Glucocorticoid treatment and endocrine pancreas function: implications for glucose homeostasis, insulin resistance and diabetes.

Running title: Glucocorticoids and pancreatic endocrine cells

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Abstract

Glucocorticoids (GCs) are broadly prescribed for numerous pathological conditions due to their anti-inflammatory, antiallergic and immunosuppressive effects, among other actions. Nevertheless, GCs can produce undesired diabetogenic side effects through interactions with the regulation of glucose homeostasis. Under conditions of excess and/or long-term treatment, GCs can induce peripheral insulin resistance (IR) by impairing insulin signalling, which results in reduced glucose disposal and augmented endogenous glucose production. Additionally, GCs can promote abdominal obesity, elevate plasma fatty acids and triglycerides and suppress osteocalcin synthesis in bone tissue. In response to GC-induced peripheral IR and in an attempt to maintain normoglycaemia, pancreatic beta-cells undergo several morphofunctional adaptations that result in hyperinsulinaemia. Failure of beta-cells to compensate for this situation favours glucose homeostasis disruption, which can result in hyperglycaemia, particularly in susceptible individuals. GC treatment does not only alter pancreatic beta-cell function; pancreatic alpha-cells are also affected by GC actions that can lead to hyperglucagonaemia, further contributing to glucose homeostasis imbalance and hyperglycaemia. Additionally, the release of other islet hormones, such as somatostatin, amylin and ghrelin, are also affected by GC administration. These undesired GC actions merit further consideration for the design of improved GC therapies without diabetogenic effects. In summary, in this review, we consider the impact of GC treatment on peripheral IR, islet function and glucose homeostasis.

1. Introduction.

Glucocorticoids (GC), such as cortisol in humans and corticosterone in rodents, are produced in the adrenal cortex and play a key role in regulating glucose homeostasis and nutrient metabolism. Synthetic GCs, which include dexamethasone and prednisolone, are used in medical practice because of their anti-inflammatory, antiallergic and immunosuppressive effects. Although synthetic GCs are broadly prescribed in numerous pathological conditions, they have important adverse metabolic effects, including peripheral insulin resistance (IR) and glucose intolerance as well as overt hyperglycaemia and diabetes. These side effects are observed particularly in susceptible individuals such as pregnant women, obese subjects, insulin-resistant individuals or first-degree relatives of diabetic patients (Van Raalte *et al.* 2009). The ability of GCs to produce peripheral IR is central to explain their impact on glucose homeostasis. It is well known that any reduction in peripheral insulin sensitivity, e.g., when GCs are administered, is adaptively compensated by augmented pancreatic beta-cell function (Beard *et al.* 1984, Nicod *et al.* 2003, Ahrén 2008, Rafacho *et al.* 2008). This islet compensation meets the principle of the disposition index, the product of insulin secretion and peripheral insulin sensitivity. When beta-cells fail to adjust to the insulin demand imposed by the GC treatment, fasting and/or postprandial hyperglycaemia may arise. The severity and progression of these alterations depend on several parameters including dosage, period and previous individual susceptibility among others (Novelli *et al.* 1999, Rafacho *et al.* 2008, Jensen *et al.* 2012). In addition to the islet's compensatory responses to IR, GCs directly affect beta-cell function, which may further complicate adequate glycaemia regulation. Although less explored than insulin release, these steroids also affect the secretion of other islet hormones with important roles in glucose homeostasis, such as glucagon, somatostatin and amylin. All

these alterations in islet hormonal secretion can exacerbate GCs' diabetogenic actions. In the next sections, we review the main effects of GCs on peripheral tissues and the endocrine pancreas and also consider the risks and limitations of their therapeutic use.

1.1. Cellular mechanisms of glucocorticoid action. Ninety-five percent of circulating cortisol is bound to corticosteroid-binding globulins and albumin (Andrews & Walker 1999). The plasma levels of the inactive form, cortisone, are approximately 50-100 nM, and the hormone is largely unbound to plasma proteins (Walker *et al.* 1999). Local conversion between active and inactive forms is catalysed by 11beta-hydroxysteroid dehydrogenase (11beta-HSD). 11beta-HSD type 1 is a reductase that converts inactive cortisone (in humans) and 11-dehydrocorticosterone (in rodents) to active cortisol and corticosterone, respectively (Low *et al.* 1994, Voice *et al.* 1996). The type 2 isoform works as a dehydrogenase that catalyses the opposite reaction (Brown *et al.* 1993). The actions of 11beta-HSD1 and 11beta-HSD2 serve as a pre-receptor control of GC action and determine local GC concentrations.

GC action at the site of cells is activated by the steroid hormone binding to its receptor. The classical GC receptor (GR), a ligand-regulated transcription factor that belongs to the superfamily of nuclear receptors, binds GCs and regulates transcription of target genes by activation or repression (Mangelsdorf *et al.* 1995). The GR is expressed in virtually all tissues; however, GR is able to regulate genes in a cell-specific manner, indicating that the response to GCs is regulated by factors beyond receptor expression. The GR is guided from the moment of synthesis to decay through signal transduction and by a variety of molecular chaperones such as HSP70 (Nelson *et al.* 2004) and HSP90 (Pratt *et al.* 2006), which facilitate folding, maturation and ligand binding. In addition, GR-mediated transcriptional activation is modulated both

positively and negatively by phosphorylation (Ismaili & Garabedian 2004) performed by kinases and phosphatases. Although the activity of the GR is often thought in terms of direct gene transactivation, considerable cross-talk also occurs between the GR and a cohort of molecules to mediate their function as transcriptional factors, including octamer transcription factors Oct1 and Oct2, CREB (cAMP response element binding protein) and STAT5 (signal transducers and activators of transcription-5) (Chen *et al.* 2012, Ratman *et al.* 2013, Engblom *et al.* 2007). Competition for limiting transcription co-activators is an important determinant of the fate of the cross-talk between the GR and other transcription factors. In addition to these genomic GC actions, the steroid hormone can induce effects on a minute time scale, which is difficult to explain by mechanisms involving gene expression changes (Long *et al.* 2005). Localised cell membrane receptors with GC affinity have recently been identified (Strehl & Buttgereit, 2014).

1.2. Glucocorticoid therapy in clinical practice. Drugs based on GCs were introduced in the 1950s and have been an important therapeutic strategy to treat rheumatic and inflammatory diseases ever since. In this regard, the relevant properties are the immunosuppressive, anti-inflammatory and anti-allergic effects that GCs exert on primary and secondary immune cells, tissues and organs (Stahn & Buttgereit 2008). Estimates suggest that between 1 and 2% of the adult population in the Western world is receiving some form of long-term GC treatment, with a clear higher usage among the geriatric patient group (Van Staa *et al.* 2000). In dermatology, GCs are the most widely used therapy, for example, to treat atopic eczema. Inhaled GCs are used to treat allergic reactions in airways and to dampen bronchial hyperreactivity in asthma. Systemically,

GCs are used to combat connective tissue inflammation, rheumatoid arthritis, bowel diseases and in allotransplantation (Thiele *et al.* 2005).

2. Diabetogenic actions of glucocorticoids in skeletal muscle and adipose, hepatic and bone tissues. There are a myriad of risks associated with excessive GC use; these risks have been recognised since GCs came into clinical use (Schäcke *et al.* 2002). Given GCs' strong capacity to counteract the action exerted by insulin and raise blood sugar levels, it is not surprising that IR and glucose intolerance is a concern in patients with Cushing's syndrome and disease (endogenous GC overproduction) and in patients prescribed GC-based therapy for immunomodulatory purposes (Raúl Ariza-Andraca *et al.* 1998). In addition, hypercortisolaemic conditions share many characteristics with metabolic syndrome, a cluster of abnormalities including hyperglycaemia, abdominal obesity, dyslipidaemia and hypertension (Anagnostis *et al.* 2009). Low-dose GC therapy is considered when the daily dose is less than 7.5 mg prednisolone or equivalent (van der Goes et al. 2010). When such a dose is administrated orally, plasma prednisolone levels peak 2-4 hours after intake at about 400-500 nM (~150-200 ng/ml) and return to baseline within 12 hours after steroid administration (Wilson *et al.* 1977, Tauber *et al.* 1984). These values are in the same range as normal endogenous cortisol levels: reference values for samples taken between 4:00 am and 8:00 am are 250-750 nM and for samples taken between 8:00 pm and 12:00 pm are 50-300 nM. This indicates that the absolute cortisol values are not as important for developing adverse effects during low-dose GC therapy as is the diurnal variation. Current knowledge gives at hand that developing diabetes after starting low-dose GC treatment seems rare but progression of already impaired glucose tolerance to overt diabetes is possible (van der Goes *et al.* 2010). Therefore, clinical recommendation states that baseline fasting glucose should be

monitored before initiating therapy and during following up according to standard patient care. Certainly, the adverse effects are more pronounced during high-dose GC therapies (>30 mg prednisolone or equivalent daily). In a retrospective study of hemoglobin A1c (HbA1c) levels in patients with rheumatic diseases subjected to prednisolone treatment, it was found that around 82% had HbA1c levels higher than 48 mmol/mol (given in IFCC standard, corresponding to 6.7% in DCCT standard). Serum HbA1c levels higher than 52 mmol/mol (7.1%), were seen in 46% of the patients and 23% of the patients had HbA1c levels as high as 57 mmol/mol (7.6%), which should be considered as a high risk factor for diabetes. Taken together, it was found that the cumulative prednisolone dose was the only factor significantly associated with the development of steroid-induced diabetes among rheumatic patients (Origuchi *et al.* 2011).

2.1. Adipose tissue. GCs regulate the maturation of pre-adipose cells into differentiated adipose cells as well as metabolism in adipose tissue (Rebuffé-scrive *et al.* 1992). Because the GR is predominantly expressed in adipose cells located in intra-abdominal fat, GCs are more highly activated in these fat deposits (Pedersen *et al.* 1994). A striking feature observed under conditions of GC excess is enhanced accumulation of visceral fat and loss of peripheral fat deposits in the arms and legs (Reynolds *et al.* 2012) (Figure 1). In the peripheral fat deposits, GCs promote expression of the key lipolytic enzyme hormone-sensitive lipase (Slavin *et al.* 1994) and, thus, acute infusion of cortisol in healthy humans induces triglyceride hydrolysis and the release of fatty acids and glycerol to the systemic circulation (Divertie *et al.* 1991). On the contrary, it has been suggested that GCs promote increased fat mass and triglyceride synthesis in visceral fat. Hence, GCs and insulin work in concert to activate lipoprotein lipase (Ottosson *et al.* 1994), which leads to relocation of fat deposits from

arms and legs to abdominal sites. Furthermore, GC treatment was shown to inhibit AMPK (5' AMP-activated protein kinase) activity specifically in rat visceral but not subcutaneous adipose tissue (Christ-Crain *et al.* 2008), which may explain the redistribution of fat deposits that occurs during GC excess. This hypothesis remains to be proven in humans but is supported by the observation that patients with Cushing's syndrome exhibited a 70% lower AMPK activity in visceral adipose tissue (Kola *et al.* 2008). Additionally, GC-induced attenuation of insulin signalling in the adipose tissue has been associated with reduced glucose uptake (Ortsäter *et al.* 2012). In summary, GCs exposure leads to impaired insulin signalling and a systemic elevation of fatty acids and triglycerides which contributes to IR. Furthermore, GCs induce abdominal obesity.

2.2. Skeletal muscle. Skeletal muscle accounts for approximately 80% of insulin-mediated glucose uptake (IMGU) and is the largest glycogen store. GCs interfere directly with insulin signalling in skeletal muscle cells. Studies have shown that administration of dexamethasone reduces expression and activity of IRS1 (insulin substrate-1) and PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) in rodent skeletal muscle cells (Saad *et al.* 1993, Morgan *et al.* 2005), which would presumably lead to a reduction in IMGU and abrogation of glycogen synthesis (Figure 1). Indeed, in a study with healthy human volunteers, prednisolone treatment for 6 days (0.8 $mg \cdot kg^{-1}$ day^{-1}) reduced insulin-induced leg glucose uptake by 65% compared to placebo treatment (Short *et al.* 2009). In support, rats treated with GCs were shown to have reduced insulin-stimulated glucose uptake, caused by attenuated insulin-induced GLUT4 (glucose transporter type 4) translocation to the cell membrane in myotubes (Dimitriadis *et al.* 1997). The condition is worsened by the accumulation of ectopic fat deposition in skeletal muscle (Fransson *et al.* 2013) (Figure 1), which originates from

the systemic GC-induced fatty acid elevation as discussed above. Taken together, these data show that GCs directly interfere with insulin signalling in skeletal muscle cells leading to reduced IMGU.

2.3. Hepatic tissue. Hepatic tissue plays a key role in controlling glucose and lipid homeostasis. Although insulin does not directly stimulate glucose uptake in liver cells, the hormone is responsible for hepatic glycogen synthesis and gluconeogenesis suppression. These insulin actions are mediated via insulin receptor signalling. As in skeletal muscle, GC excess also interferes with the insulin signalling cascade in hepatic tissue. In one study, dexamethasone-treated rats (1.5 mg/kg body weight for 6 consecutive days) exhibited an approximately 50-70% reduction in insulin receptor binding in hepatocytes (Olefsky *et al.* 1975). A significant reduction in insulin receptor density was also observed in hepatocytes from rats chronically treated with dexamethasone (Caro & Amatruda 1982). Diminished tyrosine phosphorylation in either insulin receptor or IRS1 was observed in liver from rats treated with dexamethasone for 5 consecutive days (Saad *et al.* 1993). In addition, GCs were shown to augment endogenous glucose production in several (Rizza *et al.* 1982, Pagano *et al.* 1983, Rooney *et al.* 1993) but not all (Wajngot *et al.* 1990) studies conducted in healthy humans. GC-driven glucose production may be caused by enhanced gluconeogenesis, as GCs induce rate limiting enzymes for gluconeogenesis, e.g., phosphoenolpyruvate carboxylase and glucose-6-phosphatase (Lange et al 1994, Cassuto *et al.* 2005) (Figure 1). GC-mediated expression of gluconeogenic enzymes appears to be dependent on liver X receptor (LXR) expression (Patel *et al.* 2011). Indeed, mice lacking LXRbeta (but not LXRalpha) were demonstrated to be resistant to dexamethasone-induced hyperglycaemia, hyperinsulinaemia, and hepatic steatosis but remained sensitive to dexamethasone-dependent immune system repression (Patel *et al.* 2011). Moreover,

since GCs promote muscle wasting and lipolysis, they also increase the bioavailability of substrates for gluconeogenesis (Divertie *et al.* 1991, Kim *et al.* 2012) (Figure 1). Finally, fat accumulation leads to hepatic steatosis (Fransson *et al.* 2013), which, by itself, attenuates insulin sensitivity (Kim *et al.* 2012). To summarise, elevated GC levels promote gluconeogenesis in hepatic tissue leading to fasting hyperglycaemia.

2.4. Bone tissue. Osteoporosis is a common side effect observed in patients on GC-based therapy (Hoes *et al.* 2010). GCs also suppress osteoblast function, including osteocalcin synthesis (Prummel *et al.* 1991) (Figure 1). Osteocalcin is an osteoblast-specific peptide that is reported to be involved in normal murine fuel metabolism (Ferron *et al.* 2008). In pioneering work by Lee et al (Lee *et al.* 2007), it was demonstrated, both in cell culture and in mice, that osteocalcin increased pancreatic beta-cell proliferation as well as insulin expression and release, resulting in improved glucose tolerance. In addition, uncarboxylated osteocalcin increased adiponectin expression and secretion in adipose tissue, which in turn enhanced insulin sensitivity (Lee *et al.* 2007). In human type 2 diabetes, serum osteocalcin concentrations are positively correlated with improved glucose control (Bao *et al.* 2011). In another study, osteoblast-targeted disruption of GC signalling significantly attenuated the suppression of osteocalcin synthesis and prevented the development of insulin resistance, glucose intolerance, and abnormal weight gain in corticosterone-treated mice (Brennan-Speranza *et al.* 2012). Nearly identical effects were observed in GC-treated animals following hepatic expression of both carboxylated and uncarboxylated osteocalcin. These data suggest a link between GC effects on the skeleton and the steroid hormone effects on glucose homeostasis.

3. Effects of glucocorticoid treatment on pancreatic beta-cells and insulin secretion. Pancreatic beta-cells respond to increasing plasma glucose levels by secreting insulin, which maintains glycaemia within narrow physiological ranges. This key function can be altered by GCs through direct and indirect actions and may also depend on whether GCs act as acute or chronic stimuli. In the next sections, we consider the different aspects of GCs' effects on beta-cells.

3.1. Acute effects of glucocorticoids. The direct *in vitro* effects of GCs on glucose-stimulated insulin secretion (GSIS) are generally inhibitory and occur within a few minutes, as demonstrated in isolated rat islets exposed to corticosterone (0.02-20 mg/L) (Billaudel & Sutter 1979) (Figure 2A, left). This inhibitory action involves alpha-adrenergic signalling because phentolamine (a non-selective alpha-adrenergic antagonist) blocks GCs' effect (Barseghian & Levine 1980). This rapid impact of GCs is not reproduced by synthetic steroids. GSIS inhibition in mouse (Lambillotte *et al.* 1997) and rat islets (Zawalich *et al.* 2006) is apparent only after the third hour of exposure to 1 μM dexamethasone.

GCs may also exert a negative *in vivo* effect during acute administration. A single oral dose of prednisolone (75 mg) (van Raalte *et al.* 2010) or dexamethasone (1 mg) (Schneiter & Tappy 1998) in healthy volunteers resulted in decreased insulin secretion and/or a reduced insulinogenic index (the ratio between Δinsulinaemia and Δglycaemia) during a meal or an oral glucose tolerance test (oGTT), respectively. In contrast, other studies did not demonstrate this acute GC effect in healthy men (Vila *et al.* 2010) or normal adult rats (Stojanovska *et al.* 1990) during an intravenous or oGTT, respectively. Similar to the *in vitro* observations mentioned above, increased sympathetic drive may be involved in GCs' inhibition of *in vivo* insulin secretion

(Longano & Fletcher 1983). This hypothesis is based on a study conducted with adult Swiss mice treated with hydrocortisone (300 mg/kg body weight) 1 hour prior to determining fed blood glucose and plasma insulin values. The insulinogenic index was reduced 1 hour after steroid administration in fed mice but unaltered when chlorisondamine (a ganglionic blocker) or phentolamine were given 10 minutes before GC administration (Longano & Fletcher 1983) (Figure 2B, left). Overall, acute exposure or administration of GCs appears to cause a decline in the insulinogenic index in humans and rodents, and this effect may be mediated by sympathetic activation of alpha-adrenergic receptors. It is important to highlight that 24 hours after interrupting GC administration, all beta-cell function parameters return to normal values (van Raalte *et al.* 2010).

3.2. Chronic effects of glucocorticoids. As observed in acute *in vitro* experiments, chronic incubation (hours to days) with synthetic GCs in *in vitro* conditions leads to decreased GSIS in rodent isolated islets, dispersed beta-cells and insulin-secreting cell lines (Lambillotte *et al.* 1997, Zawalich *et al.* 2006), Shao *et al.* 2004, Ullrich *et al.* 2005). GCs' deleterious effects on GSIS involve impaired glucose oxidative metabolism (Shao *et al.* 2004), activation of repolarising K^+ channels (Ullrich *et al.* 2005), generation of reactive oxygen species (Roma *et al.* 2011), endoplasmic reticulum dyshomeostasis (Linssen *et al.* 2011), activation of 11β-HSD1 (Davani *et al.* 2000) and decreased efficiency of intracellular Ca^{2+} on the secretory response (Lambillotte *et al.* 1997, Zawalich *et al.* 2006, Shao *et al.* 2004) (Figure 2A, right).

However, in contrast to the above-mentioned inhibitory effects observed in both acute and long-term GC incubation, chronic *in vivo* administration of these steroids leads to up-regulated beta-cell function as a result of the compensatory adaptation to

GC-induced IR. Administration of high doses of prednisolone (30 mg) or dexamethasone (2 to 15 mg) to healthy individuals for prolonged periods (up to 15 days and up to 4 days, respectively) resulted in normoglycaemia or a modest increase of fasting glycaemia (Beard *et al.* 1984, Schneiter & Tappy 1998, Hollindgal *et al.* 2002, Willi *et al.* 2002, Nicod *et al.* 2003, Ahrén 2008, van Raalte *et al.* 2010, Petersons *et al.* 2013). Importantly, in most of these studies, volunteers developed hyperinsulinaemia. In fact, during glucose challenging with a hyperglycaemic-clamp (Beard *et al.* 1984, Nicod *et al.* 2003) or an oGTT (Schneiter & Tappy 1998, Hollindgal *et al.* 2002, Willi *et al.* 2002) insulin release was significantly higher in GC-treated individuals compared to control groups. Plasma C-peptide values were also elevated after prednisolone treatment in healthy men at basal conditions (Hollindgal *et al.* 2002) and during a meal tolerance test (van Raalte *et al.* 2010). This enhanced beta-cell function was also observed in adult rats treated for up to 13 consecutive days with dexamethasone (0.125-2.0 mg/kg) based on basal hyperinsulinaemia (Novelli *et al.* 1999, Karlsson *et al.* 2001, Rafacho *et al.* 2008) or *in vivo* glucose challenging (Rafacho *et al.* 2008, 2011). This augmented beta-cell function occurred in a dose- (Rafacho *et al.* 2008) and time-dependent manner (Rafacho *et al.* 2011). In normal adult mice, administration of dexamethasone for 10 days or corticosterone from the first consecutive week also resulted in basal hyperinsulinaemia (Thomas *et al.* 1998, Fransson *et al.* 2013).

This hyperinsulinaemia is consistent with insulin hypersecretion observed in pancreatic islets isolated from GC-treated rats (Novelli *et al.* 1999, Karlsson *et al.* 2001, Rafacho *et al.* 2008, 2010a, 2010b). This enhanced beta-cell secretion involves an improvement in glucose responsiveness (Karlsson *et al.* 2001, Rafacho *et al.* 2008), sensitivity (Rafacho *et al.* 2008) and oxidative metabolism (Rafacho *et al.* 2010a) as well as augmented Ca^{2+} handling (Rafacho *et al.* 2010a) and an improved response to

cholinergic signals (Angelini *et al.* 2010, Rafacho *et al.* 2010,) (Figure 2B, right). The islet compensatory response is also accompanied by structural changes. It has been demonstrated that, beta-cell mass increases in a time- (Rafacho *et al.* 2011) and dose-dependent manner (Rafacho *et al.* 2009) with GC administration, according to the correspondent degree of insulin insensitivity. Taken together, these results show that when humans or animal models are exposed to prolonged steroid treatment, they develop augmented beta-cell function and mass to counteract the IR resulting from GC administration.

3.3. Glucocorticoid treatment, beta-cell dysfunction and glucose intolerance.

Depending on the GC regimen, glucose homeostasis is maintained at normal or near normal physiological conditions by adaptive beta-cell compensations. However, these adaptations do not always guarantee an adequate glucose homeostasis. Although insulin hypersecretion observed after prolonged steroid treatment appears to be consistent in most experiments performed with healthy volunteers (Beard *et al.* 1984, Schneiter & Tappy 1998, Ahrén 2008, van Raalte *et al.* 2010) and normal adult rats (Karlsson *et al.* 2001, Rafacho *et al.* 2008, 2009, 2011), glucose intolerance is also present. In these studies, hyperinsulinaemia is normally associated with normoglycaemia or modest increases in blood glucose values, but the insulin (Rafacho *et al.* 2008, 2011, Schneiter & Tappy 1998) and c-peptide hypersecretion (van Raalte *et al.* 2010) during glucose or meal challenges, respectively, do not prevent elevation in postprandial blood glucose levels. Therefore, the insulinogenic index may not necessarily match the peripheral insulin demand imposed by GCs.

The negative impact of GCs on glucose homeostasis is more apparent in individuals with any degree of susceptibility to glucose intolerance, such as those with

low insulin sensitivity (Larsson & Ahrén 1999), low insulin response to glucose (Wajngot *et al.* 1992), first-degree relatives of patients with type 2 diabetes (Jensen *et al.* 2012), obesity (Besse *et al.* 2005) and those who are older (Novelli *et al.* 1999). In these individuals, beta-cell function does not correspond to the peripheral insulin demand, and the deregulation of glucose homeostasis becomes more pronounced, reinforcing that individual background is a critical factor. Indeed, this susceptibility to beta-cell failure after treatment with dexamethasone has also been observed in animal models with an obesity background, such as *fa/fa* rats (Ogawa *et al.* 1992) and *ob/ob* mice (Khan *et al.* 1992).

In an attempt to analyse whether GCs have any direct effects on beta-cells *in vivo* independent of peripheral GC actions, a transgenic mouse model that specifically over-expresses GR in these cells was generated (Delaunay *et al.* 1997, Davani *et al.* 2004). These mice were normoglycaemic but displayed glucose intolerance associated with reduced insulin secretion during a glucose load (Delaunay *et al.* 1997). When these transgenic mice aged, hyperglycaemia developed together with marked glucose intolerance and reduced *in vivo* and *ex vivo* GSIS. Remarkably, no change in beta-cell apoptosis was observed in these mice (Davani *et al.* 2004). This deterioration in GSIS was prevented by incubating islets with benextramine (a selective α2-adrenergic receptor antagonist), suggesting the involvement of adrenergic signals. In any case, the analysis of direct GC effects on beta-cells *in vivo* is difficult because the systemic metabolic consequences of GC treatment most likely mask the GC-mediated changes in beta-cell function. Of note, almost all the morphofunctional beta-cell changes elicited by GC administration are transitory and reversible after 10 days of discontinuation of steroid treatment in rats, suggesting an unacknowledged plasticity in the regulation of beta-cell function and growth (Rafacho *et al.* 2010b).

4. Effects of glucocorticoids on glucagon release and other islet hormones.

Glucagon secretion by pancreatic alpha-cells plays a key role in glucose homeostasis. Glucagon's release is enhanced at low plasma glucose levels but decreases under hyperglycaemic conditions (Quesada *et al.* 2008; Marroqui *et al.* 2014). Glucagon is one of the most important hyperglycaemic hormones and acts as insulin's counterpart, opposing numerous anabolic insulin-mediated actions. The hyperglycaemic effect is mainly produced by activating hepatic glycogenolysis and gluconeogenesis, which results in the release of endogenous glucose into the bloodstream. This process restores normoglycaemia under hypoglycaemic conditions (Quesada *et al.* 2008; Marroqui *et al.* 2014). Hyperglucagonaemia may be present in diabetes. Additionally, inhibition of glucagon release at high glucose levels may be impaired in this metabolic condition. This impaired alpha-cell function can lead to higher hepatic glucose output, further contributing to hyperglycaemia in diabetic patients (Quesada *et al.* 2008; Marroqui *et al.* 2014). As in the case of beta-cells, in the next section we summarise the acute and chronic effects of GCs on alpha-cell function.

4.1. Acute effects of glucocorticoids on alpha-cell function and glucagon release.

One study reported that corticosterone (10^{-7} M) potentiated glucagon release induced by a glucose-free medium or arginine in isolated perfused rat pancreas (Barseghian & Levine 1980). In contrast, incubation of mouse pancreatic islets with dexamethasone (0.5-50 nM), corticosterone (50 nM) or 11-dehydrocorticosterone (50 nM) for 2 hours reduced glucagon secretion induced by low glucose levels, effects that were reversed by a GR antagonist (Swali *et al.* 2008). The inhibitory action of 11-dehydrocorticosterone was partially reversed by a selective 11beta-HSD1 inhibitor. This

fact, along with the co-localisation of this enzyme with human and rodent islet alpha-cells, indicates that this islet cell type serves an important local function in pancreatic GC metabolism (Swali *et al.* 2008). This situation may be different in other species, for example in rats, where this enzyme is expressed in non-alpha-cells (Rafacho *et al.* 2014). In contrast with the above-mentioned results, prednisolone (10^{-5} M) failed to modify glucagon secretion in mouse pancreatic islets (Marco *et al.* 1976). Likewise, incubation of rat pancreatic islets with dexamethasone (1 µM) for 3 hours did not modify glucagon secretion (Rafacho *et al.* 2014). Thus, *in vitro* experiments with acute GC exposure have reported divergent effects on glucagon secretion. These divergences may depend on different factors, including the preparation and species studied as well as the specificity and potency of the different GCs used.

4.2. Chronic effects of glucocorticoids on alpha-cell function and glucagon release. Alpha-cell growth regulation by long-term GC exposure has been explored during development. Alpha-cell mass was decreased in 21-day-old foetuses obtained from pregnant rats that received dexamethasone in drinking water (1µg/ml) either during the last week of pregnancy or throughout gestation (Dumortier *et al.* 2011). In contrast, GR inactivation in the pancreatic beta-cell (rat insulin promoter-Cre transgene) or in cells expressing pancreatic and duodenal homeobox-1 (PDX-1), which is involved in pancreas development, did not modify alpha-cell mass in adult mice (Gesina *et al.* 2004). Adult rats treated with dexamethasone (1 mg/kg) for 5 consecutive days exhibited a 50% increase in alpha-cell mass (Rafacho *et al.* 2014). Similarly, administration of corticosterone to adult rats fed a high-fat diet promoted a synergistic positive effect on alpha-cell mass (Beaudry *et al.* 2013). In general, GC administration

in adults appears to up-regulate alpha-cell mass, while the opposite effect is observed during development.

Glucagon release is also modulated by GCs. Rats treated with dexamethasone (1 mg/kg) for 5 consecutive days showed hyperglucagonaemia (Rafacho *et al.* 2014). In this model, isolated pancreatic islets exhibited impaired inhibition of glucagon release at high glucose levels. Similarly, dexamethasone (0.25 mg/kg) administered for 7 days in rhesus macaques induced fasting hyperglucagonaemia (Cummings *et al.* 2013), and prednisolone (0.2-0.3 mg daily) administered for 4 days increased basal and arginine-induced glucagon secretion in isolated mouse islets (Marco *et al.* 1976). In contrast to the above-mentioned results obtained for *in vivo* GC treatment, glucagon release was suppressed in isolated rat islet cells incubated for 18 hours with dexamethasone at 10^{-9} and 10^{-10} M, but was without effect at higher steroid concentrations (Papachristou *et al.* 1994). Thus, most *in vivo* and *ex vivo* chronic studies point to enhanced alpha-cell secretion after GC administration. The resulting hyperglucagonaemia may aggravate GC-induced hyperglycaemia by stimulating hepatic glucose release and opposing insulin actions (Quesada *et al.* 2008) (Figure 3).

Clinical studies have also examined GCs' effects on human alpha-cell function. Administration of prednisolone (40-100 mg daily) for up to 4 days induced fasting hyperglucagonaemia and glucagon hypersecretion in response to arginine (Marco *et al.* 1973). Similarly, daily dexamethasone treatment (2 mg) for 3 days led to increased basal plasma glucagon levels and enhanced alanine-induced glucagon release in non-obese subjects (Wise *et al.* 1973). Both responses were even more pronounced in obese individuals and patients with Cushing's syndrome. Moreover, administration of dexamethasone (3 mg twice daily for 2 days) and prednisolone (30 mg for 2 consecutive weeks) led to increased fasting and postprandial glucagon levels (Beard *et al.* 1984, van

raalte *et al.* 2013). In contrast, in a few studies, fasting glucagon concentrations were found to be unchanged by dexamethasone (3 mg twice daily for 2 and ½ days) (Larsson & Ahrén 1999). Thus, the majority of clinical studies show that GC treatment may up-regulate alpha-cell function, which may enhance GCs' diabetogenic actions (Figure 3).

4.3. Effects of glucocorticoids on somatostatin, amylin and ghrelin release. Pancreatic delta-cells secrete somatostatin, which indirectly affects glucose homeostasis, suppressing both insulin and glucagon release (Quesada *et al.* 2008). *In vivo* experiments showed that dexamethasone administration (0.5 mg/kg) for 3 or 8 days in rats increased somatostatin gene expression and protein content in the pancreas (Papachristou *et al.* 1994). However, plasma somatostatin levels were not measured in these conditions. In *in vitro* experiments, incubation of isolated islet-cells with dexamethasone for 18 hours produced a biphasic effect: while low doses (10^{-10} M) stimulated the somatostatin gene and protein expression, high doses (10^{-8}-10^{-5} M) produced the opposite effect (Papachristou *et al.* 1994). At this chronic exposure, the high doses reduced somatostatin release into the medium. When foetal pancreatic islets were cultured for 8 days with corticosterone (0.1 μg/ml), both the somatostatin concentration in the medium and the islet somatostatin content were increased (McEvoy *et al.* 1981). Thus, few experiments indicate that GC may regulate directly or indirectly delta-cell function (Figure 3). Elevation in plasma somatostatin concentrations should inhibit alpha and beta-cell function under normal physiological conditions. However, this appears not to be the case during GC administration, given that GC treatment results in hyperglucagonaemia and hyperinsulinaemia.

The islet amyloid polypeptide (IAPP), also called amylin, is co-secreted with insulin by pancreatic beta-cells in response to food intake, most likely via the same

445 mechanisms that allow for insulin release. This hormone decreases postprandial
446 glycaemia by inhibiting gastric emptying and suppressing glucagon secretion
447 (Westermark *et al.* 2011). However, type 2 diabetes has also been related to the
448 formation of toxic amyloid aggregates that can induce beta-cell apoptosis (Westermark
449 *et al.* 2011). This aggregation might be associated with IR and insulin (and amylin)
450 hypersecretion (Westermark *et al.* 2011), which also result from GC treatment. With
451 this enhanced hormonal release, impaired intracellular IAPP processing may initiate the
452 amyloid aggregation process. For instance, dexamethasone treatment for up to 12 days
453 led to increased levels of both proinsulin and IAPP mRNA in rat islets (Bretherton-Watt
454 *et al.* 1989, Koranyi *et al.* 1992). Similarly, both enhanced plasma amylin levels and
455 amylin secretion from isolated pancreata were found in dexamethasone-induced insulin-
456 resistant rats (Pieber *et al.* 1993, Mulder *et al.* 1995). Similar findings in amylin
457 changes have been reported in humans after dexamethasone treatment (Ludvik *et al.*
458 1993), indicating that GC administration may enhance IAPP release (Figure 3).

459 Ghrelin is released by P/D1 cells from the stomach but also by epsilon-cells
460 from the pancreas (Wierup *et al.* 2013). Only few epsilon-cells are present in each islet.
461 Ghrelin inhibits insulin and somatostatin secretion but increases glucagon release
462 (Chuang *et al.* 2011, Wierup *et al.* 2013). Additionally, this hormone potently stimulates
463 growth hormone release from the anterior pituitary gland and stimulates appetite (Malik
464 *et al.* 2008). In hypercortisolemic patients with Cushing's disease, plasma ghrelin
465 concentrations increased after successful surgery, while prednisolone administration (30
466 mg/day) for five days decreased plasma ghrelin levels in healthy individuals (Otto *et al.*
467 2004). However, no changes were observed in response to a unique bolus of
468 hydrocortisone (0.6 mg/kg) in healthy men (Vila *et al.* 2010). In a neonatal rat model,
469 dexamethasone (0.5-0.05 mg/kg) administrated for four consecutive days led to

augmented plasma ghrelin levels in newborns (Bruder *et al.* 2005). However, any of the above-mentioned studies discriminated the ghrelin source, either the stomach or the pancreas. Thus, much research is necessary to address whether GCs can affect the function of epsilon islet-cells.

5. Conclusions and future perspectives.

The diabetogenic effects of GCs are a limiting factor to their clinical use, particularly in individuals with diabetes risk factors. These side effects include unfavourable actions on peripheral tissues, such as skeletal muscle, liver, bone and adipose tissue, which mainly result, among other effects, in decreased insulin sensitivity, augmenting insulin needs. In response to this GC-induced IR, the endocrine pancreas undergoes compensatory beta-cell changes in function and mass, which lead to hyperinsulinaemia and enhanced stimulated insulin release, to maintain normoglycaemia. Despite the fact that most of these adaptations are observed in healthy subjects and animal models under GC treatment, the adaptations do not necessarily guarantee an adequate insulinogenic index to prevent glucose intolerance. These beta-cell adaptations are less efficient in susceptible individuals, increasing the risk of impaired glucose homeostasis during GC treatment. Up-regulated beta-cell function resulting from steroid treatment contrasts with the direct inhibitory actions observed in both acute and long-term *in vitro* GC exposure. Thus, the effects derived from *in vivo* GC treatment may prevail over the potential direct GC actions on beta-cells. In any case, further research is necessary to unravel the molecular mechanisms of both direct and indirect GC actions on the endocrine pancreas.

Several studies have also documented acute and chronic GC effects on non-beta pancreatic cells. The mechanisms implicated are not clear but may involve multiple

factors, including direct actions on islet cells as well as effects derived from adaptations to IR, hyperglycaemia, hyperinsulinaemia or other conditions. Remarkably, the majority of *in vivo* animal studies and clinical reports show that, in addition to hyperinsulinaemia, GC treatment induces higher plasma levels of glucagon and amylin and may probably affect somatostatin. The increased plasma amylin levels might also be considered diabetogenic because enhanced IAPP concentrations may lead to increased rates of toxic amylin aggregation (Couce *et al.* 1996). Additionally, the hyperglucagonaemia observed with GC treatment opposes insulin actions and may aggravate steroid-induced hyperglycaemia by increasing hepatic glucose output, as indicated in diabetes (Quesada *et al.* 2008). Thus, the impaired release of the different islet hormones may increase the diabetogenic effects of GCs.

The majority of studies about GC actions involve the use of murine models, and thus, prudence is required when translating this experimental data to humans. However, it is also important to mention that the prolonged duration of several GC therapies in clinical practice may exceed the safe period proposed in experimental approaches in human studies, which generally do not surpass 2-15 days of GC treatment (van Raalte *et al.* 2009). Thus, experimental data from human, although of great relevance, fail to totally mimic the conditions of clinical practice (i.e. duration). Elaboration of protocols to investigate GC actions in human volunteers is not feasible, considering the risk of irreversible negative effects, ethical issues, as well as the nature of *ex vivo* and *in vitro* techniques available for the mechanistic studies (van Raalte *et al.* 2009). In this regard, animal models are valuable tools, since part of the above-mentioned limitations can be resolved.

Improved knowledge of GCs' intracellular signalling mechanisms and effects will help to design better GC therapies. In this regard, it has been suggested that gene

transrepression accounts for the majority of therapeutic GC effects, while transactivation of metabolic target genes is mainly responsible for the side effects (Strehl & Buttgereit 2013). Using this concept, several GR agonists dissociating transrepression from transactivation were developed (Löwenberg *et al.* 2008). Some of these agonists have proven useful for maintaining GCs' anti-inflammatory and immunosuppressive effects, while reducing side effects like hyperglycaemia. However, the above-mentioned concept may be over-simplistic, and side effects may not only be explained by transactivation but also by non-genomic actions (Vandevyver *et al.* 2013). Thus, a great deal of research is still necessary to develop GR agonists with reduced drawbacks for glucose homeostasis. Moreover, the combination of GC-based therapies with glucose-lowering drugs could also be an interesting alternative to explore to minimise the disadvantages of GC treatment.

Declaration of interest.

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1. Effects of glucocorticoids on peripheral tissues involved in the control of glucose homeostasis. Excess or prolonged GC treatment may disrupt glucose homeostasis by interfering with several metabolic-related tissues. In visceral adipose tissue, GCs elevate LPL activity, leading to fat accumulation at this fat site. Fat in the limbs appears to respond to GCs with increased HSL activity, resulting in increased lipid (FFA and glycerol) release, supplying substrates for hepatic TG synthesis and gluconeogenesis, and also in intramuscular fat accumulation. These steroids may also affect insulin signalling in adipose tissue. GCs impair insulin-stimulated glucose uptake in skeletal muscles and induce muscle wasting, which, in turn, provides gluconeogenesis substrates. In the liver, GCs have a negative effect on rate-limiting enzymes controlled by insulin. Finally, GC in excess may also alter osteocalcin synthesis in osteoblast cells leading to reduced osteocalcinaemia. Abbreviations: FFA, free fatty acids; GCs, glucocorticoids; G6Pase, glucose-6-phospatase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; PEPCK, phophoenolpyruvate carboxykinase; TG, triacylglycerol.

Figure 2. Sites of the insulin secretory process affected by *in vitro* or *in vivo* (*ex vivo*) exposure to glucocorticoids. In **(A)**, the known components involved in the acute or chronic *in vitro* effects of GCs on the beta-cell insulin secretory process are highlighted with a positive signal (indicates GCs stimulate/increase that action/function) or a negative signal (indicates GCs inhibit/diminish that action/function). Most notably, GCs impair beta-cell glucose metabolism, favour repolarising K_v^+ currents, decrease PKA and PKC activation, induce ER dyshomeostasis, increase 11beta-HSD1 activity and ROS generation and impair calcium handling. Together, these effects inhibit insulin

secretion. In **(B)**, the known components involved in beta-cell function which are affected by acute or long-term *in vivo* GC treatment are highlighted with a positive signal, which indicates increased content or activity. Most notably, augmented glucose metabolism and cholinergic pathway activity cause increased calcium influx and insulin secretion. In this context, a positive GC effect on K^+ and VDCC channels could not be excluded. Abbreviations: AC, adenylyl cyclase; Ach, acetylcholine; alphaAR, alpha adrenergic receptor; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; Gi, G-coupled inhibitory protein; GLUT2, glucose transporter 2; IP_3, inositol triphosphate; K^+, ATP-dependent K^+ channel; K_v^+, voltage-dependent K^+ channel; M3R, muscarinic receptor type 3; PIP_2, phosphatidylinositol bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; VDCC, voltage-dependent Ca^{2+} channel; 11beta-HSD-1, 11beta-hydroxysteroid dehydrogenase type 1.

Figure 3. Diabetogenic effects of GC treatment: implication of islet hormones. GC treatment can induce IR in peripheral tissues. As a compensatory adaptive process, the endocrine pancreas increases insulin release, leading to hyperinsulinaemia. An adequate compensatory response to the insulin requirements imposed by IR allows for normoglycaemia. However, an insufficient beta-cell response could lead to impaired glucose tolerance, which can progress to overt hyperglycaemia and type 2 diabetes. GC treatment also induces high plasma levels of glucagon and amylin, and may affect somatostatin concentrations. Although somatostatin inhibits alpha and beta-cells, the potential changes in this hormone induced by GCs do not appear to produce a significant negative effect in these conditions. Hyperglucagonaemia increases hepatic glucose output, which exacerbates hyperglycaemia and glucose intolerance and further

opposes insulin action, decreasing the insulin effect. High amylin levels have been related to increased predisposition to amyloid formation in decreased insulin sensitivity conditions, like those generated by GCs. Amyloid aggregation is related to increased beta-cell death and malfunction. The molecular mechanisms underlying the high plasma levels of glucagon and amylin induced by GC treatment are still unknown.

Figure 1

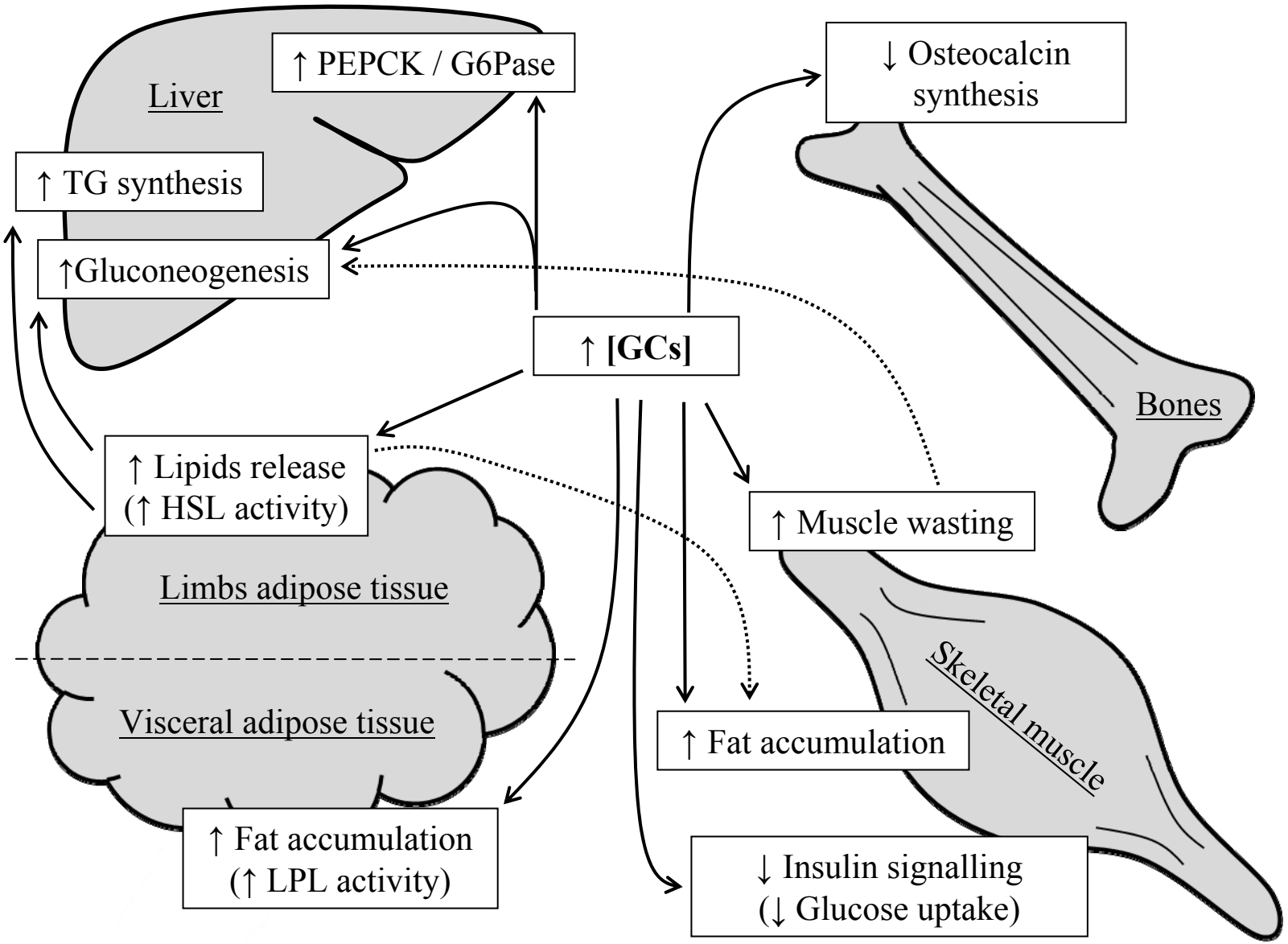

Liver
↑ PEPCK / G6Pase
↑ TG synthesis
↑Gluconeogenesis
↓ Osteocalcin synthesis
Bones
↑ [GCs]
↑ Lipids release (↑ HSL activity)
Limbs adipose tissue
Visceral adipose tissue
↑ Muscle wasting
Skeletal muscle
↑ Fat accumulation
↑ Fat accumulation (↑ LPL activity)
↓ Insulin signalling (↓ Glucose uptake)

Figure 2

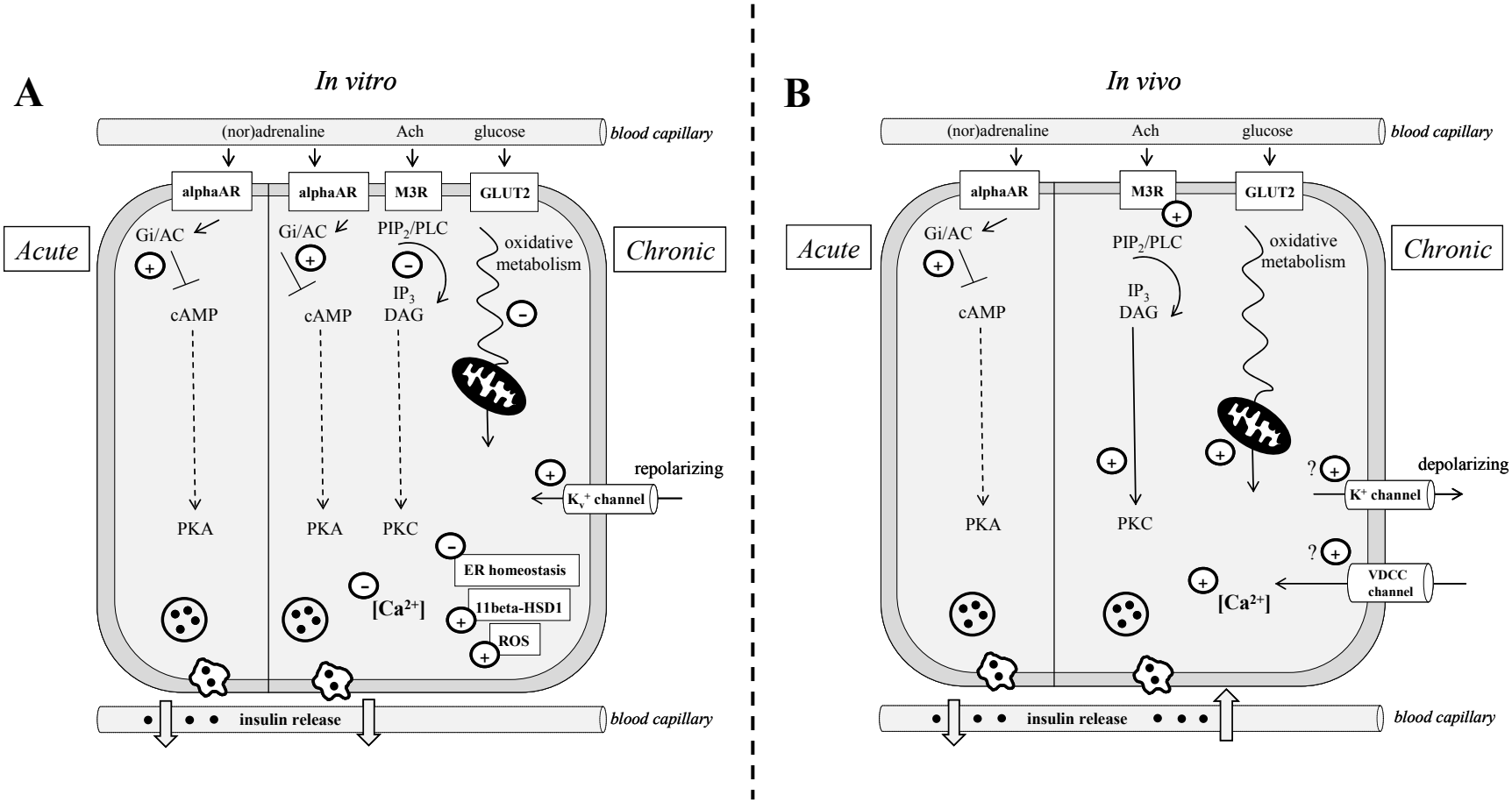
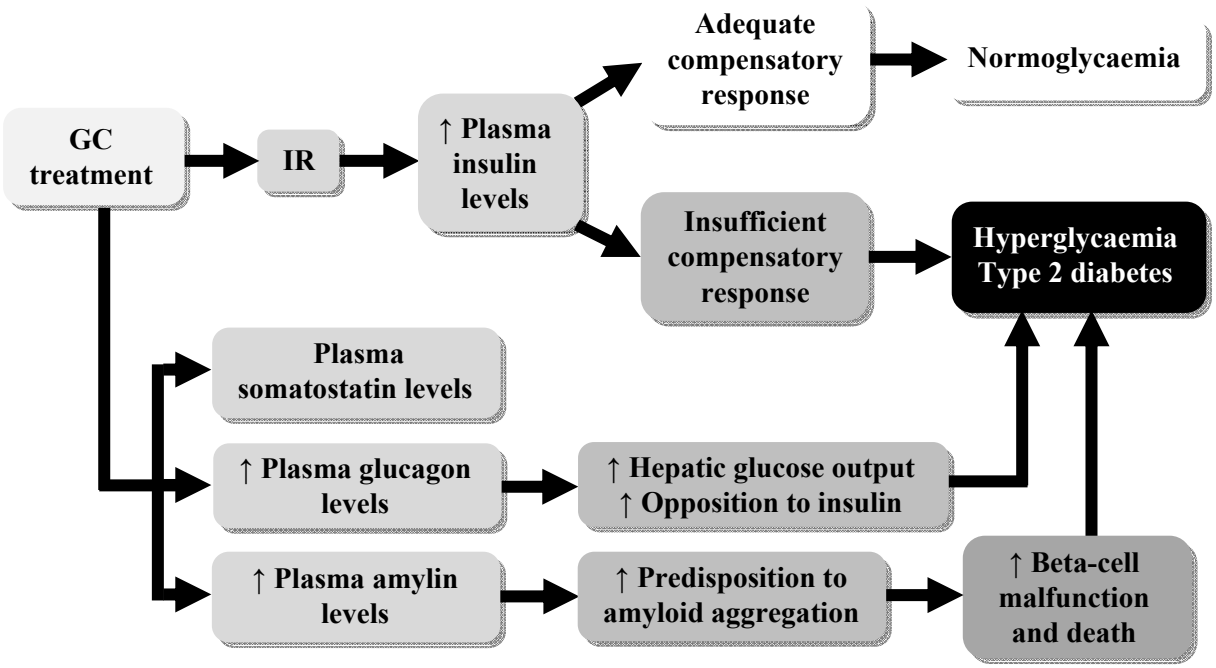

A
In vitro
(nor)adrenaline
Ach
glucose
blood capillary
alphaAR
alphaAR
M3R
GLUT2
Acute
Chronic
Gi/AC
Gi/AC
PIP2/PLC
oxidative metabolism
IP3
DAG
cAMP
cAMP
PKA
PKA
PKC
repolarizing
Kv+ channel
ER homeostasis
11beta-HSD1
ROS
[Ca2+]
insulin release
blood capillary
B
In vivo
(nor)adrenaline
Ach
glucose
blood capillary
alphaAR
M3R
GLUT2
Acute
Chronic
Gi/AC
PIP2/PLC
oxidative metabolism
IP3
DAG
cAMP
PKA
PKC
depolarizing
K+ channel
VDCC channel
[Ca2+]
insulin release
blood capillary

Figure 3


GC treatment
IR
↑ Plasma insulin levels
Adequate compensatory response
Normoglycaemia
Insufficient compensatory response
Hyperglycaemia Type 2 diabetes
Plasma somatostatin levels
↑ Plasma glucagon levels
↑ Hepatic glucose output ↑ Opposition to insulin
↑ Plasma amylin levels
↑ Predisposition to amyloid aggregation
↑ Beta-cell malfunction and death