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Hepatic glucagon receptor binding and glucose-lowering in vivo by peptidyl and non-peptidyl glucagon receptor antagonists

Qing Dallas-Yang[a,1], Xiaolan Shen[b,1], Mathias Strowski[c], Edward Brady[d], Richard Saperstein[d], Raymond E. Gibson[e], Deborah Szalkowski[a], Sajjad A. Qureshi[a], Mari Rios Candelore[a], Judith E. Fenyk-Melody[b], Emma R. Parmee[f], Bei B. Zhang[a], Guoqiang Jiang[a,*]

[a]*Metabolic Disorders-Diabetes, Merck Research Laboratories, Rahway, NJ 07065, United States*
[b]*Comparative Medicine, Merck Research Laboratories, Rahway, NJ 07065, United States*
[c]*Medizinische Klinik m. S. Hepatologie und Gastroenterologie, Charité - Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany*
[d]*Pharmacology, Merck Research Laboratories, Rahway, NJ 07065, United States*
[e]*Imaging, Merck Research Laboratories, West Point, PA 19486, United States*
[f]*Medicinal Chemistry, Merck Research Laboratories, Rahway, NJ 07065, United States*

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Abstract

Glucagon receptor antagonists have been actively pursued as potential therapeutics for the treatment of type 2 diabetes. Peptidyl and non-peptidyl glucagon receptor antagonists have been shown to block glucagon-induced blood glucose elevation in both animals and humans. How the antagonists and the glucagon receptor interact in vivo has not been reported and is the subject of the current study. Using ^{125}I-labeled glucagon as a radiotracer, we developed an in vivo glucagon receptor occupancy assay in mice expressing a human glucagon receptor in place of the endogenous mouse glucagon receptor (hGCGR mice). Using this assay, we first showed that the glucagon receptor is expressed predominantly in liver, to a much lesser extent in kidney, and is below detection in several other tissues/organs in the mice. We subsequently showed that, at 2 mg/kg body weight (mg/pk) dosed intraperitoneally (i.p.), peptidyl glucagon receptor antagonist des-His-glucagon binds to ~78% of the hepatic glucagon receptor and blocks an exogenous glucagon-induced blood glucose elevation in the mice. Finally, we also showed that, at 10 and 30 mg/kg dosed orally (p.o.), compound A, a non-peptidyl small molecule glucagon receptor antagonist, occupied 65–70% of the hepatic glucagon receptor, and significantly diminished exogenous glucagon-induced blood glucose elevation in the mice. At 3 mg/kg, however, compound A occupied only ~39% of the hepatic glucagon receptor and did not affect exogenous glucagon-induced blood glucose elevation in the mice. Taken together, the results confirmed previous reports that glucagon receptors are present predominantly in the liver, and provide the first direct evidence that peptidyl and non-peptidyl glucagon receptor antagonists bind to the hepatic glucagon receptor in vivo, and that at least 60% receptor occupancy correlates with the glucose lowering efficacy by the antagonists in vivo.

Keywords: Glucagon; Receptor; Binding; Occupancy; Antagonist

1. Introduction

Glucagon is a 29-amino acid peptide hormone that plays a key role in maintaining glucose homeostasis in vivo. Glucagon acts via the glucagon receptor, a seven *trans*-membrane G-protein coupled receptor consisting of 485 amino acids (Jelinek et al., 1993). Glucagon signaling

* Corresponding author. P.O. Box 2000, RY80N-A26, Merck and Co., Inc., Rahway, NJ 07065, United States, Tel.: +732 594 2176; fax: +732 594 3570.
E-mail address: guoqiang_jiang@merck.com (G. Jiang).
[1] These authors contributed equally to this work.

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provides a major counter regulatory mechanism for insulin by stimulating hepatic glucose output and thereby increasing glycemia (reviewed in Jiang and Zhang, 2003). There is ample evidence suggesting that the glucagon signaling pathway plays an important role in initiating and maintaining hyperglycemic conditions in diabetic animals and in humans, and that the glucagon receptor is a potential target for the treatment of diabetes. For instance, chronic hyperglucagonemia is associated with and is at least partially responsible for increased hepatic glucose output and hyperglycemia in type 2 diabetes (reviewed in Jiang and Zhang, 2003). Furthermore, glucagon-neutralizing antibodies (Brand et al., 1995; Brand et al., 1996; Brand et al., 1994; Tan et al., 1985) and antagonistic glucagon peptide analogs (Johnson et al., 1982; Unson et al., 1989; Van Tine et al., 1996) have been shown to lower glycemia in diabetic animal models.

In addition to the neutralizing antibodies and antagonistic peptides, many non-peptidyl small-molecule glucagon receptor antagonists have been reported (Cascieri et al., 1999; Chang et al., 2001; de Laszlo et al., 1999; Knudsen et al., 2001; Ladouceur et al., 2002; Ling et al., 2001; Ling et al., 2002; Madsen et al., 1999; Madsen et al., 1998; Madsen et al., 2002; Parker et al., 2000; Petersen and Sullivan, 2001). Some of these antagonists have been shown to lower fasting blood glucose (Ling et al., 2002) or to block exogenous glucagon-stimulated elevation of blood glucose in animal models (Ling et al., 2001; Madsen et al., 2002). A non-peptidyl small molecule glucagon receptor antagonist was shown to block glucagon-induced elevation of hepatic glucose production and blood glucose in human in a dose-dependent fashion (Petersen and Sullivan, 2001). More recently, it has been reported that reduction of hepatic glucagon receptor levels by antisense oligonucleotides leads to improvement of glycemia control in animal models of diabetes (Liang et al., 2004).

Whereas the glucose-lowering effects of some of the glucagon receptor antagonists have been convincingly documented as described above, how these antagonists interact with the glucagon receptor in vivo has not been fully characterized and understood. The current study was initiated to investigate the interaction between the glucagon receptor and some of the known antagonists in vivo as well as to facilitate the pharmaceutical development of novel antagonists for type 2 diabetes. We developed, optimized and validated an in vivo glucagon receptor occupancy assay in mice containing a human glucagon receptor in place of the endogenous mouse glucagon receptor (hGCGR mice) (Shiao et al., 1999). Using this assay, we demonstrate that glucagon receptors are expressed predominantly in the liver, that peptidyl and non-peptidyl glucagon receptor antagonists bind to the hepatic glucagon receptor in vivo, and that the levels of in vivo glucagon receptor occupancy by the antagonists correlate with their ability to block exogenous glucagon-induced blood glucose elevation in hGCGR mice.

2. Materials and methods

2.1. Reagents

Receptor grade radioiodinated glucagon, with a specific activity of 2200 Ci/mmol, was purchased from Amersham (Piscataway, NJ). Glucagon contained in the Glucagon Emergency Kit was purchased from Lilly (Indianapolis, IN) for in vivo experiments. Des-His-glucagon and glucagon-like peptide 2 (GLP-2) were purchased from Bachem (CA). Glucagon for in vitro binding assays was also purchased from Bachem. Bacitracin, aprotinin, EDTA, bovine serum album and polyethylenimine were purchased for Sigma. Complete™ protease inhibitor cocktail tables were purchased from Roche Diagnostic (Germany). Compound A ((+)-3,5 diisopropyl-2-(1-hydroxyethyl)-6-propyl-4′ -fluoro-1,1′ -biphenyl; $C_{23}H_{31}FO$) is a small molecule glucagon receptor antagonist (Petersen and Sullivan, 2001).

2.2. Animal preparation

Glucagon receptor knockout mice ($GCGR^{-/-}$) mice were originally obtained from Dr. M.J. Charron of Albert Einstein College of Medicine (Gelling et al., 2003). The hGGCR mice had been created through the replacement of the endogenous mouse glucagon receptor and replaced by an exogenous human glucagon receptor as previously described (Shiao et al., 1999).

Naïve, male (22–30 g) hGCCR mice were used for all animal studies. They were bred within our facility for all experimentation. Prior to this study, mice were group-housed, provided with ad libitum rodent chow (Teklad 7012, Madison, WI) and reverse osmosis water via a water bottle. The light/dark cycle was 12:12 h and the building was provided with HEPA filtered air, maintained at 72±2 °F. The rodent colony was determined to be specific pathogen free as determined by gross necropsy, endo- and ectoparasite examination and the Charles River Laboratories (Wilmington, MA) Assessment Plus serology mouse profile of sentinel animals. All animal procedures involved the humane care and use of animals were performed within an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International-accredited facility, and were approved by the Merck-Rahway Institutional Animal Care and Use Committee (IACUC).

2.3. In vitro binding assay

To prepare membranes for the binding assay, livers from male hGCGR mice were weighed and then minced in ice cold lysis buffer (50 mM Tris plus 0.1% bovine serum album) at ~4 ml/liver, homogenized with a polytron until there were no visible chunks. Ice-cold extra lysis buffer (~8 ml/liver equivalent) was then added to the homogenate, which was then subjected to centrifugation at 30,000×g using Sorval GSA rotor at 13,000 rpm for 15 min. The pellet

was resuspended in the lysis buffer (~8 ml/liver equivalent) and the suspension was then subjected to the same centrifugation again. The pellet was then resuspended in membrane freezing buffer (50 mM Tris, 5 mM $MgCl_2$, 0.01% bacitracin and aprotinin) at ~2 ml/liver equivalent. Protein concentration in the membrane preparation was determined by the Bradford method. Membranes were aliquoted, quickly frozen in dry ice and stored at −80 °C for subsequent uses.

MultiScreen 1.0 μm Glass Fiber Type B Filter plates (Millipore) were incubated with 0.2% polyethylenimine solution at 4 °C for 2 h, washed twice with 50 mM Tris pH7.5, incubated with 50 mM Tris pH7.5 plus 5% bovine serum album for 10 min at room temperature and drained right before addition of binding reactions. Binding reaction (total volume 200 μl) contained 1× binding buffer (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1% bovine serum album, 0.01% bacitracin and 1× complete protease inhibitor cocktail), 4.8 μg hGCGR mice liver membrane protein described above, 10 pM [^{125}I]glucagon and varying concentrations (from 0.1 pM to 1 μM) of unlabeled competitor glucagon. [^{125}I]Glucagon was added immediately after the addition of the unlabeled glucagon. After incubation at room temperature for 1 h, 150 μl of the reaction mixture was transferred to and filtered via the wells of the 96-well filter plate. To determine the levels of the nonspecific binding of [^{125}I]glucagon to filter, mock reactions containing the reaction buffer and 50 pM [^{125}I]glucagon but no liver membrane were also added to and filtered via the wells. The filters were washed 5× with the binding buffer, air dried, soaked in 50 μl scintillation fluid overnight, and then counted using a top-counter.

Instead of a saturation binding study using increasing concentrations of [^{125}I]glucagon, an inhibition study was performed as described above with fixed concentration of [^{125}I]glucagon and increasing concentrations of competitive unlabeled glucagon. The result from the inhibition study was used to generate a typical dose-inhibition curve, which we subsequently analyzed to provide a saturation curve. The inhibition curve was generated using 11 concentrations of glucagon (from 0.1 pM to 1 μM), using a single-site model with a nonspecific binding component: $B_i=B_o-B_o*I/(1+IC_{50})+NS$, where B_i is the radiotracer bound in the presence of a specific concentration of inhibitor, I, with apparent affinity of IC_{50}. Since we do not explicitly define the radioligand concentration in this equation, the resulting "affinity" is an IC_{50} rather than the more rigorous K_i.

To convert the inhibition data to provide a saturation curve, we assume that the affinity of glucagon is essentially the same as that of the radioligand, [^{125}I]glucagon. The specific activity of radioligand is then calculated as the sum of the mass from the radioligand (specific activity of 2200 Ci/mmol) and the added unlabeled glucagon. For example, at 10 pM added glucagon, the total mass of glucagon and radioligand reduces the effective specific activity of the [^{125}I]glucagon to 1105 Ci/mmol. The data are plotted as total radioligand bound vs. radioligand concentration, and curve fitting was done using the function $B_T=L*B_{max}/(K_d+L)+m*L$ (available in SigmaPlot©), where L is the ligand concentration and B_T is the total radioligand bound which is the sum of specific and nonspecific components. The nonspecific binding is defined by $m*L$ where m is the slope of the nonspecific binding curve.

2.4. In vivo binding assay

GCGR$^{-/-}$ and hGCGR mice were generated and prepared as described above. There were six mice per treatment group. For experiments in which glucagon receptor antagonists were not involved, the animals were dosed intravenously (i.v.) via tail vein with radioligand [^{125}I]glucagon alone, or the radioligand plus excessive unlabeled glucagon (for determination of specific binding) or GLP-2 peptide. For experiments in which peptidyl glucagon receptor antagonist des-His-glucagon was involved, the animals were dosed intraperitoneally (i.p.) with vehicle (saline with 0.5% bovine serum album) 20 min prior to tail vein i.v. dosing of either radioligand alone or the radioligand plus excessive unlabeled glucagon, or treated with des-His-glucagon (i.p.) prior to tail vein i.v. dosing of radioligand alone. For experiments in which small molecule glucagon receptor antagonist compound A was involved, animals were first dosed orally by gavage (p.o.) with vehicle (0.5% methylcellulose) 1 hr prior to tail vein i.v. dosing of radioligand alone or the radioligand plus excessive unlabeled glucagon, or dosed with compound A (p.o.) prior to tail vein i.v. dosing of the radioligand alone. All treatments (i.p., p.o. or i.v. dosing) were performed at 10 μl/g body weight. Unless specified in figure legends, the radioligand and unlabeled competitive peptides (glucagon or GLP-2) were dosed at 0.0083 μCi and 1500 ng/g body weight, respectively, and tissues were harvested 15 min after the injection of the radiotracer.

Tissues were lysed in the lysis buffer (0.1% SDS and 1% Triton-X 100) in the ratio of 5 ml buffer/g liver tissue with the aid of a Polytron. One milliliter of lysate (equivalent to 200 mg tissue) was counted using a gamma counter to determine levels of [^{125}I]glucagon in tissue (CPM per 200 mg tissue). The protocol for the in vivo binding assay in animals was approved by IACUC.

2.5. In vivo pharmacodynamic assay

Male hGCGR mice were maintained as described above for in vivo receptor binding experiment. There were 10 mice per treatment group. The animals were first treated with des-His-glucagon or vehicle (i.p., saline plus 0.5% bovine serum album) (Fig. 5A), or with compound A or vehicle (p.o., 5% methylcellulose) (Fig. 6A). Twenty minutes after des-His-glucagon or 60 min after compound

A dosing, one group of the vehicle-treated animals was challenged with saline (i.p.). Another group of the vehicle-treated animals and all groups of the des-His-glucagon or compound A-treated animals were challenged with glucagon (i.p., 15 ng/g body weight). Solutions were prepared and dosing was done as described above in the in vivo binding assay. Blood was sampled via tail vein bleeding at various intervals throughout the experiments for measuring blood glucose.

3. Results

3.1. Hepatic glucagon receptor density and ligand binding affinity in hGCGR mice

The overall goal of the study was to confirm that the glucose-lowering effects of glucagon receptor antagonists were mediated by direct binding of the antagonists to the glucagon receptor in vivo. Accordingly, we hoped to establish an in vivo receptor occupancy assay capable of measuring the binding of glucagon receptor antagonists to the glucagon receptor in vivo. To test the feasibility of developing such an assay, we first determined the ligand binding affinity and the hepatic expression levels (or density) of glucagon receptor in hGCGR mice. The rationale for this was, first, it is our experience that, to achieve a reasonable window in in vivo receptor binding assays, the receptor must be expressed in target tissues at sufficiently high levels and bind to the radioligand with adequately high affinity, such that the ratio of receptor density to binding affinity is 5 or greater (Eckelman et al., 1979). Second, it is generally thought that the glucagon receptor is expressed predominantly in the liver and that increased hepatic glucose output is the key mechanism via which glucagon regulates blood glucose in vivo (reviewed in Jiang and Zhang, 2003). Finally, we chose hGCGR mice because several glucagon receptor antagonists which were developed against the human glucagon receptor have significantly weaker affinity for rodent receptors (Cascieri et al., 1999; Ling et al., 2001).

Using cell membrane prepared from the liver of hGCGR mice, we performed an in vitro competition binding assay using a fixed concentration of [^{125}I]glucagon and increasing concentrations of unlabeled glucagon. The inhibition curve shown in Fig. 1A is well described by a single-binding site model with IC_{50}=0.33 nM, indicating a high affinity binding of glucagon to the receptor. While nonspecific binding appears to be extensive (~50% of total binding), it is attributed predominantly to the nonspecific interaction between radiotracer and the glass-fiber filters (data not shown). In fact, excluding such nonspecific binding of radiotracer to the glass fiber filter, approximately 90% of the total binding of the radiotracer to the membrane was inhibited at the 10 nM or higher concentrations of unlabeled glucagon (data not shown). These results are consistent with the notion that glucagon binds to the glucagon receptor with high specifically.

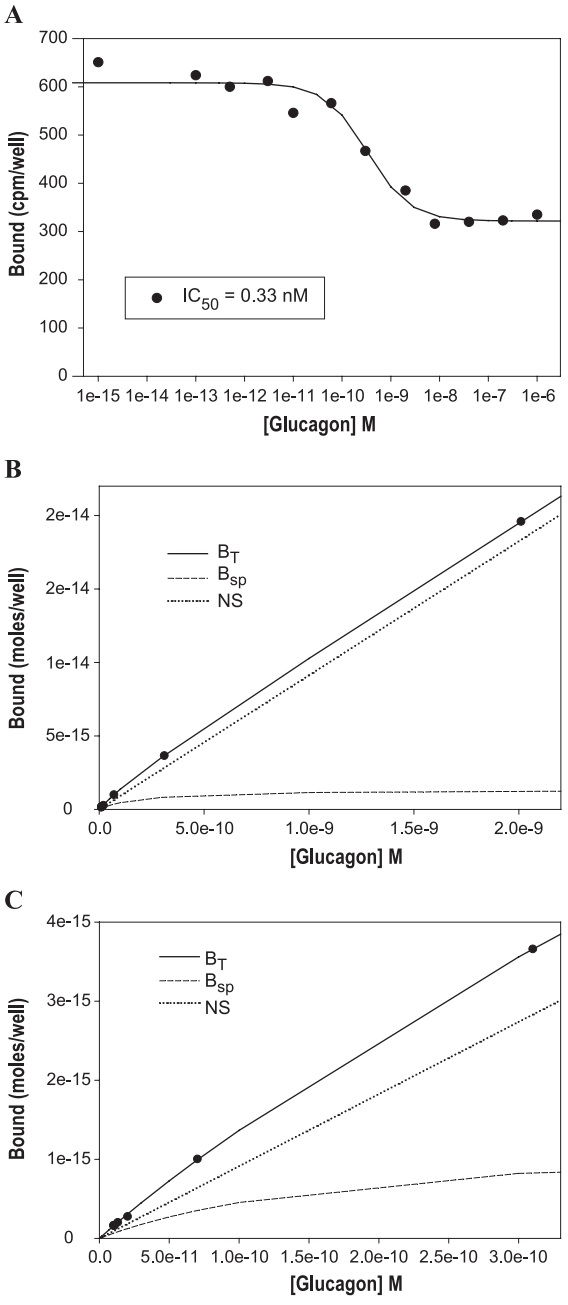


Fig. 1. Glucagon inhibition of [^{125}I]glucagon binding to hGCGR mice liver membranes in vitro. In vitro binding assay used hGCGR mice liver membrane, fixed concentration (10 pM) of [^{125}I]glucagon and increasing concentrations (0.1 pM to 1 μM) of unlabeled glucagon as competitor. (A) A dose response inhibition curve of the [^{125}I]glucagon binding at the presence of increasing concentrations of unlabeled glucagon. (B) Saturation binding curve using 6 data points. (C) Expansion of lower concentrations of the unlabeled glucagon in B. For both B and C, solid line represents the total binding (B_T), dashed line specific binding (Bsp), and dotted line nonspecific binding (NS) estimated from curving fitting. Note that B and C have the appearance of a saturation curve but are originated from a displacement experiment instead of a typical saturation experiment as described in the main text. The experiments were repeated three times with similar results.

To estimate the density of hepatic glucagon receptors, the data was recalculated via the specific activity dilution method as described in Section 2, resulting in typical total binding saturation curves shown in Fig. 1B and C. Curve fitting the data to a single-site saturation model with nonspecific binding provides the B_T curve (solid line). The parameters obtained were used to generate each component of the total binding curve: the specific binding (Bsp, dashed line) and the nonspecific binding (NS, dotted line). Knowing the amount of tissue added to each well, we converted the parameter for B_{max} to an effective concentration in tissue (assuming uniform distribution of receptor and a tissue specific gravity of 1.0). Thus, the effective B_{max} for glucagon receptor in liver is approximately 14 nM. Taken together, the results indicate that glucagon receptor is indeed expressed at high levels in the liver and binds to glucagon with high affinity, and that, with a 44:1 ratio of receptor density (14 nM) to ligand binding affinity (0.33 nM), it therefore appeared likely that we would be able to develop an in vivo receptor occupancy assay for the glucagon receptor.

3.2. Binding of [^{125}I]glucagon to hepatic glucagon receptor in hGCGR mice in vivo

As a first step toward developing a receptor occupancy assay, we determined whether specific binding of [^{125}I]glucagon to liver tissue of hGCGR mice can be demonstrated in vivo, and attempted to optimize the conditions under which such specific binding could be maximized to obtain the best signal/noise ratios. [^{125}I]glucagon at 0.083 μCi/g body weight without or with excessive unlabeled glucagon at 1500 ng/g body weight was injected into hGCGR mice via the tail vein. Livers were harvested at 5, 15 and 30 min after the tail vein i.v. dosing and lysed in 5 ml lysis buffer per gram tissue. One milliliter of the lysate from each of the tissue samples (equivalent to 200 mg liver tissue) was counted in a gamma counter. As shown in Fig. 2A, at all three different time points, the levels of [^{125}I]glucagon in the livers from animals injected with [^{125}I]glucagon alone were about three- to four-fold higher than those from animals injected with both [^{125}I]glucagon and unlabeled glucagon. These results provided the first indication that there is indeed significant specific binding of glucagon to the glucagon receptor in the liver. We decided to perform subsequent experiments by obtaining the liver at 15 min after tail vein i.v. dosing of [^{125}I]glucagon for the following reasons. The ratio of the counts from animals injected with [^{125}I]glucagon alone (total binding) vs. the counts from animals injected with [^{125}I]glucagon plus unlabeled glucagon (specific binding) (~5-fold) was higher than those at 5 and 30 min post tail vein i.v. dosing, providing a larger window for the assay. Additionally, hepatic glucagon receptor binding at 15 min post glucagon injection is probably more physiologically relevant since, in pharmacodynamic assays, elevation of blood glucose by exogenous glucagon peaked at 15 min (see Figs. 5 and 6).

Having decided on the timing of dissection as described above, another experiment was performed to determine the optimal amount of [^{125}I]glucagon needed in the assay. [^{125}I]Glucagon at 0.0083, 0.083 and 0.167 μCi/g body weight with or without 1500 ng/g body weight unlabeled glucagon was administrated to different groups of hGCGR mice via tail vein i.v. dosing. Livers were harvested at 15 min post-injection and processed as described above. The results, as shown in Fig. 2B, indicate that [^{125}I]glucagon at 0.0083 μCi/g body weight gives the largest window (~4-fold). Accordingly, for all the subsequent experiments

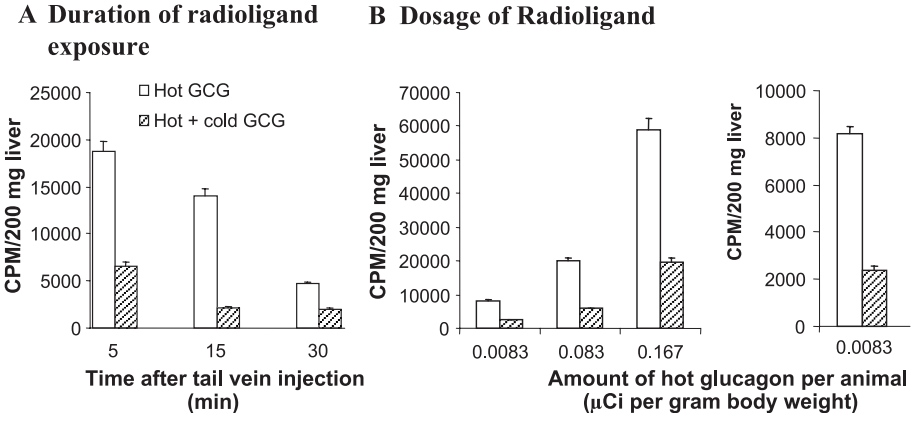
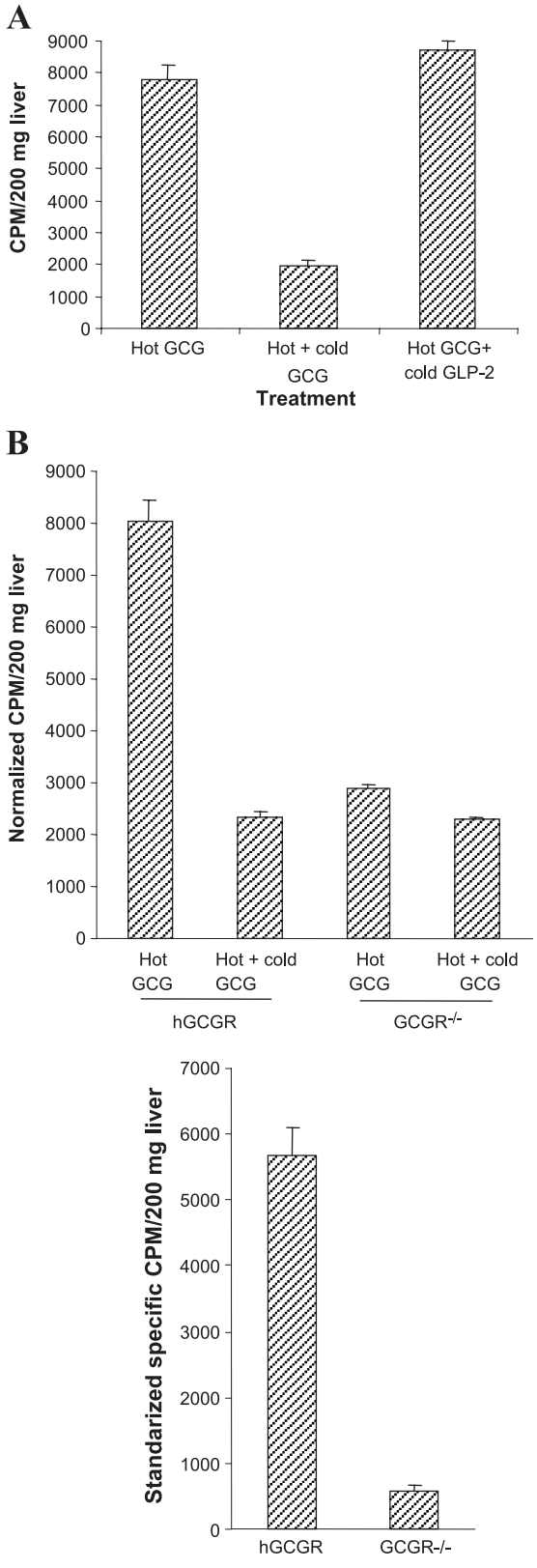


Fig. 2. Optimization of radioligand exposure duration and levels in hGCGR mice. (A) hGCGR mice were injected via tail vein 0.083 μCi [^{125}I]glucagon without or with excess unlabeled glucagon, and the livers were then harvested at indicated time after the tail vein i.v. injection. (B) hGCGR mice were injected via tail vein 0.0083, 0.083 or 0.167 μCi [^{125}I]glucagon per gram body weight without or with excess unlabeled glucagon, and the livers were then harvested at 15 min post tail vein i.v. dosing. The counts in animals injected with 0.0083 μCi [^{125}I]glucagon/g body weight are also shown in the right figure with a different scale to facilitate visualization. Hot GCG: tail vein i.v. dosing with [^{125}I]glucagon alone; hot+cold GCG: tail vein i.v. dosing with [^{125}I]glucagon plus unlabeled glucagon. The experiments for both A and B were repeated three times with similar results.

except those experiments involving hGCGR$^{-/-}$ mice (Fig. 3B), we used [^{125}I]glucagon and unlabeled glucagon at the level of 0.0083 μCi/g and 1500 ng/g body weight, respectively, and obtained tissues 15 min post [^{125}I]glucagon injection.



3.3. Specificity of the binding of [^{125}I]glucagon to the liver of hGCGR mice in vivo

As we discussed above, the ability of excessive unlabeled glucagon to reduce the level of [^{125}I]glucagon in liver indicates that [^{125}I]glucagon binds to hepatic glucagon receptor specifically. To further confirm the specificity of the binding of [^{125}I]glucagon to the liver tissue, we first tested the ability of unlabeled glucagon-like peptide 2 (GLP-2) to compete with [^{125}I]glucagon for binding to the liver tissue. While both GLP-2 and glucagon are proteolytically processed from the same prohormone proglucagon, GLP-2 is known to bind to the GLP-2 receptor but not the glucagon receptor (Furuta et al., 2001; Rouille et al., 1997; Rouille et al., 1994) and should serve as a proper control for this experimental setting. hGCGR mice were co-injected via tail vein with [^{125}I]glucagon without or with unlabeled glucagon or GLP-2 peptide. As expected, the results (Fig. 3A) showed that excessive unlabeled glucagon but not GLP-2 significantly reduced the level of [^{125}I]glucagon in hGCGR mice livers.

We subsequently compared the binding of [^{125}I]glucagon in the liver of hGCGR mice with that of glucagon receptor knock-out mice (GCGR$^{-/-}$ mice). hGCGR mice and the GCGR$^{-/-}$ mice were injected via tail vein with [^{125}I]glucagon without or with excess unlabeled glucagon. However, GCGR$^{-/-}$ mice were injected with 0.083 μCi [^{125}I]glucagon/g body weight (10-fold more the [^{125}I]glucagon used in the hGCGR mice) because GCGR$^{-/-}$ mice manifest hyperglucagonemia (Gelling et al., 2003; Parker et al., 2002). As shown in Fig. 3B, while the level of [^{125}I]glucagon was relatively high in hGCGR mice livers and effectively competed with excessive unlabeled glucagon, it was low in GCGR$^{-/-}$ mice and was not affected by unlabeled glucagon. This provides final confirmation that the binding of [^{125}I]glucagon in hGCGR mice is indeed specific.

Fig. 3. Specificity of [^{125}I]glucagon binding to liver tissues in vivo. (A) hGCGR mice were injected via tail vein with [^{125}I]glucagon without or with excess unlabeled glucagon or GLP-2 peptide. (B) hGCGR mice and GCGR$^{-/-}$ mice were injected via tail vein with 0.0083 and 0.083 μCi [^{125}I]glucagon g^{-1} body weight, respectively, without or with excess unlabeled glucagon. For B, the counts for hGCGR mice and GCGR$^{-/-}$ mice were normalized by dividing the counts from GCGR$^{-/-}$ by 10 to account for the 10-fold higher levels of [^{125}I]glucagon used in the GCGR$^{-/-}$ mice than in the hGCGR mice. Normalized specific binding=normalized counts from animals injected with [^{125}I]glucagon alone−normalized counts from animals injected with [^{125}I]glucagon plus unlabeled glucagon. Hot GCG: tail vein i.v. dosing with [^{125}I]glucagon alone; hot+cold GCG: tail vein i.v. dosing with [^{125}I]glucagon plus unlabeled glucagon; hot GCG+Cold GLP-2: tail vein i.v. dosing with [^{125}I]glucagon plus unlabeled GLP-2 peptide.

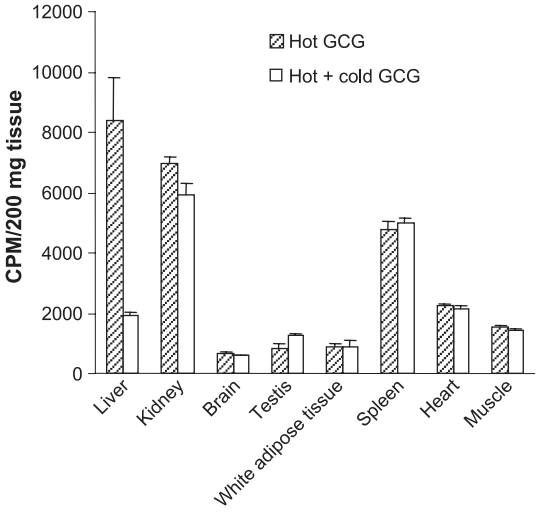


Fig. 4. Tissue distribution of glucagon binding sites in hGCGR mice. hGCGR mice were injected via tail vein [^{125}I]glucagon without or with excess unlabeled glucagon. The liver, kidney, brain, testis, white adipose tissues, spleen, heart and muscle were then harvested at 15 min after the tail vein i.v. dosing. Shown are the counts in various tissues. Hot GCG: tail vein i.v. dosing with [^{125}I]glucagon alone; hot+cold GCG: tail vein i.v. dosing with [^{125}I]glucagon plus unlabeled glucagon.

3.4. Tissue distribution of the glucagon receptor in vivo

It was reported that, although the glucagon receptor is present in multiple tissues, it is expressed predominantly in liver (Burcelin et al., 1996; Christophe, 1996). To confirm this, we determined the level of the specific binding of [^{125}I]glucagon in various tissues in hGCGR mice using the in vivo binding assay. Fifteen minutes after tail vain i.v. injection of [^{125}I]glucagon without and with excessive unlabeled glucagon, liver, kidney, brain, testis, white adipose tissues, spleen, heart and soleus muscle were collected and processed as described previously. As shown in Fig. 4, specific binding of [^{125}I]glucagon was found predominately in liver and to a much lesser extent in kidney. On the other hand, no significant specific binding of [^{125}I]glucagon was detected in other tissues including brain, testes, adipose, heart, spleen and muscle. Although the receptor may be present in these tissues, the concentration may be too low to be detected by this in vivo methodology. These results led us to concentrate only on hepatic glucagon receptors in the subsequent experiments.

3.5. Glucose lowering efficacy and hepatic glucagon receptor occupancy of peptidyl glucagon receptor antagonist des-His-glucagon in hGCGR mice

Some peptidyl glucagon receptor antagonists including des-His-glucagon have been reported to lower blood glucose in animal models in vivo (Johnson et al., 1982; Unson et al., 1989). We first performed a pharmacodynamic assay in which hGCGR mice were treated with vehicle (i.p., saline plus 0.5% bovine serum album) or des-His-glucagon (i.p., 2 mg/kg) and, 20 min later, challenged with vehicle or exogenous glucagon (i.p., 15 ng/g body weight). The results showed that glucagon challenge resulted in significant elevation of blood glucose that peaked at about 15–20 min post-challenge, and such elevation was largely blocked by prior treatment with des-His-glucagon (Fig. 5A). We

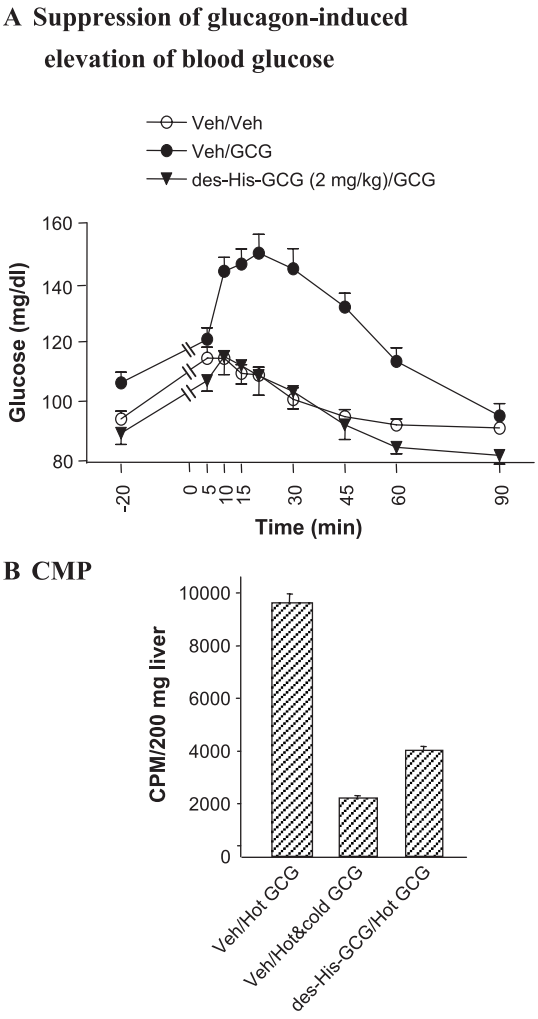


Fig. 5. Glucose lowering efficacy and hepatic glucagon receptor occupancy by peptidyl glucagon receptor antagonist des-His-glucagon in hGCGR mice. (A) Glucose lowering efficacy. Pharmacodynamic assays on hGCGR mice were performed as described in Section 2. Veh/Veh: hGCGR mice first treated with vehicle (i.p., saline plus 0.5% bovine serum album) and then treated with vehicle again 20 min later (i.p.). Veh/GCG: hGCGR mice first treated with vehicle (i.p.) and then challenged with exogenous glucagon (i.p.). des-His-GCG/GCG: hGCGR mice first treated with des-His-glucagon (i.p., 2 mg/kg) and then challenged with exogenous glucagon (i.p.). Time 0 is the time when exogenous glucagon was administrated. There were 10 animals per treatment group. (B) Receptor occupancy. Veh/Hot GCG: hGCGR mice first treated with vehicle (i.p.) and then injected with [^{125}I]glucagon (i.v. tail vein injection). Veh/Hot and cold GCG: hGCGR mice first treated with vehicle (i.p.) and then and then injected with both [^{125}I]glucagon and unlabeled glucagon (tail vein i.v.). des-His-GCG/hot GCG: hGCGR mice first treated with des-His-glucagon (i.p.) and then injected with [^{125}I]glucagon (tail vein i.v.). Receptor occupancy for des-His-glucagon (%)=100*(CPM for Veh/Hot GCG−CPM for des-His-GCG/hot GCG)/(CPM for Veh/hot GCG−CPM for Veh/hot and cold GCG) (data not shown). The experiments were repeated twice with similar results.

subsequently performed an in vivo receptor occupancy assay on hGCGR mice, which were similarly treated with des-His-glucagon. The results showed that, at 35 min post i.p. dosing of des-His-glucagon (equivalent to the time when glucagon-induced blood glucose elevation peaks in the pharmacodynamic assay), des-His-glucagon at 2 mg/kg occupied ~78% hepatic glucagon receptor in the hGCGR mice (Fig. 5B).

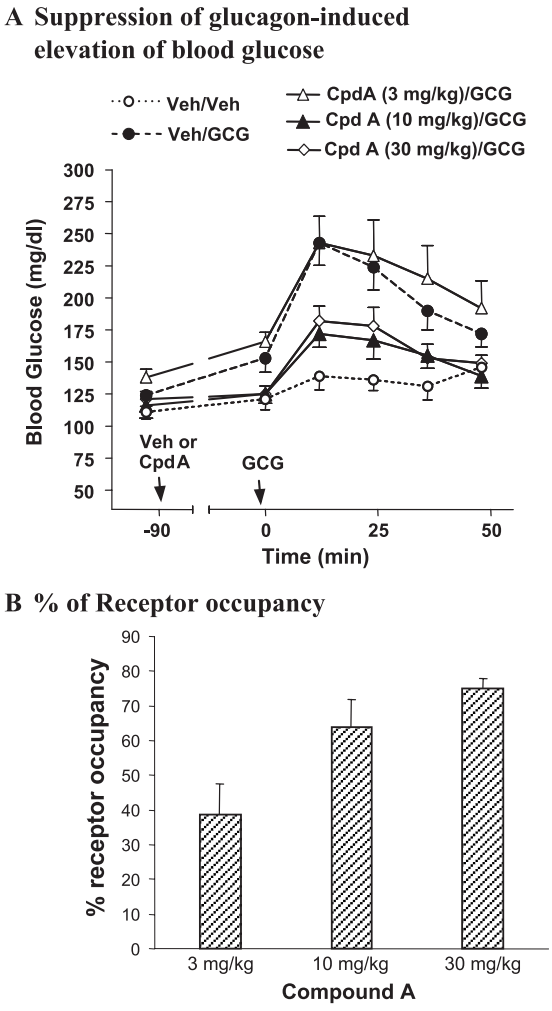


Fig. 6. Glucose lowering and hepatic glucagon receptor occupancy by small molecular glucagon receptor antagonist compound A in hGCGR mice. (A) Glucose lowering efficacy. Pharmacodynamic assays on hGCGR mice were performed as described in Section 2. Vehi/Veh: hGCGR mice first treated with 5% methylcellulose (p.o.) and then treated with saline plus 0.5% bovine serum album (i.p.). Veh/GCG: hGCGR mice first treated with 5% methylcellulose (p.o.) and challenged with exogenous glucagon (i.p.). Compound A (3, 10 and 30mg/kg)/GCG: hGCGR mice first treated with compound A (p.o.) at the indicated doses and then challenged with exogenous glucagon (i.p.). There were 10 animals per treatment. (B) Receptor occupancy. Veh/hot GCG: hGCGR mice first treated with 5% methylcellulose (p.o.) and then injected with [^{125}I]glucagon (tail vein i.v.). Veh/hot and cold GCG: hGCGR mice first treated with 5% methylcellulose (p.o.) and then injected with both [^{125}I]glucagon and unlabeled glucagon (tail vein i.v.). Cpd A (3, 10 or 30 mg/kg)/GCG: hGCGR mice first treated with compound A (p.o.) at the indicated dosage and then injected with [^{125}I]glucagon (tail vein i.v.). Receptor occupancy for compound A (%)=100*(CPM for Veh/hot GCG−CPM for Cpd A/hot GCG)/(CPM for Veh/hot GCG−CPM for Veh/hot and cold GCG). The experiments were repeated twice with similar results.

3.6. Glucose lowering efficacy and hepatic glucagon receptor occupancy of a small molecule glucagon receptor antagonist (compound A) in hGCGR mice

Compound A, a glucagon receptor antagonist, was reported to block glucagon-induced glucose elevation in humans when it was dosed p.o. (Petersen and Sullivan, 2001). We first performed a pharmacodynamic experiment in which hGCGR mice were dosed with either vehicle (p.o., 5% methylcellulose) or compound A (p.o., 3, 10 and 30 mg/kg) and, 1 h later, challenged with either vehicle (tail vein i.v. dosing, saline plus 0.5% bovine serum album) or exogenous glucagon (tail vein i.v. dosing, 15 ng/g body weight). The results showed that the glucagon challenge resulted in a significant elevation of blood glucose that peaked at about 15–20 min post-challenge, and such elevation was significantly diminished (>50%) by prior treatment with compound A at 10 and 30 but not at 3 mg/kg (Fig. 6A).

We subsequently performed an in vivo receptor occupancy assay on hGCGR mice which were similarly treated with compound A. The result showed that, at 75 min post p.o. dosing of compound A (equivalent to the time when glucagon-induced blood glucose elevation peaks in the pharmacodynamic assay), the compound occupied the glucagon receptor in a dose dependent fashion, with approximately 39%, 65% and 70% occupancy at 3, 10 and 30 mg/kg, respectively (Fig. 6B).

4. Discussion

Given the important role of glucagon in glucose homeostasis in normal physiology and the hyperglucagonemia or increased glucagon/insulin ratios observed in type 2 diabetic states, it is not surprising that there is significant interest in discovering and developing glucagon receptor antagonists for the treatment of the type 2 diabetes. In fact, peptidyl as well as non-peptidyl glucagon receptor antagonists have been shown to lower blood glucose in both animal models and man (reviewed in Jiang and Zhang, 2003; Ling, 2002; Madsen et al., 1999). By developing an in vivo glucagon binding assay, the current study investigated the interaction between the glucagon receptor and its peptidyl and non-peptidyl small molecule glucagon receptor antagonists in vivo, and the relationship between such interactions at the molecular level and the glucose lowering effects in whole animals.

It is generally thought that, for successful in vivo occupancy assays, the radioligand and the receptor should interact with high affinity and the receptor should be expressed at reasonably high levels in the target tissues, with the ratios of receptor density vs. binding affinity being

5 or higher (Eckelman et al., 1979). Accordingly, we performed in vitro binding experiments and subsequently demonstrated that radiolabeled tracer, [^{125}I]glucagon, binds with high affinity (EC_{50}=0.3 nM) to the glucagon receptor which is expressed at relatively high levels (~14 nM) in liver (Fig. 1). We subsequently showed that, in hGCGR mice livers, excessive unlabeled glucagon significantly reduced the levels of [^{125}I]glucagon, providing the first evidence that binding of [^{125}I]glucagon to the liver tissue is specific and can be readily detected in vivo (Fig. 2). Furthermore, we demonstrated that excessive unlabeled glucagon, but not GLP-2 peptide, was able to reduce [^{125}I]glucagon levels in the hGCGR mice (Fig. 3A), and that the reduction of [^{125}I]glucagon by unlabeled glucagon in liver occurs in hGCGR mice but not in GCGR$^{-/-}$ mice (Fig. 3B). These studies firmly established the specific binding of [^{125}I]glucagon to the hGCGR mouse liver and provided validation for the in vivo glucagon receptor occupancy assay.

Although glucagon binding sites have been demonstrated in multiple tissues including liver, brain, pancreas, kidney, intestine and adipose tissue, it was shown to be located predominantly in the liver (Burcelin et al., 1996; Christophe, 1996). Consistent with the previous reports, the results from the current study showed that glucagon binding sites are present predominantly in the liver and to a much less extent in kidney (Fig. 4). It is important to note however that the lack of specific binding in other tissues in this assay does not indicate its absence. However, it does suggest significantly lower expression levels in these tissues. It is generally thought that glucagon mediates its glycemic control mainly by targeting the hepatic glucagon receptor (reviewed in Jiang and Zhang, 2003). In consideration of the predominant expression of glucagon receptor in the liver tissues and the central role of the hepatic glucagon receptor in glucagon-mediated glycemic control, it is therefore reasonable to assume that blocking the hepatic glucagon receptor will be the key mode of action of glucagon receptor antagonists in vivo.

The peptidyl glucagon receptor antagonist des-His-glucagon (2 mg/kg, i.p.) largely blocked exogenous glucagon-induced blood glucose elevation in a pharmacodynamic assay (Fig. 5A) and occupied ~78% of the hepatic glucagon receptor in an in vivo binding assay (Fig. 5B) in hGCGR mice. We also showed that compound A, a non-peptidyl small molecule glucagon receptor antagonist, significantly blocked glucagon-induced elevation of blood glucose at 10 and 30 but not 3 mg/kg in a pharmacodynamic assay (Fig. 6A), and occupied 39%, 65% and 70% of the hepatic glucagon receptor at 3, 10 and 30 mg/kg, respectively, in a receptor occupancy assay (Fig. 6B). Taken together, these results suggest that both glucagon receptor antagonists directly interact with the glucagon receptor in vivo and that such interaction is correlated with and most likely responsible for the metabolic effects of the antagonists in vivo. Based on the results with des-His-glucagon and compound A as well as many other glucagon receptor antagonists (data not shown), it appears that, by occupying a minimum of 50–60% hepatic glucagon receptors, glucagon receptor antagonists can effectively block exogenous glucagon-induced blood glucose elevation in the hGCGR mice.

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