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Long-term hyperglucagonaemia induces early metabolic and renal phenotypes of Type 2 diabetes in mice

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Abstract

Clinical studies have shown that patients with early Type 2 diabetes often have elevated serum glucagon rather than insulin deficiency. Imbalance of insulin and glucagon in favouring the latter may contribute to impaired glucose tolerance, persistent hyperglycaemia, microalbuminuria and glomerular injury. In the present study, we tested the hypothesis that long-term glucagon infusion induces early metabolic and renal phenotypes of Type 2 diabetes in mice by activating glucagon receptors. Five groups of adult male C57BL/6J mice were treated with vehicle, glucagon alone (1 μ g/h via an osmotic minipump, intraperitoneally), glucagon plus the glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon (5 μ g/h via an osmotic minipump), [Des-His¹-Glu⁹]glucagon alone or a high glucose load alone (2 % glucose in the drinking water) for 4 weeks. Glucagon infusion increased serum glucagon by 129 % ($P < 0.05$), raised systolic BP (blood pressure) by 21 mmHg ($P < 0.01$), elevated fasting blood glucose by 42 % ($P < 0.01$), impaired glucose tolerance ($P < 0.01$), increased the kidney weight/body weight ratio ($P < 0.05$) and 24 h urinary albumin excretion by 108 % ($P < 0.01$) and induced glomerular mesangial expansion and extracellular matrix deposition. These responses were associated with marked increases in phosphorylated ERK1/2 (extracellular-signal-regulated kinase 1/2) and Akt signalling proteins in the liver and kidney ($P < 0.01$). Serum insulin did not increase proportionally. Concurrent administration of [Des-His¹-Glu⁹]glucagon with glucagon significantly attenuated glucagon-increased BP, fasting blood glucose, kidney weight/body weight ratio and 24 h urinary albumin excretion. [Des-His¹-Glu⁹]glucagon also improved glucagon-impaired glucose tolerance, increased serum insulin by 56 % ($P < 0.05$) and attenuated glomerular injury. However, [Des-His¹-Glu⁹]glucagon or high glucose administration alone did not elevate fasting blood glucose levels, impair glucose tolerance or induce renal injury. These results demonstrate for the first time that long-term hyperglucagonaemia in mice induces early metabolic and renal phenotypes of Type 2 diabetes by activating glucagon receptors. This supports the idea that glucagon receptor blockade may be beneficial in treating insulin resistance and Type 2 diabetic renal complications.

Keywords

glucagon; glucose tolerance; insulin deficiency; kidney; microalbuminuria; Type 2 diabetes

INTRODUCTION

Insulin and glucagon are two of the most important pancreatic hormones in the regulation of blood glucose metabolism and homeostasis. The primary action of insulin is to decrease blood

glucose levels by promoting tissue glucose uptake and glycogen synthesis by the liver in response to rising blood glucose levels (hyperglycaemia), while glucagon acts to counter the action of insulin in response to a fall in blood glucose levels (hypoglycaemia) by inducing glycogenolysis and gluconeogenesis [1–3]. A fine balance between the actions of these two hormones is crucial for the physiological regulation of body glucose supply and consumption [1–3]. Thus it is not surprising that insulin deficiency leads to the development of Type 1 diabetes, which can be treated with insulin replacement or β -cell transplantation. By contrast, the causes or factors responsible for the development of Type 2 diabetes are often multifactorial, with genetics, lifestyles and insulin deficiency all playing an important role. The role of glucagon in the development of Type 1 or Type 2 diabetes mellitus remains poorly understood. Previous studies in humans or animal models have shown that Type 2 diabetes could develop in the presence of normal, subnormal and even elevated blood insulin levels at the early stages of the disease [4,5]. An excess of the counter-regulatory hormone glucagon (or hyperglucagonaemia) has long been implicated, but has received much less attention in the pathogenesis of Type 2 diabetes [3,6,7]. Indeed, some clinical studies have shown that subjects with Type 2 diabetes often have inappropriately elevated fasting glucagon levels that are unable to decrease appropriately in response to increased glucose or food intake [8–10]. Increased serum glucagon levels may in theory contribute to elevated serum and tissue glucose levels and, consequently, increased insulin resistance in these patients [2–4].

However, whether long-term glucagon excess or hyperglucagonaemia may directly induce Type 2 diabetic metabolic abnormalities and glomerular injury has not been explored in humans or animals. Most previous studies have chronically infused (or injected) glucagon in humans or animals for only a few minutes, hours or days to determine the effects of glucagon on blood glucose levels, serum insulin and glucagon responses, hepatic glucose production and tissue uptake of glucose [6,11–13]. None of these studies have lasted long enough to determine whether long-term hyperglucagonaemia may induce target tissue injury in a human or animal model of Type 2 diabetes. Neither are there any studies designed specifically to determine whether glucagon receptor antagonists can attenuate the effects of long-term hyperglucagonaemia, although glucagon receptor antisense oligonucleotides were found to exert antidiabetic effects in *db/db* diabetic fatty mice or genetically modified mice [14,15].

In the present study, we tested the hypothesis that long-term administration of glucagon to produce hyperglucagonaemia can induce early metabolic and renal phenotypes of Type 2 diabetes in a glucagon-infused mouse model, and that the metabolic and renal effects of glucagon are mediated by specific glucagon receptors. We reasoned that glucagon stimulates G_s -protein-coupled receptors to activate glycogenolytic and gluconeogenic pathways, resulting in hyperglycaemia [16,17]. Hyperglycaemia is generally thought to be highly pro-growth and pro-hypertrophic [3–5]. Moreover, glucagon is known to cause glomerular hyperfiltration in the kidney, a characteristic of early Type 2 diabetic glomerular injury [18–21]. Persistent hyperglycaemia and glomerular hyperfiltration induced by long-term hyperglucagonaemia may lead to glomerular injury by stimulating proliferation and hypertrophy of mesangial cells with subsequent mesangial expansion and glomerular injury in Type 2 diabetes.

Part of this work was presented at the 60th Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research in association with the Council on the Kidney in Cardiovascular Disease, held in San Antonio, TX, U.S.A., 4–7 October 2006, and subsequently published in abstract form [21a].

MATERIALS AND METHODS

Animals

A total of 40 adult male C57BL/6J mice (approx. 25 g; 10 weeks of age) were purchased from Jackson Laboratories, and were maintained on a normal rodent chow with free access to tap water. Upon arrival, mice were trained for 1 week for measurement of SBP [systolic BP (blood pressure)] via a computerized tail-cuff method (Visitech) [22]. At 2 days before surgery, mice were housed individually in a metabolic cage for measurement of 24 h fluid intake and collection of 24 h urine samples [22], followed by overnight fasting (i.e. food not provided from 18:00 hours to 09:00 hours the next morning) for measuring fasting blood glucose levels and performing a GTT (glucose tolerance test), as described below. Animals were divided into five groups ($n = 8$) and were treated as follows. Group 1 was treated with saline via an osmotic minipump (Alzet Model 2004; 0.25 $\mu\text{l/h}$ for 28 days) as a control, as described previously in mice [22] or rats [23]. The pump was implanted inside the abdominal cavity via a mid-line incision. Group 2 were treated with glucagon alone (1 $\mu\text{g/h}$; Bachem) via an osmotic minipump for 4 weeks to induce early Type 2 diabetic phenotypes. Previous studies have shown that infusion of glucagon at 0.5 $\mu\text{g/h}$ in mice increased blood glucose by 60% above basal levels [24]. In humans, infusion of glucagon at approx. 4 $\mu\text{g} \cdot \text{kg}^{-1}$ of body weight $\cdot \text{day}^{-1}$ more than doubled plasma glucagon levels and increased blood glucose by 75% compared with placebo controls [25]. Group 3 were treated concurrently with glucagon, as in Group 2, plus the glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon (5 $\mu\text{g/h}$; Bachem), via an osmotic minipump for 4 weeks [26]. This antagonist is one of the most potent and selective antagonists of glucagon receptors that inhibits glucagon receptor-mediated glucose synthesis and release at nanomolar concentrations *in vivo* and *in vitro* [1,26]. Group 4 were treated with [Des-His¹-Glu⁹]glucagon alone for 4 weeks and served as the control for Group 3. Group 5 were treated with 2% (w/v) glucose in the drinking water, which maintained constant blood glucose concentrations at postprandial levels for 4 weeks. This was used as a control for Group 2 to determine whether the effects of hyperglucagonaemia are dependent on its hyperglycaemic actions alone. After starting treatment, body weight, 24 h drinking and urine output, SBP, fasting blood glucose and glucose tolerance were measured weekly.

All protocols and procedures in the present study were approved by Henry Ford Health System's Institutional Animal Care and Use Committee.

Measurement of fasting blood glucose levels and GTT

Mice fasted overnight before basal fasting blood glucose levels were measured, and a GTT was performed 1 day before and then weekly after the minipump was implanted or high glucose administration was initiated. Glucose was injected at 1 mg/g of body weight (intraperitoneally), and tail blood glucose levels were measured continuously using a glucose analyser (Accu-Chek; Roche) at 30 min intervals for 2 h, as described previously [14,15].

Measurement of serum glucagon and insulin concentrations

Serum glucagon and insulin concentrations were measured only at the end of study, because it was difficult to collect enough blood samples for weekly measurements of these hormones without compromising BP and cardiovascular and renal function. Mice were killed by decapitation, and trunk blood samples were collected into tubes containing a protease inhibitor cocktail for measurement of serum glucagon and insulin levels. After centrifugation, serum samples were collected, and the peptides were extracted and stored at -80°C for measurements of plasma glucagon and insulin levels using rat glucagon (Wako) or rat/mouse insulin (Linco) ELISA kits respectively [5,27,28]. The glucagon ELISA is specific to rat, mouse and human pancreatic glucagon and does not cross-react with intestinal glucagon or glucagon-like peptides. It can detect levels as low as 50 pg of glucagon/ml with an inter-assay variation of

approx. 4%. The insulin ELISA is specific to rat and mouse insulin (100%), but less specific to human or rat proinsulin (< 50%), with a sensitivity of 0.2 ng/ml and an inter-assay variation of approx. 6%.

Assessment of glomerular injury

Glomerular injury was evaluated by two approaches, albuminuria and PAS (periodate–Schiff) staining, to score glomerular mesangial expansion and extracellular matrix deposition [5]. Urinary albumin excretion was measured using a mouse albumin ELISA kit (Alpha Diagnostic) [5], and cryostat kidney sections (6 μ m) were stained by PAS for histological analysis by a pathologist blinded to the treatment groups. Glomerular lesions were analysed using the MetaMorph Imaging System (Universal Imaging) to measure the area of PAS-stained mesangial expansion and extracellular matrix deposition as a percentage of total glomerular area.

Western blot analysis of ERK1/2 (extracellular-signal-regulated kinase 1/2) and Akt phosphorylation

Liver and kidney samples were lysed with Nonidet P40 lysis buffer [50 mmol/l Tris/HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EDTA, 0.25% sodium deoxycholate, 1 mmol/l PMSF, 1 mmol/l sodium orthovanadate, 1 mmol/l sodium fluoride and 1% Nonidet P40] containing a protease inhibitor cocktail (1 μ g/ml each of aprotinin, leupeptin and pepstatin), as described previously for rat glomerular mesangial cells [29,30]. Protein concentrations were measured using a BCA (bicinchoninic acid) protein assay kit (Pierce). Portions (100 μ g of protein) from each sample were separated by SDS/PAGE [8–16% (w/v) polyacrylamide gel] and transferred semi-dry on to an Immobilon-P membrane (Millipore). Total and phosphorylated ERK1/2 proteins were detected by immunoblotting using a rabbit polyclonal ERK1/2 antibody (Cell Signaling) and a mouse monoclonal anti-[phospho-ERK1/2 (Tyr²⁰⁴)] antibody (Santa Cruz Laboratories), as described previously [29,30]. Total and phospho-Akt (Ser⁴⁷³) proteins were measured by Western blots using specific antibodies against total Akt or phospho-Akt (Ser⁴⁷³) (Cell Signaling). Western blotting of β -actin (Sigma) on the same membranes was used to confirm equal protein loading. Immunoblots were visualized by enhanced chemiluminescence using HRP (horseradish peroxidase)-conjugated secondary antibodies (Santa Cruz Laboratories), scanned and analysed using a microcomputer imaging device (MCID; Imaging Research) [29,30].

Statistical analysis

Results are means $\pm$ S.E.M. The differences in all parameters between basal (week 0) and weekly measurements within the same group of mice were analysed using one-way ANOVA, followed by Newman–Keuls multiple comparison post-hoc test if $P < 0.05$. The differences in the same parameters between groups of mice were analysed using an unpaired Student's t test. As the results were distributed normally, a sample size of approx. eight mice/group gave approx. 80% power to detect differences of 2.1 S.D. for the Newman–Keuls test, with 3–5 treatments compared with the control. The statistical differences were set at a P value < 0.05 .

RESULTS

Effects of long-term glucagon infusion on serum glucagon and insulin levels

Infusion of glucagon at 1 μ g/h for 4 weeks increased serum glucagon levels by 129% ($P < 0.01$; Table 1), which was increased further by concurrent administration of glucagon and a selective peptide receptor antagonist [Des-His¹-Glu⁹]glucagon ($P < 0.01$ compared with glucagon alone; Table 1). The latter response was due in part to global occupancy of glucagon receptors by the antagonist, resulting in higher serum glucagon levels. At the end of the 4 week infusion period,

glucagon did not significantly alter serum insulin levels (Table 1); however, concurrent administration of glucagon and [Des-His¹-Glu⁹]glucagon significantly increased serum insulin levels by approx. 50% ($P < 0.01$ compared with glucagon-infused mice; Table 1). In mice treated with [Des-His¹-Glu⁹]glucagon alone, serum glucagon levels were at a similar level to that of glucagon-infused mice (Table 2). By contrast, serum insulin levels were higher in these mice than control and glucagon-infused mice (Table 2).

Effects of long-term glucagon infusion on SBP

Before glucagon infusion, basal SBP was similar for all groups of mice, and there were no changes in BP in control mice during the 4 week study period (Figure 1). In glucagon-infused mice, SBP increased gradually by an average of 10 mmHg during week 2 of glucagon infusion ($P < 0.05$ compared with control), it peaked at week 3 ($P < 0.01$ compared with control), and then fell back slightly to a level still significantly higher than at week 1 of glucagon administration ($P < 0.05$ compared with control). In mice treated with concurrent infusion of glucagon and the antagonist [Des-His¹-Glu⁹]glucagon, SBP was normalized to control (not significant compared with control; Figure 1). There were no significances in heart rate between the treatments or groups (Table 1). The antagonist [Des-His¹-Glu⁹]glucagon alone had no effect on BP (Table 2).

Effects of long-term glucagon infusion on fasting blood glucose levels

Although there were no differences in basal fasting blood glucose levels between the three groups, infusion of glucagon for 4 weeks markedly increased fasting blood glucose levels in mice ($P < 0.01$; Figure 2A). Concurrent administration of [Des-His¹-Glu⁹]glucagon with glucagon significantly attenuated the glucagon-increased fasting blood glucose levels ($P < 0.01$; Figure 2A). Interestingly, mice treated with [Des-His¹-Glu⁹]glucagon alone had significantly lower fasting blood glucose levels without altering postprandial blood glucose levels (Table 2).

Effects of long-term glucagon infusion on glucose tolerance

As shown in Figure 2(B), long-term glucagon administration significantly impaired glucose tolerance in mice. In control mice, injection of glucose (1 mg/g of body weight, intraperitoneally) increased blood glucose levels at 30 min ($P < 0.01$), which returned to basal levels 60 min after glucose injection (Figure 2B). In glucagon-infused mice, injection of glucose increased blood glucose levels at 30 min ($P < 0.01$), which was significantly higher than control mice ($P < 0.01$). At 60 min, blood glucose returned to a level that was still significantly higher than its basal value as well as the corresponding time-point in control mice ($P < 0.01$), suggesting an impairment of glucose tolerance in these mice. By contrast, glucagon receptor antagonist completely prevented the effect of glucagon on glucose tolerance (Figure 2B). Again, mice treated with the antagonist [Des-His¹-Glu⁹]glucagon alone had a significant improvement in glucose tolerance at 60 min after glucose injection (Table 2).

Effects of long-term glucagon infusion on renal hypertrophy

Figure 3(A) shows that long-term hyperglucagonaemia increased the kidney weight/body weight ratio, an index of renal hypertrophy, by more than 30% ($P < 0.05$ compared with control), which was significantly inhibited by concurrent administration with the glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon ($P < 0.05$ compared with glucagon alone; Figure 3A). Administration of [Des-His¹-Glu⁹]glucagon alone did not have an effect on the kidney weight/body weight ratio (Table 2).

Effects of long-term glucagon infusion on 24 h urinary albumin excretion

Basal 24 h urinary albumin excretion/mg of creatinine was similar for all of the three groups of mice (Figure 3B). Infusion of glucagon increased 24 h urinary albumin excretion by more than 300% in mice ($P < 0.01$ compared with control). The concurrent administration of glucagon and its receptor antagonist [Des-His¹-Glu⁹]glucagon decreased 24 h urinary albumin excretion to a level not significantly different from the control (Figure 3B).

Effects of long-term glucagon infusion on glomerular injury

Figure 4 shows representative glomerular micrographs from a control, glucagon-infused and glucagon + [Des-His¹-Glu⁹]glucagon-treated mouse kidney respectively. Compared with the control kidney (Figure 4A), there was appreciable glomerular mesangial expansion and extracellular matrix deposition in glucagon-infused mouse kidney (Figure 4B), and this effect of glucagon was significantly attenuated by [Des-His¹-Glu⁹]glucagon (Figure 4C), suggesting a glucagon-receptor-mediated response. Quantification of the percentage glomerular area stained by PAS for control, glucagon-infused and glucagon + [Des-His¹-Glu⁹]glucagon-treated kidney is shown in Figure 4(D). Compared with control (Figure 4A), glucagon-infused mice had a higher percentage of PAS-stained glomerular area ($P < 0.01$ compared with control; Figure 4B). Blockade of glucagon receptors by [Des-His¹-Glu⁹]glucagon prevented glucagon-induced mesangial expansion and extracellular matrix deposition ($P < 0.01$ compared with glucagon; Figure 4C).

Effects of long-term glucagon infusion on activation of liver and kidney ERK 1/2 and Akt signalling

To study the cellular mechanisms underlying glucagon-induced target organ injury in diabetes, we measured the effects of long-term hyperglucagonaemia on the phosphorylation of ERK1/2 (at Tyr²⁰⁴) and the insulin signalling protein Akt (at Ser⁴⁷³) in the liver and kidney. Long-term glucagon infusion markedly increased phospho-ERK1/2 (Tyr²⁰⁴) by 182% in the liver ($P < 0.01$ compared with control; Figure 5A) and 358% in the kidney ($P < 0.01$ compared with control; Figure 5B). Concurrent administration of glucagon with its receptor antagonist [Des-His¹-Glu⁹]glucagon completely prevented these responses (Figure 5). Total ERK1/2 levels were not affected. Glucagon also induced the phosphorylation of Akt, with a 459% increase in phospho-Akt (Ser⁴⁷³) in the liver ($P < 0.01$ compared with control; Figure 6A) and a 223% increase in the kidney ($P < 0.01$ compared with control; Figure 6B). Total Akt levels were also unaltered by glucagon. Again, the phosphorylation of Akt in the liver and kidney by glucagon was blocked by [Des-His¹-Glu⁹]glucagon, suggesting a specific effect of glucagon (Figure 6).

Effects of long-term administration of high glucose intake alone on SBP and metabolic and renal phenotypes

Table 3 summarizes the overall effects of long-term administration of a high glucose load alone for 4 weeks on systemic, metabolic and renal phenotypes in mice. Long-term high glucose intake alone did not significantly change body weight, SBP, heart and kidney weight, and fasting basal and postprandial blood glucose levels (Table 3). There were also no significant differences in GTTs between basal and during weeks 2 and 4 of high glucose administration. As expected, serum glucagon levels were slightly decreased, whereas serum insulin levels were increased by high glucose intake (Table 3). No apparent glomerular mesangial expansion and extracellular matrix deposition in response to high glucose administration was evident, as determined by PAS staining of kidney sections (results not shown). There were no significant increases in 24 h urine albumin excretion in mice treated with a high glucose load compared with control mice.

DISCUSSION

In the present study, we demonstrate for the first time that long-term administration of glucagon via an osmotic minipump to produce hyperglucagonaemia led to manifestations of early Type 2 diabetic metabolic and renal phenotypes in a mouse model. The major glucagon-induced metabolic and renal abnormalities include a moderate increase in SBP, increased fasting blood glucose levels, impaired glucose tolerance, increased the kidney weight/body weight ratio, an index of renal hypertrophy due to glomerular hyperfiltration, and development of microalbuminuria, mesangial expansion and extracellular matrix deposition. These effects were associated with marked increases in phospho-ERK1/2 (Tyr²⁰⁴) and Akt (Ser⁴⁷³) signalling proteins in the liver and kidney. We believe that the observed metabolic and renal effects were specific to glucagon, because concurrent administration of glucagon with a selective glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon prevented and/or attenuated these responses to glucagon stimulation. Furthermore, long-term administration of the antagonist [Des-His¹-Glu⁹]glucagon or a high glucose load alone did not significantly increase basal fasting blood glucose levels or impair weekly glucose tolerance throughout the 4 week study period (Tables 2 and 3). Our results, therefore, suggest that long-term hyperglucagonaemia, as often seen in some patients with Type 2 diabetes, may induce early Type 2 diabetic metabolic and renal phenotypes in mice by activating specific glucagon receptors, and that glucagon receptor blockade may be beneficial in treating insulin resistance and renal injury in early Type 2 diabetes.

Increased fasting blood glucose levels, impaired glucose tolerance and development of microalbuminuria and glomerular injury are the hallmarks of early Type 2 diabetic nephropathy in humans [2–4,8–10]. The classic view is that increased insulin resistance at the early stage and progression to insulin deficiency at the late stage contribute to Type 2 diabetes, and that glucagon plays little, if any, role in the pathogenesis and target organ injury of Type 2 diabetes [1,4]. However, many studies in human Type 2 diabetes found inappropriately elevated serum glucagon levels (also named hyperglucagonaemia) [8–10]. Moreover, serum glucagon levels fail to fall in response to increased glucose or food intake in Type 2 diabetes [10]. This suggests that glucagon may play an important role in causing hyperglycaemia and, therefore, may contribute to increased insulin resistance in early Type 2 diabetes [2,31]. Indeed, many previous studies in humans or animals have demonstrated that glucagon administration for several hours or days was associated with or without increased fasting blood glucose levels and impaired glucose tolerance [11–13]. However, it has been very complicated to determine whether glucagon plays a direct role in these responses, because it is difficult to infuse glucagon to induce hyperglucagonaemia and then block its effects with its receptor antagonist [Des-His¹-Glu⁹]glucagon in human subjects for weeks or months. An indirect role of glucagon in Type 2 diabetes was implicated in studies on *db/db* diabetic fatty mice, a genetically modified rodent model of human Type 2 diabetes, in which blockade of glucagon receptors with antisense oligonucleotides attenuated the development of hyperglycaemia and metabolic abnormalities [14,15]. As glucagon was not infused in these studies, it is not possible to confirm a direct role of hyperglucagonaemia in causing diabetes in these animals.

In the present study, we employed a novel approach by chronically infusing glucagon via an osmotic minipump for 4 weeks to induce hyperglucagonaemia [24]. This approach has a unique advantage that hyperglucagonaemia can be induced at a level that is commonly observed in patients with Type 2 diabetes with hyperglucagonaemia without manipulating serum insulin levels [8–10]. We found that infusion of glucagon at 1 µg/h for 4 weeks more than doubled serum glucagon levels without significantly altering serum insulin levels. The degree of hyperglucagonaemia is consistent with a previous study in which administration of glucagon at 0.5 µg/h for 1 week doubled serum glucagon levels in mice with impaired glucagon synthesis [24]. Although glucagon is known to be a potent stimulator of insulin release in acute settings,

serum insulin did not increase proportionally in response to long-term glucagon stimulation in the present study. This strongly implies that, if left untreated, long-term hyperglucagonaemia may eventually impair insulin synthesis and release from pancreatic β -cells. However, because we did not examine pancreatic α - and/or β -cell morphology in these animals, it is not known whether long-term hyperglucagonaemia causes β -cell injury. Nevertheless, mice treated concurrently with glucagon and a selective glucagon receptor antagonist had a significant increase in serum insulin levels compared with control and glucagon-infused mice (Table 1). This result suggests that blockade of glucagon receptors may have improved pancreatic β -cell function by removing the influence of glucagon infusion in these mice. Indeed, long-term administration of the antagonist [Des-His¹-Glu⁹]glucagon alone decreased basal fasting blood glucose levels and improved glucose tolerance at 60 min during weeks 3 and 4 of administration (Table 2). Further histological studies are required to determine whether long-term hyperglucagonaemia with or without glucagon receptor blockade alters the number of pancreatic α - and/or β -cells or the levels of glucagon and insulin immunostaining in the pancreas.

The present study also shows that long-term hyperglucagonaemia in mice was associated with the development of some early metabolic and renal phenotypes of human Type 2 diabetes [4, 8–10]. Increased fasting blood glucose levels and impaired glucose tolerance due to increased insulin resistance are often used to diagnose early Type 2 diabetes [4,8–10]. The cellular mechanisms underlying the development of impaired glucose tolerance and increased insulin resistance in early Type 2 diabetes are not well understood, but genetics, lifestyles and hormonal factors are commonly cited. Our present results obtained from glucagon-infused mice suggest that long-term hyperglucagonaemia at least plays a partial role in some patients with Type 2 diabetes, because the primary action of glucagon is to activate glycogenolytic and gluconeogenic pathways, resulting in hyperglycaemia [1,2,31]. Although acute hyperglycaemia initially stimulates insulin release in order to bring blood glucose levels under control, persistent hyperglycaemia due to hyperglucagonaemia would eventually damage pancreatic β -cell function and increase insulin resistance [2–4]. One of major consequences of persistent hyperglycaemia due to hyperglucagonaemia and increased insulin resistance is the development of target organ injury in cardiovascular and renal tissues [4,31]. In the present study, we focused on the development of early renal glomerular injury by long-term hyperglucagonaemia because glomerular hyperfiltration and microalbuminuria are two major complications of early Type 2 diabetic renal glomerular injury [5,32,33]. We [20] and others [18,19,21] have shown previously that glucagon induced glomerular hyperfiltration in anaesthetized rats. Long-term increases in GFR (glomerular filtration rate) by hyperglucagonaemia may lead to renal hypertrophy, glomerular injury and development of microalbuminuria [32]. Indeed, in addition to increasing the kidney weight/body weight ratio, glucagon more than doubled 24 h urinary albumin excretion, and this response was completely prevented in mice treated concurrently with glucagon and its selective receptor antagonist [Des-His¹-Glu⁹]glucagon. The development of glomerular injury induced by hyperglucagonaemia is indicated further by comparing PAS staining to evaluate glomerular mesangial expansion and extracellular matrix deposition [5]. Although glucagon was administered for only 4 weeks, appreciable glomerular mesangial expansion was already observed in mice receiving glucagon infusion, which was blocked by co-administration of [Des-His¹-Glu⁹]glucagon, indicating a receptor-mediated effect.

Our findings of increased phosphorylation of ERK1/2 (Tyr²⁰⁴) and Akt (Ser⁴⁷³) signalling proteins in the liver and kidney by glucagon suggest that activation of these two signalling pathways by long-term hyperglucagonaemia may play an important role in the development of target organ injury in Type 2 diabetes [29–31,34]. We have shown recently [29–31] that glucagon induced growth and proliferation of rat glomerular mesangial cells via activation of ERK 1/2, which was mediated by PLC (phospholipase C)/Ins(1,4,5) P_3 /Ca²⁺ signalling, PKA

(cAMP-dependent protein kinase) as well as PI3K (phosphoinositide 3-kinase). Activation of PKA/Akt and PI3K by glucagon is highly relevant to interactions or cross-talk with insulin-induced Akt signalling [1,2,34]. The latter pathway is known to play a critical role in promoting cell growth, hypertrophy and proliferation by hyperinsulinaemia in Type 2 diabetes [2,15,34]. Long-term hyperglucagonaemia may induce target organ injury directly via activation of ERK1/2 and interactions with insulin-activated Akt signalling.

Finally, it is widely assumed that impaired glucose tolerance, increased insulin resistance and the development of target organ injury is caused primarily by hyperglycaemia secondary to insulin deficiency or glucagon excess in Type 2 diabetes [2–4,8–10]. However, few studies have been performed in humans or animals to determine whether long-term hyperglycaemia can replicate the metabolic and renal phenotypes of Type 2 diabetes. In the present study, we fed a separate group of mice a high glucose load (2%) in order to maintain blood glucose at postprandial levels or levels found in glucagon-infused mice for 4 weeks (Table 3). We reasoned that if high-glucose-fed mice also developed similar metabolic and renal abnormalities after 4 weeks of glucose administration, as demonstrated in glucagon-infused mice, then we can reasonably conclude that hyperglycaemia alone, rather than hyperglucagonaemia, was responsible for causing those effects in glucagon-infused mice. However, long-term administration of a high glucose load did not significantly increase basal fasting blood glucose levels or impair weekly glucose tolerance in mice, as widely expected (Table 3). With the exception of 24 h fluid intake and urine excretion, most abnormal metabolic and renal phenotypes, as demonstrated in glucagon-infused mice, were not reproduced in mice treated with a high glucose load alone (Table 3). The mechanisms responsible for the apparent lack of Type 2 diabetic metabolic and renal phenotypes in mice treated with high glucose intake in the present study are not known. Nevertheless, three factors may be considered. First, the high glucose load or the level of hyperglycaemia was not sufficiently high or severe enough, as occur in Type 1 diabetes. Secondly, the duration of high glucose load or hyperglycaemia was not sufficiently long enough, which may take several months or years, as occurs in human diabetes. And thirdly, in the presence of a healthy pancreas, high glucose intake or hyperglycaemia may induce insulin secretion while suppressing glucagon secretion (Table 3). Our results support the hypothesis that the non-hyperglycaemic actions of glucagon may play an important role in the development of target organ injury in Type 2 diabetes. These issues require further studies.

In summary, the present study has demonstrated for the first time in a mouse model that long-term glucagon administration for 4 weeks induces important early metabolic and renal phenotypes of Type 2 diabetes by activating specific glucagon receptors. The metabolic and renal abnormalities include a moderate increase in SBP, elevated fasting blood glucose, impaired glucose tolerance, and the development of renal hypertrophy, glomerular injury and microalbuminuria. These effects of glucagon were largely blocked by concurrent administration with a selective glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon. By contrast, administration of the antagonist or high glucose intake alone for 4 weeks did not produce the metabolic and renal phenotypes of glucagon-infused mice. Our results suggest that long-term hyperglucagonaemia may play an important role in increased insulin resistance and development of renal glomerular injury in Type 2 diabetes [31]. Thus hyper-glucagonaemia in mice induced by long-term glucagon infusion may be a novel tool to study the role of glucagon in Type 2 diabetes and assist in the development of orally potent glucagon receptor antagonists for treating cardiovascular and renal complications of Type 2 diabetes.

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Abbreviations

BP	blood pressure
ERK1/2	extracellular-signal-regulated kinase 1/2
GTT	glucose tolerance test
PAS	periodate–Schiff
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
SBP	systolic BP

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Figure 1. Effects of long-term hyperglucagonemia on SBP in glucagon-infused mice
 Values are means $\pm$ S.E.M. ($n = 8$). Note that glucagon induced moderate, but time-dependent, increases in SBP. ** $P < 0.01$ compared with control mice. ++ $P < 0.01$ compared with glucagon-infused mice. Ant, [Des-His¹-Glu⁹]glucagon.

Figure 2. Effects of long-term hyperglucagonaemia on fasting blood glucose (A) and glucose tolerance (B) in glucagon-infused mice
 Values are means $\pm$ S.E.M. ($n = 8$). ** $P < 0.01$ compared with basal (week 0) for fasting blood glucose (in A) or compared with 0 min for the GTT within the same group (in B). ++ $P < 0.01$ compared with glucagon-infused mice at corresponding periods. Ant, [Des-His¹-Glu⁹] glucagon.

Figure 3. Effects of long-term hyperglucagonaemia on renal hypertrophy (A) and microalbuminuria (B) in glucagon-infused mice

Values are means $\pm$ S.E.M. ($n = 8$) * $P < 0.05$ compared with control mice; + $P < 0.05$ and ++ $P < 0.01$ compared with glucagon-infused mice. ** $P < 0.01$ compared with week 0 before glucagon infusion. Glu, glucagon; Ant, [Des-His¹-Glu⁹]glucagon.

Figure 4. Effects of long-term hyperglucagonaemia on glomerular mesangial expansion and extracellular matrix deposition in glucagon-infused mice

Kidney sections from control (A), glucagon-infused mice (B) and mice treated concurrently with glucagon and [Des-His¹-Glu⁹]glucagon (C) were stained with PAS. Mesangial expansion and extracellular matrix deposition are indicated by the arrows. The extent of glomerular injury was expressed as a percentage of glomerular volume (D). Values are means $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ compared with control mice; ++ $P < 0.01$ compared with glucagon-infused mice. Ant, [Des-His¹-Glu⁹]glucagon.

Figure 5. Effects of long-term glucagon infusion on total and phospho-ERK 1/2 in mouse liver (A) and kidney (B), as measured using Western blotting
p-ERK1/2, phospho-ERK1/2 (Tyr²⁰⁴); t-ERK1/2, total ERK1/2. Quantification of phospho-ERK1/2 is shown below the immunoblots. Values are means $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ compared with control; ++ $P < 0.01$ compared with glucagon-infused mice. Ant, [Des-His¹-Glu⁹]glucagon.

Figure 6. Effects of long-term glucagon infusion on total and phospho-Akt in mouse liver (A) and kidney (B), as measured using Western blotting
 p-Akt, phospho-Akt (Ser⁴⁷³); t-Akt, total Akt. Quantification of phospho-Akt is shown below the immunoblots. Values are means $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ compared with control; ++ $P < 0.01$ compared with glucagon-infused mice. Ant, [Des-His¹-Glu⁹] glucagon.

Table 1

Effects of the glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon on long-term hyperglucagonaemia-induced SBP, and metabolic and renal phenotypes in glucagon-infused mice

Parameter	Control	Glucagon	Glucagon + [Des-His ¹ -Glu ⁹]glucagon
Body weight (g)	24.2 ± 0.4	23.3 ± 0.5	21.9 ± 0.6
Water intake (ml/24 h)	3.9 ± 0.3	3.9 ± 0.2	3.1 ± 0.4 [*]
SBP (mmHg)	111 ± 3	134 ± 5	104 ± 4 ⁺⁺
Heart rate (beats/min)	619 ± 18	658 ± 16 [*]	611 ± 25 ⁺
Heart weight (mg)	146.9 ± 4.8	160.5 ± 6.2 [*]	140.5 ± 4.3 ⁺
Kidney weight (mg)	117.6 ± 4.4	126.2 ± 3.3 [*]	110.5 ± 3.9 ⁺
Urine excretion (ml/24 h)	1.2 ± 0.1	1.6 ± 0.2 [*]	1.1 ± 0.1 ⁺
Haematocrit (%)	40.2 ± 1.0	44.1 ± 1.0 [*]	43.2 ± 1.0 [*]
Serum glucagon (pg/ml)	240.9 ± 37.6	445 ± 45.6 ^{**}	882.5 ± 50.5 ^{**++}
Serum insulin (pg/ml)	303 ± 63.1	249.9 ± 50.8	454 ± 71.3 ⁺

* $P < 0.05$ or

** $P < 0.01$ compared with control mice;

⁺ $P < 0.05$ and

⁺⁺ $P < 0.01$ compared with glucagon-infused mice.

Table 2

Effects of long-term administration of the glucagon receptor antagonist [Des-His¹-Glu⁹]glucagon alone for 4 weeks on SBP, and metabolic and glucose tolerance in mice

Parameter	Basal	Week 1	Week 2	Week 3	Week 4
Body weight (g)	21.6 ± 0.8	21.8 ± 0.5	22.9 ± 0.4	23.8 ± 0.6	24.1 ± 0.4
SBP (mmHg)	116 ± 3	115 ± 5	115 ± 3	113 ± 4	115 ± 3
Water intake (ml/24 h)	4.0 ± 0.4	4.0 ± 0.2	3.6 ± 0.2	3.4 ± 0.2	3.4 ± 0.2
Urine excretion (ml/24 h)	1.01 ± 0.14	0.38 ± 0.15	0.73 ± 0.12	0.62 ± 0.11	0.57 ± 0.21
Heart weight (mg)	n/a	n/a	n/a	n/a	105.4 ± 2.5
Kidney weight (mg)	n/a	n/a	n/a	n/a	116.3 ± 2.1
Fasting blood glucose (mg/dl)	85.3 ± 8.8	95.3 ± 4.2	84.3 ± 1.7	80.7 ± 3.6	73.7 ± 2.2
Postprandial blood glucose (mg/dl)	145.6 ± 4.8	n/a	130.5 ± 3.9	n/a	142 ± 7.4
GTT (mg/dl)					
30 min	263.5 ± 9.4	n/a	256.7 ± 8.9	n/a	252.7 ± 16.1
60 min	194.8 ± 10.3	n/a	154.7 ± 4.8	n/a	159.3 ± 8.4
90 min	145.3 ± 5.6	n/a	128.8 ± 9.6	n/a	133.5 ± 8.4
120 min	136.7 ± 6.5	n/a	117.5 ± 6.8	n/a	120.7 ± 7.8
Serum glucagon (pg/ml)	n/a	n/a	n/a	n/a	407.3 ± 92.3
Serum insulin (pg/ml)	n/a	n/a	n/a	n/a	949 ± 133

n/a, not measured at this time-point.

* $P < 0.05$ and

** $P < 0.01$ compared with basal.

Effects of long-term administration of a high glucose load alone (2 % in drinking water) for 4 weeks on SBP, and metabolic and glucose tolerance in mice

Parameter	Basal	Week 1	Week 2	Week 3	Week 4
Body weight (g)	22.7 ± 0.6	24.5 ± 0.7	24.8 ± 0.7	24.9 ± 0.7	25.2 ± 0.7
SBP (mmHg)	119 ± 3	120 ± 4	118 ± 3	117 ± 3	117 ± 3
Water intake (ml/24 h)	4.7 ± 0.2	5.7 ± 0.4 *	5.3 ± 0.2	5.6 ± 0.3 *	4.5 ± 0.3
Urine excretion (ml/24 h)	1.1 ± 0.2	1.7 ± 0.4 *	2.1 ± 0.4 *	2.1 ± 0.2 *	1.3 ± 0.2
Heart weight (mg)	n/a	n/a	n/a	n/a	111.1 ± 3.2
Kidney weight (mg)	n/a	n/a	n/a	n/a	116.8 ± 5.5
Fasting blood glucose (mg/dl)	81.4 ± 5.2	88.2 ± 6.3	78.5 ± 5.6	78.4 ± 2.7	75.9 ± 4.3
Postprandial blood glucose (mg/dl)	154.6 ± 11.5	n/a	163.4 ± 5.9	n/a	164.2 ± 5.7
GTT (mg/dl)					
30 min	237 ± 7.9	n/a	230.0 ± 17	n/a	258.3 ± 17.1
60 min	178.4 ± 6.8	n/a	171.7 ± 11	n/a	182.5 ± 14.7
90 min	147.6 ± 2.2	n/a	153.7 ± 5.2	n/a	148.0 ± 11.8
120 min	132.1 ± 4.6	n/a	138.6 ± 3.7	n/a	133.4 ± 8.9
Serum glucagon (pg/ml)	n/a	n/a	n/a	n/a	208.8 ± 47.6
Serum insulin (pg/ml)	n/a	n/a	n/a	n/a	1335 ± 103

n/a, not measured at this time-point.
* *P* < 0.05 compared with basal.