Granulocyte-Colony Stimulating Factor activates JAK2/PI3K/PDE3B Pathway to Inhibit Corticosterone Synthesis in a Neonatal Hypoxic-ischemic Brain Injury Rat Model

Mélissa S. Charles^{a,b}, Pradilka N Drunalini Perera^b, Desislava Met Doycheva^b, and Jiping Tang^b

^a Department of Microbiology and Molecular Genetics, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA

^bDepartment of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA

Abstract

Objective—Our previous study demonstrated that granulocyte-colony stimulating factor (G-CSF)-induced neuroprotection is accompanied by an inhibition of corticosterone production in a neonatal hypoxic-ischemic (HI) rat model. The present study investigates how G-CSF inhibits corticosterone production, using adrenal cortical cells and HI rat pups.

Methods—Cholera toxin was used to induce corticosterone synthesis in a rodent Y1 adrenal cortical cell line by increasing cyclic adenosine monophosphate (cAMP). Both corticosterone and cAMP were quantitatively measured using a commercial enzyme-linked immunosorbent assay (ELISA). The downstream signaling components of the G-CSF receptor, including Janus Kinase 2 (JAK2)/Phosphatidylinositol-3-kinase (PI3K)/Protein kinase B (Akt) and Phosphodiesterase 3B (PDE3B), were detected by western blot. Sprague-Dawley rat pups at the age of 10 days (P10) were subjected to unilateral carotid artery ligation followed by hypoxia for 2.5 hours. Brain infarction volumes were determined using 2,3,5-triphenyltetrazoliumchloride monohydrate (TTC) staining.

Results—G-CSF at 30ng/ml inhibited corticosterone synthesis but lost its inhibitory effect at higher doses. The inhibitory effect of G-CSF was conferred by interfering with cAMP signaling via the activation of the JAK2/PI3K/PDE3B signaling pathway. The degradation of cAMP by G-CSF signaling reduced corticosterone production. This mechanism was further verified in the neonatal HI brain injury rat model, in which inhibition of PDE3B reversed the protective effects of G-CSF.

Corresponding Author: Jiping Tang, MD, Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Risley Hall, Room 219 Loma Linda, CA, 92350, USA, Phone: (909) 558-7693, Fax: (909) 558-0119, jtang@llu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of Interest: None.

Conclusion—Our data *suggest* that the neuroprotective G-CSF reduces corticosterone synthesis at the adrenal level by degrading intracellular cAMP via activation of the JAK2/PI3K/PDE3B pathway.

Keywords

Hypoxia-Ischemia; Granulocyte Colony Stimulating Factor; Y1; Corticosterone; JAK2; PDE; cholera toxin; adrenal cortical cells; hypothalamic-pituitary-adrenal axis

Introduction

Previous reports indicated that granulocyte-colony stimulating factor (G-CSF), a hematopoietic protein, was involved in regulating hormones of the hypothalamic pituitary axis (HPA), primarily adrenocorticotrophic hormone (ACTH) and the rodent specific glucocorticoid, corticosterone (Mucha et al 2000, Charles et al, 2012). G-CSF reduced brain infarct volume in a neonatal hypoxia-ischemia (HI) rat model, partially by decreasing corticosterone in the blood (Charles et al, 2012). The paucity of studies investigating the effect of G-CSF on neuroendocrine activity highlights an important area that necessitates investigation (Zyliska et al, 1999; Tringali et al, 2007). Particularly in light of the devastating clinical conditions that increase HPA activity such as a cerebrovascular event (Charles et al, 2012; Weidenfeld et al, 2011; Krugers et al, 2000; Fassbender et al, 1994; Ruan et al, 2014), understanding how G-CSF influences HPA activity may become a beneficial area of study. Glucocorticoid treatment is commonly administered in infants with neonatal HI, and considering the previous report suggesting that G-CSF may lose its protective effect when co-administered with a glucocorticoid (Charles et al, 2012), understanding the mechanism on the HPA may impact its translational potential.

The G-CSF receptor belongs to a family of long-chain helical cytokine receptors; the activation of these receptors with ligands such as erythropoietin leptin downregulates the ACTH-induced synthesis of glucocorticoids (Hiroike et al, 2000; Mashburn and Atkinson, 2008; Hsu et al, 2006; Roubos et al, 2012; Hsu et al, 2006; Tringali et al, 2007; Tokgöz et al, 2002). G-CSF signals the Janus Kinase 2 (JAK2)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway, which has been shown to inhibit steroidogenic products in cell culture models, and to regulate their transcription and translation (Lefrancois-Martinez et al, 2011; Li et al, 2003; Hsu et al, 2006). Thus, we hypothesized that G-CSF exerts its steroidogenic influences via the JAK2/PI3K/Akt pathway.

ACTH stimulates steroidogenesis in adrenal cortical cells by upregulating intracellular levels of its second messenger, cyclic adenosine monophosphate (cAMP) (Rainey et al, 2004; Cooke, 1999; An et al, 2014). In the widely used Y1 rodent adrenal cortical cell line, steroidogenesis can be initiated by any stimulant of cAMP production such as cholera toxin (Forti et al, 2002; Yasumura et al, 1966). The production of cAMP leads to the activation of protein kinase A (PKA), ultimately leading to steroidogenesis (Lin et al, 1995; Lopez et al, 2001; Clark et al, 2000). Previous reports have shown that JAK2 activation can suppress steroidogenesis by inhibiting cAMP via the upregulation of phosphodiesterase 3B (PDE3B) (Hsu et al, 2006; Johnsen et al, 2009). Therefore, our corollary hypothesis is that G-CSF

inhibits corticosterone synthesis by activating the JAK2/PI3K/Akt/PDE3B pathway. We tested this in the present study by using cholera toxin to increase cAMP production, and inhibiting the JAK2/PI3K/PDE3B pathway with appropriate inhibitors in an adrenal cortical cell line. The mechanism observed *in vitro* was further verified in rat pups with HI brain injury.

Material and Methods

Animal model

The Institutional Animal Care and Use Committee of Loma Linda University approved all experiments done in this study. A modified Rice–Vannucci model was used, as previously described (Charles et al., 2012; Rice et al., 1981). In brief, Sprague–Dawley rat pups were ordered and allowed 3–5 days to acclimate to the new facility. At 10 days old (P10), pups underwent unilateral right common carotid artery ligation under isoflurane anesthesia. After recovery for 1 hour, the animals were placed in a hypoxic chamber (submerged in a 37°C water bath) with 8% O₂, balance N₂ for 2.5 hours. All rat pups were returned to their mothers at the same time after hypoxic exposure, and all surgeries were conducted at the same time of day, to ensure consistency.

Drug Administration

A total of 55 animals were used in this study, with 4 animals expiring in the hypoxic chamber, giving a mortality of 7.27%. The 51 remaining animals were randomly divided into the following groups: Vehicle (n=7); DMSO (n=6); G-CSF 50 µg/kg (n=8) (Doycheva et al, 2013); ACTH 0.5 mg/kg (n=8) (Wen et al, 2000); PDE3B inhibitor (3-isobutyl-1-methylxanthine (IBMX) 10 µg/kg (n=6) (Tilley and Maurice, 2002); G-CSF + ACTH (n=8); and G-CSF + IBMX (n=8). The drugs were administered subcutaneously in a total volume of 30µL 1 hour after hypoxia.

Infarct Volume and Body Weight

At 24 hours after HI, brains were collected and the infarct volumes were determined with 2,3,5-triphenyltetrazoliumchloride monohydrate (TTC) (Sigma Aldrich, St-Louis, MO USA) staining, as previously described (Charles et al, 2012; Yamauchi et al, 2014; Fathali et al, 2013; Kunze et al, 2014). Briefly, the brains were sectioned in 2 mm slices, incubated in 2% TTC solution for 5 min in the dark, washed in phosphate buffered saline (PBS), and fixed in 10% formaldehyde. Each infarct was traced and analyzed with Image J Software (Version 1.43u; National Institutes of Health, Bethesda, MD, USA). The animals were weighed on a high precision balance before surgery and at 24 hours after surgery, immediately before being euthanized. The weight difference was calculated as (weight 24 hours after HI — weight before surgery).

Cell culture

Rodent Y1 adrenal cortical cells (ATCC, Manassas, VA) were grown in F12K medium (ATCC) supplemented with 2.5% fetal bovine serum (ATCC), 15% horse serum (Fisher Scientific, St-Louis, MO), and 1% penicillin/streptomycin (Thermo Scientific, Rockford, IL). They were grown as a monolayer in a humidified atmosphere at 37°C in 5% CO₂ in T75

flasks (BD Biosciences, San Jose, CA). The medium was changed every 4 days, and cells were sub-cultured after 8 days and split into a 1:3 ratio. They were then stored in liquid nitrogen (5% dimethyl sulfoxide (DMSO) growth medium) or plated for experiments. All experiments were conducted in passage 4 - passage 6 cells. Cells were counted using the TC10™ Automated Cell Counter (Bio-Rad Life Science, Hercules, CA) and seeded in 12-well plates at a concentration of 1×10^6 live cells/well. The cells were grown in 2ml of growth media/well for 48 hours. They were then serum-starved for 8 hours, as previously described (Calejman et al, 2011), and subsequently incubated for 24 hours with growth medium containing the appropriate chemicals for the respective groups.

Chemicals and Treatment

Cholera toxin, the JAK2 inhibitor (Tyrphostin AG490 (AG490), the PI3K inhibitor (LY-294002), ACTH, and the PDE3B inhibitor (IBMX), were purchased from Sigma Aldrich (St-Louis, MO). The inhibitors, AG490, LY-294002, and IBMX, were respectively diluted in DMSO for stock solutions of 1mg/ml, 5mg/ml, and 0.5 M. A G-CSF receptor chimera (Fc Chimera Active) was purchased from Abcam (Cambridge, MA), and G-CSF was obtained from Loma Linda University Pharmacy; both were diluted in phosphate buffered saline. The following concentrations were used: cholera toxin (50 ng/ml) (Hsu et al, 2006); AG490 (50 μ M) (Chen et al, 2005); LY-294002 (20 μ M) (Williams et al, 2010); and IBMX (10 μ M) (Montero-Hadjadje et al, 2006). A dose-response study of G-CSF (using 30, 100, and 300 ng/ml) (Hsu et al, 2006), and the G-CSF receptor chimera (at 10, 30, and 100 ng/ml) after cholera toxin treatment was conducted. At 24 hours, growth media was collected from each well for subsequent assays and analysis. G-CSF-treated cells were collected at the following time points after treatment initiation: 0, 5, 15, 30, 60, and 120 minutes, to determine the activity of the JAK/PI3K/Akt/PDE3B pathway.

Cell Viability Assay

At 24 hours post treatment, growth media was removed from each well and the cells were trypsinized. The trypan blue cell exclusion test, used to determine cell viability, was assayed and recorded using the TC10™ Automated Cell Counter (Bio-Rad) (Wang et al, 2014).

Corticosterone and cAMP Quantitative Assay

Corticosterone was measured in the growth media 24 hours after treatment (Astort et al, 2009, Calejman et al, 2011), using a commercial ELISA kit (Enzo Life Sciences). The minimum detection limit of the assay was 27.0 pg/ml. A 1:20 dilution of the media was assayed according to the manufacturer's instructions. After 2 hours of treatment (Hsu et al, 2006), growth media was removed and hydrochloric acid added to the cells to stop the activity of phosphodiesterases. The level of cAMP in the media was quantitatively analyzed using a colorimetric competitive ELISA kit (Enzo Life Sciences) with a sensitivity of 0.30 pmol/ml.

Western Blot Analysis

Cells were washed with phosphate buffered saline (PBS) and incubated in 100 μ l of RIPA cell lysis buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 20 minutes. They were

then snap-frozen in liquid nitrogen, thawed, and centrifuged at 125g for 10 minutes. The supernatant was collected, and assayed for protein concentration using the spectrophotometric Bradford protein Assay (Bio-Rad). Approximately 30 µg of proteins were electrophoresed in 10% SDS-PAGE gel, and transferred on a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat blocking grade milk (Bio-Rad) and probed with a 1:1000 dilution of primary antibody overnight. The following antibodies were used: G-CSF receptor (Abcam), JAK-2 (Abcam), phospho-JAK2 (Abcam), PI3K (Cell Signaling Technology, Danvers, MA), phospho-PI3K (Cell Signaling Technology), Akt (Cell Signaling Technology), phospho-Akt (Cell Signaling Technology), PDE3B (Abcam), and actin (Santa Cruz Biotechnology). After washing three times, the membranes were probed with a 1:1000 dilution of secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. The protein bands were visualized with ECL Plus, Chemiluminescence (GE Healthcare and Life Sciences, Piscataway, NJ). The densities were analyzed using Image J Software (Version 1.43u; National Institutes of Health, Bethesda, MD) and normalized to actin.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA), followed by post-hoc Tukey multiple comparison test, was used. A probability value <0.05 was considered statistically significant.

Results

G-CSF influences cholera toxin-induced steroidogenesis via its own receptor on Y1 cells

The G-CSF receptor was expressed in Y1 cells (Fig. 1A). The administration of cholera toxin resulted in a 5-fold increase of corticosterone production ($p<0.001$, Fig. 1B). G-CSF co-administration with the cholera toxin inhibited corticosterone synthesis at the low dose of 30 ng/ml ($p<0.05$ vs. cholera toxin alone) but lost its inhibitory property at the higher doses (100 to 300 ng/ml, Fig. 1B). The concentration of corticosterone in the cholera toxin + G-CSF 300 ng/ml group was significantly increased compared to that in the cholera toxin + G-CSF 30 ng/ml group ($p<0.05$). To determine whether G-CSF receptor activation was responsible for the inhibitory effect observed on corticosterone synthesis, the cells were incubated with cholera toxin + GCSF + G-CSF Receptor Chimera (10, 30, 100 ng/ml). The inhibition of corticosterone synthesis was lost when the G-CSF receptor chimera was added (Fig. 1C). This effect reached significance at the dose of 30 ng/ml. Thus, G-CSF 30 ng/ml was used for subsequent molecular assays and hormone analysis.

G-CSF activates the JAK2/PI3K/Akt pathway in Y1 cells

After G-CSF (30 ng/ml) was administered, the protein expression of JAK2, PI3K, and Akt, as well as their active phosphorylated forms, were analyzed at 0, 5, 15, 30, 60, and 120 minutes after treatment initiation. JAK2 phosphorylation, as well as the total JAK2 protein, increased over the time course (Fig. 2A). The ratio of phospho-JAK2/total JAK2 peaked at 60 minutes, but did not reach significance (Fig. 2B). The increase in total JAK2 after G-CSF treatment significantly peaked 30 minutes after treatment ($p<0.05$ vs. 0 minutes). The expression of phospho-PI3K increased over the time course and peaked 30 minutes after

treatment (phospho-PI3K/total PI3K) ($p < 0.05$ vs. 0 minutes, Fig. 2C). Total PI3K protein expression remained constant (Fig. 2D). The ratio of phospho-Akt/total Akt significantly increased and peaked 15 minutes post treatment ($P < 0.05$ vs. 0 minutes) (Fig. 2E), while the total Akt protein expression remained constant (Fig. 2F). PDE3B, a downstream component of PI3K/Akt signaling, was significantly increased 5 minutes after G-CSF treatment ($p < 0.05$ vs 0 minutes, Fig. 3)

G-CSF inhibits corticosterone synthesis via the JAK2/PI3K/PDE3B pathway

To determine whether JAK2, PI3K, and the downstream element, PDE3B, are involved in G-CSF-induced inhibition of corticosterone synthesis, the JAK2 inhibitor, AG490, the PI3K inhibitor, LY-294002, and PDE3B inhibitor, IBMX, were added to the cholera toxin + G-CSF 30ng/mL treatment. Since the inhibitors were dissolved in DMSO, a control group with DMSO (0.2%) was added for comparative analysis. DMSO at 0.2% did not increase corticosterone synthesis compared to control. The addition of each inhibitor in combination with cholera toxin + G-CSF 30 ng/ml to the media blunted the inhibitory effect of G-CSF-induced corticosterone synthesis ($p < 0.05$ vs. cholera toxin + G-CSF 30 ng/mL) (Fig. 4A). Corticosterone was significantly higher ($p < 0.05$) in the media of cells in the following groups, compared to cholera toxin + GCSF 30 ng/ml: cholera toxin, cholera toxin + G-CSF 30 ng/mL + AG490, cholera toxin + G-CSF 30 ng/ml + LY-294002, cholera toxin + G-CSF 30 ng/mL + IBMX. Administration of the drugs did not affect the viability of the cells, as assessed with the Trypan Blue Exclusion Test (Fig. 4B)

G-CSF Inhibits Cholera Toxin-induced cAMP Upregulation via a JAK2/PI3K/PDE3B pathway

The primary function of phosphodiesterases (PDE) is to cleave the phosphodiester bond of cAMP, the main second messenger and mediator of steroidogenesis (Mehats et al, 2002). The expression of cAMP was assessed 2 hours after initiating treatment, as previously described (Hsu et al, 2006). Administration of cholera toxin dramatically increased cAMP levels, compared to control, from 3.85 ± 0.20 pmol/L to 22.2 ± 2.84 pmol/ml ($p < 0.01$ vs. Control and DMSO) (Fig. 5). G-CSF administration alone did not significantly affect cAMP levels, when compared with Control and DMSO-treated cells. The combination of cholera toxin and G-CSF 30 ng/ml significantly reduced the cholera toxin-induced upregulation of cAMP, from 22.2 ± 2.84 pmol/mL to 14.47 ± 0.60 pmol/mL (Fig 5). The inhibition of JAK2 with AG490 in the cholera toxin + G-CSF 30 ng/ml+ AG490-treated cells significantly antagonized the inhibitory effect of G-CSF ($p < 0.01$ vs. cholera toxin + G-CSF 30 ng/mL); the average concentration was 24.83 ± 2.60 pmol/mL. Inhibiting PI3K with LY-294002 in the cells treated with cholera toxin + G-CSF 30 ng/ml yielded a cAMP level of 19.18 ± 1.38 pmol/mL, which was significantly higher than cells without LY-294002 in the growth medium ($p < 0.01$ vs. cholera toxin + G-CSF 30 ng/mL). The inhibition of PDE3B with IBMX substantively increased cAMP levels in cells treated with cholera toxin + G-CSF, to 61.15 ± 27.08 pmol/mL ($p < 0.01$ vs. cholera toxin + G-CSF 30 ng/mL). All the inhibitors reversed the G-CSF-induced reduction of cAMP levels in cholera toxin-treated cells. Treatment with G-CSF 30 ng/mL and the inhibitors alone did not significantly change cAMP levels when compared to control and DMSO-treated cells.

Infarct volume and body weight 24 hours post HI after G-CSF, ACTH and IBMX administration

The administration of G-CSF (50 µg/kg) 1 hour after HI was able to significantly reduce infarct volumes at 24 hours (Fig. 6A) ($p < 0.05$ G-CSF vs Vehicle) from $28.62 \pm 1.862\%$ to $18.71 \pm 2.26\%$. The infarct volumes were significantly increased in animals treated with ACTH ($32.50 \pm 1.47\%$) and IBMX ($32.35 \pm 3.13\%$). Moreover, G-CSF lost its neuroprotective effect when co-administered with ACTH and IBMX (Fig. 6A); the infarct volumes were, respectively, $25.17 \pm 1.87\%$ and $27.49 \pm 1.76\%$.

Since a characteristic of HI injury is weight loss (Charles et al, 2012, Chen et al, 2011), the weight difference among groups was measured. Vehicle-treated animals lost a mean weight of 0.97 ± 0.24 g and DMSO-treated animals lost a mean weight of 1.87 ± 0.24 g (Fig. 6B). G-CSF treatment significantly reversed the weight loss compared to all the other groups; these animals gained 1.03 ± 0.21 g ($p < 0.001$ vs all other groups). ACTH and IBMX-treated animals lost mean weights of 1.464 ± 0.17 g and 1.617 ± 0.26 g, respectively. The protective effects of G-CSF on body weight was reversed in the GCSF+ ACTH group (0.60 ± 0.17) and in G-CSF + IBMX group (0.88 ± 0.17) (Fig. 6B).

Discussion

In this study, we investigated the direct effects of G-CSF on adrenal steroidogenesis, and explored the probable mechanism by which G-CSF inhibits this process. We examined the effect of G-CSF on steroidogenic products in a well-characterized rodent Y1 adrenal cortical cell line. We found that G-CSF at 30 ng/mL inhibits the cholera toxin-induced corticosterone synthesis in Y1 cells and upregulates cAMP. We also demonstrated that the G-CSF receptor is expressed in Y1 adrenal cortical cells. By co-administering G-CSF with a G-CSF receptor chimera, the inhibitory effect of G-CSF against cholera toxin was lost. Additionally, we demonstrated that GCSF activates the JAK2/PI3K/Akt/PDE3B pathway in adrenal cells, which is responsible for the inhibition of cAMP and steroidogenic signaling, in turn blunting corticosterone biosynthesis. Lastly, we evaluated the effect of G-CSF *in vivo* with exogenous ACTH, which, when administered, mimics the activation of the HPA axis. We showed that ACTH and the PDE3B inhibitor reversed G-CSF-induced neuroprotection (50 µg/kg) *in vivo*.

Expression of G-CSF receptor on adrenal cortical cells

No previous reports have shown the expression of the G-CSF receptor in Y1 adrenal cortical cells. After observing this (Fig. 1A), a dose response study of G-CSF was conducted to determine its influence on rodent corticosterone synthesis (Fig. 1B). Our results show that low dose G-CSF (30 ng/mL) inhibits corticosterone synthesis, but with increased concentrations, the inhibitory effect was lost. It was previously illustrated that G-CSF receptor can be downregulated by its own ligand (Jilma et al, 2000). Therefore, we postulate that the loss of effects is probably a result of G-CSF receptor down-regulation and saturation. Hence, other signaling pathways would be activated to oppose the inhibitory effect, or to drive steroidogenesis. Based on the premise that increasing G-CSF concentration reverses the inhibition, it is probable that increasing the concentration beyond

the ones used for this study could potentially increase steroidogenesis. This explanation would help to reconcile the results of Mucha and colleagues (Mucha et al, 2000), which indicate that G-CSF increases blood plasma levels of corticosterone after chronic administration in naïve rats. However, this remains to be elucidated.

Role of G-CSF in adrenal corticosterone synthesis

After demonstrating that the G-CSF receptor is expressed on Y1 adrenal cells, we verified its role in adrenal corticosterone synthesis by blocking it with a G-CSF receptor chimera. The G-CSF receptor chimera, at various concentrations, resulted in increased corticosterone production (Fig. 1C), indicating that the inhibitory effect of G-CSF on corticosterone synthesis was induced by the activation of its own receptor in adrenal cortical cells. This is the first report demonstrating that G-CSF has direct interaction with cells in the adrenal gland, a part of the HPA axis. This finding is critically important for elucidating the mechanisms of G-CSF-induced neuroprotection; especially if the effect can potentially cause adverse or beneficial therapeutic effects in a clinical setting.

G-CSF activation of the JAK2/PI3K/Akt pathway

G-CSF receptor activation is known in other cell types to activate JAK2 and PI3K/Akt downstream signaling (Nakamae-Akahori et al, 2006; Schneider et al, 2005). Whether this is conserved in adrenal cells was unknown. The PI3K/Akt pathway (Lai et al, 2014; Deng et al, 2014) is upstream of PDE3B, a molecule that lowers intracellular cAMP (Hsu et al, 2006; Johnsen et al, 2009). Therefore, we measured the total and activated forms of JAK2, PI3K, and Akt, and the expression of PDE3B over 2 hours after G-CSF administration (Fig. 2). Our results indicate that JAK2 phosphorylation increased over 2 hours, as did the total JAK2 expression (Fig. 2A). Total JAK2 protein expression significantly increased and peaked 30 minutes after treatment initiation. The activated forms of PI3K and Akt also increased over 2 hours, peaking from 15-30 minutes. However, unlike JAK2, the total PI3K and Akt expressions were not affected (Fig. 2B, 2C). G-CSF also increased PDE3B over the 120 minutes time course, with the greatest peak at 5 minutes (Fig. 3). PDE3B peaked at a much earlier time course than its upstream regulators. This suggests that the upstream JAK2 may utilize a feed-forward mechanism that synergistically amplifies PDE3B upregulation. This however remains to be elucidated. Overall, these results show that the JAK2/PI3K/Akt pathway activation upon G-CSF stimulation is preserved in rodent adrenal cortical cells.

Effect of treatments on cell viability

Cholera toxin, a potent stimulator of cAMP production, induced corticosterone synthesis, which was blocked by G-CSF. The inhibitors of the JAK2/PI3K/PDE3B pathway were able to reverse this blocking effect (Fig. 4A), suggesting that the JAK2/PI3K/PDE3B pathway mediates G-CSF-induced corticosterone synthesis inhibition. We verified, using Trypan Blue Exclusion, that the differences reported among each treated group were not due to changes in cell viability (Fig. 4B). There was no significant difference observed in cell viability among the groups. Therefore, the observed effects on corticosterone synthesis and other molecular events were attributed to the drug's influence on signaling pathways, and not cell viability.

G-CSF activation of JAK2/PI3K/PDE3B inhibits steroidogenesis

The elevation of cAMP that was observed 2 hours after cholera toxin administration was inhibited by G-CSF (Fig. 5). This finding suggests that the activation of JAK2/PI3K/PDE3B signaling by G-CSF inhibits steroidogenesis via reduction of cAMP levels. Furthermore, the inhibitors of JAK2/PI3K/PDE3B signaling individually reversed the G-CSF-induced inhibition of cAMP levels. The expression of cAMP was higher in cells treated with cholera toxin + G-CSF 30 ng/mL + IBMX compared to cells treated with cholera toxin alone. This occurrence may be the result of inhibiting the basal activity of PDEs by IBMX, as steroidogenic signaling has been shown to enhance the basal activity of PDEs (Mehats et al, 2002).

Phosphodiesterase and G-CSF neuroprotection after brain injury

Since the HPA axis and PDEs appear to play a crucial role in the mechanism of G-CSF treatment; we verified G-CSF's effect *in vivo* after exposing neonatal pups to HI injury. The animals treated with G-CSF had smaller infarct volumes in the brain compared to animals given vehicles (PBS and DMSO), as previously seen (Charles et al, 2012) (Fig. 6A). Consistent with our finding, the protective effects of G-CSF were also seen in adult rats with cerebral ischemic injury (Solaroglu et al, 2009; Liu et al, 2014). ACTH, by increasing the synthesis of corticosterone (Wen et al, 2000, Johnson et al, 2013), reversed the protective effect of G-CSF on brain infarction in rat pups. We found that the protective effect of G-CSF was also reversed by IBMX (Fig. 6A), which is consistent with the results from our *in vitro* study, suggesting that PDEs mediate GCSF-induced neuroprotection. As body weight loss in rat pups results from HI brain injury (Fathali et al, 2010), it is not surprising that both ACTH and IBMX reversed GCSF-induced protection against body weight loss (Fig. 6B).

Significance and Future Studies

Concerning the limited studies that look at the relationship between G-CSF and HPA activity in naïve and hypoxic ischemic rats, one must understand the complexity of what is at hand. Markedly, the putative ability of G-CSF to influence HPA activity ought to be thoroughly investigated at the adrenal, pituitary, and hypothalamic levels. It is highly probable that G-CSF may directly act on other organs along the HPA axis, as reports have indicated that other hematopoietic growth factors with similar signaling pathways can inhibit corticotropin-releasing hormone (CRH), which is responsible for ACTH synthesis at the pituitary level (Tringali et al, 2007; Zylinska et al, 1999). In light of our previous report (Charles et al, 2012), which indicates that ACTH was not increased in spite of corticosterone inhibition, one must also consider the delicate negative feedback and the probable interaction of G-CSF with the pituitary gland. This physiological response necessitates a thorough understanding of the negative feedback, and how it may also influence brain injury outcomes. Future studies investigating functional outcomes such as behavioral studies (Fathali et al, 2010) are warranted to better understand the G-CSF treatment and its effect of HPA axis in the neonate population. Determining the manner in which G-CSF influences HPA activity in both naïve and post HI states merits further exploration, particularly since agonizing the physiological effects of HPA activity appear to attenuate the protective effects of G-CSF.

To better address the probable effect of G-CSF on HPA organs, we report in our *in vitro* study that the G-CSF receptor is expressed on adrenal cortical cells. This discovery opens a new area of study that would expand our current knowledge of GCSF in the neurological and hematopoietic fields, into that of adrenal functions.

Conclusion

In conclusion, we demonstrated that G-CSF has non-hematopoietic functions in the HPA axis by regulating steroidogenesis in the Y1 adrenal cortical cell line. G-CSF was able to abate the upregulation of cholera toxin-induced cAMP via the JAK2/PI3K/Akt/PDE3B pathway, which ultimately inhibited the steroidogenic product, corticosterone. G-CSF was demonstrated to protect the neonatal brain from HI injury, but lost its protective effect when co-administered with IBMX and ACTH. We propose that a greater understanding of G-CSF's neuroendocrine properties may better translate its efficacy in the clinical setting, particularly when investigating diseases such as neonatal hypoxia-ischemia that involve the over-activation of the HPA axis (Charles et al, 2012).

Acknowledgments

Funding:

This study was supported by NIH grants NS060936 to Jiping Tang.

References

- Astort F, Repetto EM, Martínez Calejman C, Cipelli JM, Sánchez R, Di Gruccio JM, Mercu M, Pignataro OP, Arias P, Cymeryng CB. High glucose-induced changes in steroid production in adrenal cells. *Diabetes Metab Res Rev*. Jul; 2009 25(5):477–86. [PubMed: 19489000]
- An C, Shi Y, Hu X, Gan Y, Stetler RA, Leak RK, Gao Y, Sun BL, Zhong P, Cheng J. Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. *Prog Neurobiol*. Apr.2014 115:6–24. [PubMed: 24374228]
- Calejman MC, Astort F, Di Gruccio JM, Repetto EM, Mercu M, Giordanino E, Sanchez R, Pignataro O, Arias P, Cymeryng CB. Lipopolysaccharide stimulates adrenal steroidogenesis in rodent cells by a NFκB-dependent mechanism involving COX-2 activation. *Mol Cell Endocrinol*. Apr 30; 2011 337(1-2):1–6. [PubMed: 21300135]
- Charles MS, Ostrowski RP, Manaenko A, Duris K, Zhang JH, Tang J. Role of the pituitary-adrenal axis in granulocyte-colony stimulating factor-induced neuroprotection against hypoxia-ischemia in neonatal rats. *Neurobiol Dis*. Jul; 2012 47(1):29–37. [PubMed: 22779090]
- Chen YC, Chang MF, Chen Y, Wang SM. Signaling pathways of magnolol-induced adrenal steroidogenesis. *FEBS Lett*. Aug 15; 2005 579(20):4337–43. [PubMed: 16061232]
- Clark BJ, Ranganathan V, Combs R. Post-translational regulation of steroidogenic acute regulatory protein by cAMP-dependent protein kinase A. *Endocr Res*. Nov; 2000 26(4):681–9. [PubMed: 11196444]
- Cooke BA. Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. *Mol Cell Endocrinol*. May 25; 1999 151(1-2):25–35. [PubMed: 10411317]
- Deng J, Lei C, Cheng Y, Fang Z, Yang Q, Zhang H, Cai M, Shi L, Dong H, Xiong L. Neuroprotective gases—fantasy or reality for clinical use? *Prog Neurobiol*. Apr.2014 115:210–45. [PubMed: 24440817]
- Doycheva D, Shih G, Chen H, Applegate R, Zhang JH, Tang J. Granulocyte-colony stimulating factor in combination with stem cell factor confers greater neuroprotection after hypoxic-ischemic brain

damage in the neonatal rats than a solitary treatment. *Transl Stroke Research*. Apr; 2013 4(2):171–8.

- Fassbender K, Schmidt R, Mössner R, Daffertshofer M, Hennerici M. Pattern of activation of the hypothalamic-pituitary-adrenal axis in acute stroke. Relation to acute confusional state, extent of brain damage, and clinical outcome. *Stroke*. Jun; 1994 25(6):1105–8. [PubMed: 8202965]
- Fathali N, Lekic T, Zhang JH, Tang J. Long-term evaluation of granulocyte-colony stimulating factor on hypoxic-ischemic brain damage in infant rats. *Intensive Care Med*. Sep; 2010 36(9):1602–8. [PubMed: 20461500]
- Fathali N, Ostrowski R, Hasegawa Y, Lekic T, Tang J, Zhang J. Splenic Immune Cells in Experimental Neonatal Hypoxia–Ischemia. *Transl. Stroke Research*. Jul 26.2013 4:208–219.
- Forti FL, Schwindt TT, Moraes MS, Eichler CB, Armelin HA. ACTH promotion of p27(Kip1) induction in mouse Y1 adrenocortical tumor cells is dependent on both PKA activation and Akt/PKB inactivation. *Biochemistry*. Aug 6; 2002 41(31):10133–40. [PubMed: 12146978]
- Gallo-Payet N, Payet MD. Mechanism of action of ACTH: beyond cAMP. *Microsc Res Tech*. Jun 15; 2003 61(3):275–87. [PubMed: 12768543]
- Hiroike T, Higo J, Jingami H, Toh H. Homology modeling of human leptin/leptin receptor complex. *Biochem Biophys Res Commun*. Aug 18; 2000 275(1):154–8. [PubMed: 10944457]
- Hsu HT, Chang YC, Chiu YN, Liu CL, Chang KJ, Guo IC. Leptin interferes with adrenocorticotropin/3',5'-cyclic adenosine monophosphate (cAMP) signaling, possibly through a Janus kinase 2-phosphatidylinositol-3-kinase/Aktphosphodiesterase 3-cAMP pathway, to down-regulate cholesterol side-chain cleavage cytochrome P450 enzyme in human adrenocortical NCI-H295 cell line. *J Clin Endocrinol Metab*. Jul; 2006 91(7):2761–9. [PubMed: 16684834]
- Solaroglu I, Cahill J, Tsubokawa T, Beskonakli E, Zhang JH. Granulocyte colony-stimulating factor protects the brain against experimental stroke via inhibition of apoptosis and inflammation. *Neurol Res*. Mar; 2009 31(2):167–72. [PubMed: 19298757]
- Jilma B, Hergovich N, Homoncik M, Jilma-Stohlawetz P, Kreuzer C, Eichler HG, Zellner M, Pugin J. Granulocyte colony-stimulating factor (G-CSF) downregulates its receptor (CD114) on neutrophils and induces gelatinase B release in humans. *Br J Haematol*. Oct; 2000 111(1):314–20. [PubMed: 11091218]
- Johnsen IK, Kappler R, Auernhammer CJ, Beuschlein F. Bone morphogenetic proteins 2 and 5 are down-regulated in adrenocortical carcinoma and modulate adrenal cell proliferation and steroidogenesis. *Cancer Res*. Jul 15; 2009 69(14):5784–92. [PubMed: 19584291]
- Johnson K, Bruder ED, Raff H. Adrenocortical control in the neonatal rat: ACTH- and cAMP-independent corticosterone production during hypoxia. *Physiol Rep*. Aug.2013 1(3):e00054. [PubMed: 24303136]
- Krugers HJ, Maslam S, Korf J, Joëls M, Holsboer F. The corticosterone synthesis inhibitor metyrapone prevents hypoxia/ischemia-induced loss of synaptic function in the rat hippocampus. *Stroke*. May; 2000 31(5):1162–72. [PubMed: 10797181]
- Kunze A, Zierath D, Drogomiretskiy O, Becker K. Variation in behavioral deficits and patterns of recovery after stroke among different rat strains. *Transl Stroke Res*. Oct; 2014 5(5):569–76. [PubMed: 24711015]
- Lai TW, Zhang S, Wang YT. Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Prog Neurobiol*. Apr.2014 115:157–88. [PubMed: 24361499]
- Lefrancois-Martinez AM, Blondet-Trichard A, Binart N, Val P, Chambon C, Sahut-Barnola I, Pointud JC, Martinez A. Transcriptional control of adrenal steroidogenesis: novel connection between Janus kinase (JAK) 2 protein and protein kinase A (PKA) through stabilization of cAMP response element-binding protein (CREB) transcription factor. *J Biol Chem*. Sep 23.2011 286(38)
- Li J, Feltzer RE, Dawson KL, Hudson EA, Clark BJ. Janus kinase 2 and calcineurin are required for angiotensin II-dependent activation of steroidogenic acute regulatory protein transcription in H295R human adrenocortical cells. *J. Biol. Chem*.2003 278:52355–52362.
- Lin D, Sugawara T, Strauss JF 3rd, Clark BJ, Stocco DM, Saenger P, Rogol A, Miller WL. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science*. Mar 24; 1995 267(5205):1828–31. [PubMed: 7892608]

- Liu J, Wang Y, Akamatsu Y, Lee CC, Stetler RA, Lawton MT, Yang GY. Vascular remodeling after ischemic stroke: mechanisms and therapeutic potentials. *Prog Neurobiol.* Apr.2014 115:138–56. [PubMed: 24291532]
- Lopez D, Nackley AC, Shea-Eaton W, Xue J, Schimmer BP, McLean MP. Effects of mutating different steroidogenic factor-1 protein regions on gene regulation. *Endocrine.* Apr; 2001 14(3): 353–62. [PubMed: 11444433]
- Mashburn KL, Atkinson S. Variability in leptin and adrenal response in juvenile Steller sea lions (*Eumetopias jubatus*) to adrenocorticotrophic hormone (ACTH) in different seasons. *Gen Comp Endocrinol.* Jan 15; 2008 155(2):352–8. [PubMed: 17888915]
- Mehats C, Andersen CB, Filopanti M, Jin SL, Conti M. Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol Metab.* Jan-Feb;2002 13(1):29–35. [PubMed: 11750860]
- Montero-Hadjadje M, Delarue C, Fournier A, Vaudry H, Yon L. Involvement of the adenylyl cyclase/protein kinase A signaling pathway in the stimulatory effect of PACAP on frog adrenocortical cells. *Ann N Y Acad Sci.* Jul.2006 1070:431–5. [PubMed: 16888205]
- Mucha S, Zylinska K, Pisarek H, Komorowski J, Robak T, Korycka A, Stepie H. Pituitary-adrenocortical responses to the chronic administration of granulocyte colony-stimulating factor in rats. *J Neuroimmunol.* Jan 3; 2000 102(1):73–8. [PubMed: 10626669]
- Nakamae-Akahori M, Kato T, Masuda S, Sakamoto E, Kutsuna H, Hato F, Nishizawa Y, Hino M, Kitagawa S. Enhanced neutrophil motility by granulocyte colony-stimulating factor: the role of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *Immunology.* Nov; 2006 119(3):393–403. [PubMed: 16903868]
- Rainey WE, Saner K, Schimmer BP. Adrenocortical cell lines. *Mol Cell Endocrinol.* Dec 30; 2004 228(1-2):23–38. [PubMed: 15541570]
- Rice JE 3rd, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann Neurol.* Feb; 1981 9(2):131–41. [PubMed: 7235629]
- Roubos EW, Dahmen M, Kozicz T, Xu L. Leptin and the hypothalamo-pituitary-adrenal stress axis. *Gen Comp Endocrinol.* May 15; 2012 177(1):28–36. [PubMed: 22293575]
- Ruan L, Lau BW, Wang J, Huang L, Zhuge Q, Wang B, Jin K, So KF. Neurogenesis in neurological and psychiatric diseases and brain injury: from bench to bedside. *Prog Neurobiol.* Apr.2014 115:116–37. [PubMed: 24384539]
- Schneider A, Krüger C, Steigleder T, Weber D, Pitzer C, Laage R, Aronowski J, Maurer MH, Gassler N, Mier W, Hasselblatt M, Kollmar R, Schwab S, Sommer C, Bach A, Kuhn HG, Schäbitz WR. The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *J Clin Invest.* Aug; 2005 115(8):2083–98. [PubMed: 16007267]
- Stocco DM. The role of the StAR protein in steroidogenesis: challenges for the future. *J Endocrinol.* Mar; 2000 164(3):247–53. [PubMed: 10694364]
- Tilley DG, Maurice DH. Vascular smooth muscle cell phosphodiesterase (PDE) 3 and PDE4 activities and levels are regulated by cyclic AMP in vivo. *Mol Pharmacol.* Sep; 2002 62(3):497–506. [PubMed: 12181425]
- Tokgöz B, Uta C, Do ukan A, Oymak O, Kele timur F. Influence of long term erythropoietin therapy on the hypothalamic-pituitary-thyroid axis in patients undergoing capd. *Ren Fail.* May; 2002 24(3):315–23. [PubMed: 12166698]
- Tringali G, Pozzoli G, Lisi L, Navarra P. Erythropoietin inhibits basal and stimulated corticotropin-releasing hormone release from the rat hypothalamus via a nontranscriptional mechanism. *Endocrinology.* Oct; 2007 148(10):4711–5. [PubMed: 17615151]
- Wang Q, Chao D, Chen T, Sandhu H, Xia Y. δ -Opioid Receptors and Inflammatory Cytokines in Hypoxia: Differential Regulation between Glial and Neuron-Like Cells. *Transl. Stroke Res.* Mar. 2014 5:476–483. [PubMed: 24788819]
- Weidenfeld J, Leker RR, Gai N, Teichner A, Bener D, Ovadia H. The function of the adrenocortical axis in permanent middle cerebral artery occlusion: effect of glucocorticoids on the neurological outcome. *Brain Res.* Aug 17.2011 1407:90–6. [PubMed: 21741032]

- Wen C, Li M, Fraser T, Wang J, Turner SW, Whitworth JA. L-arginine partially reverses established adrenocorticotrophin-induced hypertension and nitric oxide deficiency in the rat. *Blood Press.* 2000; 9(5):298–304. [PubMed: 11193135]
- Williams TA, Monticone S, Morello F, Liew CC, Mengozzi G, Pilon C, Asioli S, Sapino A, Veglio F, Mulatero P. Teratocarcinoma-derived growth factor-1 is upregulated in aldosterone-producing adenomas and increases aldosterone secretion and inhibits apoptosis in vitro. *Hypertension.* Jun; 2010 55(6):1468–75. [PubMed: 20385969]
- Yamauchi T, Saito H, Ito M, Shichinohe H, Houkin K, Kuroda S. Platelet lysate and granulocyte-colony stimulating factor serve safe and accelerated expansion of human bone marrow stromal cells for stroke therapy. *Transl Stroke Res.* Dec; 2014 5(6):701–10. [PubMed: 25059633]
- Yasumura Y, Buonassisi V, Sato G. Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. *Cancer Res.* Mar; 1966 26(3):529–35. [PubMed: 5930699]
- Zylinska K, Mucha S, Komorowski J, Korycka A, Pisarek H, Robak T, Stepie H. Influence of granulocyte-macrophage colony stimulating factor on pituitary-adrenal axis (PAA) in rats in vivo. *Pituitary.* Nov; 1999 2(3):211–6. [PubMed: 11081156]

Figure 1. G-CSF influences cholera toxin-induced steroidogenesis via its own receptor *in vitro*
A) The G-CSF receptor is expressed in Y1 adrenal cortical cells. **B)** GCSF significantly inhibits the cholera toxin (CTX)-induced synthesis of corticosterone at a low dose of 30 ng/ml, but loses its inhibitory effect at 100 and 300 ng/ml. G-CSF at 30 ng/ml does not affect basal corticosterone synthesis. (N=8/ group) **C)** The addition of the G-CSF receptor chimera reversed the inhibitory effect of G-CSF and reached significance at 100 ng/ml. #= $p < 0.01$ vs Vehicle, * = $p < 0.05$ vs CTX ** = $p < 0.05$ vs CTX + G-CSF 30 ng/ml N=6/ group.

Figure 2. G-CSF activates the JAK2/PI3K/Akt pathway in *in vitro*

A) & B) Phospho-JAK2/total JAK2 increased over the course of 120 minutes after G-CSF treatment and peaked at 60 minutes. The total JAK2 protein expression was increased over 120 minutes and significantly peaked at 30 minutes ($p < 0.05$ vs 0 minutes). **C) & D)** Phospho-PI3K/total PI3K peaked 30 minutes after G-CSF treatment ($p < 0.05$ vs 0 minutes). Total PI3K expression was not affected by G-CSF treatment. **E) & F)** Phospho-Akt/total Akt protein activation was increased after G-CSF treatment and peaked at 15 minutes ($p < 0.05$ vs 0 minute). Total Akt remained unchanged. N=4/group.

Figure 3. G-CSF activates PDE3B expression in Y1 cells
PDE3B was activated after GCSF 30 ng/ml treatment and significantly peaked 5 minutes after treatment ($p<0.05$ vs 0 minute). PDE3B levels decreased over time. N=4/group.

Figure 4. G-CSF inhibits corticosterone synthesis via the JAK2/PI3K/PDE3B pathway *in vitro*
A) The JAK2 inhibitor (AG490), PI3K inhibitor (LY-294002), and PDE3B inhibitor (IBMX), significantly reversed the inhibition conferred by G-CSF on cholera toxin-induced steroidogenesis (corticosterone production) ($p < 0.05$ vs CTX + G-CSF 30 ng/ml). Treatment with DMSO (0.2%), G-CSF 30 ng/ml, AG490, LY-294002, and IBMX alone, did not significantly affect basal corticosterone synthesis compared to Control. **B)** The Trypan Blue Cell Exclusion assay illustrated that the viability of each treated group did not change ($p > 0.05$ vs. control). N=6/group.

Figure 5. G-CSF abates cholera toxin-induced cAMP upregulation via the JAK2/PI3K/PDE3B pathway *in vitro*

CTX significantly increased cAMP levels from 3.85 ± 0.20 pmol/l to 22.2 ± 2.84 pmol/ml ($p < 0.001$ vs Control and DMSO (0.2%)). G-CSF decreased the upregulation of cAMP to 14.47 ± 0.60 pmol/ml. The JAK2, PI3K, and PDE3B inhibitors (AG490, LY-294002, IBMX) antagonized this effect. DMSO (0.2%), G-CSF 30 ng/ml, AG490, LY-294002, or IBMX alone did not significantly affect basal cAMP levels. N= 6/group.

Figure 6. Infarct Volume 24 hours post HI after G-CSF, ACTH and IBMX administration

A) TTC-stained coronal brain sections of treated groups after HI. * = p < 0.05 G-CSF vs Vehicle ** = p < 0.01 G-CSF vs ACTH *** = p < 0.001 G-CSF vs IBMX. Each line on the left hand side of the brain images demarks 1 mm. **B)** Body weight differences at 24 hours post HI.