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# Activation of PPAR-γ reduces HPA axis activity in diabetic rats by up-regulating PI3K expression

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ABSTRACT

Increased hypothalamus-pituitary-adrenal axis (HPA) activity in diabetes is strongly associated with several morbidities noted in patients with the disease. We previously demonstrated that hyperactivity of HPA axis under diabetic conditions is associated with up-regulation of adrenocorticotrophic hormone (ACTH) receptors (MC2R) in adrenal and down-regulation of glucocorticoid receptors (GR and MR) in pituitary. This study investigates the role of peroxisome proliferator-activated receptor (PPAR)-γ in HPA axis hyperactivity in diabetic rats. Diabetes was induced by intravenous injection of alloxan into fasted rats. The PPAR-γ agonist rosiglitazone and/or PI3K inhibitor wortmannin were administered daily for 18 consecutive days, starting 3 days after diabetes induction. Plasma ACTH and corticosterone were evaluated by radioimmunoassay, while intensities of MC2R, proopiomelanocortin (POMC), GR, MR, PI3K p110α and PPAR-γ were assessed using immunohistochemistry. Rosiglitazone treatment inhibited adrenal hypertrophy and hypercorticoidism observed in diabetic rats. Rosiglitazone also significantly reversed the diabetes-induced increase in the MC2R expression in adrenal cortex. We noted that rosiglitazone reduced the number of corticotroph cells and inhibited both anterior pituitary POMC expression and plasma ACTH levels. Furthermore, rosiglitazone treatment was unable to restore the reduced expression of GR and MR in the anterior pituitary of diabetic rats. Rosiglitazone increased the number of PPAR-γ$^+$ cells and expression of PI3K p110α in both anterior pituitary and adrenal cortex of diabetic rats. In addition, wortmannin blocked the ability of rosiglitazone to restore corticotroph cell numbers, adrenal hypertrophy and plasma corticosterone levels in diabetic rats. In conclusion, our findings revealed that rosiglitazone down-regulates HPA axis hyperactivity in diabetic rats *via* a mechanism dependent on PI3K activation in pituitary and adrenal glands.



## 1. Introduction

Increased HPA axis activity has been reported in both diabetic patients and laboratory animals subjected to poor glycemic control and is associated with some diabetic complications, including wound healing deficiency, neuropathy and retinopathy (Bitar et al., 1999; Bruehl et al., 2007; Chan et al., 2001; Chiodini et al., 2007). Hyperactivity of the diabetic HPA axis is marked by elevated circulating ACTH and glucocorticoids, increased free urinary glucocorticoids levels, decreased negative feedback following dexamethasone suppression test and impaired responsiveness to stress associated with decreased pituitary and adrenal responses to both CRH and ACTH administration (Chan et al., 2002; e Silva et al., 2009; Roy et al., 1998). High levels of ACTH and glucocorticoids in diabetic subjects are, in part, consequence of a hypothalamic drive, manifested by an increased expression of CRH mRNA in the paraventricular nucleus. Insulin treatment is able to normalize both ACTH and glucocorticoids levels in streptozotocin-diabetic animals, although it failed to reduce the hypothalamic drive (Chan et al., 2002). We previously demonstrated that the greater responsiveness to ACTH and the reduction in HPA axis negative feedback observed in alloxan-diabetic rats compared to non-diabetic rats are associated with increased expression of the ACTH receptor (MC2R) in adrenal glands and decreased expression of glucocorticoid receptors in pituitary (GR and MR), respectively (Torres et al., 2013).

Peroxisome proliferator-activated receptor (PPAR)-γ is an isoform of the PPAR family of nuclear hormone receptors, which can regulate gene expression (Ahmadian et al., 2013). Endogenous agonists of PPAR-γ include unsaturated fatty acids, eicosanoids and oxidized-LDL. Thiazolidinediones, including rosiglitazone, have a high binding affinity to PPAR-γ and are used clinically as anti-diabetic agents due to anti-hyperglycemic activity (Raji and Plutzky, 2002). PPAR-γ is highly expressed in both brown and white adipose tissue and possesses a transcriptome that regulates genes involved in lipid metabolism, energetics and adipocyte differentiation (Jain et al., 1998). However, PPAR-γ

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expression can be observed in other organs, including hypothalamus (Qi et al., 2012), pituitary (Wiesner et al., 2004) and adrenals (Zaripheh et al., 2006), all of which comprise the HPA axis. Moreover, PPAR-γ ligands inhibit POMC transcription induced by CRH in mouse corticotroph pituitary tumor cells *in vitro* and reduce the circulating levels of ACTH and corticosterone in mice inoculated with corticotroph pituitary tumor cells (Heaney et al., 2002). In addition, treatment with rosiglitazone was able to normalize cortisol secretion in patients with Cushing disease (Ambrosi et al., 2004). PPAR-γ is closely associated to PI3K/AKT signaling pathway (Honda et al., 2009; Jones et al., 2010). PPAR-γ activation *in vitro* induces increased expression of PI3K in myotubes (Anandharajan et al., 2006). Furthermore, induction of PI3K pathway is related to HPA axis inhibition, once some antipsychotic drugs down-regulated human CRH gene promoter function through induction of PI3K/AKT pathway, and blocked of PI3K pathway increased ACTH-induced cortisol production by fetal adrenal cortical cells *in vitro* (Basta-Kaim et al., 2006; Newby et al., 2015).

Previously, we demonstrated that alloxan-diabetic rats presented a reduction in plasma levels of the natural ligand of PPAR-γ, 15-Deoxy-$\Delta^{12,14}$-$PGJ_2$, and rosiglitazone inhibited diabetes-induced hypercorticoidism through PPAR-γ dependent manner (Torres et al., 2012). Here, we evaluated the molecular mechanisms involved in normalization of HPA axis function after treatment with a synthetic PPAR-γ agonist rosiglitazone.

## 2. Materials and methods

### 2.1. Animals and diabetes induction

In accordance with the guidelines of the Committee on Use of Laboratory Animals of FIOCRUZ (CEUA-FIOCRUZ, license LW 23/11), male Wistar rats obtained from Oswaldo Cruz Foundation breeding colony were used. Diabetes was induced in 12-h-fasted rats (water *ad libitum*) by a single i.v. injection of alloxan monohydrate (40 mg/kg) diluted with sterile saline (0.9% NaCl). The analyses of kinetics of glycaemia and GR expression in anterior pituitary were performed 1, 2, 3 days after having given alloxan, while all other studies were made 21 days following the diabetes induction. Since the half-life of alloxan is short, just few minutes (Patterson and Lazarow, 1949), we conducted our analysis 21 days after diabetes induction (Carvalho et al., 2003; Carvalho et al., 2006) to ensure that the increase of corticosterone levels noted in diabetic rats was not associated with any stress caused by injection of alloxan directly. Rats injected with vehicle and subjected to similar experimental conditions were used as negative controls. Blood glycaemia was determined with a glucose monitor (Johnson & Johnson, New Brunswick, USA) from samples obtained from the tail vein. Three days after alloxan injection, only rats with blood glucose levels above 11 mmol/L were used in further experiments.

### 2.2. Treatments

Twenty male rats were randomly assigned into 4 groups as follows: non-diabetic untreated (n = 5); non-diabetic treated with rosiglitazone (n = 5); diabetic untreated (n = 5); and diabetic treated with rosiglitazone (n = 5). In another set of experiments, 47 male rats were randomly divided into 7 experimental groups: non-diabetic untreated (n = 5); non-diabetic treated with wortmannin (n = 7); non-diabetic treated with rosiglitazone (n = 7); diabetic untreated (n = 7); diabetic treated with wortmannin (n = 7); diabetic treated with rosiglitazone (n = 7); and diabetic treated with wortmannin plus rosiglitazone (n = 7). Three days after diabetes induction, animals were treated daily with the PPAR-γ agonist rosiglitazone (0.5 mg/Kg, i.p.) and/or the PI3K inhibitor wortmannin (0.1 mg/Kg, i.p.) for 18 consecutive days. Non-diabetic rats received the same treatment and were used as a control group. Untreated non-diabetic and diabetic rats received an equal volume of vehicle (DMSO 0.05%, i.p.).

### 2.3. Determination of macro and microscopic adrenal hypertrophy indexes

Adrenal glands from each experimental group were dissected and placed in Millonig fixative solution for 48 h. Tissues were dehydrated and cleared with xylene before paraffin embedding. Adrenal sections were stained with hematoxylin and eosin (H&E). A Light microscope (Olympus BX50) with a 1000× magnification coupled to a video camera (Olympus UC30) was employed for analysis of at least five different fields from *zona fasciculata* of each adrenal gland section. Cell nuclei were counted and data expressed as cell number per high power field (HPF), as reported (Beuschlein et al., 2002). An additional hypertrophy index was obtained by determining the mean area of *zona fasciculata* cells obtained from the HPF using the software Image Pro-Plus 6.2 (Media Cybernetics Inc., Rockville, USA). To assess adrenal gland hypertrophy macroscopically, the ratio between adrenal weight and body weight was determined.

### 2.4. Immunohistochemistry staining

Paraffin-embedded sections of rat pituitary and adrenals were deparaffinized with xylene, rehydrated by a graded series of ethanol washes and boiled in 10 mM sodium citrate (pH 6.0) for 15 min to enhance antigen retrieval. Tissue sections were incubated with 3% $H_2O_2$ in methanol for 15 min to block endogenous peroxidase. To prevent non-specific binding, the sections were then incubated for 3 h with a solution containing 2.5% bovine serum albumin (BSA), 8% fetal bovine serum (FBS) and 1% of non-fat milk dissolved in Tris-buffered saline enriched with 0.1% Tween 20 (TBST). Sections were incubated overnight with the specific antibody (polyclonal rabbit anti-rat POMC, GR, or MC2-R from Santa Cruz Biotechnology, Santa Cruz, USA; polyclonal goat anti-rat PI3K p110α or MR from Santa Cruz Biotechnology, Santa Cruz, USA; monoclonal mouse anti-rat ACTH from Santa Cruz Biotechnology, Santa Cruz, USA, and monoclonal rabbit anti-rat PPAR-γ from Cell Signaling Technology, Danvers, USA) diluted in TBST with 1% BSA. Primary antibody binding was detected after incubating the sections with a horseradish peroxidase conjugated-secondary antibody (polyclonal anti-goat, anti-rabbit or anti-mouse IgG HRP, R&D System, Minneapolis, USA), followed by a 15 min exposure to the enzyme substrate 3-amino-9-ethylcarbazole (AEC). Sections were washed with TBST between all steps and weakly counterstained with hematoxylin for easy identification of tissue structures.

Tissue sections were mounted in aqueous medium and images captured *via* light microscope (Olympus BX50) coupled to a video camera (Olympus UC30), and images obtained from anterior pituitary or *zona fasciculata* of adrenal cortex were analyzed with the software Image Pro Plus 6.2 (Media Cybernetics Inc., Rockville, USA). Briefly, the red to brown colored pixels associated with a positive immunohistochemistry stain were selected in a model image and applied to the remaining fields. The number of positive pixels was divided by the field area and expressed as pixels/μm$^2$.

### 2.5. Corticotroph and PPAR-γ$^+$ cells quantification

After performing an immunohistochemistry to ACTH or PPAR-γ in pituitary and adrenal glands, images from anterior lobe were acquired in high power field (HPF) using a light microscope (Olympus BX50) with a 400× magnification coupled to a video camera (Olympus UC30). We considered all ACTH positive cells as corticotroph cells, as previously described (Harvey et al., 1993). We analyzed at least five different fields from glands section.

### 2.6. Hormone evaluation

21 days after diabetes induction, animals were killed in a $CO_2$ chamber, during the nadir (08:00 h) of the circadian rhythm as described previously (Chan et al., 2002), and blood was immediately collected from