomach, appears to be more strongly associated with the perception of postprandial fullness [35], and suppression of subsequent energy intake [36].

The exposure of the small intestine to nutrients triggers a powerful inhibitory feedback to slow gastric emptying and

small intestinal transit—the magnitude of this feedback is dependent on the type of nutrient [37], as well as both the length [38–40] and region [40–42] of small intestine exposed. GLP-1 and peptide YY (PYY), coexpressed with GLP-1 in the entero-endocrine L cells predominutesantly (but not exclusively) from the distal gut, appear to be potent mediators of the so-called "ileal brake" [43]. This small intestinal feedback mechanism results in highly regulated gastric emptying of nutrients, including carbohydrate, from the stomach to the small intestine at an overall rate of about 1 to 4 kcal/minutes [44, 45]. The release of GLP-1 from the small intestine is critically dependent on the carbohydrate load in both healthy subjects and type 2 diabetic patients [45–48]. When glucose is infused intraduodenally at the rate of 1 kcal/minutes there is a minutesimal, and transient, release of GLP-1, whereas there is a substantial, and sustained, GLP-1 response to infusion at the rate of 4 kcal/minutes [49], probably reflecting a greater length of small intestinal exposure [38]. Fat (mediated by free fatty acids), protein [50], and bile acids [51] are also potent stimuli of GLP-1 release from the L cells. For carbohydrate, it has been suggested that stimulation of intestinal "sweet taste" receptors triggers GLP-1 release [52], although in humans, the artificial sweetener, sucralose, does not induce GLP-1 secretion [53]. However, secretion of GLP-1 in response to sucrose is increased when malabsorption is induced by the α-glucosidase inhibitor, acarbose, presumably due to exposure of the L cell—bearing distal gut to larger amounts of carbohydrate [54].

The terminutesal aspect of the gastrointestinal tract, the colon, is characterized by the presence of haustra (formed by nonpropagated phasic contractions), which compartmentalise the luminutesal compartment and favour stool formation by water and electrolyte absorption. Colonic motility is discontinuous, and mostly slow, which ensures prolonged retention of contents, though occasionally rapid activity allows large amounts of residue to be transferred across the colon within seconds. Expulsion of contents is regulated by the ano-rectum.

3. The Interrelationship of Gastric Emptying with Postprandial Glycaemia

Postprandial hyperglycaemia is being increasingly recognised as an independent predictor of cardiovascular mortality in both diabetic and nondiabetic populations [55, 56]. The rate of gastric emptying influences postprandial glycaemic excursions and vice versa: a situation comparable to the "chicken and egg" relationship [57]. Gastric emptying is a major determinutesant of postprandial glycaemic excursions in healthy subjects [45] as well as type 1 and type 2 diabetic patients [46] so that slower gastric emptying is associated with reduction in blood glucose, especially in the first hour after ingestion of a meal [58, 59] (Figure 2). Conversely, gastrointestinal motor function is highly sensitive to changes in the glycaemic state [57]. For example, in both type 1 [60] and type 2 diabetic patients [61], an increase in postprandial blood glucose levels is associated with proportional slowing

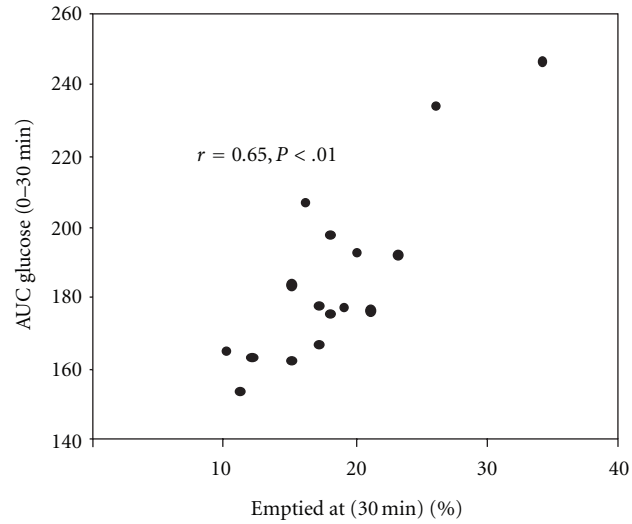

Figure 2: Relationship between the area under the plasma glucose concentration curve between 0 and 30 minutes and the retention of the meal in the stomach at 30 minutes ($r = 0.65$, $P < .01$) (adapted from Horowitz et al. 1993 [45] and used with the permission of the publisher).

of gastric emptying. Even changes within the physiological postprandial blood glucose range (i.e., 4 mmol/L versus 8 mmol/L) affect gastric emptying in healthy as well as uncomplicated type 1 diabetic patients [62]. The slowing of gastric emptying by acute hyperglycaemia reflects the stimulation of pyloric motility [62, 63], suppression of antral motility [64], and reduction in proximal gastric tone [65]. Acute hyperglycaemia also attenuates the ability of erythromycin [66], and probably other prokinetic drugs, to accelerate gastric emptying. In contrast to the effect of hyperglycaemia, insulin-induced hypoglycaemia accelerates gastric emptying substantially, probably representing a counter-regulatory mechanism [67].

4. Effects on Gastric Motility

It has been well established that GLP-1 slows gastric emptying. The following section reviews the effects of GLP-1 and incretin-based therapies on gastric motility.

4.1. Exogenous GLP-1. Exogenous GLP-1 slows gastric emptying in healthy [16, 19, 68], obese [69], type 2 diabetic [12], and critically ill subjects [70]. Infusion of GLP-1 slows gastric emptying of both solid and liquid components of a meal and alters intragastric meal distribution so that a greater proportion of the meal is retained in the distal stomach [19] (Figure 3). Even at "low" doses (0.3 pmol/kg/minutes, designed to reflect "physiological" postprandial GLP-1 plasma concentrations) intravenous adminutesistration of GLP-1 profoundly slows gastric emptying in a substantial proportion of healthy subjects into the "gastroparetic" range [19]. In both healthy subjects [16, 19, 71] and type 2 diabetic patients [12], the effect of exogenous GLP-1 on gastric emptying appears to be dose-related. Furthermore,

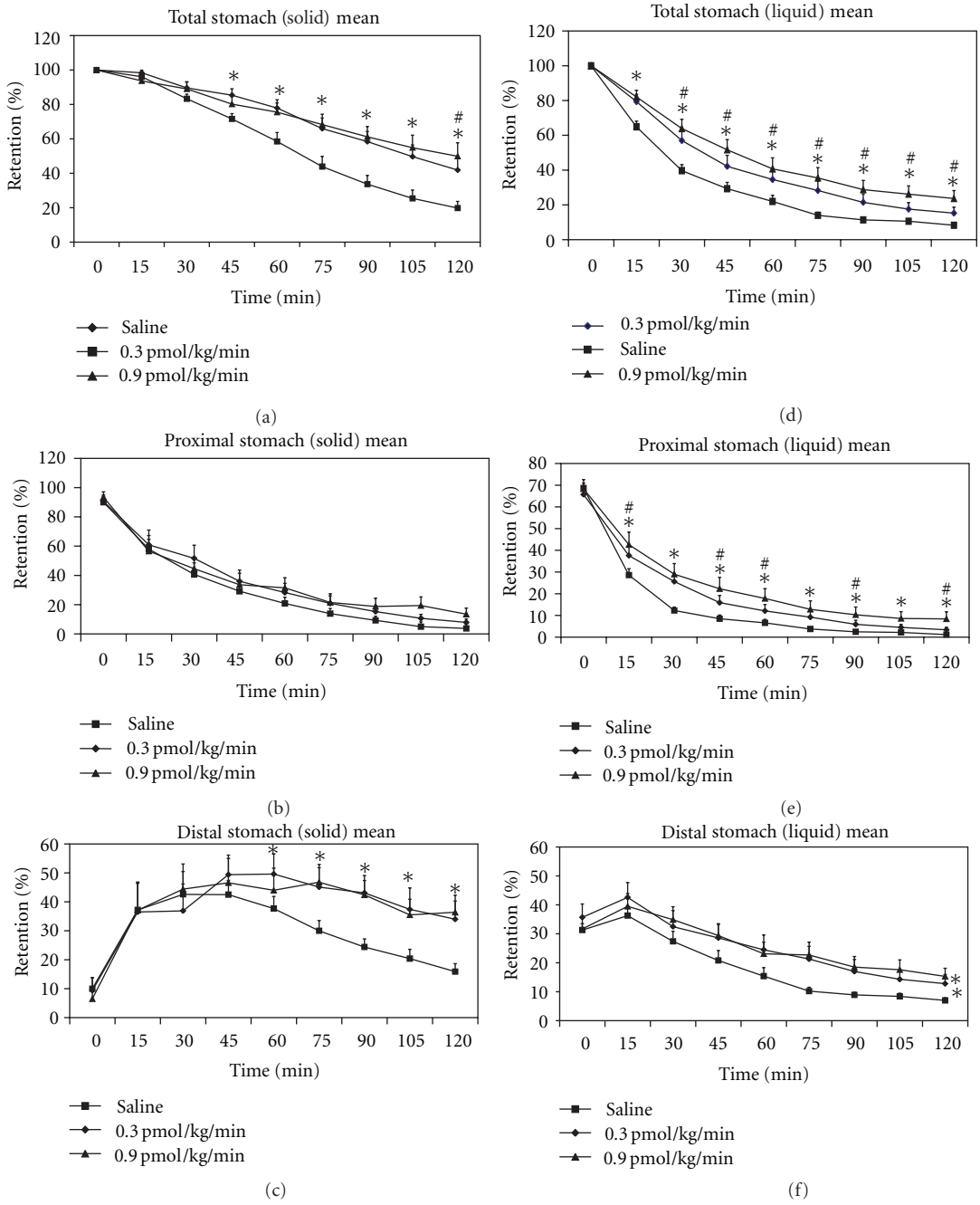

Figure 3: Gastric emptying curves for the solid and liquid components of a meal for the total, proximal, and distal stomach during IV infusion of GLP-1 at 0.3 and 0.9 pmol/kg/minutes, or 0.9% saline, in 10 healthy humans. Data are means ± SEM. *GLP-1 0.3 and 0.9 versus saline: $P < .05$; #GLP-1 0.9 versus GLP-1 0.3: $P < .05$ (adapted from Little et al. 2006 [19] and used with permission from the publisher).

an inverse relationship between the early postprandial rise in blood glucose and the rate of gastric emptying, following infusion of exogenous GLP-1, indicates the importance of the gastric motor actions of GLP-1 in its glucose-lowering effect [19]. As would be predicted by the slowing of gastric emptying, exogenous GLP-1 relaxes the proximal stomach in a dose-dependent manner [72], reduces antral and duodenal motility, and increases pyloric tone in both the fasted and the fed states [71].

4.2. Endogenous GLP-1. While a number of studies have employed infusions of exogenous GLP-1 at "low" rates, designed to reflect physiological postprandial GLP-1 concentrations, a more valid approach to assess the role of endogenous GLP-1 is the use of a specific GLP-1 antagonist such as exendin (9-39) amide. It appears that endogenous GLP-1 has a modest effect to slow gastric emptying and thereby delay carbohydrate absorption, given a sufficient caloric load [73], by mechanisms that include antral inhibition and

stimulation of pyloric motility [34]. Three other studies employing exendin (9-39) failed to show an effect of endogenous GLP-1 on gastric emptying [86–88], but this is likely to reflect methodological differences, including the use of a suboptimal technique (plasma D-xylose) for measuring gastric emptying in one study [87]. Furthermore, in one of these studies [88] although no difference in gastric emptying was observed, exendin 9-39 did change the intragastric distribution of the meal, supporting the role of endogenous GLP-1 in regulating gastric motility.

4.3. GLP-1-Based Therapies. The insulinotropic property of GIP is markedly diminutesished in type 2 diabetic patients [89–91], probably in part as an effect of hyperglycaemia. On the other hand, GLP-1 retains its properties (at supraphysiological doses) in type 2 diabetic patients with potent effects on gastric motility and postprandial glycaemic control [12]. Consequently, this hormone has been an important target for the pharmaceutical industry in the treatment of diabetes [12, 81, 92]. This has stimulated the development of synthetic GLP-1 analogues, which are resistant to rapid degradation, and inhibitors of the enzyme DPP-4 (which boost concentrations of the active fraction of endogenous GLP-1). Both classes of drugs are now used widely in the management of type 2 diabetes.

4.3.1. GLP-1 Receptor Agonists. There are a number of GLP-1 receptor agonists on the market or in development. Of the established agents, exenatide and liraglutide, the former has been best studied in regards to motor effects. It appears that an important mechanism contributing to the action of exenatide, in reducing postprandial glycaemia, is by slowing gastric emptying [81, 93]. Exenatide, derived from the saliva of the Gila monster *Heloderma suspectum,* has been shown to induce a dose-dependent deceleration of gastric emptying in healthy subjects [80] and type 2 diabetic patients [82, 94]. Exenatide slows gastric emptying of both solid and liquid components of a meal, irrespective of the presence of established autonomic neuropathy [81]. However, in both type 2 diabetic patients [81] and patients with critical illness [70], the effects of GLP-1 or incretin-based therapies appear to be dependent on the prior rate of gastric emptying, so that there is little further slowing in those with delayed emptying at baseline. The relevance of this for selecting the most appropriate patients to be treated with exenatide and other GLP-1 agonists remains to be clarified, but it is clearly an important issue for further study. While animal studies with long acting exenatide (LAR exenatide) have failed to show evidence of tachyphylaxis (i.e., reduction in pharmacological response over time) [95], a recent trial in type 2 diabetic patients indicated that gastric emptying may be more strongly slowed by twice daily exenatide than once weekly LAR exenatide [96], suggesting that continuous GLP-1 exposure could result in a diminutesution of pharmacological response, potentially reflecting changes in receptor activation and/or changes in vagal function.

The major adverse effects of exenatide and liraglutide are nausea and vomiting. While these could relate to its effects on gastric motor function, and antral distension in particular [35, 36, 97], the occurrence of adverse effects seems not to relate closely to the delay in gastric emptying [81, 96], and it is possible that central mechanisms are important. The effects on gastric emptying have not been comprehensively established for incretin-based therapies other than exenatide, but liraglutide also slows gastric emptying—the magnitude of which is uncertain [83, 84]. Evaluation of this aspect of drug action represents an important research priority for these agents.

4.3.2. Dipeptidyl Peptidase-4 Inhibitors. Dipeptidyl peptidase-4 inhibitors, including sitagliptin and vildagliptin, result in an increase in circulating active GLP-1 concentrations [27, 98] but appear to have, at most, a modest effect on gastric emptying [27]. Some deceleration in gastric emptying was observed in a study of obese insulin resistant monkeys treated with vildagliptin [85], but human studies published to date have not demonstrated an effect of DPP-4 inhibitors on the rate of gastric emptying [82, 99, 100], possibly because the elevation in active GLP-1 concentrations is relatively modest. It should be noted that upper gastrointestinal adverse effects such as nausea and vomiting are less commonly encountered with DPP-4 inhibitors than with GLP-1 receptor agonists, and the relative lack of effects of the former on gastric emptying could well be relevant in this regard.

5. Effects on Small Intestinal Motility

The effects of GLP-1 on small intestinal motility have not been extensively studied. Exogenous, intravenous GLP-1 has been shown to inhibit murine fasted and fed small bowel motility in a dose-dependent manner and appears to have an additive effect when combined with intravenous GLP-2 in the fasted state [74]. Exendin (9-39) blocks the inhibition of murine small intestinal motility induced by intraduodenal infusion of peptone [75]. Suppression of fasting small intestinal motility by exogenous GLP-1 is also evident in healthy humans and those with irritable bowel syndrome, manifested by a reduction in the frequency of MMCs in a dose-dependent manner [76]. Indeed, the GLP-1 analogue, ROSE-010, has been reported to be more effective than placebo at relieving abdominutesal pain in irritable bowel syndrome patients [101]. None of the human studies have hitherto evaluated the effects of GLP-1 or its analogues on postprandial small intestinal motility, but this could represent an additional mode of glucose-lowering by these agents, given that pharmacological inhibition of small intestinal flow events has been shown to reduce the rate of small intestinal glucose absorption in healthy humans [102].

6. Effects on Colonic Motility

Only a handful of animal studies have specifically evaluated the effects of GLP-1 (exogenous or endogenous) on colonic motility. Adminutesistration of intra-cerebroventricular GLP-1 was reported to increase rat fecal pellet output and

Table 1: Summary of motor effects of GLP-1 and incretin-based therapies on the gastrointestinal tract.

	Gastric motility (delayed gastric emptying)	Small intestinal motility (delayed small intestinal transit)	Large intestinal motility (delayed colonic transit)
Endogenous GLP-1 (physiological dose)	One positive study [73]	No studies available	No studies available
Exogenous GLP-1 (pharmacological dose)	Strong evidence in human studies; healthy [16], obese [69], type 2 diabetic [12], critically ill [70]	Positive evidence in animal studies [74, 75]. Positive effect on fasting motility in humans [76]	Positive evidence in animal studies [77]. Only indirect evidence in humans [78, 79].
GLP-1 receptor agonists (e.g., exenatide, liraglutide)	Strong evidence with exenatide (healthy) [80], (type 2 diabetes) [80–82]. Some evidence with liraglutide [83, 84]	No studies available	No studies available
DPP-4 inhibitors (e.g., sitagliptin, vildagliptin)	Positive evidence with animal studies only [85]	No studies available	No studies available

this was reversed by the GLP-1 receptor antagonist, exendin (9-39) [77]. Evidence for a role for GLP-1 in the regulation of colonic transit in humans has been limited to the reports of GLP-1 secreting tumors and their association with severe constipation and markedly delayed colonic transit [78, 79].

7. Mechanism of Action of GLP-1 and Incretin-Based Therapies on Gut Motility

The mechanisms by which GLP-1, or incretin-based therapies, exert their motor actions on the gut have not yet been fully elucidated but appear to be complex. A number of studies have indicated involvement of the vagal nerves in mediating some of these effects of GLP-1 [103–105]. Gastric relaxation [68, 105] and postprandial gastric accommodation [68], in response to exogenous GLP-1, are mediated by vagal cholinergic pathways; antro-pyloro-duodenal motility apparently is not [103]. Inhibition of fasting small bowel motility in rats by exogenous GLP-1 is mediated via endogenous nitric oxide (NO), while suppression of fed motility is independent of NO [106]. Studies of the rodent duodenum and colon suggest that GLP-1 can decrease excitatory cholinergic neurotransmission in the enteric nervous system via presynaptic GLP-1 receptors, which modulate NO release [107].

Some gastrointestinal motor effects of GLP-1 appear to be centrally mediated—GLP-1 can readily diffuse through the blood-brain barrier [77, 108] to gain access to GLP-1 receptors in the circumventricular organs, the subfornical organ, and area postrema [109]; the latter in particular controls vomiting. Albiglutide (or Albugon) is a newer GLP-1 receptor agonist that does not readily diffuse into the area postrema and has a low prevalence of gastrointestinal adverse effects, possibly for this reason [110, 111].

8. Conclusion

Exploiting the properties of GLP-1 to the fullest for therapeutic purposes will require an in-depth understanding, not only of its incretin effects but also of its impact on gut motility. Although the last decade and a half has seen some important steps in that direction, particularly in understanding the impacts of GLP-1 and incretin-based therapies on gastric emptying, it is clearly a work in progress (Table 1). Further research is needed to gain a better understanding of the actions of GLP-1 and incretin-based therapies on small bowel motility, the extent of the role of endogenous GLP-1 on gut function, and how strongly the motor effects of GLP-1-based therapies are maintained with long-term use. The implications of effects on gastric emptying and small intestinal motility for glycaemic control in diabetes are clinically significant, as the former are often disordered in long-standing diabetes.

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Review Article

From Theory to Clinical Practice in the Use of GLP-1 Receptor Agonists and DPP-4 Inhibitors Therapy

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Promoting long-term adherence to lifestyle modification and choice of antidiabetic agent with low hypoglycemia risk profile and positive weight profile could be the most effective strategy in achieving sustained glycemic control and in reducing comorbidities. From this perspective, vast interest has been generated by glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 inhibitors (DPP-4i). In this review our ten-year clinical and laboratory experience by *in vitro* and *in vivo* studies is reported. Herein, we reviewed available data on the efficacy and safety profile of GLP-1 receptor agonists and DPP-4i. The introduction of incretin hormone-based therapies represents a novel therapeutic strategy, because these drugs not only improve glycemia with minimal risk of hypoglycemia but also have other extraglycemic beneficial effects. In clinical studies, both GLP-1 receptor agonists and DPP-4i, improve β cell function indexes. All these agents showed trophic effects on beta-cell mass in animal studies. The use of these drugs is associated with positive or neucral effect on body weight and improvements in blood pressure, diabetic dyslipidemia, hepatic steazosis markets, and myocardial function. These effects have the potential to reduce the burden of cardiovascular disease, which is a major cause of mortality in patients with diabetes.

1. Introduction

The global prevalence of type 2 diabetic (T2DM) patients estimated at 6.4% is expected to be close to 8% by 2030 [1]. The overall total predicted increase is thought to be due largely to rising rates of overweight, obesity, physical inactivity, and population aging [2]. Improving glycaemic control remains the most effective therapeutic approach to reduce the risk of development and/or progression of microvascular complications. Furthermore, a recent meta-analysis of long-term, prospective randomized controlled clinical trials (UKPDS, PROactive, ADVANCE, VADT, and ACCORD) revealed a significant association between intensive blood glucose control and incident cardiovascular events: a 0.9% HbA1c decrease was related to a reduction of 17% in nonfatal MI (odds ratio (OR): 0.83, 95% confidence interval (CI): 0.75–0.93) and 15% in coronary heart disease (OR: 0.85, 95% CI: 0.77–0.93) versus conventional therapy [3]. In a metaregression analysis, higher body mass index (BMI), duration of diabetes, and incidence of severe hypoglycaemia were associated with greater risk of cardiovascular death in intensive treatment groups [4]. Altogether, these results underline the importance of achieving and maintaining good glycemic control, from the time of diagnosis, predominantly through a tailored approach. Promoting long-term adherence to lifestyle modification and choice of antidiabetic agent with low hypoglycemia risk profile and positive weight profile could be the most effective strategy in achieving sustained glycemic control and in reducing comorbidities. From this perspective, vast interest has been generated by glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 inhibitors (DPP-4i) based on many clinical studies revealing long-term glucose-lowering efficacy related to low hypoglycemic rates, positive/neutral weight effects, and amelioration of β cell function [5–7].

2. Background: A Ten-Year Clinical and Laboratory Experience

GLP-1 is a gastrointestinal hormone, mainly secreted in a nutrient-dependent manner, which enhances glucose-induced insulin secretion and induces satiety. It has been

reported that GLP-1 levels after an oral glucose load are reduced in patients with T2DM [8] even if more recent data suggest a controversial point of view [9]. The reduction of oral glucose-stimulated active GLP-1 levels in T2DM patients has also been observed during euglycaemic hyperinsulinemic clamp. This impairment, which is not the result of differences in glycaemia or insulinaemia during assessment, could contribute to the pathogenesis of hyperglycaemia in T2DM [8] and in particular to the reduction of early postprandial insulin secretion; in fact, the administration of GLP-1 receptor antagonists to healthy volunteers elicits both an impairment of meal-induced insulin secretion and an increase of postprandial glycaemia similar to that observed in T2DM. GLP-1 is rapidly inactivated by dipeptidyl peptidase-4 (DPP-4), an enzyme produced by endothelial cells in different districts and that circulates in plasma. The reduction of meal or oral-glucose-stimulated GLP-1 levels in T2DM patients is probably due to both an impairment of secretion and an increased degradation. The major limitation of using native GLP-1 to treat diabetic patients is the short half-life. There are now several compounds in various stages of preclinical or clinical development for the treatment of T2DM that utilize the GLP-1 signaling pathway; these include GLP-1 receptor agonists with extended half-lives and DPP-4i that increase circulating levels of endogenous, intact, and bioactive GLP-1 [10].

Metformin, first drug of choice in the treatment of T2DM, induced a significant increase of GLP-1(7–36) amide/(7–37) at 30 and 60 min after the oral glucose load in obese nondiabetic subjects. In pooled human plasma, metformin (0.1–0.5 microg/mL) significantly inhibited degradation of GLP-1(7–36) amide after a 30 min incubation at 37°C; similar results were obtained in a buffer solution containing DPP-4. This effect could be due to an inhibition of GLP-1 degradation [11]. This effect was also present in obese drug-naïve T2DM patients. In fact 4 weeks after treatment with metformin 850 mg three times daily, post-load GLP-1 levels was significantly increased [12]. Furthermore the relationship between meal-induced GLP-1 secretion and postprandial hyperglycemia was studied in 21 drug-naïve T2DM patients. Blood glucose and active GLP-1 levels were measured 0, 30, 60, 90, and 120 min after a standard meal test, and a continuous glucose monitoring (CGM) system was applied for the following 3 days. A significant inverse correlation between GLP-1 response and postprandial glucose levels was observed for each additional unit of total energy or carbohydrate intake. A lower GLP-1 response is associated with higher levels of HbA1c and with a greater degree of meal-induced hyperglycemia, both during a meal test and "real-life" condition [13].

GLP-1 and DPP-4 activity levels have been subsequently studied during an oral glucose tolerance test (OGTT), in order to investigate their modification in patients with different glucose tolerance degree. At 30 min GLP-1 levelswere significantly lower in subjects with impaired glucose tolerance and T2DM compared to those with normal glucose tolerance. The area under the curve of GLP-1 levels during OGTT was significantly different among the three groups with a significant decrease between subjects with normal and impaired glucose tolerance and between those with normal glucose tolerance and type 2 diabetes mellitus. DPP-4 showed no significant difference between the groups. Therefore, an increase of GLP-1 degradation seems to be not involved in the early stages of diabetes [14]. However, chronic hyperglycaemia was significantly associated to an increased DPP-4 activity and mRNA expression in human glomerular endothelial cells in vitro [15]. In order to assess effects of chronic hyperglycaemia on circulating DPP-IV activity in vivo, we have analyzed diabetic patients not adequately controlled. T2DM subjects with HbA1c levels >8.5% showed significantly higher DPP-4 activity than patients affected by newly diagnosed diabetes and Impaired Glucose Tolerance (IGT). Variations in DPP-4 activity over 3 months in T2DM showed a significant positive correlation with HbA1C levels. Therefore, chronic hyperglycaemia induced a significant increase in DPP-4 activity in type 2 diabetic patients with poor metabolic control, probably contributing to the reduction in circulating active GLP-1 and subsequently to postprandial hyperglycaemia [16]. Moreover, the effects of insulin sensitizers drugs, such as Metformin and Rosiglitazone, on the modulation of GLP-1 circulating levels, DPP-4 activity, and mRNA expression was evaluated in human aortic endothelial cells (HAECs) and human microvascular dermal endothelial cells (HMVECs) exposed to high glucose concentration. Hyperglycemia is associated to a significant DPP-4 activity increase only in microvascular endothelial cells. Rosiglitazone is able to modulate in a negative manner the expression of DPP-4 but not its activity in macrovascular endothelial cells, while at 24 h of exposure it is able to increase significantly DPP-4 activity but not its expression in microvascular endothelial cells. Metformin at 48 h in microvascular endothelial cells is able to reduce in a significant manner the activity of DPP-4 but not its expression [17]. Therefore, the modulation of DPP-4 seems to be site specific.

3. Rationale and Extraglycaemic Effects of Incretin Therapy

GLP-1 receptor agonists that resist degradation by DPP-4 and have protracted-action kinetics have been developed, and DPP-4i that slow the enzymatic cleavage of native GLP-1 provide alternative approaches to enhancing incretin-mediated glucose control.

However, GLP-1 receptor agonists and DPP-4i are premised on highly divergent mechanisms of action. DPP-4 is ubiquitously expressed in many tissues and is involved in a wide range of processes in addition to its influence on incretin hormone biological effects. GLP-1 receptor agonists provide pharmacologic levels of GLP-1 receptor stimulation, whereas DPP-4i appear to increase circulating levels of GLP-1 to within the physiologic range [18].

GLP-1 receptors are additionally expressed in extra-pancreatic tissue, having potential for the treatment of obesity and for beneficial cardio- and endothelioprotective effects. Because diabetic patients are prone to cardiovascular disease, T2DM treatment strategies should address the cardiovascular risk profile, including blood pressure, lipids,

and body weight profile, in addition to intensive glycaemic control.

Incretin-based therapies, both GLP-1 receptor agonists and DPP-4i, unlike many other antidiabetic therapies (such as sulphonylureas and insulin in particular), they do not induce weight gain. Moreover therapy with GLP-1 receptor agonists results in progressive and sustained significant weight loss in most patients [19, 20]. These agents exert physiological effect similar to those of native GLP-1, including enhancement of glucose-dependent insulin secretion and suppression of inappropriately high glucagon secretion. Furthermore, they also slow gastric emptying and reduce food intake. DPP-4i have similar actions to that of GLP-1 receptor agonists; they also enhance glucose-dependent insulin secretion and suppress glucagon, but they do not delay gastric emptying or reduce food intake. In controlled clinical trials the effect of DPP4i on weight was neutral [21].

In a recent large retrospective study exenatide twice daily was compared with other glucose-lowering agents in terms of their impact on cardiovascular events incidence. Despite the higher prevalence of coronary artery disease (CAD), obesity, hyperlipidemia, hypertension, and/or other comorbidities at baseline, exenatide-treated patients were less likely to have a CVD event than non-exenatide-treated ones (HR: 0.81, 95% CI: 0.68–0.95; $P = .01$). Furthermore, exenatide-treated patients demonstrated lower rates of CVD-related hospitalization (HR: 0.88, 95% CI: 0.79–0.98; $P = .02$) and all-cause hospitalization (HR: 0.94, 95% CI: 0.91–0.97; $P < .001$) than those not having received exenatide [22].

Emerging data suggest a cardioprotective effect of DPP-4i in humans. These drugs reduce HbA1c, although to a lesser extent than sulphonylureas, with no weight gain and low hypoglycaemic risk. The risk of cardiovascular events and all-cause death, during DPP-4i treatment, is 0.76 [0.46–1.28] and 0.78 [0.40–1.51], respectively [23]. In particular, sitagliptin administration at a single dose of 100 mg in patients with CAD and preserved left ventricular (LV) function enhanced LV response to stress, attenuated postischaemic stunning, and improved global and regional LV performance compared to placebo [24]. Encouraging results have also been published from a phase III randomized placebo-controlled trial regarding the granulocyte-colony stimulating factor- (G-CSF) based stem cell mobilization in combination with sitagliptin in patients after acute MI. During the first 6 weeks of followup, sitagliptin along with G-CSF seems to be safe and effective for myocardial regeneration representing a new therapeutic option in the future [25].

There are also data regarding the impact of GLP-1 receptor agonists and DPP-4i on endothelial function and cardiovascular diseases biomarkers. In particular, an additional effect of liraglutide on inflammatory process has been reported, since it was related to a significant reduction of high-sensitivity C-reactive protein (hsCRP) circulating levels in T2DM patients through a dose-dependent pathway [26]. Similar inhibitory effects on vascular cell adhesion molecule-1 (VCAM-1) and hsCRP have been reported for exenatide [27, 28]. Protective effects on endothelial function have been also described for sitagliptin, mainly through induction of NOS activity, and to greater extent in comparison to pioglitazone [29].

Table 1

	GLP-1 receptor agonists	DPP-4 inhibitors
Administration	Injection	Oral
GLP-1 concentration	Pharmacologic	Physiologic
Insulin Secretion	++	+
Glucagon secretion	– –	– –
Action	↑ GLP-1	↑ GLP-1 and GIP
Gastric emptying	–	±
Weight loss	++	=
β-cell protection	++*	++*
Immunogenicity	+°	–
Nausea and vomiting	+	–

* In animal model.
°Not similar for different GLP-1 receptor agonists according to their homology to native peptide.

Substantial evidence demonstrates that both GLP-1 receptor agonists and DPP-4i produce modest reductions in systolic blood pressure and, in some cases, diastolic blood pressure. The effect of the incretin hormones on serum lipids are either neutral or beneficial, with small, nonsignificant decreases in low-density lipoprotein cholesterol, increases in high-density lipoprotein cholesterol, and occasionally significant decreases in fasting triglyceride levels. Furthermore, GLP-1 receptor agonists showed positive effects on hepatic steatosis [30, 31].

T2DM is a progressive chronic disease characterized by insulin resistance and impaired beta-cell function. Treatments that prevent further beta-cell decline are therefore essential for the management of type 2 diabetes. In vitro and animal studies showed that GLP-1 promotes β-cell neogenesis and preservation and inhibits β-cell apoptosis [32]. Various human studies confirmed GLP-1 receptor agonists and DPP-4i effects in β-cell function improvement [33–35] but there is no evidence supporting β-cell proliferation and/or antiapoptosis effect in human yet.

Table 1 summarizes the differences between GLP-1 receptors agonists and DPP-4 inhibitors.

4. Clinical Use of Glucagon-Like Peptide 1 Receptor Agonists and Dipeptidyl Peptidase 4 Inhibitors

4.1. GLP-1 Receptor Agonists. GLP-1 receptor agonists have, to date, varying degrees of evidence to support their positive effects on glycated hemoglobin (HbA1c) reduction in T2DM patients.

Efficacy and safety data for exenatide were derived from three phase III Diabetes Management for Improving Glucose Outcomes (AMIGO) trials in patients with T2DM who were inadequately controlled with either a sulfonylurea, metformin, or a sulfonylurea plus metformin [36–38].

In each of these trials, twice daily exenatide was shown to reduce HbA1c significantly, by approximately 0.9% from

baseline over the 30-week trial period compared with placebo. A pooled analysis of trial data plus two 52-week extension studies, which were completed by 314 of 1446 patients in an intention-to-treat group from the 30-week randomized trials, found that the reduction in HbA1c was sustained over 2 years [39].

In T2DM patients not adequately controlled with metformin, exenatide showed noninferiority effects on HbA1c in comparison to basal insulin glargine add-on therapy, but a better beta-cell function improvement and weight profile (difference −4.6 kg, $P < .0001$) [40].

In two open-label trials, exenatide was compared with insulin treatment in patients inadequately controlled by metformin plus sulphonylurea. In the first study, 551 patients were randomized to receive glargine once daily or exenatide twice daily. After 26 weeks, HbA1c fell by 1.1% points in both groups (baseline: 8.2%). Exenatide reduced postprandial glucose excursions more than insulin glargine, while insulin glargine had a more pronounced effect on fasting plasma glucose. Body weight increased, as expected, by 1.8 kg with glargine while a 2.3 kg weight loss was obtained with exenatide. Nocturnal hypoglycaemia was less frequent with exenatide [41]. Barnett et al. confirmed these differences in T2DM not adequately controlled with metformin or sulphonylurea [42]. In the second 52-week trial, exenatide was compared with twice daily biphasic insulin aspart. Exenatide treatment resulted in HbA1c reduction similar to biphasic insulin aspart and provided better postprandial glycaemic control and weight profile (−2.5 kg versus +2.9 kg) [43].

A recent meta-analysis of placebo-controlled clinical trials assessing efficacy of incretin-based medications in T2DM patients showed that liraglutide both at 1.2 and 1.8 mg is associated with greater reductions in HbA1c in comparison to exenatide (weighted mean differences, WMD = −0.75, 95% CI = −0.83 to −0.67, $P < .001$), vildagliptin (WMD = −0.67, 95% CI = −0.83 to −0.52, $P < .001$), or sitagliptin (WMD = −0.79, 95% CI = −0.93 to −0.65, $P < .001$) [44]. In T2DM not adequately controlled with metformin, exenatide and sulphonylurea are associated to similar improvement in HbA1c levels, fasting and post prandial glycemia. However exenatide add-on therapy induced weight loss, improvement of insulin resistance indexes, and high-sensitivity C-reactive protein (Hs-CRP) reduction versus glibenclamide [45].

Liraglutide's efficacy and safety in combination with other hypoglycemic treatment has been extensively investigated in the phase III Liraglutide Effect and Action in Diabetes (LEAD) clinical development programme. Trials duration ranged from 26 to 52 weeks, and several trials have ongoing extension phases. In T2DM patients not adequately controlled with sulphonylurea, liraglutide add-on therapy resulted, in comparison to rosiglitazone, in significantly greater HbA1c reduction (treatment differences for liraglutide 1.8 mg −0.7% (95% CI 1.6; 1.1) in comparison to rosiglitazone, and −0.6% (95% CI 1.5; 1.1) for liraglutide 1.2 mg), and in a significantly better weight profile [46]. Liraglutide in addition to metformin showed a noninferior glycemic amelioration in comparison to sulphonylurea, associated to a relatively low rate of reported minor hypoglycemia (0.03–0.14 events/year) significantly less than for the glimepiride group (1.23 events/year; $P = .001$) [47]. In T2DM patients not adequately controlled with metformin and sulphonylurea, liraglutide addition was significantly associated to greater reduction in HbA1c in comparison to insulin glargine (treatment differences for liraglutide versus insulin glargine −0.24%, 95% CI −0.39, −0.08; $P = .0015$), positive weight effects (mean treatment difference of −3.43 kg (95% CI −4.00, −2.86; $P < .0001$) and waist circumference reductions (treatment difference −2.40 cm, 95% CI −3.14, −1.65; $P < .0001$) [48].

LEAD-6 study (26 week) confirmed, during liraglutide (1.8 mg) add-on therapy, a significantly greater reduction of HbA1c and fasting glycemia, less effective postprandial glucose control, and similar weight effects (liraglutide −3.24 kg versus exenatide −2.87 kg) in comparison to exenatide (10 μg) [49].

Currently, concomitant use of GLP-1 receptor agonist and insulin is not recommended by the manufacturer. Potential concerns with combination therapy include additive hypoglycemia, reduced patient adherence to another subcutaneously administered medication, and an estimated $200–300 additional monthly cost for the patient. In a retrospective analysis evaluating the effectiveness of exenatide and insulin combination ($N = 76$) for 1 year, statistically significant reductions in HbA1c (mean 0.87% reduction) and weight change (mean 5.2 kg reduction) were observed. As reported in the other clinical trials, there were significant reductions especially in bolus insulin dose, with 45% of patients discontinuing short-acting insulin and a 35% reduction in the mean daily dose of short-acting insulin [50]. These compounds were safe; in fact the most common side effects are nausea, vomiting and diarrhea reported especially at the beginning of the therapy; side effects as pancreatitis and QT prolongation during exenatide treatment are rare [18].

4.2. Dpp-4 Inhibitors. DPP-4i, also called incretin enhancers, may be used as monotherapy or in combination with other antidiabetic compounds. Sitagliptin, vildagliptin, and saxagliptin are already available in many countries, either as single agents or in fixed-dose combined formulations with metformin. Other DPP-4i, such as alogliptin and linagliptin, are currently in late phase of development.

All together gliptins have a good oral bioavailability which is not significantly influenced by food intake. Pharmacokinetic/dynamic characteristics, as sufficiently prolonged half-life and sustained DPP-4 enzyme inactivation, generally allow one single oral administration per day for the management of T2DM; the only exception is vildagliptin for which a twice daily administration is recommended because of a shorter half-life. DPP-4i are in general not substrates for cytochrome P450 (except saxagliptin that is metabolized via CYP 3A4/A5) and do not act as inducers or inhibitors of this system [51, 52]. Several metabolites have been documented but most of them are inactive; however, the main metabolite of saxagliptin also exerts a significant DPP-4 inhibition and is half as potent as the parent compound. Renal excretion is the most important elimination pathway,

except for linagliptin whose excretion in the liver appears to be predominant. PK properties of gliptins, combined with their good safety profile, explain why no dose adjustment is necessary in elderly patients or in patients with mild to moderate hepatic impairment. As far as patients with renal impairment are concerned, significant increases in drug exposure for sitagliptin and saxagliptin have been reported so that appropriate reductions in daily dosages are recommended according to estimated glomerular filtration rate. The PK characteristics of DPP-4i suggest that these compounds are not exposed to a high risk of drug-drug interactions [53]. However, the daily dose of saxagliptin should be reduced when coadministered with potent CYP 3A4 inhibitors. The DPP-4i improve glycaemic control, reducing both fasting and postprandial glucose levels to lower HbA1c levels, without weight gain and with an apparently good adverse event profile. At present, there seems to be little to distinguish between the different inhibitors in terms of their efficacy as antidiabetic agents and their safety. In particular, vildagliptin monotherapy resulted in improved glycemic control in drug-naïve patients with type 2 diabetes [54]. Although the hypothesis of noninferiority to gliclazide was not borne out statistically, the reductions in HbA1c were similar over a two-year period and vildagliptin had significant benefits in terms of less weight gain and less hypoglycemia. Vildagliptin improves islet function in T2DM under fasting conditions [55]. This suggests that DPP-4 inhibition has metabolic benefits in addition to enhancing meal-induced GLP-1 and GIP activity. The weight neutrality of vildagliptin likely results in part from its intrinsically low risk for hypoglycemia [56]. Recent studies point to additional potential mechanisms. One study found that drug-naïve patients randomized to vildagliptin exhibited significantly lower chylomicron lipid and apolipoprotein levels than placebo patients, suggesting that vildagliptin may inhibit intestinal fat extraction. A meta-analysis indicates that vildagliptin was not associated with increased risk of hepatic events or hepatic enzyme elevations indicative of drug-induced liver injury, pancreatitis, infections, or skin-related toxicity [57]. The safety of vildagliptin in renal transplant patients has been recently assessed [58, 59].

Sitagliptin monotherapy improved glycemic control compared to placebo and was generally well tolerated in patients with type 2 diabetes. The glycemic response to treatment with sitagliptin 100 mg/day was similar between 100-mg once daily and 50-mg twice daily dose regimens [60]. Recent trials have shown that the combinations offer additive efficacy in reducing blood glucose when given as initial antidiabetic therapy and as add-on therapy when pioglitazone alone fails to maintain glycemic control. The combination of sitagliptin and pioglitazone was well tolerated and would appear to be suited to a fixed-dose single-tablet combination for once daily administration [61].

Preclinical and clinical trial data with sitagliptin to date do not indicate an increased risk of pancreatitis [62].

Saxagliptin, 5 mg once daily, has been shown to be effective in patients with type 2 diabetes treated with diet alone, metformin, sulfonylurea, or glitazone, with a favourable tolerance profile. Reduction in HbA1c levels averaged 0.6−0.8%, without increasing the risk of hypoglycaemia or promoting weight gain [63]. In clinical trials, saxagliptin as monotherapy or in combination with metformin, a sulphonylurea (glyburide), or thiazolidinedione (pioglitazone or rosiglitazone) significantly improved glycemic control without increasing hypoglycaemic events and weight versus comparator [64–68]. Furthermore, recent clinical data supported the efficacy and favorable tolerability profile of a reduced daily dose of saxagliptin (2.5 mg) in T2DM patients with a significant renal impairment [69]. In conclusion, DPP-4i have emerged as alternatives to sulfonylureas, commonly used as add-on to metformin in treatment of T2DM. They showed similar efficacy to sulfonylureas but with lower risk of hypoglycemia and reduction or no change in body weight, and if confirmed in humans, they may preserve islet function thereby minimizing the risk for secondary failure. Their limitation at present is the lack of long-term experience on durability and safety [21].

5. Future Perspective

Few head-to-head clinical trials comparing different incretin-based drugs as add-on treatment are performed. Scheen et al. showed similar efficacy on glycaemia and tolerability between saxagliptin and sitagliptin added to metformin in T2DM patients inadequately controlled [70]. Liraglutide provided significant better improvement in glycemic control, especially in fasting condition, in comparison to exenatide twice daily [49]. Exenatide once weekly was associated with significant greater HbA1c reduction (−1.5%, 95% CI −1.7 to −1.4 versus −0.9%, −1.1 to −0.7) and weight loss (−2.3 kg, 95% CI −2.9 to −1.7 versus −1.5 kg, 95% CI −2.4 to −0.7) in comparison to sitagliptin as add-on treatment to metformin [71].

The profile of action of GLP-1 receptor agonists and DPP-4 inhibitors suggests the possibility of an actual reduction in cardiovascular risk, which needs to be confirmed by large long-term clinical trials, and beta cell protection, to date only proven in animal models [72]. The use of incretins is suggested very early in the type 2 diabetic history, but some studies suggest a potential use of them also in obese patients and in prediabetic patients [73, 74]. Moreover, recently liraglutide efficacy was evaluated in the management of obese patients. This treatment was well tolerated during 20 weeks and associated to a significant higher weight loss in comparison to placebo and orlistat [73].

At present, GLP-1 receptor agonists need to be administered by subcutaneous injection once or twice daily. Several once weekly GLP-1 receptor agonists are in phase 3 clinical trial development as exenatide once weekly, taspoglutide, albiglutide, LY2189265, and CJC-1134-PC. A greater reduction in HbA1c and fasting plasma glucose was found with the once weekly GLP-1 receptor agonists compared with exenatide BID, while the effect on postprandial hyperglycemia was modest. The reduction in HbA1c was in most studies greater compared to oral antidiabetic drugs and insulin glargine. The reduction in weight did not differ between the short- and long-acting agonists. The gastrointestinal side

effects were less with the once weekly agonists compared with exenatide BID, except for taspoglutide. Antibodies seem to be most frequent with exenatide once weekly, while hypersensitivity has been described in few patients treated with taspoglutide. The development of taspoglutide has been stopped because of safety problems. Injection site reactions differ among the long-acting GLP-1 receptor agonists and are observed more frequently than with exenatide BID and liraglutide. The once weekly GLP-1 receptor agonists are promising candidates for the treatment of type 2 diabetes although their efficacy may not be superior to once daily analogue liraglutide [75].

Hyperglycemia plays a major role in the development of the microvascular and macrovascular complications of diabetes. With the exception of metformin, traditional agents used for the treatment of type 2 diabetes are able to improve glycemia, but their use is often limited by treatment-associated side effects, including hypoglycemia, weight gain, and edema and they do not have any positive effect on beta-cell mass or function. The introduction of incretin hormone-based therapies represents a novel therapeutic strategy, because these drugs not only improve glycemia with minimal risk of hypoglycemia but also have other extraglycemic beneficial effects. In clinical studies, both exenatide and liraglutide, such as sitagliptin, vildagliptin, and saxagliptin, improve β cell function and glycemia with minimal hypoglycemia. All these agents have trophic effects on beta-cell mass in animal studies. The use of these agents is also associated with reduced or neutral effect on body weight and improvements in blood pressure, diabetic dyslipidemia, hepatic steatosis markers, and myocardial function. These effects have the potential to reduce the burden of cardiovascular disease, which is a major cause of mortality in patients with diabetes.

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Review Article

Glucagon-Like Peptide-1, Diabetes, and Cognitive Decline: Possible Pathophysiological Links and Therapeutic Opportunities

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Metabolic and neurodegenerative disorders have a growing prevalence in Western countries. Available epidemiologic and neurobiological evidences support the existence of a pathophysiological link between these conditions. Glucagon-like peptide 1 (GLP-1), whose activity is reduced in insulin resistance, has been implicated in central nervous system function, including cognition, synaptic plasticity, and neurogenesis. We review the experimental researches suggesting that GLP-1 dysfunction might be a mediating factor between Type 2 diabetes mellitus (T2DM) and neurodegeneration. Drug treatments enhancing GLP-1 activity hold out hope for treatment and prevention of Alzheimer's disease (AD) and cognitive decline.

1. Insulin Resistance and Cognitive Decline

During the last years, Alzheimer's disease (AD) and clinical syndromes associated to insulin resistance have shown an ever-increasing prevalence in Western countries. These conditions pose a great threat to present and future population's health and represent two of the main causes of disability and health expenditures. Several research lines during the last decade have suggested an association among Type 2 diabetes mellitus (T2DM), insulin resistance, and cognitive decline, both in cross-sectional and in longitudinal studies.

Cross-sectional studies have found that older subjects with T2DM on average show a poorer cognitive performance than age-matched controls [1]. This association seems independent of other vascular risk factors and is attributable not only to a greater extent of white matter lesions but also to a more severe cortical atrophy [2], especially in temporomesial areas (hippocampus, amygdala) [3]. Moreover insulin resistance is associated to a worse cognitive performance in nondiabetic subjects too [4]. On the other hand cross-sectional studies have observed a significant association of dementia, AD in particular, with T2DM [5] and insulin resistance [6].

Also several longitudinal studies have observed an association of T2DM with dementia risk over years [7]. Moreover it has been observed that older nondiabetic subjects with metabolic syndrome and increased level of inflammatory markers have an increased risk of subsequent cognitive decline [8]. Recently published data have shown that, among nondiabetic nondemented older subjects, insulin resistance is associated with AD incidence after a few years [9]. In keeping with this observation, insulin resistance has been associated recently with a greater extent of AD-like neuropathology at autopsy [10]. Therefore it is plausible that, among older subjects with asymptomatic AD, a coexistent

impairment of insulin metabolism can hasten symptoms expression.

This association might be linked to different biological mechanisms, first of all the presence of brain insulin resistance. In fact brain insulin receptor activity might have several neuroprotective effects, via PI3K (phosphatidylinositol-3-kinase)/Akt and ERK1/2 signalling pathways [11, 12]: decreased inflammation and apoptosis, increased synaptic plasticity, and inhibition of glycogen synthase kinase-(GSK-)3, with subsequent decreased tau phosphorylation, which is a hallmark of AD neuropathology. It has been shown in vitro that insulin receptor activation is able to decrease synaptic binding sites through which amyloid oligomers produce their toxic activity, with resulting reduction of oxidative stress and dendritic spines loss [13]. Moreover postmortem analyses of AD patients brain have shown an impairment of insulin and IGF-1 receptors signalling, especially evident in neurons with neurofibrillary tangles, suggesting that degenerating neurons are resistant to insulin/IGF-1 action [14]. Some authors have even proposed the existence of a "Type 3 diabetes mellitus", limited to central nervous system (CNS), as a cause for AD, as they were able to produce an AD-like neurodegeneration in a mouse model after intracerebroventricular injection of streptozotocin, inducing a depletion of CSF insulin without any change in peripheral insulin metabolism [15]. This hypothesis is supported by a pilot study, which has shown a significant cognitive improvement after intranasal insulin, without change of peripheral glucose metabolism [16].

On the other hand, experimental data have associated peripheral insulin resistance with reduced insulin activity inside the CNS, due to a reduced hormone transport through the blood-brain barrier [17], and with increased brain Aβ production in murine models of AD [18]. Moreover in studies of normal subjects with euglycemic clamp, the infusion of high insulin doses, mimicking insulin resistance, raises Aβ-42 levels, probably due to a reduced catabolism, and CNS inflammatory markers [19].

2. Metabolic Effects of GLP-1, T2DM, and the "Gut-to-Brain" Axis

Glucagon-like peptide-1 (GLP-1), a member of the incretins family, is a 30-aminoacid peptide, which is derived from preproglucagon molecule and is secreted by intestinal endocrine epithelial L-cells. It is the most potent stimulator of oral glucose-induced insulin secretion, it is released in response to meal intake and is rapidly metabolized and inactivated by dipeptidyl-peptidase-4 [20]. GLP-1 transmembrane receptor (GLP-1R) is a G-protein-coupled receptor and is expressed not only in pancreatic islets, but also in gastrointestinal tract, kidney, lung, vascular system, heart, and brain [21].

GLP-1R activation stimulates adenylate cyclase, with formation of cyclic adenosine monophosphate (cAMP) and subsequent phosphorylation of protein kinase A; moreover it activates PI3-kinase pathway, with downstream activation of Akt kinase, MAP-kinases, and src-kinases [22, 23]. Via these pathways, GLP-1 stimulates pancreatic β-cells, activating insulin secretion and inducing insulin gene expression [21]; moreover it has been shown that GLP-1 stimulates proliferation and differentiation, and reduces apoptosis of β-cells [23]. It seems interesting that, at least in pancreatic islets, GLP-1 activity seems synergic with insulin action in promoting β-cell survival [24].

Beyond its main activity, GLP-1 reduces plasma glucagon, inhibits gastrointestinal motility, and promotes satiety, reducing food intake [21]. Moreover it has a wide range of functions on glucose metabolism and cardiovascular system. In fact it improves insulin sensitivity, reduces appetite, modulates heart rate and blood pressure, reduces vascular tone, ameliorates endothelial function, and increases myocardial contractility, with preliminary data suggesting clinical benefit in heart failure [25].

It has been known for many years that T2DM is characterized by a severely reduced incretin effect, defined as the difference between insulin responses to oral and intravenous glucose administration [26]. Reduced GLP-1 levels have been observed after a mixed meal in Type 2 diabetes compared with controls [27], with a marked reduction especially of the late-phase response [28]. Moreover an altered GLP-1 response both to mixed meal [29] and to oral glucose load [30] has been observed in insulin resistance.

It has been proven that at least part of the metabolic effect of GLP-1 is mediated by CNS [31]. In fact brain GLP-1R are partly responsible not only for food intake control, but also for control of glucose homeostasis, with coordinate actions on pancreas and liver [32]. It has been observed in mice that GLP-1 secreted into the hepatoportal vein increases the firing rate of the vagus nerve, sending signals to the brainstem nucleus of the tractus solitarius, which on the other hand releases GLP-1 in hypothalamic regions, inducing reflex insulin secretion and muscle glycogen synthesis [33]. These data support the existence of a "gut-to-brain axis", with a central role of GLP-1 released both by intestinal cells and by neurons, involved in the regulation of systemic glucose metabolism, whose activity seems to be blunted in high-fat fed, insulin-resistant mice [33].

On the other hand, it has been hypothesized that GLP-1 can influence brain metabolism. In fact a small human study with FDG-PET (positron emission tomography with 18-fluorodeoxyglucose) has shown a possible effect of GLP-1 on brain glucose metabolism. In this study GLP-1 infusion in normoglycemic conditions reduced glucose transport across blood-brain barrier in specific brain areas while a trend of decrease of cerebral metabolic rate was also observed, thus maintaining brain glucose concentration unchanged. This observation leads the authors to hypothesize that GLP-1 may exert a neuroprotective effect by limiting intracerebral glucose fluctuation in postprandial periods, when plasma glucose is increased [34].

3. GLP-1, Neuroprotection, and Alzheimer's Disease

Beyond its metabolic role, several studies have clarified a role of GLP-1 in CNS function. Experimental studies have identified a widespread expression of GLP-1R across a large number of rat brain regions, not directly involved

in metabolic control, including hippocampus, thalamus, striatum, substantia nigra, amygdala, nucleus basalis Meynert, subventricular zone, and temporal cortex [35]. GLP-1R expression has been observed in specific cellular subtypes which are crucial for memory and learning functions, including pyramidal neurons of CA region and granule cells of dentate gyrus in hippocampus, and in large neocortical neurons [36]. Other authors have observed GLP-1R expression also on glial cells (microglia and astrocytes), proposing a role for them as modulators of CNS inflammation [37].

The neurotrophic effect of GLP-1R has been strongly suggested by studies of mice knockout (KO) for GLP-1R, which show an impairment of contextual memory, as assessed by the passive avoidance test, which measures the ability of the animal to learn and remember that an instinctive behavior causes a punishment. Memory impairment of KO mice was reversible after GLP1-R gene DNA transfer with a viral vector [38]. These data were confirmed in a subsequent study of cognitive functions in a GLP-1R KO mouse model: a reduced recognition memory and spatial memory has been shown while other behavioural parameters, including exploration and anxiety, were unchanged. Interestingly a neurophysiological study of hippocampus CA1 area mice showed a severe impairment of long-term potentiation, which is the synaptic process associated to consolidation of long-term memory [39].

Adding to these observations, it has been demonstrated that GLP-1 analogues, which have greater metabolic stability than the native molecule, also cross blood-brain barrier when administered peripherally [40]. As only small amounts of native GLP-1 reach CNS if peripherally administered, due to rapid catabolism, much of the pharmacologic research has focused on analogues of the molecule, which are more resistant to degradation, while retaining the stimulatory effect on GLP-1R.

Several experimental evidences have demonstrated a neuroprotective role for GLP-1 and its analogues. In cultured rat pheochromocytoma cells, some authors observed that GLP-1 and exendin-4, (*Ex-4*) a long-acting GLP-1 analogue, stimulated neurite outgrowth in a similar fashion to nerve-growth-factor (NGF). Besides, *Ex-4* was able to augment NGF-induced neuronal differentiation, and apparently attenuated neural degeneration following NGF withdrawal [41]. Other authors confirmed these data on cultured neural cells, finding that GLP-1 exposure protected cells from death promoted by NGF deprivation, by suppressing the proapoptotic protein Bim (Bcl-2 interacting mediator of cell death) [42].

Part of the neuroprotective effect of GLP-1R agonists is probably related to reduced neuronal damage due to amyloid metabolism. In fact *Ex-4* has been shown to reduce the synthesis of amyloidogenic Aβ fragment and to protect cells from β-amyloid toxicity in cultured neural cells [43]. Moreover intracerebroventricular injection of GLP-1 or *Ex-4* has been shown to decrease levels of brain amyloid fragment in control mice [44].

The efficacy of peripherally administered GLP-1 analogues has been shown also in experimental models. In normal adult rats *Ex-4* improves hippocampus-based cognitive performance, namely, spatial learning and working memory, as assessed by the "radial arm maze," which allows the measurement of the time necessary for the animal to find food, placed at the end of several equidistantly spaced arms, which radiate form a central platform [45]. In the same paper the repeated administration of *Ex-4* was effective in ameliorating mood and reducing hopelessness, as measured by the immobility time in the "forced swim test," during which animals are forced to swim in a cylinder filled with water, from which they cannot escape [45].

The previously mentioned behavioural effects are paralleled by several histochemical changes observed "ex vivo." Intraperitoneal administration of *Ex-4* has increased both the number of proliferating cells and the expression of neuronal differentiation markers in adult rat hippocampus and in subventricular zone [45, 46].

A neuroprotective effect of *Ex-4* has been observed recently in experimental models of neurodegeneration. In the triple transgenic AD-mouse, which is an experimental model of the human disease, the induction of diabetes with streptozotocin was associated with an increase of β-amyloid brain load, consistently with evidence linking T2DM with AD neuropathology, and subcutaneous administration of *Ex-4* prevented this increase [43]. The results of this study suggests that *Ex-4* may have a therapeutic role in AD, alone or with T2DM. Moreover in an animal model of Parkinson's disease, in which *Ex-4* was able to increase the number of dopaminergic neurons in the substantia nigra, a contemporary reduction of extrapyramidal signs was observed [46].

Another long-acting GLP-1 analogue used for T2DM, liraglutide (*Lir*), is able to cross the blood-brain barrier [47], and has shown neuroprotective effects in experimental models. This is also the case for other GLP-1 mimetics, Asp(7)GLP-1, N-glyc-GLP-1, and Pro(9)GLP-1, which, like *Lir*, are able to increase synaptic plasticity, measured as long-term potentation in CA1 hippocampal region of rats [47].

Both *Ex-4* and *Lir* were recently tested for their neuroprotective effect in mouse models of T2DM. In this study GLP-1 analogues were injected subcutaneously to three mouse models of diabetes (ob/ob mice, db/db mice, and high-fat-diet-fed mice). At the histochemical analysis a greater number of proliferating neurons in hippocampal dentate gyrus was found in diabetic mice compared with nondiabetic controls, and this number was further enhanced by both drugs [48]. The increased neurogenesis in T2DM models was interpreted by the authors as a response to increased brain cell death which is associated with the disease; this compensatory process would be supported by GLP-1 mimetics. This interpretation is supported by another paper published by the same authors, regarding the cognitive effect of *Lir* in the mouse model of high-fat-diet-induced obesity. In parallel with metabolic changes (weight loss, increased glucose tolerance), mice treated with *Lir* subcutaneous injections showed an improvement of learning and memory ability, assessed with "object recognition test." The test measures the extent of exploratory activity of a previously presented object, which is expected to be lower in comparison with newly presented objects, and is therefore

considered a measure of recognition memory. Furthermore, *Lir* reduced negative effects of high-fat diet on hippocampal long-term potentation [49].

Another analogue of GLP-1, Val(8)-GLP-1(7-36), was studied in rats, and its intracerebroventricular injection reversed the impairment of spatial memory induced by injection of β-amyloid fragment Aβ1–40. Moreover pretreatment with Val8-glucagon-like peptide-1 prevented the impairment of hippocampal long-term potentiation that is induced by the presence of Aβ1–40 [50]. These data are consistent with a different research, performed with the same molecule, on AD-like mice with a double mutation of amyloid precursor protein (APP) and presenilin 1 (PS1). A beneficial effect was observed on long-term potentiation, both in young (9 months) and in older animals (18 months) while β-amyloid plaques and inflammatory microglia activation was unchanged in treated animals [51]. These data support the hypothesis that GLP-1R agonists might partly prevent toxic effect of β-amyloid deposition, with obvious interest for possible AD treatment.

With the background of the previously discussed preclinical data, *Ex-4* is now being studied as a treatment for AD and PD in Phase 2 studies (see http://www.clinicaltrials.gov/, NCT01255163 and NCT01174810). Of notice, the hypoglycemic effect of GLP-1 analogues in normoglycemic subjects seems minimal [52, 53] and should not constitute a major concern for the treatment of nondiabetic subjects.

4. Conclusions

The available evidences strongly support the hypothesis that the observed association between insulin resistance/T2DM and cognitive decline/dementia is mediated not only by well-known vascular changes, but also by direct neurotoxic effect of glucose metabolism impairment.

Incretin activity, and GLP-1 in particular, which is reduced in insulin resistance conditions, represents a possible pathophysiological link between metabolism disorders and neurodegeneration. The reduction of GLP-1 levels, characteristic of T2DM, might be associated to a reduced neuroprotection, which seems particularly relevant for hippocampal regions [39], where AD neuropathology is most evident. It is tempting to speculate that GLP-1 and insulin have synergistic activity in promoting neuron survival, as it has been shown in pancreatic β-cells, both for native GLP-1 [24] and for GLP-1 synthetic analogues [54, 55]. Human studies evaluating the association between GLP-1 levels and cognitive function, controlling for insulin resistance status, are needed to support the hypothesis of a direct neuroprotective effect of incretins.

GLP-1 analogues has been shown to enhance cognitive function in control animals [38, 45], to prevent cognitive impairment in models of T2DM [48, 49], and to counteract β-amyloid toxicity in models of AD [43, 51]. These experimental data support the effort of testing such molecules both for the prevention of cognitive decline in T2DM and for treatment of AD patients in randomized controlled trials.

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Research Article

Glucagon-Like Peptide-1 Receptor Agonists and Cardiovascular Events: A Meta-Analysis of Randomized Clinical Trials

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Objective. Data from randomized clinical trials with metabolic outcomes can be used to address concerns about potential issues of cardiovascular safety for newer drugs for type 2 diabetes. This meta-analysis was designed to assess cardiovascular safety of GLP-1 receptor agonists. *Design and Methods*. MEDLINE, Embase, and Cochrane databases were searched for randomized trials of GLP-1 receptor agonists (versus placebo or other comparators) with a duration ≥12 weeks, performed in type 2 diabetic patients. Mantel-Haenszel odds ratio with 95% confidence interval (MH-OR) was calculated for major cardiovascular events (MACE), on an intention-to-treat basis, excluding trials with zero events. *Results*. Out of 36 trials, 20 reported at least one MACE. The MH-OR for all GLP-1 receptor agonists was 0.74 (0.50–1.08), $P = .12$ (0.85 (0.50–1.45), $P = .55$, and 0.69 (0.40–1.22), $P = .20$, for exenatide and liraglutide, resp.). Corresponding figures for placebo-controlled and active comparator studies were 0.46 (0.25–0.83), $P = .009$, and 1.05 (0.63–1.76), $P = .84$, respectively. *Conclusions*. To date, results of randomized trials do not suggest any detrimental effect of GLP-1 receptor agonists on cardiovascular events. Specifically designed longer-term trials are needed to verify the possibility of a beneficial effect.

1. Introduction

Cardiovascular safety is a growing concern for drugs used for chronic conditions, such as diabetes. Among glucose-lowering agents, sulfonylureas [1, 2], insulin [3, 4], and thiazolidinediones [5–7], have been suspected of adverse cardiovascular effects, although some of those preoccupations have not been confirmed [8–11]. Following these concerns, the Food and Drug Administration issued a guidance for companies submitting new chemical entities as treatments for type 2 diabetes, requiring that, either in phase II-III trials, or in a subsequent phase IV specifically designed randomized clinical trial, a sufficient amount of information is collected so as to exclude a risk increase of over 30% (i.e., the upper limit—two-sided—of 95% confidence interval for major cardiovascular events, in comparison with placebo and/or other treatments, should not exceed 1.30; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM071627.pdf).

Two GLP-1 receptor agonists (exenatide and liraglutide) have been approved for human use, and several others are currently under clinical development. It has been observed that chronic stimulation of GLP-1 receptors could produce beneficial effects on several cardiovascular risk factors [12]; furthermore, preliminary data on humans suggest that GLP-1 could have direct effects on myocardial function [13]. However, no major trial assessing the effects of GLP-1 receptor agonists on cardiovascular morbidity and mortality is available to date, nor will it be for a few years. In the meantime, the information on incident cases recorded as adverse

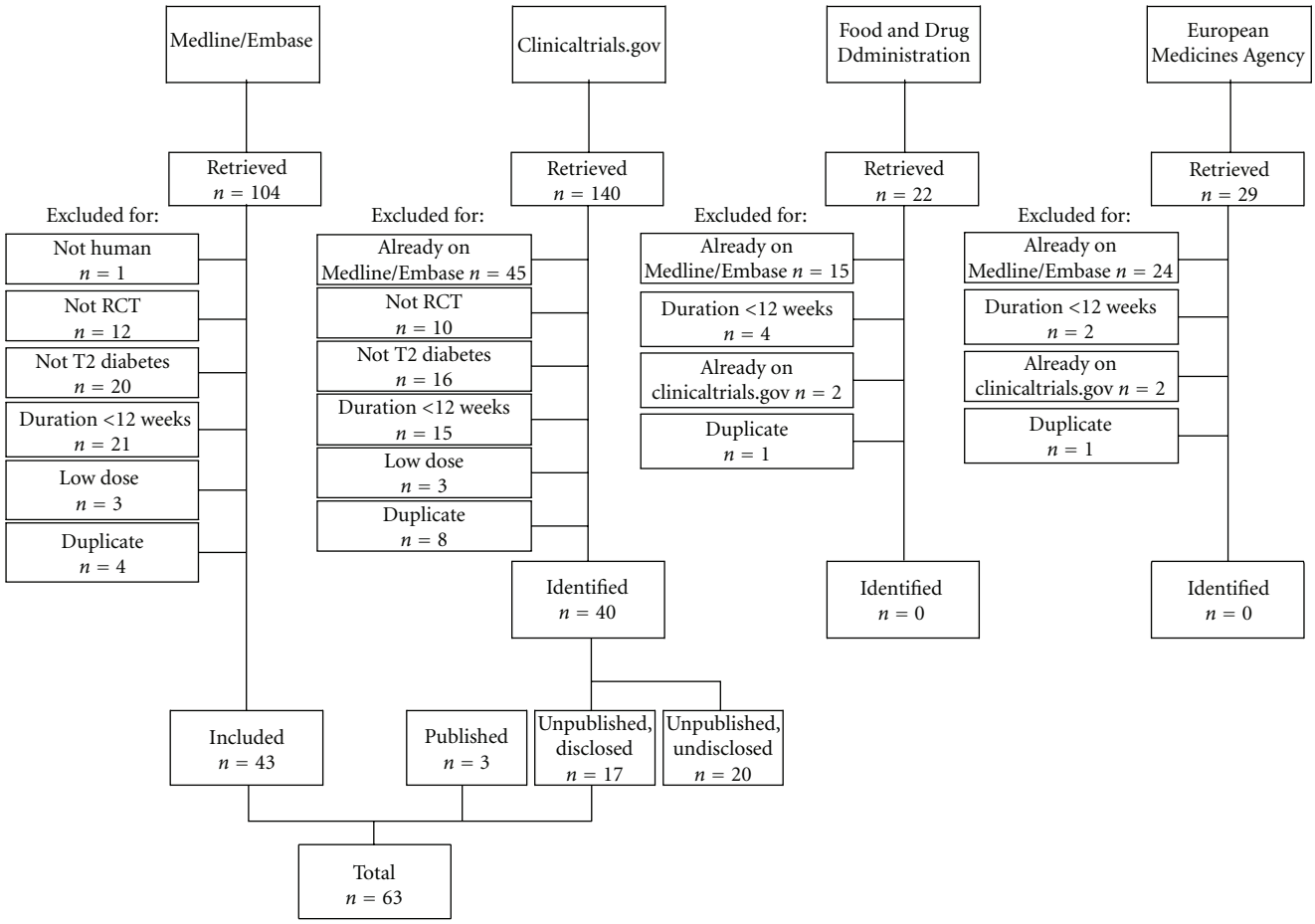

Figure 1: Trial flow diagram. RCT: randomized clinical trial; T2: type 2.

events during trials designed for metabolic endpoints could provide some hints on the possible cardiovascular profile of these drugs. This meta-analysis was designed to assess the effect of GLP-1 receptor agonists, compared with placebo or active hypoglycemic drugs, on major cardiovascular events in type 2 diabetic patients, as derived from randomized controlled trials.

2. Research Design and Methods

2.1. Data Sources and Searches. An extensive Medline, Embase, and Cochrane database search for "exenatide," "liraglutide," "albiglutide," "taspoglutide," "lixisenatide," and "semaglutide" was performed, collecting all randomized clinical trials on humans up to November 1th, 2010. The identification of relevant abstracts, the selection of studies based on the criteria described above, and the subsequent data extraction were performed independently by two of the authors (E. Mannucci and M. Monami), and conflicts resolved by the third investigator (N. Marchionni). Completed but still unpublished trials were identified through a search of http://www.clinicaltrials.gov/ website. Food and Drug Administration (FDA, http://www.fda.gov/) and European Medicines Agency (EMEA, http://www.ema.europa.eu/) reviews of approved drugs, as well as published information provided to FDA in response to queries during the approval process, were also searched for retrieval of unpublished trials.

2.2. Study Selection. A meta-analysis was performed including all randomized clinical trials with a duration of at least 12 weeks, either with a cross-over or a parallel series design, enrolling patients with type 2 diabetes, comparing glucagon-like peptide-1 (GLP-1) receptor agonists with placebo or active drugs (oral hypoglycemic agents and/or insulin) of other classes. Trials enrolling nondiabetic, or type 1 diabetic, subjects were also excluded. No review protocol was published elsewhere.

2.3. Data Extraction and Quality Assessment. Results of unpublished trials (characteristics of patients enrolled, treatments, and major cardiovascular events) were retrieved, if available, on http://www.clinicaltrials.gov/, http://www.novonordisk-trials.com/website/content/trial-results.aspx, http://www.lillytrials.com/results/results.html, or http://www.clinicalstudyresults.org/; Food and Drug Administration (FDA, http://www.fda.gov/) and European Medicines Agency (EMEA, http://www.ema.europa.eu/) reviews of approved drugs, as well as published information provided to FDA in

Table 1: Characteristics of the unpublished and undisclosed studies.

Study	Number of patients planned	Comparator	Add-on to	Trial duration (wks)	Design	Study end date*	Sponsor
Exenatide							
NCT00434954	488	Aspart	Metformin	26	PS, DB	August 2009	Amylin
Liraglutide							
NCT00696657	415	Placebo	None	12	PS, DB	February 2009	Novo
Taspoglutide							
NCT00809705	60	Placebo	None	12	PS, DB	February 2010	Hoff-Roche

PS: parallel series; DB: double blind; Hoff. Roche: Hoffman-La Roche; Novo: Novo Nordisk.

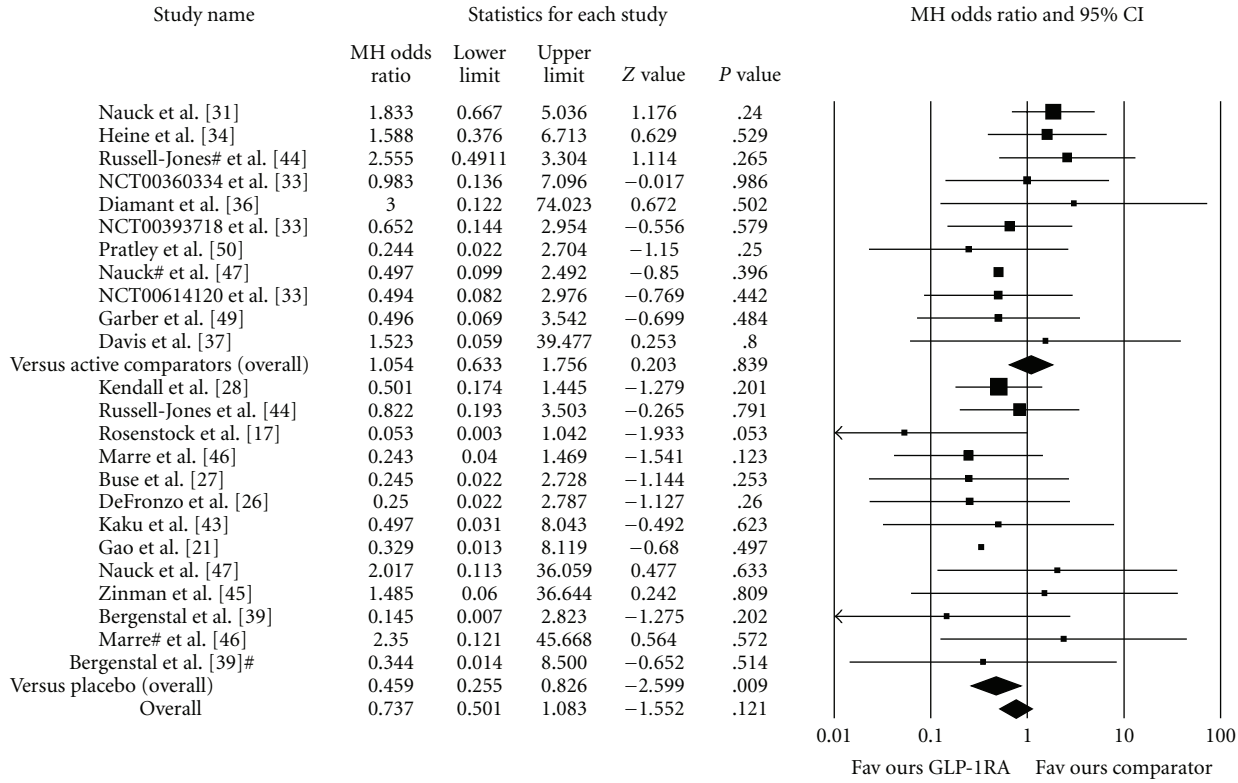

Figure 2: Effect of GLP-1 receptor agonists on fatal and nonfatal major cardiovascular events (MACE). Forest plot of individual studies. GLP-1 RA: glucagon-like peptide-1 receptor agonists. #Studies with multiple comparators.

response to queries during the approval process, were also searched for retrieval of unpublished information. All those sources were also used to complete information on results of published trials, when not reported in publications. For all published trials, results reported in papers were used as the primary source of information, when available.

The quality of trials was assessed using some of the parameters proposed by Jadad et al. [14]. The score was not used as a criterion for the selection of trials whereas some items were used only for descriptive purposes.

2.4. Data Synthesis and Analysis. The principal outcome was the effect of GLP-1 receptor agonists, compared with other hypoglycemic agents or placebo, on major cardiovascular events (MACE) as defined in the list provided by FDA for this purpose (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/EndocrinologicandMetabolicDrugsAdvisoryCommittee/UCM148659.pdf), including cardiovascular death, nonfatal myocardial infarction and stroke, and hospitalizations due to acute coronary syndromes and/or heart failure.

Predefined separate analyses were performed for trials with different GLP-1 receptor agonists, whenever possible.

Mantel-Haenszel odds ratio with 95% confidence interval (MH-OR) was calculated for each of the events defined above, on an intention-to-treat basis, excluding trials with

Table 2: Characteristics of the studies included in the meta-analysis.

Study* (ref.)	NCT/FDA-reference	Add-on to	Description of randomization	Description of allocation	Description of blinding	Reporting of drop-out	Intention-to-treat
Albiglutide versus placebo							
Rosenstock et al. [19]	NCT00518115	None/Metf.	NA	NA	A	A	Yes
Exenatide versus placebo							
Gill et al. [20]	NCT00516074	Metf./TZD	NA	NA	A	A	Yes
Kadowaki et al. [21]	NCT00382239	Sulfonylurea	A	NA	A	A	Yes
Zinman et al. [22]	NCT00099320	TZD	A	A	A	A	Yes
Gao et al. [23]	NCT00324363	SU + Metf.	A	NA	A	A	Yes
DeFronzo et al. [24]	NCT00135330	Rosiglitazone	A	NA	OL	A	Yes
Apovian et al. [25]	NR	Multiple	A	A	A	A	Yes
Moretto et al. [26]	NCT00381342	None	A	A	A	A	Yes
Liutkus et al. [27]	NR	Metf/TZD + Met	A	A	A	A	Yes
DeFronzo et al. [28]	NCT00039013	Metformin	A	NA	A	A	Yes
Buse et al. [29]	NCT00039026	Sulfonylurea	A	NA	A	A	Yes
Kendall et al. [30]	NCT00035984	SU + Metf.	NA	NA	A	A	Yes
Exenatide versus rosiglitazone							
DeFronzo# et al. [24]	NCT00135330	None	A	NA	OL	A	Yes
Exenatide versus glibenclamide							
Derosa et al. [31]	NCT00135330	None	A	NA	OL	A	Yes
Exenatide versus BiAsp 30/70							
Bergenstal et al. [32]	NCT00097877	SU + Metf.	A	A	OL	A	Yes
Nauck et al. [33]	NCT00082407	SU + Metf.	A	A	OL	A	Yes
Exenatide versus glargine							
Barnett et al. [34]	NCT00099619	SU + Metf.	A	A	OL	A	Yes
NCT00360334 [35]	NCT00360334	OAD	NR	NR	OL	A	Yes
Heine et al. [36]	NCT00082381	SU + Metf.	A	A	OL	A	Yes
Bunck et al. [37]	NCT00097500	Metformin	A	NA	OL	A	Yes
Diamant et al. [38]	NCT00641056	SU + Metf./Metf	A	A	OL	A	Yes
Exenatide versus insulin							
Davis et al. [39]	NCT00099333	SU/Metf.	NA	NA	OL	A	Yes
Exenatide LAR versus placebo							
Kim et al. [40]	NCT00103935	Metf./None	A	A	A	A	Yes
Exenatide LAR versus pioglitazone							
Bergenstal et al. [41]	NCT00637273	None	A	A	A	A	Yes
Exenatide LAR versus sitagliptin							
Bergenstal et al. [41]#	NCT00637273	None	A	A	A	A	Yes
Liraglutide versus placebo							
Madsbad et al. [42]	FDA_1310	None	NA	NA	A	A	Yes
Vilsbøll [43]	NCT00154401	None	NA	NA	A	A	Yes
Seino et al. [44]	FDA_1334	None	A	A	A	A	Yes
Kaku et al. [45]	NCT00395746	Sulfonylurea	NA	NA	NA	NA	Yes
Russell-Jones et al. [46]	NCT00331851	SU + Metf.	A	A	A	A	Yes
Zinman et al. [47]	NCT00333151	Metf. + TZD	A	A	A	A	Yes
Marre et al. [48]	NCT00318422	Sulfonylurea	NA	NA	A	A	Yes
Nauck et al. [49]	NCT00318461	Metformin	A	A	A	A	Yes

Table 2: Continued.

Study* (ref.)	NCT/FDA-reference	Add-on to	Description of randomization	Description of allocation	Description of blinding	Reporting of drop-out	Intention-to-treat
Liraglutide versus metformin							
Feinglos et al. [50]	NR	None	NA	NA	NA	A	No
Liraglutide versus rosiglitazone							
Marre# et al. [48]	NCT00318422	Sulfonylurea	NA	NA	A	A	Yes
Liraglutide versus glimepiride							
Madsbad# et al. [42]	NR	None	NA	NA	OL	A	Yes
NCT00614120 [35]	NCT00614120	Metformin	NR	NR	OL	NR	NR
Nauck# et al. [49]	NCT00318461	Metformin	A	A	OL	A	Yes
Garber et al. [51]	NCT00294723	None	A	A	OL	A	Yes
Liraglutide versus glibenclamide							
NCT00393718 [35]	NCT00393718	None	NR	NR	OL	NR	NR
Liraglutide versus sitagliptin							
Pratley et al. [52]	NCT00700817	None	A	A	OL	A	Yes
Liraglutide versus glargine							
Russell-Jones# et al. [46]	NCT00331851	SU + Metf.	A	A	A	A	Yes

*All the studies are multicenter and designed as parallel series, with the exception of NCT00099619 which is a cross-over trial; #studies with multiple comparators. Metf.: metformin; NA: not adequate or not adequately reported; A: adequate; TZD: thiazolidinediones; TZD + Met.: thiazolidinediones + metformin; SU + Metf.: sulfonylureas and metformin; OL: open-label; OAD: oral antidiabetic drugs; NR: not reported; SU/Metf: sulfonylureas or metformin; LAR: long-acting release.

zero events. A random effect model was used because of the impossibility of a reliable assessment of heterogeneity, due to the small number of events in each trial [15]. Publication bias was not assessed, considering that the small number of adverse cardiovascular events in each study was irrelevant for the decision to publish trials with metabolic endpoints. The main expected bias is represented by the fact that the trials included were designed for noncardiovascular (metabolic) endpoint; this means that cardiovascular events were reported only as adverse events, without any systematic screening or predefined diagnostic criteria. The meta-analysis was reported following the PRISMA checklist [16]. All analyses were performed using Comprehensive Meta-analysis Version 2, Biostat (Englewood, NJ, USA) and SPSS 16.0.

This research was performed independently of any funding, as part of the institutional activity of the investigators.

3. Results

The trial flow is summarized in Figure 1. A total of 36 trials, 3 of which unpublished, were retrieved. Information on major cardiovascular events was reported in 33 trials, 20 of which with at least one event. The analysis on MACE was therefore performed on 20 trials, enrolling 6,490 and 3,995 patients (3.467 and 2.172 patient* years) in the GLP-1 receptor agonist and comparator groups, respectively. The characteristics of the retrieved trials, and of those which resulted to be complete but were undisclosed, or did not report information on MACE, are summarized in Tables 1, 2, and 3.

The total number of patients with events was 65 (0.01%) and 49 (0.01%) in the GLP-1 receptor agonists and comparator groups, respectively. Treatment with the experimental drugs was not associated with an increased incidence of MACE (MH-OR.0.74 (0.50–1.08); $P = .12$). A significant reduction of cardiovascular events with GLP-1 receptor agonists was observed in placebo-controlled trials but not in studies versus active comparators (Figure 2). No consistent pattern suggesting differences between exenatide and liraglutide emerged across analyses. In comparisons with insulin (5 trials with events) and sulfonylureas (4 trials with events), the MH-OR for GLP-1 receptor agonists was 1.77 (0.91–3.44), $P = .09$, and 0.49 (0.22–1.10), $P = .085$, respectively.

All-cause mortality was reported in 33 trials, 9 of which with at least one event (8 and 7) in GLP-1 receptor agonists and comparator, respectively; MH-OR for experimental drugs was 0.67 [0.26–1.78], $P = .43$.

4. Conclusions

The reduction of cardiovascular morbidity and mortality is one of the main aims of long-term treatment of hyperglycemia in type 2 diabetes. Therefore, the possibility of an increased cardiovascular risk associated with some hypoglycemic treatments [1, 3–7] is almost paradoxical. Although some data on adverse cardiovascular effects of specific drugs were not confirmed by subsequent investigations [8–11], the concerns of health authorities about the safety of new compounds appear to be justified (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM071627.pdf).

Table 3: Moderators and outcome variables in individual studies included in the meta-analysis.

Study (ref.)	Number of patients (ID/C)	Trial duration (wks)	Age (ys)	Duration of DM (ys)	HbA1c/FPG baseline (%/mmol/L)	BMI baseline (Kg/m^2)	MACE (n,ID/C)	All-cause mortality (n,ID/C)	Cardiovasc. mortality (n,ID/C)
Albiglutide versus placebo									
Rosenstock et al. [19]	128/50	16	54	5	8.0/9.7	32.0	0/3	NR/NR	NR/NR
Exenatide versus placebo									
Gill et al. [20]	27/25	12	55	NR	7.3/NR	NR	0/0	0/0	0/0
Kadowaki et al. [21]	115/40	12	59	11	8.0/9.1	25.9	0/0	0/0	0/0
Zinman et al. [22]	121/112	16	56	8	7.9/8.9	34.0	0/0	0/0	0/0
Gao et al. [23]	234/232	16	55	8	8.3/9.3	26.2	0/1	0/0	0/0
DeFronzo et al. [24]	47/45	20	56	NR	7.9/NR	NR	0/0	0/0	0/0
Apovian et al. [25]	96/98	24	55	5	7.6/8.6	33.7	0/0	0/0	0/0
Moretto et al. [26]	155/77	24	54	1	7.8/8.7	31.5	0/0	0/0	0/0
Liutkus et al. [27]	111/54	26	54	6	8.2/9.1	33.5	0/0	0/0	0/0
DeFronzo et al. [28]	223/113	30	53	6	8.2/9.4	34.0	1/2	0/0	0/0
Buse et al. [29]	248/123	30	55	6	8.6/10.3	33.5	1/2	0/0	0/0
Kendall et al. [30]	486/247	30	55	9	8.5/9.9	34.0	7/6	0/1	0/1
Exenatide versus rosiglitazone									
DeFronzo[#] et al. [24]	45/45	20	56	NR	7.9/NR	NR	0/0	0/0	0/0
Exenatide versus glibenclamide									
Derosa et al. [31]	63/65	52	56	NR	8.8/7.9	28.6	NR/NR	0/0	0/0
Exenatide versus BiAsp 30/70									
Bergenstal et al. [32]	124/248	24	52	NR	10.1/11.4	33.8	NR/NR	0/1	0/1
Nauck et al. [33]	253/248	52	58	10	8.6/11.1	30.4	10/5	2/1	1/1
Exenatide versus glargine									
Barnett et al. [34]	138/138	16	55	7	8.9/12.0	31.3	0/0	0/0	0/0
NCT00360334 [35]	118/116	26	56	NR	8.6/10.8	34.1	2/2	NR/NR	NR/NR
Heine et al. [36]	282/267	26	59	9	8.2/10.2	31.3	5/3	0/0	0/0
Bunck et al. [37]	36/33	52	58	5	7.5/9.1	30.6	NR/NR	NR/NR	NR/NR
Diamant et al. [38]	233/232	26	58	8	8.3/9.8	32.0	1/0	0/0	0/0
Exenatide versus insulin									
Davis et al. [39]	33/16	16	53	11	8.1/8.7	34.0	1/0	0/0	0/0
Exenatide LAR versus placebo									
Kim et al. [40]	30/14	15	53	4	8.4/10.7	36.0	0/0	0/0	0/0
Exenatide LAR versus pioglitazone									
Bergenstal et al. [41]	160/165	26	52	6	8.5/9.1	32.0	0/3	0/0	0/0
Exenatide LAR versus sitagliptin									
Bergenstal et al. [41][#]	160/166	26	52	6	8.5/9.1	32.0	0/1	0/1	0/0
Liraglutide versus placebo									
Madsbad et al. [42]	135/29	12	57	4	7.5/NR	30.4	0/0	0/0	0/0
Vilsbøll [43]	123/40	14	56	4	8.3/11.8	30.1	0/0	0/0	0/0
Seino et al. [44]	180/46	14	57	8	8.3/NR	23.9	0/0	0/0	0/0
Kaku et al. [45]	176/88	24	60	10	8.4/NR	24.9	1/1	0/0	0/0
Russell-Jones et al. [46]	232/115	26	57	9	8.3/9.2	30.6	5/1	1/2	0/2

Table 3: Continued.

Study (ref.)	Number of patients (ID/C)	Trial duration (wks)	Age (ys)	Duration of DM (ys)	HbA1c/FPG baseline (%/mmol/L)	BMI baseline (Kg/m^2)	MACE (n,ID/C)	All-cause mortality (n,ID/C)	Cardiovasc. mortality (n,ID/C)
Zinman et al. [47]	355/175	26	55	9	8.5/10.1	33.7	1/0	0/0	0/0
Marre et al. [48]	695/114	26	56	6	8.4/9.7	29.7	3/2	0/0	0/0
Nauck et al. [49]	724/121	26	57	7	8.4/10.0	31.2	6/0	1/0	0/0
Liraglutide versus metformin									
Feinglos et al. [50]	176/34	12	53	5	7.0/NR	34,5	0/0	0/0	0/0
Liraglutide versus rosiglitazone									
Marre# et al. [48]	695/232	26	56	6	8.4/9.7	29.7	3/0	0/0	0/0
Liraglutide versus glimepiride									
Madsbad# et al. [42]	135/26	12	57	4	7.5/NR	30.4	0/0	0/0	0/0
NCT00614120 [35]	698/231	16	53	7	NR/NR	25.5	3/2	0/0	0/0
Nauck# et al. [49]	724/121	26	57	7	8.4/10.0	31.2	6/2	1/0	0/0
Garber et al. [51]	498/248	52	53	5	8.3/9.4	33.0	2/2	0/1	0/0
Liraglutide versus glibenclamide									
NCT00393718 [35]	268/132	24	58	8	8.3/NR	24.8	4/3	1/0	0/0
Liraglutide versus sitagliptin									
Pratley et al. [52]	446/219	26	55	6	8.4/10.0	32.8	1/1	1/1	0/1
Liraglutide versus glargine									
Russell-Jones# et al. [46]	232/234	26	57	9	8.3/9.2	30.6	5/1	1/1	0/1

Studies with multiple comparators; DM: diabetes mellitus; FPG: fasting plasma glucose; MACE: major cardiovascular events; cardiovasc.: cardiovascular; NR: not reported.

In order to reach definitive conclusions on cardiovascular safety of any drug, large-scale, long-term trials should be performed prior to marketing; unfortunately, this effort would be economically unfeasible for pharmaceutical companies. The FDA accepted a compromise, allowing the organization of such trials after drug approval, as a condition for the maintenance of marketing authorization. The limit of this approach is that cardiovascular safety of new drugs will be established only several years after their approval, leaving clinicians without reliable information on this critical point in the meantime.

Meta-analyses of cardiovascular events recorded as adverse events in randomized clinical trials designed for other purposes can represent an additional source of information. This approach has several limitations, most notably the lack of predefined diagnostic criteria and screening methods for incident cardiovascular disease, with the risk of misdiagnosis and underdiagnosis. It should also be recognized that in some of the trials included, cardiovascular events were reported only as adverse events, without being prospectively adjudicated. Moreover, the limited duration of trials designed for metabolic purposes can impair their ability to detect longer-term effects on atherogenesis. Furthermore, the meta-analysis of small trials with few events each poses some specific, and complex, statistical problems [17]. All these limitations affected the reliability of results of some meta-analyses [6, 7] on cardiovascular safety of hypoglycemic drugs [10, 17, 18].

Those considerations should be taken into account when interpreting the results of the present meta-analysis, which exclude, at least in the short term, any major adverse effect of GLP-1 receptor agonists on cardiovascular morbidity. Interestingly, those drugs, as a class, are below to the 1.3 threshold chosen by the FDA for the upper limit of 95% confidence interval to establish the cardiovascular safety of a new drug.

Interestingly, a significant reduction of cardiovascular morbidity with GLP-1 receptor agonists was observed in comparison with placebo. This result should be discussed with great caution, considering the limitations highlighted above; in fact, a meta-analysis of trials performed for different (noncardiovascular) endpoints provides reliable information on safety, but not on efficacy. Speculatively, several mechanisms could underlie a beneficial effect of GLP-1 receptor agonists on cardiovascular risk. Reduction of blood glucose, body weight, and blood pressure, as well as favorable effects on lipid profile, have all been reported. Direct myocardial effects of GLP-1 receptor stimulation could theoretically reduce the functional impact of myocardial ischemia [13], leading to clinical improvements. However, the possibility of a beneficial action of GLP-1 receptor agonists on cardiovascular events should be confirmed through specifically designed randomized clinical trials.

In conclusion, GLP-1 receptor agonists do not appear to increase cardiovascular morbidity in comparison with placebo or other active drugs. Any possible beneficial action should be assessed in further trials.

Author Contributions

M. Monami organized the collection of clinical data, prepared and revised the paper, and performed data analysis. F. Cremasco collected clinical data and assisted in study design and data analysis. C. Lamanna collected clinical data and revised the paper. C. Colombi collected clinical data and assisted in study design. S. Zannoni collected clinical data. I. Iacomelli collected clinical data N. Marchionni reviewed/edited the paper. E. Mannucci designed the study, prepared and revised the paper, and took part in data analysis.

Conflict of Interests

The corresponding author confirms that he had full access to all the data in the study and had final responsibility for the decision to submit for publication. M. Monami has received speaking fees from Eli Lilly and Sanofi-Aventis. F. Cremasco is currently employed by Eli Lilly. N. Marchionni has received speaking fees from Eli Lilly, Novo Nordisk, and Sanofi-Aventis, and research grants from Eli Lilly, Novo Nordisk, and Sanofi-Aventis. E. Mannucci has received consultancy fees from Eli Lilly and Novo Nordisk, speaking fees from Eli Lilly, Novo Nordisk, and Sanofi-Aventis, and research grants from Eli Lilly, Novo Nordisk, and Sanofi-Aventis.

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Review Article

Glucagon-Like Peptide-1 Gene Therapy

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Glucagon-like peptide 1 (GLP-1) is a small peptide component of the prohormone, proglucagon, that is produced in the gut. Exendin-4, a GLP-1 receptor agonist originally isolated from the saliva of *H. suspectum* or Gila monster, is a peptide that shares sequence and functional homology with GLP-1. Both peptides have been demonstrated to stimulate insulin secretion, inhibit glucagon secretion, promote satiety and slow gastric emptying. As such, GLP-1 and Exendin-4 have become attractive pharmaceutical targets as an adjunctive therapy for individuals with type II diabetes mellitus, with several products currently available clinically. Herein we summarize the cell biology leading to GLP-1 production and secretion from intestinal L-cells and the endocrine functions of this peptide and Exendin-4 in humans. Additionally, gene therapeutic applications of GLP-1 and Exendin-4 are discussed with a focus on recent work using the salivary gland as a gene therapy target organ for the treatment of diabetes mellitus.

1. Introduction

A great deal of research has been performed on glucagon-like peptide-1 (GLP-1) since the initial characterization of glucagon-like substances secreted from the intestine in response to oral glucose [1]. A search of the literature identifies many articles describing original data as well as many excellent reviews (Holst [2], Riedel and Kieffer [3], and Brubaker [4]). Therefore, it is not the intent of this review to repeat what has previously been written, but to summarize what is known about GLP-1 with a focus on gene therapy using expression vectors of GLP-1 and other incretin mimetics in the salivary gland for the treatment of type 2 diabetes mellitus (T2DM).

2. Cell Biology

Glucagon-like peptide-1 (GLP-1(7–37)) is a 30 amino acid peptide that is initially synthesized as part of proglucagon, a prohormone composed of 180 amino acids (NCBI reference for human proglucagon, NP 002045.1). Within the proglucagon peptide lie the sequences of several smaller peptide hormones, such as glucagon, GLP-1, GLP-2, glicentin, and oxyntomodulin. Proglucagon is expressed in pancreatic α-cells within the Islets of Langerhans and also in intestinal endocrine L cells [5, 6]. In these specialized cells, proglucagon is intracellularly trafficked to the regulated secretory pathway where it can be processed into the smaller peptide hormone products indicated above. However, due to differential expression of the prohormone convertases (PCs) in each of these tissues [7–10], proglucagon is processed such that glucagon is produced in the pancreas by PC2 [11, 12] and GLP-1 is produced in the gut by PC1 [13–15]. The GLP-1 peptide can then undergo additional processing at both termini. At the amino terminus, six amino acids are removed to generate a new, mature N-terminus that is involved in activating the GLP-1 receptor in target tissues [16, 17]. The carboxyl terminus is trimmed by carboxypeptidase E [18] to remove two arginine residues, allowing a newly exposed

glycine residue to be amidated by the enzyme, peptidyl alpha amidating mono-oxygenase [19]. GLP-1 is stored within secretory ranules of L cells until it is released in a stimulus dependent manner. GLP-1 is secreted into the bloodstream in response to taste receptor activation [20] and nutrients present in the digestive tract after a meal [21, 22] including glucose, amino acids, and, as recently demonstrated, some selected tetrapeptides [23]. In addition, neuroendocrine input [24] involving signaling by leptin, insulin, and gastric inhibitory peptide as well as muscarinic receptor activation [25] are involved in stimulated GLP-1 secretion from L cells.

3. Endocrinology

Following secretion of GLP-1 in vivo, it is estimated that approximately 30% of circulating GLP-1 survives long enough to reach the pancreas [26]. GLP-1 has a biological half-life of 2-3 min, similar to that of insulin, and is rapidly degraded by dipeptidyl aminopeptidase IV (DPP IV). Upon reaching the pancreas, GLP-1 induces the secretion of insulin from β-cells in a glucose-dependent manner as well as inhibits glucagon secretion from α-cells. Importantly, it has also been shown that GLP-1 enhances β-cell proliferation with a subsequent increase in β-cell mass (reviewed in [27]). This results in an increase in insulin availability under conditions of high demand and recovery of β-cell mass previously lost by a progressive reduction of pancreatic β-cell mass and function as a consequence of T2 DM. Additionally, GLP-1 restores glucose sensitivity to β-cells [28] and potentiates insulin-stimulated glucose utilization in pancreatized dogs [29]. GLP-1 also functions to slow gastric emptying [30], allowing a more controlled efflux of nutrients into the intestine and to the circulation. The controlled release of nutrients to the circulation in turn results in a more controlled nutrient uptake response by the tissues in the body. Additionally, a central role of GLP-1 in the regulation of satiety has been shown [31].

4. Exendin-4

Exendin-4 is a 39 amino acid peptide that shares similar functional properties with GLP-1 and was identified in the venom of the lizard, *Heloderma suspectum*, commonly known as the Gila monster (reviewed in [32]). While not a GLP-1 ortholog, exendin-4 shares 53% amino acid homology with full-length GLP-1. Due to a glycine residue in position 8, it is more resistant to degradation by DPP IV and hence has a longer biological half-life ($\sim$3-4 h) in the circulatory system [33]. As mentioned, exendin-4 acts as a GLP-1 receptor agonist and was shown to increase cAMP in guinea pig pancreas [34] and stimulate insulin secretion not only from mouse insulinoma cell lines but also rat Islet isolates [35]. Similar to that of GLP-1 in humans and mammals, exendin-4 has been shown to inhibit glucagon secretion, stimulate insulin secretion, protect against β-cell apoptosis, promote β-cell proliferation, promote satiety, and inhibit gastric emptying [32].

5. Gene Delivery of GLP-1 and Exendin-4 Constructs

The ability of GLP-1 and its analogues to stimulate insulin secretion in a glucose-dependent manner makes these molecules attractive potential therapies for T2 DM. However, the short half-life of GLP-1 in serum would necessitate repeated dosing, making injection with a recombinant peptide an expensive and inconvenient treatment strategy. To circumvent these shortcomings, many groups have explored alternative methods for expression and delivery of modified GLP-1 peptides and analogues including cell engineering [3, 36] and, more commonly, gene therapeutic approaches.

Several groups have demonstrated expression and secretion of GLP-1 following delivery of viral or plasmid gene therapy vectors either via intravenous (IV) injection [37–42] or intraperitoneal injection [43, 44]. As a whole, these studies illustrate that in vivo expression of a transgenic GLP-1 peptide had positive effects on glucose homeostasis and could delay the onset of diabetes in both T1 and T2 DM models. Additionally, Samson and colleagues demonstrated that IV injection of a helper-dependent adenovirus encoding exendin-4 was able to improve glucose homeostasis in a T2 DM model [45]. While effective in animal models, the inherent risks involved in systemic administration of gene therapy vectors make targeted delivery of vectors an appealing alternative for treatment of patients.

Several studies from the laboratory of Dr. Q. Wang have used "plasmid-based, electroporation-enhanced intramuscular gene therapy" to target delivery of vectors encoding GLP-1 or exendin-4 fusion proteins into mouse models of DM [46–48]. In initial studies, an active GLP-1 peptide or an exendin-4 peptide was fused to the heavy chain constant regions (Fc regions) of mouse IgG to generate bivalent peptides that have putative increased half-life and potency in vivo. Plasmids encoding these constructs were directly injected into the muscle of mice with T1 DM (streptozotocin-induced) or T2 DM (db/db mice) and circulating fusion peptides were detected in plasma for at least 3-4 weeks following injection. The GLP-1 fusion peptide was shown to improve glucose tolerance in both models of DM [46, 47] and the exendin-4 fusion peptide was also shown to improve glucose homeostasis and ameliorate T1 DM symptoms when tested in mice treated with chronic low doses of streptozotocin [46].

The studies of the GLP-1 fusion peptide were taken a step further in 2010 when Liu and colleagues directed site-specific integration of the GLP-1/Fc fusion peptide plasmid DNA into the genome of transgenic mice carrying the human adeno-associated virus S1-(AAVS1-) integrating region [48]. Peak levels of circulating bivalent GLP-1 peptide were achieved 2 weeks following intramuscular injection and subsequent integration of the plasmid construct into genomic DNA, with sustained plasma levels through 10 weeks of analysis. When tested in high-fat-diet-challenged mice, those expressing the GLP-1 fusion peptide from muscle had decreased weight gain and food intake over time, retained normal levels of circulating leptin and ghrelin, and demonstrated an improved insulin response in an insulin

tolerance test. When taken together, these studies demonstrate the potential of targeted gene therapy for treatment of diabetes and other endocrine deficiencies.

6. The Salivary Gland as a Gene Therapy Target Organ for the Treatment of T2 DM

The salivary gland (SG) has been demonstrated to be an effective target organ for gene therapy vectors in several animal models, and a Phase I/II clinical trial evaluating the safety and efficacy of recombinant adenoviral vector delivery to the parotid SG is currently ongoing [49]. Salivary glands possess many characteristics that make them suitable for gene transfer (reviewed in [50]). Perhaps foremost of these characteristics is that unlike liver or lung, SGs are not critical for life organs, and in the event of an adverse reaction a single SG may be removed with little effect on patient morbidity. Furthermore, because SGs are encapsulated, the likelihood of systemic viral vector spread and subsequent adverse reactions is much reduced.

Salivary glands can produce and secrete large quantities of protein, an attribute making SGs highly suitable for gene therapy to treat single protein deficiency disorders. In addition to treating inducible models of endocrine disorders, it has recently been demonstrated that gene therapeutic vector delivery to SGs can be used to treat inborn genetic errors such as Fabry disease which exhibits a deficiency in the lysosomal enzyme alpha-galactosidase A [51]. Although SG epithelial cells are primarily exocrine, it is hypothesized that the SG constitutive secretory pathway (CSP) conveys transgenic proteins to the circulation via the basolateral membrane (reviewed in [49]). This has been supported by animal studies from our group demonstrating that therapeutically relevant levels of growth hormone and parathyroid hormone were present in serum following SG transduction, indicating the potential for SG gene therapy to treat endocrine disorders [52, 53].

As a proof-of-principle for SG-mediated treatment of diabetes, we have recently shown that transduction of mouse SGs with a recombinant adenovirus encoding a GLP-1 peptide is able to delay the onset of T1 DM in mice [54]. The adenovirus used in these studies (Ad-GLP-1) contains a modified human GLP-1 cDNA sequence encoding the active GLP-1(7–37) peptide with an Ala to Gly substitution at position eight to confer resistance to DPP-IV. This construct was demonstrated to produce a bioactive GLP-1 peptide that was resistant to DPP-IV degradation and was able to stimulate insulin secretion from pancreatic β-cells in vitro.

Ad-GLP-1 was then delivered to the submandibular glands of intact mice by retroductal instillation [55] to determine the route of GLP-1 peptide secretion and the capacity for bioactivity in vivo. GLP-1 expressed by the SGs was detected in the serum of these mice and was able to rapidly reduce serum glucose levels in a glucose tolerance test when compared to animals treated with a control (Luciferase-containing, Ad-Luc) adenovirus. Furthermore, in keeping with the glucose-dependent mechanism of GLP-1 action, the blood glucose levels of fasted animals treated with Ad-GLP-1 were indistinguishable from those treated with Ad-Luc.

After establishing that bioactive transgenic GLP-1 was secreted to the circulation, we tested the ability of SG-derived GLP-1 to ameliorate diabetes in an inducible model. In these studies, Ad-GLP-1 or Ad-Luc was delivered to SGs, one day later, all mice were then treated with the beta-cell-specific toxin alloxan, and blood glucose values of Ad-GLP-1- or Ad-Luc-treated mice were followed throughout the course of the experiment. As anticipated from previous experiments, GLP-1 expressed and secreted from SGs delayed the onset of alloxan-diabetes as demonstrated by significantly lower serum glucose levels than Ad-Luc-treated mice 48–72 h after alloxan treatment. We are currently generating adeno-associated viral (AAV) vectors capable of stable, long-term GLP-1 and exendin-4 expression in order to test the efficacy of SG-derived GLP-1 and its agonist in treating genetic models of T2 DM and obesity. Preliminary data strongly suggest that long-term expression may be achieved.

Thus, the use of the SG as a tissue to express GLP-1 or exendin-4 by adeno- or adeno-associated viral vectors, demonstrate a means to accomplish a noninvasive delivery of gene therapy vectors for the treatment of DM.

7. Conclusion

The biology of GLP-1 and its potential use in gene therapy for the treatment of T2 DM is evident from the cache of papers available on the topic. GLP-1 appears to have all the requisite properties to maintain homeostatic levels of glucose in order to effectively treat T2 DM. Indeed, DPP IV inhibitors (e.g., Sitagliptin), which prolong the half-life of a patient's endogenous GLP-1, and synthetic GLP-1 receptor agonists (e.g., Exenatide and Liraglutide) are approved and on the market as adjunctive antidiabetic treatments. It is only a matter of time until a gene therapy approach appropriate for the clinic will develop and catch up to the small molecule industry of agonists, antagonists, and inhibitors. The first and most important barrier to cross in the transition from the laboratory to the clinic is demonstration of the safety of this approach. The use of the SG as a bioreactor represents one such tissue that would allow this question to be addressed with fewer complications, both physiologically and regulatory, compared to other potential gene therapy target tissues. It is apparent from the many successful attempts using a gene therapy approach to express GLP-1 or exendin-4 in animal models, that success is dependent on their sustained and, in the future, regulated expression.

Authors Contributions

A. M. Rowzee and N. X. Cawley contributed equally to this paper.

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