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Role of the Pituitary-Adrenal Axis in G-CSF Therapy after Neonatal Hypoxia-Ischemia

Melissa Stephanie Charles
Loma Linda University

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The Role of the Pituitary-Adrenal Axis in G-CSF Therapy after Neonatal Hypoxia-Ischemia

by

Mélissa Stéphanie Charles

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

_____, Chairperson

Jiping Tang, Professor of Physiology

_____

Stephen Ashwal, Professor of Pediatrics and Neurology

_____

Hansel Fletcher, Vice-Chair of Basic Sciences and Professor of Microbiology and Molecular Genetics

_____

Daila Gridley, Professor of Microbiology and Molecular Genetics, Associate Director of Radiation Research Laboratories

_____

John H. Zhang, Chair and Professor of Physiology

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CONTENTS

FIGURES

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
Akt	Protein kinase B
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bax	Bcl-2 associated protein X
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CORT	Corticosterone
CREB	cAMP response element binding protein
CRH	Corticotropin-releasing hormone
CTX	Cholera toxin
DEX	Dexamethasone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte-colony stimulating factor
G-CSF-R	G-CSF receptor
HI	Hypoxia-ischemia
HPA	Hypothalamic-pituitary adrenal

IBMX	3-isobutyl-1-methylxanthine
JAK2	Janus kinase 2
MAPK	Mitogen-activated protein kinase
MET	Metyrapone
P7	Post-natal day 7
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDE3B	Phosphodiesterase 3B
pH	Potential hydrogen
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A
POMC	Proopiomelanocortin
PVN	Paraventricular nuclei
SEM	Standard error mean
Star	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TTC	2,3,5-Triphenyltetrazoliumcholoride monohydrate

ABSTRACT OF THE DISSERTATION

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Mélissa Stéphanie Charles

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, June 2012
Dr. Jiping Tang, Chairperson

Several reports indicate that the activity of the hypothalamic-pituitary-adrenal axis (HPA) as measured by the increased level of adrenocorticotropic hormone (ACTH), and corticosterone is increased after a brain insult. These hormones are the effectors secreted respectively by the pituitary and adrenal glands. It has been shown that the down-regulation of corticosterone levels can improve detrimental outcomes associated with ischemic brain injuries. Neonatal hypoxia-ischemia (HI) is a devastating perinatal event with a grim prognosis and limited therapeutic strategies. In recent studies, granulocyte-colony stimulating factor (G-CSF) has shown promise in neonatal HI investigations by improving neuromotor function and reducing apoptosis in the brain. Furthermore, G-CSF is shown in the naïve rat to regulate hormones of the HPA axis. Therefore we hypothesized that G-CSF may in part confer its neuroprotective properties by influencing the pituitary-adrenal response after neonatal HI. To test our hypothesis, metyrapone was administered to inhibit the release of rodent specific glucocorticoid, corticosterone, at the adrenal level. Dexamethasone, a synthetic glucocorticoid, was administered to agonize the effects of corticosterone. Following the Rice-Vannucci model, seven-day old rats (P7) were subjected to unilateral carotid ligation followed by

2.5 hours of hypoxia. Our results show that both G-CSF and metyrapone significantly reduced infarct volume via anti-apoptotic properties but lost its protective effect when combined with dexamethasone. Additionally, G-CSF suppressed the increase of corticosterone in the blood after HI. To investigate the mechanism by which G-CSF modulated corticosterone synthesis, we evaluated its effect in adrenal cortical cells *in vitro.* Our results indicate that G-CSF activated the JAK/PI3K/Akt/PDE3B pathway, which in turn inhibited corticosterone synthesis. The inhibitors of JAK/PI3K/PDE3B respectively Tyrphostin AG490, LY-294002, and 3-isobutyl-1methylxanthine reversed the inhibitory effect of G-CSF on corticosterone synthesis. We report that G-CSF was neuroprotective in neonatal HI by reducing infarct volume, by suppressing the HI-induced increase of the Bax/Bcl-2 ratio, and by decreasing corticosterone at the adrenal level via the JAK/PI3K/PDE3B pathway. Metyrapone was able to confer similar neuroprotection as G-CSF while dexamethasone reversed the effects of G-CSF. In conclusion, we show that reducing corticosterone was neuroprotective after neonatal HI, which can be achieved by administering G-CSF.

CHAPTER ONE

INTRODUCTION

Neonatal Hypoxia Ischemia

Neonatal hypoxia-ischemia (HI) is a major public health concern affecting both term and preterm infants. It is characterized by exposure to low oxygen (hypoxia) and decreased blood flow (ischemia) before, during, or after labor (Covey and Levison, 2006). This event is clinically defined as asphyxia and diagnosed as hypoxic-ischemic encephalopathy (Zanelli et al, 2009). This clinical diagnosis is a tragic occurrence that will afflict the infants with lifelong complications. For instance, inveterate conditions such as cerebral palsy, mental retardation, and epilepsy are part of the grim prognosis, to name a few (Hill and Volpe, 1989). Undoubtedly, this clinical event is increasingly becoming burdensome not only for the patients, but also for the families, and communities that care for them. This alarming problem is a financial issue as much as it is a health complication. The lifelong projected costs for cerebral palsy alone are estimated at $11.5 billion dollars in the United States (Derrick et al, 2007). For this reason it has become imperative to investigate the causes and prevalence of this particular condition in the population. Thereupon, one can establish how to approach an issue that transcends medical science, public health, and the economy.

Epidemiology

HI is a major contributor to global child mortality and morbidity (Wachtel et al, 2011). HI has an overall incidence of 1-8 cases per 1000 births affecting 60% of preterm infants (Vannucci et al, 1999; Zanelli et al, 2009). With the increasing rate of premature births in the United States (Callaghan et al, 2006), the projected costs associated with HI are bound to increase. The chronic complications induced by HI affect a staggering 80% of survivors (Derrick et al, 2007; Zanelli et al, 2009). Because premature infants have an underdeveloped cerebral circulation, a high density of excitatory neurons, and more vulnerable neurons to reactive oxygen species and reactive nitrogen species, they experience a more severe injury than full-term infants (Takeoka et al, 2002). HI injury is caused by a cascade of cytotoxic, oxidative and inflammatory stresses leading to brain infarct, cerebral edema and neurological deficits (Vannucci et al, 2000).

Pathophysiology of HI

The development of brain injury in the immature brain can be divided in two phases; acute phase, and the reperfusion (delayed) phase (Lai and Yang, 2011). The magnitude of the damage depends on the duration of the insult, the age of the infant, reperfusion injury, and apoptosis (Zanelli et al, 2011). The initial effect of the depleted oxygen concentration and reduced blood flow to the brain is energy failure (Vannucci et al, 2005). The deprivation of glucose and the oxygen supply forces the cells to generate energy via glycolysis through anaerobic respiration. This increases lactate production and creates an acidic environment for the cells in the central nervous system (CNS) (Verklan, 2009). The deleterious effect of this primary energy failure impairs vascular

autoregulation, and the low pH halts the activity of cellular enzymes (Johnston et al, 2001). The sodium potassium ATP pump will then begin to fail, and cause an intracellular accumulation of sodium and calcium ions (Barks and Silverstein, 1992). The intracellular accumulation of these ions will cause the neurons to depolarize and release a large amount of excitatory amino acid including glutamate (Wyatt et al, 1989). During the reperfusion period, free radical damage will be initiated and the reactive oxygen species will combine with the synthesized nitric oxide from glutamate receptor activation to form toxic oxidants (Zanelli et al, 2011; Lai and Yang, 2011; Pacher et al, 2007). Calcium ion accumulation will activate a series of enzymes including endonucleases, proteases and phospholipases that will damage and degrade protein, and DNA (Ankarcrona et al, 1995). The latter will initiate the apoptotic and necrotic cascade which will cause the cells to die. The releasing of pro-inflammatory cytokines by resident immune cells of the CNS, and peripheral immune cells extravasating to the area of injury will initiate the inflammatory response (Iadecola and Anrather, 2011; Ferriero, 2004). All of which contribute to cerebral edema, subsequently leading to cell death. Early cell death is typically necrotic and the continuum will make delayed cell death apoptotic (Northington et al, 2001). Apoptosis will occur over days as a result of a less severe insult, thus being a potential therapeutic target (Varklan, 2009). The penumbra, the area surrounding the ischemic necrotic core, is not exposed to the same intensity of energy failure placing these neurons in a critical stage where they can be recovered (Nakajima et al, 2000; Pulera et al, 1998). Nevertheless, if these neurons are not rescued, within hours the damage will eventually become irreversible as energy failure propagates towards the rescuable cells in the penumbra.

Clinical Presentation

Within the first hours after the injury, the neonate will be unresponsive to sensory input, and have a low level of consciousness (Volpe, 2003; Sarnat and Sarnat, 1976). Hypotonia, pupillary response may be intact depending on the severity and propagation of brain injury. The infant will also exhibit abnormal respiratory patterns (Ferriero, 2004). In severe cases, 50-60% of those affected will have seizures. Subtle seizures can be observed as rowing, bicycling, sucking movements, and apnea (Verklan, 2009). Eventually, in the cases of moderate-severe injury, within 24-72 hours the infant will succumb and deteriorate (Volpe, 2003). Respiratory failure, deep stupor, and fixed pupils may occur prior or immediately before death (ibid; Varklan, 2009).

Therapeutic Options

In spite of the critical mortality, morbidity, and socio-economic hardship accompanied with HI, therapeutic avenues are still lacking. Various anticonvulsants, hypothermic treatments, and fluid and electrolytes management constitute current clinical treatment, however, they have proven only some degree of success (Koenisgsberger, 2000; Zanelli et al, 2009). Due to the complexity of timing, time-window of experimental therapeutic options, and a lack of thorough mechanistic studies, it has become crucial to investigate novel and effective treatment plans for HI. Since the body's compensatory response often contributes to the injury, it has become increasingly imperative to understand the physiological cascades that preclude the pathophysiological sequelae. For example, one physiological response that is known to exacerbate injury, is the increase of the activity of the hypothalamic-pituitary-adrenal axis.

Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA axis is an intrinsic neuroendocrine regulatory system found in vertebrates that allows the organism to respond and adapt to internal or external stressors (Tsigos and Chrousos, 2002). It is a system that helps the organism maintain homeostasis particularly after a challenge and is vital for supporting normal physiological functioning (Kudielka and Kirschbaum, 2005). For the interest of clarity, we chose a simplified definition of stress as internal or external stimuli that can disrupt the biological equilibrium. This can include a cerebrovascular event such as HI (Krugers et al, 2000; Fassbender et al, 1994).

Anatomy of the HPA Axis

The brain circuits that initiate the stress response start in the paraventricular nuclei (PVN) of the hypothalamus (Chrousos, 1992). The parvocellular corticotropin-releasing hormone (CRH) and arginine-vasopressin are secreted and released in the hypophyseal portal system where it can act on the anterior lobe of the pituitary gland (Lamberts et al, 1984; Vale et al, 1981). The PVN has neurons that project to the median eminence where a hypophyseal system of capillaries transports CRH in the vascularized anterior pituitary gland (Turnbull and Rivier, 1999). CRH is a 41-amino acid peptide that drives the adrenocorticotropic hormone (ACTH) secretion and synthesis (Vale et al, 1981). Arginine-vasopressin has little secretagogue activity, it acts as a synergistic factor for CRH activity at the pituitary level. In normal conditions, when there is no stress, CRH is released in a pulsatile fashion resulting in a diurnal rhythm peaking in the early hours of the morning and nadir during the first few hours of sleep (Turnbull and River, 1999;

Tsigos and Chrousos, 2002; Horrocks et al, 1990). The circadian changes are influenced by a variety of factors, such as lighting, activity, and stress. CRH binds to its G-protein coupled receptor on the corticotrope cells of the anterior pituitary lobe to stimulate the synthesis of proopiomelanocortin (POMC), the ACTH precursor (Turnbull and Rivier, 1996; Turnbull and Rivier, 1999). ACTH is then secreted in the systemic circulation, and binds to its receptor, also G-protein coupled, in the zona fasciculata of the adrenal cortex. The downstream signaling pathway of ACTH receptor activation leads to the synthesis and secretion of glucocorticoids in the blood (Ottenweller and Meier, 1982). In humans the major glucocorticoid is cortisol, and in rodents it is corticosterone. Glucocorticoids are the final effectors of the HPA axis that control a variety of physiological functions. These include the immune response, cardiac output, and metabolism, all of which participate in maintaining homeostasis (Pratt, 1990). Glucocorticoids also interact with the pituitary gland and the hypothalamus in the classical feedback endocrine system, to terminate the stress response by inhibiting the synthesis of CRH and ACTH.

Steroidogenesis

Glucocorticoids are products of steroidogenesis, the biological process where steroids are generated from cholesterol. When ACTH binds to its receptor the melanocortin receptor-2, a G-protein coupled receptor, it activates adenylyl cyclase, which will increase cyclic (cAMP) (Mountjoy et al, 1992; Liakos et al, 1998). The increase of intracellular cAMP will activate protein kinase A (PKA), which will release its catalytic subunit and activate the cAMP response element binding protein (CREB) (Sun et al, 2003). CREB will then activate the transcription, and translation of the

steroidogenic acute regulatory (Star) protein responsible for the rate-limiting step of steroidogenesis: transporting cholesterol from the outer to the inner mitochondrial membrane (Manna et al, 2002; Rainey et al, 2004). Once in the mitochondria, the cholesterol is cleaved by cytochrome P450 enzyme (Privalle et al, 1983). Through a series of hydroxylation mediated by enzymes located in the smooth endoplasmic reticulum and mitochondria, cortisol and corticosterone are synthesized. Once secreted in the circulatory system, it then exerts its physiological function by activating its ubiquitous glucocorticoid cytoplasmic receptor, a 94 kDa polypeptide (Smith and Toft, 1993; Pratt, 1990).

Glucocorticoid Functions

The binding of glucocorticoids to the glucocorticoid receptor will cause it to phosphorylate and translocate to the nucleus, where it interacts with glucocorticoid responsive elements (Pratt, 1990). This will lead to the repression or activation, transcription, and translation of genes, which will cause pleiotropic physiological effects (Scheinmann et al, 1995). The effects of glucocorticoids target metabolism, arousal, cognition, development, and the immune system.

Glucocorticoids, as the name suggests, is involved in glucose metabolism (Chrousos, 2000). Extra-hepatical amino acids are mobilized as a response to increased circulating glucocorticoids and used as substrate for gluconeogenesis (Tsigos et al, 1997). The increase of glucocorticoids will inhibit glucose uptake and induce insulin resistance (Tsigos and Chrousos, 2002). Chronic activation of stress will eventually increase visceral adiposity, decrease lean body mass, and suppress osteoblastic activity (ibid).

These effects can become clinically problematic for diseased patients, who may be subjected to a stressor whether physiological or emotional.

Events associated with strong emotions or deemed stressful are often clearly remembered by the subjects who experienced it. This "flash bulb" memory of the event is attributed to the action of glucocorticoid on hippocampus, amygdala, and frontal lobes (Cahill and McGaugh, 1999). The effect of glucocorticoid on cognitive performance and memory is substantiated by extensive animal research (ibid). However the effects of glucocorticoid follow an inverted-U-shape dose response curve (Lupien et al, 2007). Too much glucocorticoid and too little will lead to poor memory consolidation, and will inhibit the retrieval of previously stored information (de Quervain et al, 1998).

Glucocorticoids are also critical for lung maturation and development (Olson et al, 1979). This rings particularly true for preterm infants. Premature babies do not have adequate surfactant in their lungs, which is necessary to decrease the air-liquid surface tension interface (Bolt et al, 2001). Due to this insufficiency, these infants are at increased risk for respiratory distress syndrome (Crowley, 2000). If the respiratory distress syndrome is treated with long term mechanical ventilation it can lead to bronchopulmunary dysplasia (Northway et al, 1967). These conditions are treated with synthetic glucocorticoids to increase surfactant production. However, as previously mentioned, glucocorticoids increase glucose synthesis, and reduce its uptake, therefore some side effects include hyperglycemia, hypertension, inhibition of somatic growth and a myriad of other physiological changes (Halliday and Ehrenkranz, 2000). Although beneficial for lung maturation, the detrimental effects of chronic administration of glucocorticoids may outweigh the benefits.

In addition to their effects on memory, glucose metabolism, and development, glucocorticoids are well characterized for their immune suppressing capabilities (Franchimont, 2004). Glucocorticoids have been in use for over half a century to treat inflammatory and autoimmune diseases (ibid). This class of hormones suppresses cellular immunity, and promotes humoral immunity. Although the anti-inflammatory effects of glucocorticoids are reported in the peripheral system, this effect does not translate to the CNS. In fact, it is reported to exacerbate injury and increase inflammation after a neuronal insult (Sorrells et al, 2009). The activity of HPA axis after a cerebrovascular event, and the effect of glucocorticoid after such a stressor do not align with its anti-inflammatory properties.

Glucocorticoids and Brain Injury

Recent advances reveal that glucocorticoids are detrimental to neuronal repair and survival after an insult (Sapolsky, 1999; Dinkel et al, 2003). Notably, it is shown to increase pro-inflammatory cytokines, induce apoptosis, and increase neurotoxicity (Caso et al, 2007; Macpherson et al, 2005; Zoloaga et al, 2011; Arya et al, 2006). It was further demonstrated that treatment with the rodent specific glucocorticoid, corticosterone, worsened hippocampal neurons after global ischemia (Sapolsky and Pulsinelli, 1985). It appears that glucocorticoids have opposite effects in the CNS. This raises the question whether these reports found in adult studies are true to neonatal models of brain injuries.

Studies have shown that administering a synthetic glucocorticoid, dexamethasone, before a hypoxic-ischemic event in neonatal rats is beneficial (Feng et al, 2011; Ikeda et al, 2005; Felszeghy et al, 2004; Ekert et al, 1997; Tuor et al, 1996). However, these

studies overlook that most incidences of HI can occur *in utero*, thus making the time of occurrence difficult to detect (Perlman, 2006). Particularly since these studies suggest that the protective effects only are seen when dexamethasone is administered at least 6 hours prior to the insult. Because synthetic glucocorticoid administration has not previously been investigated in neonatal HI after the insult, it is highly probable that post-treatment, may result in hyperglycemia, hypertension, and decrease of somatic growth as reported in preterm infants treated for bronchopulmunary dysplasia (Halliday and Ehrenkranz, 2000). Also, since glucocorticoids are the last effector of the HPA axis it may be likely that agonizing an already stressed HPA with dexamethasone could prove detrimental.

The HPA activity is increased after a cerebrovascular event. Numerous studies focusing on the pituitary-adrenal response show that ACTH and corticosterone is upregulated after HI and that inhibiting glucocorticoid synthesis can reduce brain damage (Krugers et al, 1998; Krugers et al, 2000). Clinical studies have also shown that glucocorticoids reduce cerebral cortical grey matter volume (Murphy et al, 2001), and impair long-term neuromotor and cognitive function (Yeh et al, 2004). Understanding the implication of the last effector of the HPA axis is clinically paramount since synthetic glucocorticoids are often administered systemically in premature infants. The hormones of the HPA axis appear to be beneficial or detrimental within certain confines and limitations warranting a better understanding of its regulation and modulation. A recent report showed that a neuroprotective agent widely used in the clinic for hematopoietic purposes, could modulate the levels of ACTH and corticosterone in naïve adult rats

(Mucha et al, 2000). This promising neuroprotective drug is granulocyte-colony stimulating factor (G-CSF).

Granulocyte-Colony Stimulating Factor (G-CSF)

Functions of G-CSF

G-CSF is a glycoprotein with a molecular weight of 19 kDa produced by a wide range of cells including bone marrow cells, fibroblasts, and endothelial cells (Demetri and Griffin, 1991). It is a hematopoietic growth factor that initiates proliferation, differentiation, and proliferation of granulocytes (Nicola, 1990). The G-CSF protein is encoded by a single gene located on chromosome 17 q11-12 (Solaroglu et al, 2006; Le Beau et al, 1987). G-CSF confers its action by activating its receptor (G-CSF-R), a transmembrane protein of the class I cytokine receptor family (Bazan, 1990). The receptor has an immunoglobulin-like domain, a cytokine receptor-homologous domain and three fibronectin type III domains in the extracellular region (Fukunaga et al, 1991). The downstream signaling pathway of G-CSF-R activation include the Janus Kinase (JAK)/ signal transducer and activator of transcription (STAT), the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathway (Tian et al, 1994; Shimoda et al, 1997; Ward et al, 2000). G-CSF-R is expressed on a myriad of cells including those in the CNS; neurons, glial cells, and endothelial cells (Demetri and Griffin, 1991). The cloning of G-CSF established its clinical application were it is now commonly used to treat chemotherapy-induced neutropenia (Schneider et al, 2005; Neidhart et al, 1989). The wide use of G-CSF for hematopoietic recovery has

made this drug potentially translatable to neurological brain injury, particularly since its pharmacokinetic profile is well known.

Neuroprotective Capabilities of G-CSF

Numerous studies give account of G-CSF as a neuroprotective agent (Solaroglu et al, 2006; Schäbitz et al, 2010; Popa-Wagner et al, 2010). It was first investigated *in vitro* where it protected neurons from glutamate-induced cell death (Schäbitz et al, 2003). Furthermore, it was shown to be upregulated in the penumbra after an ischemic insult (ibid; Kleinschnitz et al, 2004). This suggests that the organism has a compensatory response to increase endogenous neuroprotective proteins. Other studies using various models of brain injury reported that G-CSF reduced infarct volume, mortality, brain edema, and apoptosis (Six et al, 2003; Park et al, 2005; Whalen et al, 2000). It was postulated that G-CSF may confer its protective properties by increasing neutrophils in addition to activating its receptor in the CNS. However, reports have shown that the increase of neutrophils or bone marrow stromal cells do not improve neurological function after a brain insult (Chopp and Li, 2002; Hudome et al, 1997). Therefore suggesting that neutrophil increase is not involved in G-CSF induced neuroprotection. Additionally, it was further demonstrated that 5 daily doses of G-CSF (50 μg/kg) improved neurological behavior 28 days after ischemia, as measured by cognitive and sensorimotor tests (Gibson et al. 2005; Fathali et al, 2010). The protective properties of G-CSF are pleiotropic as it was demonstrated that it could improve neural plasticity and vascularization, reduce pro-inflammatory cytokines, and protect against excitotoxicity (Zavala et al, 2002; Schäbitz et al, 2003; Shyu et al, 2004). In light of these observations,

G-CSF has promising therapeutic potential due to its multimodal effects. Whether these results translate to the pediatric population is questioned since these studies were mostly conducted in adults. Although cues are taken from the vast body of literature of adult ischemic injury, the nature of neonatal injury is substantially different (Vannucci, 2007). The brain of term infants is susceptible to injury in areas of high metabolic demands, and high density of excitatory neurons (Takeoka et al, 2002). Preterm infants sustain a more severe injury because of their underdeveloped circulatory system (ibid). Therefore to adequately assess the therapeutic potential of G-CSF in the pediatric population, specifically low birth weight and premature infants, age appropriate animal models are required.

G-CSF has shown promise in neonatal studies where it protected the brain from apoptosis, increased development, and improved neurobehavioral outcomes (Yata et al, 2007; Fathali et al, 2010). There is one study conducted by Schlager and colleagues, which shows that G-CSF did not improve neurobehavioral outcomes or brain injuries (2011). The contradicting results reported by Schlager and colleagues could be attributed to their experimental design. They used 4 times the dose reported in previous studies, and administered the drug 60 hours after the initial insult whereas other studies started therapy as early as 1 hour after the insult (Yata et al, 2007; Fathali et al, 2010). All things considered, the dearth of neonatal studies looking at G-CSF after HI indeed shows an area meriting scientific exploration.

The mechanism of G-CSF action remains to be adequately elucidated. The evidence that G-CSF may modulate (ACTH) and glucocorticoids in naïve rats (Mucha et al, 2000) suggests that it has a probable interaction with the neuroendocrine system.

These hormones are effectors of the HPA axis, which are reported to play a critical role in ischemic brain injury. Determining if G-CSF regulates these hormones in a neonatal model with a different HPA response than adults, after a diseased state known to increase HPA activity warrants much needed examination.

Significance of Studies

The complexity of the neurodevelopmental sequelae of HI necessitates a drug with multimodal properties. Therefore, to address the limitations clinically observed for the treatment of HI, we propose to study for the first time the interaction between a hematopoietic neuroprotective growth factor, G-CSF, with the neuroendocrine pituitary-adrenal activation in a neonatal model of HI brain injury. G-CSF and its mechanistic properties on the HPA axis have not previously been studied in a neonatal HI model. Furthermore, the effects of G-CSF on infarct volume have not previously been reported in neonates. The novel theoretical concept that this study sets forth will challenge the clinical practice paradigm that administers synthetic glucocorticoids to pre-term neonates with high propensity to HI complications. Equally important, is the fact that no previous reports examined the mechanism of G-CSF on steroidogenesis. For this purpose, our proposed investigation will add fundamental information that may guide future research in a wide range of directions. Particularly since G-CSF is already in clinical use for neonatal neutropenia, this compound can feasibly be introduced into practice for neonatal neuroprotection (Schlager et al, 2011; Kuhn et al, 2009; Carr et al, 2003). Herein lies the novelty where we aim at investigating the mechanism of G-CSF via the HPA effector hormone, corticosterone, in a rat neonatal model of HI.

The supposition that G-CSF may influence glucocorticoid synthesis is highly probable due to its ability to activate class I cytokine receptors (Aritomi et al, 1999). Class I cytokine receptors are transmembrane proteins expressed on the surface of cells including the steroidogenic adrenal cortical cells (Hoggard et al, 1997; Malendowicz et al, 2003). Studies have shown that class I cytokine receptor ligands, such as leptin, can inhibit corticosterone synthesis *in vitro* (Hsu et al, 2006; Salzmann et al, 2004). Because G-CSF and leptin share sequence homology, we propose that G-CSF may inhibit glucocorticoid synthesis. No previous reports indicate that the G-CSF receptor is expressed on adrenal glands; therefore we postulate that its activity is via the cytokine family receptor. Also, a variety of intracellular cascades activated by G-CSF decrease apoptotic markers caspase-3 and Bax (Solaroglu et al, 2009); these intracellular cascades are also regulated by glucocorticoids. Based on this evidence our central hypothesis is that is that G-CSF will protect the neonatal brain from apoptosis by suppressing the activity of the pituitary-adrenal response via the activation of its cytokine receptor after HI.

Approach of Studies

The **rationale** of this study is that identifying the mechanisms involved in G-CSF therapy for neonates will extend into adequately designed clinical applications for infants victimized by HI. We plan to achieve the goal of this study with the following aims which is summarized in a schematic diagram (Figure1.1).

AIM 1: Evaluate the anti-apoptotic properties of G-CSF via the pituitary-adrenal axis after neonatal HI.

Because reports indicate that glucocorticoids can exacerbate apoptosis we hypothesize that G-CSF reduces brain infarct and does so by inhibiting corticosterone synthesis.

AIM 2: Determine the mechanism by which G-CSF inhibits corticosterone synthesis in vitro.

We hypothesize that G-CSF activates class I cytokine receptors on the adrenal glands which reduces corticosterone. This study will be conducted in well-characterized rodent Y1 adrenal cortical cells.

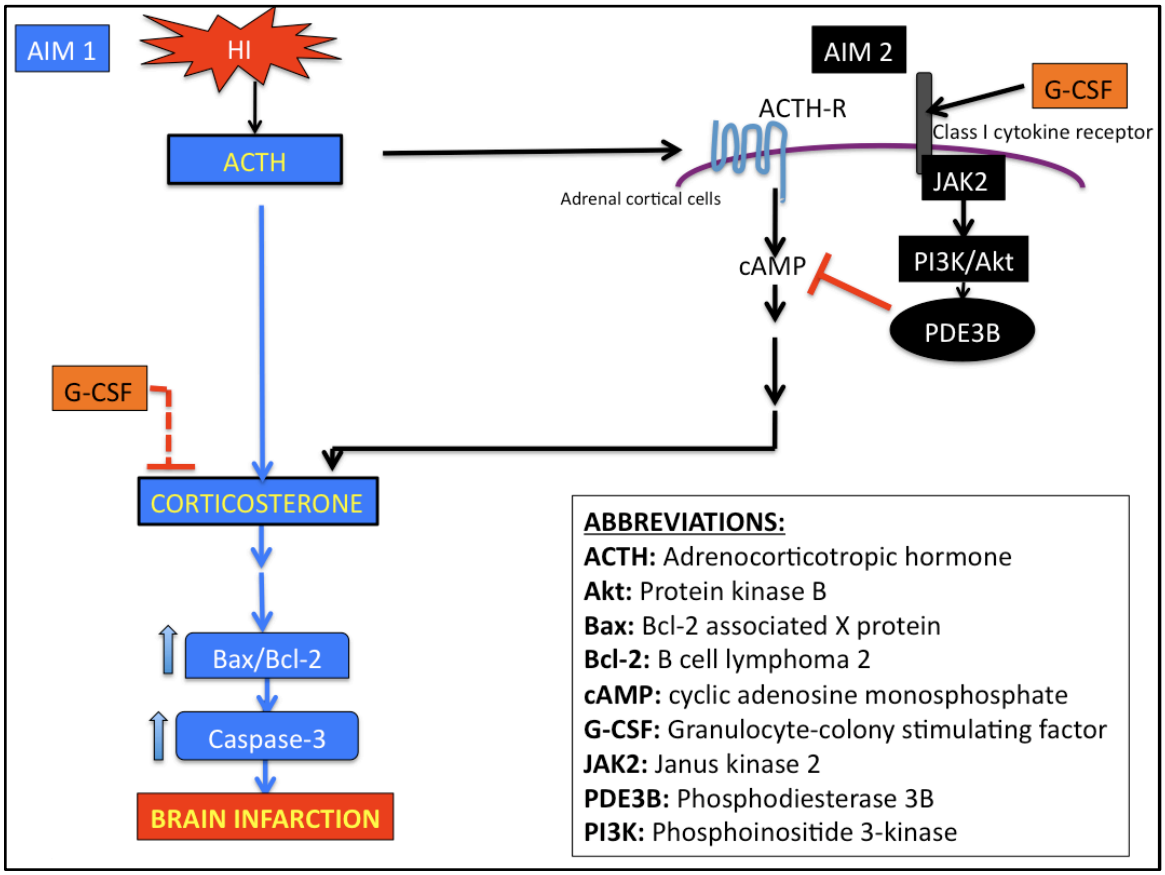

Figure 1.1. Schematic representation of the central hypothesis and specific aims. Aim 1 as illustrated in blue will investigate the effect of G-CSF on corticosterone synthesis *in vivo* after neonatal HI, measure the apoptotic markers in the brain and assess infarct volume. Aim 2 (black) will study the effect of G-CSF *in vitro* on corticosterone synthesis. This aim proposes that G-CSF activates the JAK2/PI3K/Akt/PDE3B signaling pathway to inhibit corticosterone synthesis by degrading cAMP.

CHAPTER TWO

ROLE OF THE PITUITARY-ADRENAL AXIS IN GRANULOCYTE-COLONY STIMULATING FACTOR-INDUCED NEUROPROTECTION AGAINST HYPOXIA-ISCHEMIA IN NEONATAL RATS

Mélissa S. Charles[a,b], Robert P. Ostrowski[b,e], Anatol Manaenko[b], Kamil Duris[b], John H. Zhang[b,c,d], Jiping Tang[b,d].

[a] Department of Microbiology and Molecular Genetics, [b] Department of Physiology and Pharmacology, [c] Department of Neurosurgery, [d] Department of Anesthesiology, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA, [e] Department of Experimental and Clinical Neuropathology, Medical Research Centre, Polish Academy of Sciences, 02-106 Warsaw, Poland

Corresponding Author: Jiping Tang, MD
Department of Physiology and Pharmacology
Loma Linda University School of Medicine
Risley Hall, Room 214
Loma Linda, CA, 92350, USA
Phone: (909) 558-7693
Fax: (909) 558-0119
Email: jtang@llu.edu, jipingtang@yahoo.com

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Abstract

Several reports indicate that the activity of the hypothalamic-pituitary-adrenal axis (HPA) is increased after a brain insult and that its down-regulation can improve detrimental outcomes associated with ischemic brain injuries. Granulocyte-colony stimulating factor (G-CSF) is a neuroprotective drug shown in the naïve rat to regulate hormones of the HPA axis. In this study we investigate whether G-CSF confers its neuroprotective properties by influencing the HPA response after neonatal hypoxia-ischemia (HI). Following the Rice-Vannucci model, seven-day old rats (P7) were subjected to unilateral carotid ligation followed by 2.5 hours of hypoxia. To test our hypothesis, metyrapone was administered to inhibit the release of rodent specific glucocorticoid, corticosterone, at the adrenal level. Dexamethasone, a synthetic glucocorticoid, was administered to agonize the effects of corticosterone. Our results show that both G-CSF and metyrapone significantly reduced infarct volume while dexamethasone treatment did not reduce infarct size even when combined with G-CSF. The protective effects of G-CSF do not include blood brain barrier preservation as suggested by the brain edema results. G-CSF did not affect the pituitary released adrenocorticotropic hormone (ACTH) levels in the blood plasma at 4 hours, but suppressed the increase of corticosterone in the blood. The administration of G-CSF and metyrapone increased weight gain, and significantly reduced the Bax/Bcl-2 ratio in the brain while dexamethasone reversed the effects of G-CSF. The combination of G-CSF and metyrapone significantly decreased caspase-3 protein levels in the brain, and the effect was antagonized by dexamethasone. We report that G-CSF is neuroprotective in neonatal HI by reducing infarct volume, by suppressing the HI-induced increase of the

Bax/Bcl-2 ratio, and by decreasing corticosterone in the blood. Metyrapone was able to confer similar neuroprotection as G-CSF while dexamethasone reversed the effects of G-CSF. In conclusion, we show that decreasing HPA axis activity is neuroprotective after neonatal HI, which can be conferred by administering G-CSF.

Introduction

Neonatal hypoxia ischemia (HI) remains the leading cause of perinatal brain injury, which ultimately leads to cerebral palsy, mental retardation, and epilepsy (Vannucci et al, 1999). It is a major public health concern with a prevalence of 60% in preterm infants and an incidence of 1-8 cases per 1000 births (Vannucci, 2000). In spite of its critical mortality and morbidity rate, current therapeutic avenues are still lacking; thus necessitating alternative strategies to amplify current therapeutic potential. Several reports suggest that the activity of the hypothalamic-pituitary adrenal (HPA) axis is increased after HI, and that its down-regulation can reduce brain damage (Krugers et al, 1998, Krugers et al, 2000). HPA axis activation involves the upregulation of the adrenocorticotropic hormone (ACTH) and glucocorticoids. ACTH is released from the pituitary gland in the blood stream where it acts on the adrenal glands to induce synthesis and release of glucocorticoids. Glucocorticoids are reported to play an important role in neurological function (Sorrells et al, 2009; Dumas et al, 2010), and neuronal damage in the hippocampus after ischemic insults (Roy and Sapolsky, 2003). Studies have shown that supraphysiological levels of glucocorticoids can exacerbate excitotoxic effects (Stein-Behrens et al, 1992), elevate levels of reactive oxygen species (McIntosh and Sapolsky, 1996), and increase neuroinflammation and apoptosis (MacPherson et al, 2005;

Kuchinsky and Gillardon, 2000). This is of clinical relevance, since synthetic glucocorticoids are often administered systemically in premature infants (Thébaud et al, 2001). Clinical studies have shown that premature infants treated with synthetic glucocorticoids have reduced cerebral cortical grey matter volume (Murphy et al, 2001), and impaired long-term neuromotor and cognitive function (Yeh et al, 2004). Accordingly, targeting the HI induced elevation of glucocorticoids may be a promising therapeutic target.

A promising stroke drug candidate, granulocyte-colony stimulating factor (G-CSF), has been shown to modulate ACTH and glucocorticoids in rats (Mucha et al, 2000). G-CSF is a glycoprotein, and a growth factor known to confer neuroprotection in various models of brain injury (Yata et al, 2007; Popa-Wagner et al, 2010). It is currently in Phase II clinical trial for adult ischemic stroke, and well tolerated at high doses (Schäbitz et al, 2010). In various animal studies, G-CSF has been reported to have anti-apoptotic, anti-inflammatory, excitoprotective, and neurotropic properties (Solaroglu et al, 2006, Gibson et al, 2005; Schäbitz et al, 2003; Konishi et al 1993). Furthermore, it has shown promise in neonatal HI studies where it protected neurons from apoptosis, and improved long term neurobehavior outcome (Yata et al, 2007; Fathali et al, 2010). A variety of intracellular cascades activated by G-CSF decrease apoptotic markers caspase-3, Bax (Solaroglu et al, 2009); these intracellular cascades are also regulated by glucocorticoids. Whether G-CSF confers its neuroprotective effects through the HPA axis in neonatal HI has yet to be determined.

On the basis of these observations, we hypothesize that G-CSF attenuates apoptosis partially by down-regulating the activity of the HPA axis. Herein we chose to

focus on the pituitary-adrenal response by measuring ACTH and rodent specific glucocorticoid, corticosterone. To test this hypothesis we used metyrapone, an inhibitor of glucocorticoid synthesis, which suppresses circulating levels corticosterone. Metyrapone reduces corticosterone production by inhibiting the 11-β hydroxylation of 11-deoxycorticosterone. In addition, we used dexamethasone, a synthetic glucocorticoid that agonizes the effects of circulating corticosterone. Here we investigate whether G-CSF reduces neuronal apoptosis partially by regulating the ACTH and corticosterone response following experimental neonatal HI.

Materials and Methods

Animal Model

The Institutional Animal Care and Use Committee of Loma Linda University approved all the experiments used in this study. A modified Rice-Vannucci model was used as previously described (Rice et al, 1981; Chen et al, 2009). In brief, Sprague-Dawley 7-day old rat pups (P7) underwent unilateral right common carotid ligation under isoflurane anesthesia at 0300 hours. Because P7 rats do not have an established diurnal rhythm of plasma corticosterone or ACTH, (Allen and Kendall, 1966; Leal et al, 1999) we chose a time that was in the dark cycle consistent with the nocturnal activities of rats (Levin and Levine, 1975). For consistent hormonal results, the ordered animals were allowed 3 days to acclimate to the new facility and the surgeries were all conducted at the same time. After recovery for 1 hour, the animals were placed in a hypoxic chamber submerged in a 37^{o}C water bath, subjected to 8% O_2 balanced in N_2 for 2.5 hours. Sham animals underwent anesthesia and neck incision, the carotid was exposed without the

ligation and was exposed to normoxic conditions. All P7 rats were returned to their mothers at the same time after hypoxic exposure.

Drug Administration

A total of two hundred and fourteen animals were used in this study. Sixteen animals expired in the hypoxic chamber giving this study a mortality of 7.47 percent. The remaining one hundred and ninety-eight animals were randomly divided into the following groups: Sham (n=24), Vehicle (n=27), G-CSF 50 μg/kg (n=29), Metyrapone 10 mg/kg (n=8), Metyrapone 30 mg/kg (n=29), G-CSF + Metyrapone 30 mg/kg (n=27), Dexamethasone 0.1 mg/kg (n=7), Dexamethasone 0.5 mg/kg (n=23), G-CSF + Dexamethasone 0.5 mg/kg (n=24). The drugs were administered subcutaneously in a total volume of 30 μL one hour after hypoxia.

Blood and Collection

Blood was sampled 3 hours after drug administration and 24 hours after HI. Briefly the animals were deeply anesthetized with isoflurane, and the blood was collected via cardiac puncture with a 22-gauge needle. Blood was transferred in EDTA coated tubes, and centrifuged at 3,000 rpm for 5 minutes. Blood plasma was immediately removed and stored at -80^{o}C until assayed (Mucha et al, 2000).

Infarct Volume and Body Weight

At 24 hours, brains were collected and infarct volume was determined with 2,3,5-triphenyltetrazoliumchloride monohydrate (TTC) (Sigma Aldrich, St-Louis, MO USA)

staining and analyzed by Image J software as previously described (Zhou et al, 2009). Briefly, the brains were sectioned in 2 mm slices, incubated in 2% TTC solution for 5 minutes in the dark, washed in phosphate buffered saline (PBS), and fixed in 10% formaldehyde. The infarct volume 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 immediately before being euthanized. The weight difference was calculated as (weight 24 hrs after HI – weight before surgery).

Brain Water Content

Pups were euthanized 24 hours after HI and the brains were divided in three parts (ipsilateral and contralateral hemispheres, and cerebellum). Each part was immediately weighed (wet weight) on a high precision balance (Denver Instrument, sensitivity ± 0.001 g) and again after drying in a 100^{o}C oven for 24 hours (dry weight) as previously described (Chen et al, 2008). The percentage was calculated as [(wet weight-dry weight)/wet weight] x 100.

Hormone Assay

ACTH was measured with a two-site enzyme-linked immunosorbent assay (ELISA) kit (MD Bioproducts, St.-Paul, MN, USA) following the manufacturer's protocol. This assay used a goat polyclonal antibody to ACTH, and a mouse monoclonal antibody to ACTH that respectively bound the C-terminal (34-39) and the N-terminal of ACTH (1-24). The minimum detection limit of the assay was 0.22 pg/mL.

Corticosterone was measured with an ELISA kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) with a sensitivity of 27.0 pg/ml according to the manufacturer's instructions.

Western Blot

Animals were euthanized at 24 hours after HI. The brain hemispheres were immediately collected and snap frozen in liquid nitrogen and stored at -80^{o}C until analyzed. Whole cell protein extracts were obtained from brain samples by homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and centrifuging for 25 minutes in 4^{o}C at 14,000 g. Ten percent SDS-PAGE gels were used, and 50 mg of denatured protein extracts were loaded in each well. The gel was electrophoresed and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat blocking grade milk (Bio-Rad) and probed with appropriate dilution of primary antibody overnight. The following primary antibodies used were: cleaved caspase-3 (Santa Cruz Biotechnology, 1: 1000), Bax (Cell Signaling Technology, Danvers, MA, USA, 1:1000), Bcl-2 (Cell Signaling Technology, 1:1000), and actin (Santa Cruz Biotechnology, 1:1000). After washing the membranes three times, they were probed with specie-specific secondary antibodies (Santa Cruz Biotechnology) at 1:1000 dilutions for 1 hour at room temperature and visualized using ECL Plus, Chemiluminescence (GE Healthcare and Life Sciences, Piscataway, NJ). The optical densities of the bands were analyzed with Image J Software (Version 1.43u; National Institutes of Health, Bethesda, MD) and normalized to actin as the loading control.

Statistical Analysis

The results are presented as the mean ± standard error mean (SEM). Statistical differences among groups were analyzed by using one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test (Graph Pad Prism Version 5.0d). Probability value<0.05 was considered statistically significant.

Results

Determining the Optimal Dose of Metyrapone and Dexamethasone

The metyrapone clinical dose for adrenal insufficiency is 30 mg/kg (Giordano et al, 2008) hence the starting dose of choice. We used two doses 10 mg/kg and 30 mg/kg to determine which dose would optimally reduce infarct volume compared to the control groups. Dexamethasone on the other hand has previously been studied in neonatal HI using the two following doses: 0.1 mg/kg and 0.5 mg/kg (Tuor et al, 1993, Ikeda et al, 2002). However, these reports use dexamethasone as pretreatment, our study will use it as post-treatment. Our infarct volume results indicate that metyrapone reduces the infarct volume in a dose dependent manner and the effect is significant with the 30 mg/kg dosage (Figure 2.1). The vehicle group has a mean of 29.42% ± 3.40 and metyrapone 30 mg/kg significantly reduced the infarct percentage to 16.08% ± 2.57 ($p<0.01$vs HI +Vehicle). The administration of dexamethasone did not significantly reduce infarct volume when compared to vehicle; low dose (0.1 mg/kg) mean infarct percentage was 23.99% ± 1.25 and high dose (0.5 mg/kg) was 27.69% ± 1.69 (Figure 2.1). Dexamethasone 0.5 mg/kg produced a significantly larger infarct volume compared to that produced by metyrapone 30 mg/kg treatment ($p<0.05$) (Figure 2.1). Based on these

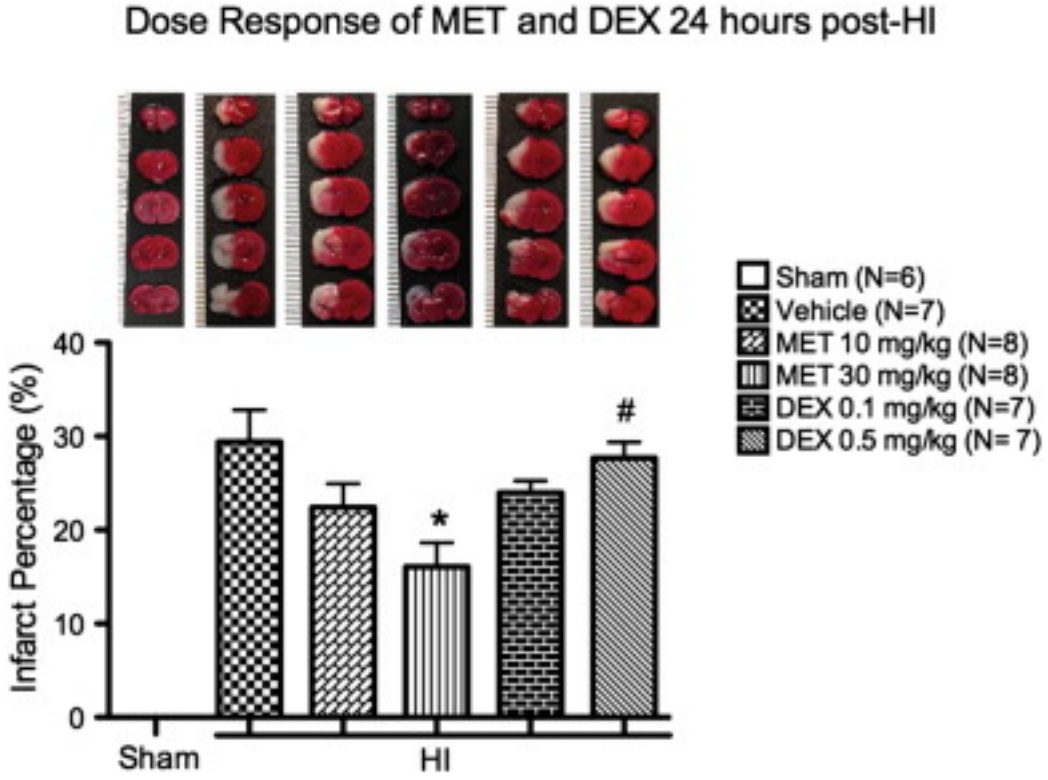

Figure 2.1 Dose responses of metyrapone and dexamethasone. Representative TTC stained coronal brain section of the dose response analysis for metyrapone (MET) and dexamethasone (DEX) at 24 h. (* = $p < 0.01$ vs Vehicle, # = $p < 0.05$ vs MET 30 mg/kg, all HI groups $p < 0.01$ vs Sham). Each line on the left hand side of the brain images demarks 1 mm.

results metyrapone 30 mg/kg and dexamethasone 0.5 mg/kg were considered as the appropriate dosages for all the following experiments and molecular studies. The F value for the ANOVA analysis conducted is 22.36.

Infarct Volume and Body Weight after G-CSF, Metyrapone and Dexamethasone Administration

The administration of G-CSF (50 mg/kg) 1 hour after HI was able to significantly reduce infarct percentage at 24 hours from 29.42% ± 3.40 to 14.70% ± 3.63 ($p<0.05$ vs. HI + Vehicle, Figure 2.2A). Metyrapone (30 mg/kg) and the Metyrapone + G-CSF groups similarly conferred neuroprotection by significantly reducing infarct volume compared to the Vehicle group to 16.08% ± 2.57 and 15.08% ± 3.47 respectively ($p<0.05$ vs. HI + Vehicle). Furthermore, agonizing the HPA axis with dexamethasone (0.5 mg/kg) administration did not significantly affect infarct volume compared to the Vehicle group (27.69% ± 1.69 infarct percentage) and G-CSF lost its neuroprotective effect when co-administered with dexamethasone yielding an infarct percentage of 26.36% ± 2.78 (Figure 2.2A).

Since a characteristic of HI injury is weight loss (Chen et al, 2011), the weight difference at 24 hours after HI was measured. The sham animals gained a mean weight of 2.11 g ± 0.22 after 24 hours while the HI vehicle treated pups lost an average 0.78 g ± 0.42. The animals treated with G-CSF lost 0.63 g ± 0.49 and the group treated with metyrapone lost 1.07 g ± 0.26 which were not significantly different compared to the vehicle treated group. Co-administration of G-CSF and metyrapone significantly reversed the weight loss compared to all the other HI groups, the animals gained 1.25 g ± 0.39

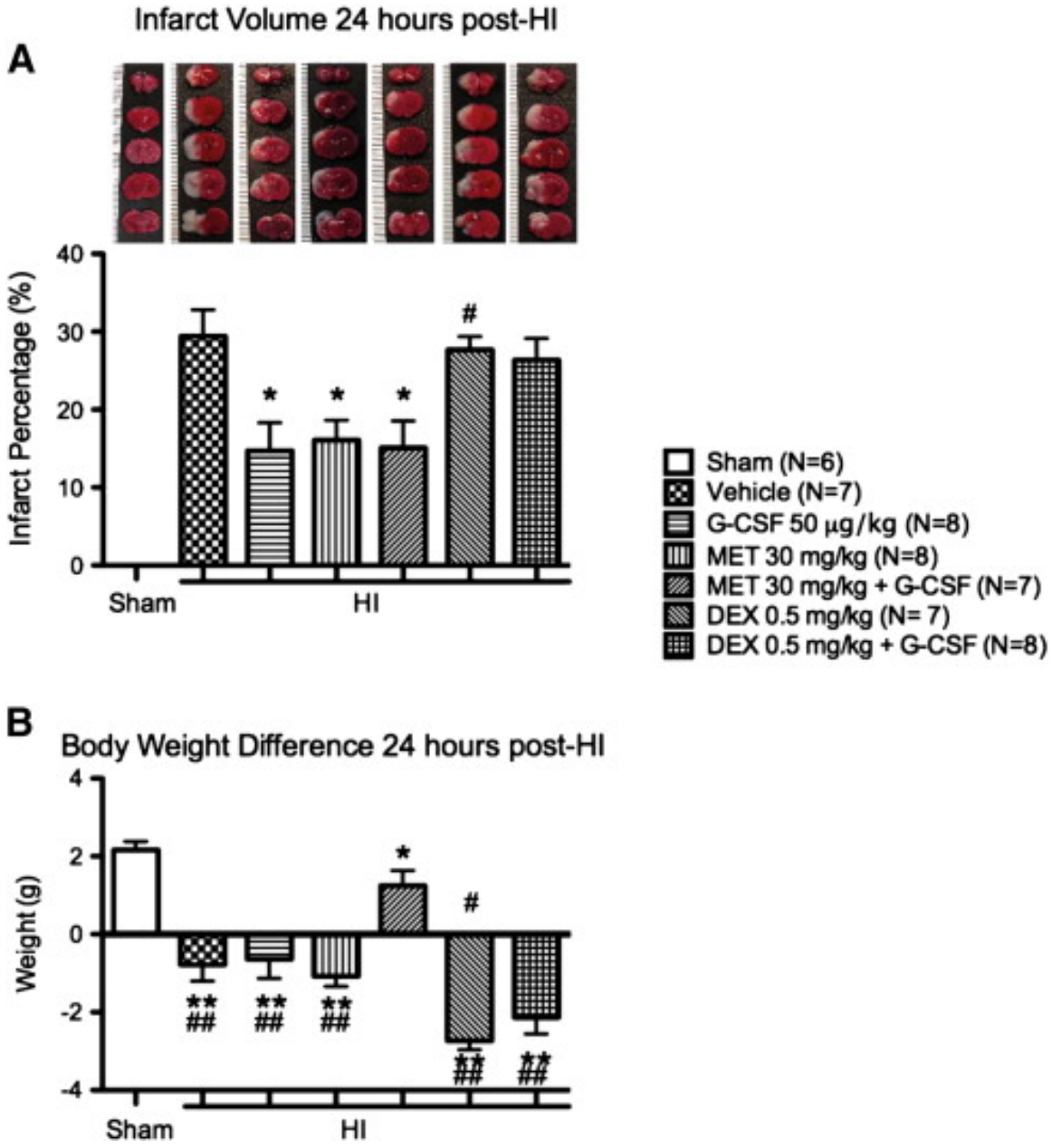

Figure 2.2 Infarct volume and body weight at 24 h after G-CSF, metyrapone and dexamethasone administration. A. TTC stained coronal brain section of Sham, and G-CSF, MET, DEX treated groups after HI. (* = $p < 0.05$ vs Vehicle, # = $p < 0.05$ vs G-CSF, all HI groups $p < 0.01$ vs Sham). **B.** Body weight gain was measured on a high precision balance scale and calculated as such: (Weight 24 h post-HI) − (Weight before HI). (* = $p < 0.01$ vs Vehicle, # = $p < 0.05$, ** = $p < 0.001$ vs Sham, ## = $p < 0.01$ vs MET + G-CSF). Each line on the left hand side of the brain images demarks 1 mm

(P<0.01, Figure 2.2B). The administration of dexamethasone significantly exacerbated weight loss compared to vehicle treated group by losing a mean 2.73 g ± 0.23 (p<0.01, Figure 2.2B). Even when co-administered with G-CSF, the animals lost a mean of 2.14 g ± 0.42 (Figure 2.2B). All the groups that underwent HI significantly lost weight compared to Sham group (p<0.001) except for the HI + G-CSF + Metyrapone group.

Measuring the Effect of G-CSF, Metyrapone and Dexamethasone on Brain Edema After HI

Brain edema was assessed by measuring brain water content, which was elevated in the ipsilateral hemisphere of the Vehicle treated group compared to Sham (p<0.05) (Figure 2.3). G-CSF did not significantly reduce brain edema, but was significantly different than the dexamethasone treated group (p<0.01), which also had elevated brain edema when compared to Sham (p<0.01). Dexamethasone increased brain water content even when co-administered with G-CSF (p<0.05 vs. Sham). The administration of metyrapone alone or in combination with G-CSF did not significantly alter brain water content. No significant changes in the brain water content amongst all the groups were observed in the contralateral hemisphere and the cerebellum (Figure 3).

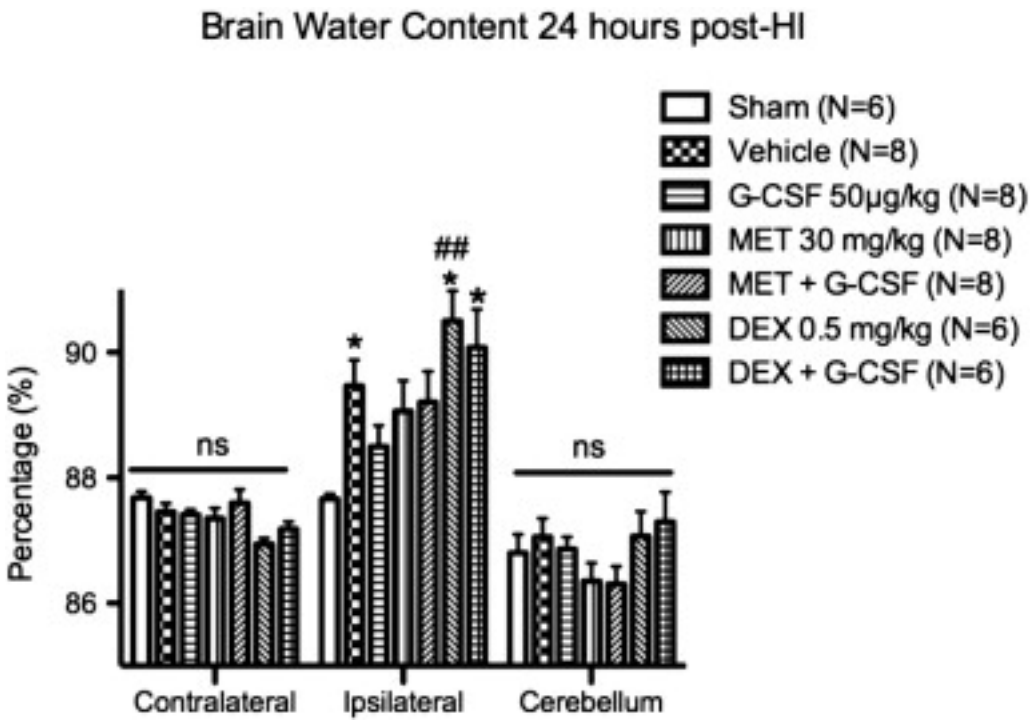

Figure 2.3. Brain water content after G-CSF, metyrapone, and dexamethasone treatment after HI. Quantification of brain water content 24 h after HI in the cerebellum, contralateral, and ipsilateral brain hemisphere. Brain water content was markedly increased by HI + Vehicle, DEX and DEX + G-CSF treated groups. (* = $p < 0.05$). No significance was observed in G-CSF, MET and MET + G-CSF. DEX treated group had higher brain water content than the G-CSF treated group (## = $p < 0.05$ vs G-CSF). No significant difference was observed amongst the groups in the cerebellum and contralateral hemisphere.

ACTH Blood Plasma Level 4 Hours and 24 Hours after HI

The ACTH level in the blood was increased after HI ($p<0.05$ vs. Sham), and administering G-CSF did not influence ACTH levels (Figure 2.4A). The administration of metyrapone increased ACTH levels, while dexamethasone significantly reduced it as a result of both drugs interfering with the negative feedback mechanism of the HPA axis ($p<0.01$) (Figure 2.4A). At 24 hours, no significant differences were observed amongst all the groups (Fig 2.4B).

Corticosterone Blood Plasma Level 4 Hours and 24 Hours after HI

The levels of corticosterone in the blood significantly increased after HI, and the administration of G-CSF reduced corticosterone (Figure 2.5A). Metyrapone reduced corticosterone levels while the administration of dexamethasone alone or in combination with G-CSF increased corticosterone levels 4 hours after HI. At 24 hours, corticosterone levels decreased in all the groups. It must be noted, that the groups that had G-CSF in their treatment regimen had lower corticosterone levels than their control groups (Figure 2.5B). The dexamethasone+G-CSF group had significantly lower corticosterone levels than the dexamethasone treated group.

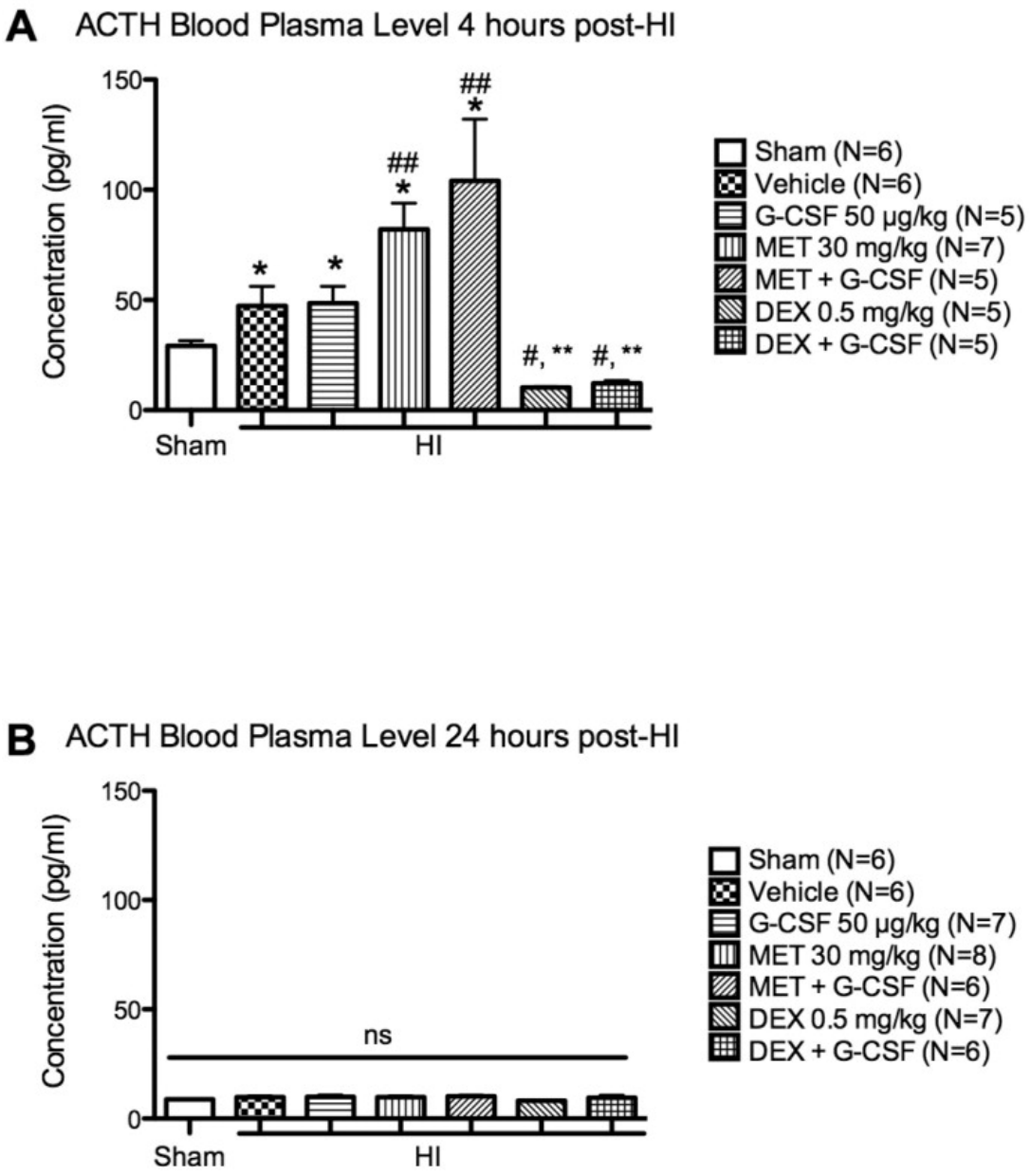

Figure 2.4 ACTH blood plasma level 4 h and 24 h after HI. A. At 4 h, ACTH levels were significantly increased by HI (* = $p < 0.05$ vs Sham). MET and MET + G-CSF groups had markedly increased ACTH level compared to Vehicle (## = $p < 0.05$). DEX and DEX + G-CSF had significantly decreased ACTH levels compared to MET and MET + G-CSF groups (# = $p < 0.01$ vs MET 30 mg/kg, ** = $p < 0.01$ vs MET + G-CSF). **B.** ACTH blood plasma level is normalized to Sham at 24 h after HI.

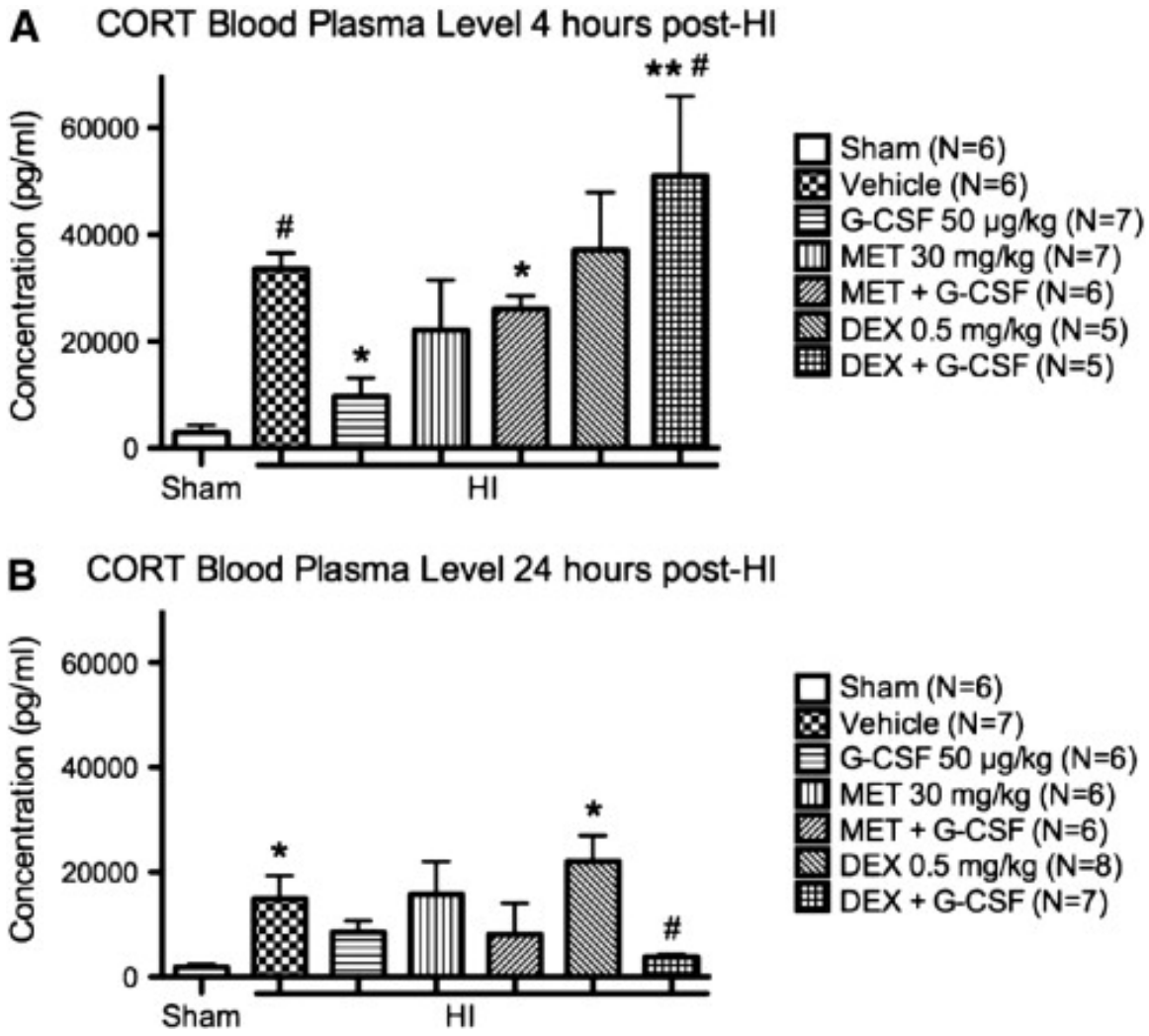

Figure 2.5 Corticosterone blood plasma level 4 h and 24 h after HI. A. HI considerably increases CORT levels and G-CSF robustly decreases CORT levels at 4 h (# = $p < 0.05$ vs Sham, * = $p < 0.01$ vs Vehicle). MET + G-CSF decreased CORT compared to Vehicle group while the DEX + G-CSF group increased CORT levels significantly (** = $p < 0.05$). **B.** CORT levels remain higher in Vehicle and DEX treated groups (* = $p < 0.05$ vs Sham). All groups with G-CSF in their regimen have lower CORT levels than their control group (# = $p < 0.05$ vs DEX 0.5 mg/kg).

Apoptotic Markers Expression in the Brain After Treatment

The western blot results indicate that G-CSF reduced pro-apoptotic marker Bax (Figure 2.6A), and increased Bcl-2 (Figure 2.6B) in the ipsilateral hemisphere, however the data did not yield statistical significance. HI induced an increase in the Bax/Bcl-2 ratio ($p<0.05$); this ratio was significantly lowered by the treatment of G-CSF, metyrapone and the combination of metyrapone and G-CSF ($p<0.05$) (Figure 2.6C). The HI-induced increase of cleaved caspase-3 was significantly reduced by the combination of G-CSF and metyrapone treatment. Dexamethasone and dexamethasone + G-CSF co-treatment further increased cleaved caspase-3 levels ($p<0.05$ vs. Sham, $p<0.05$ vs. MET+G-CSF).

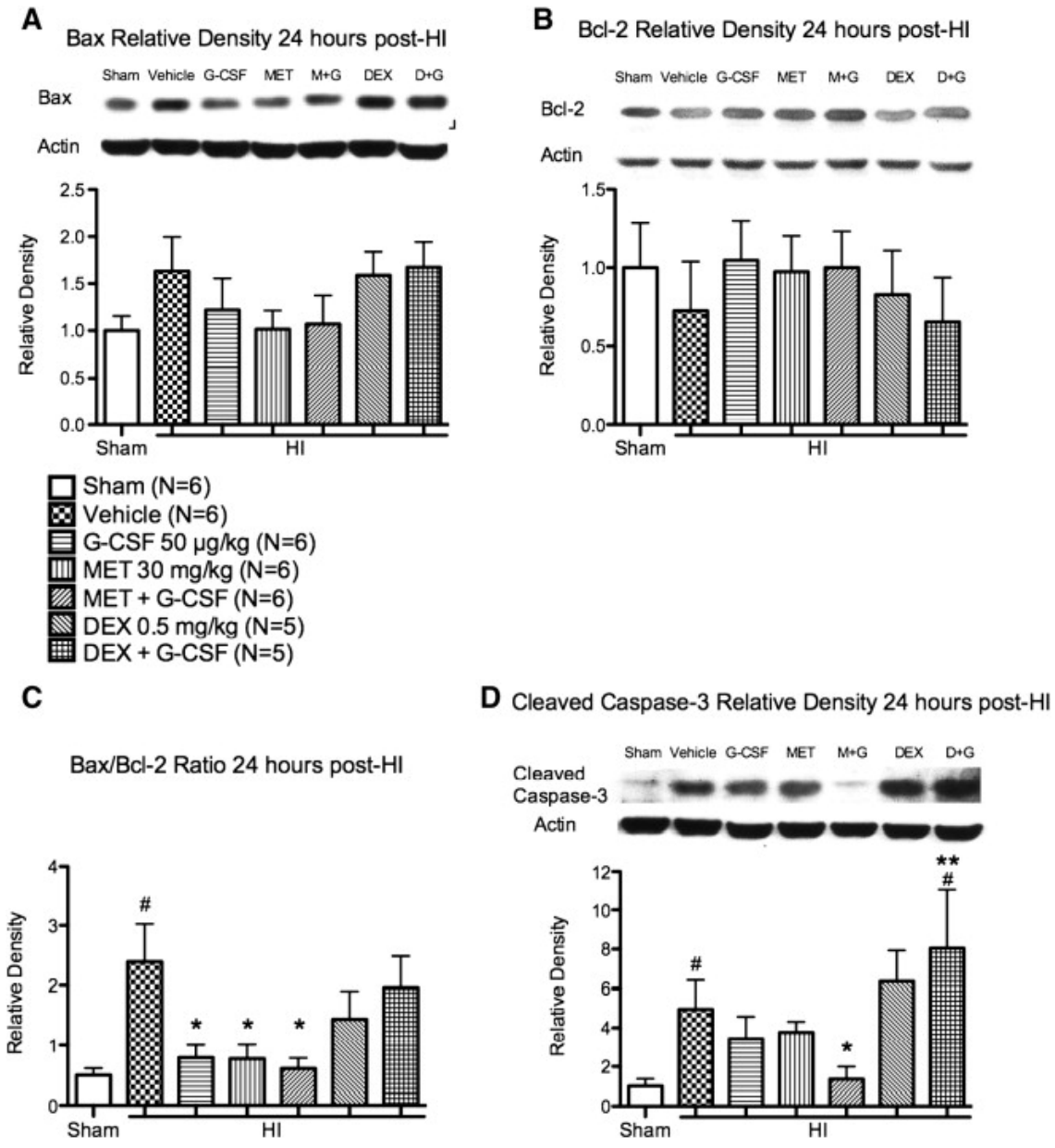

Figure 2.6 The expression of apoptotic markers in the ipsilateral hemisphere 24 h post-HI. A. HI increased Bax protein expression. No significant difference is reported amongst all groups. **B.** Bcl-2 relative density normalized to actin is decreased by HI but no significant difference is observed between groups. **C.** Bax/Bcl-2 ratio was significantly increased by HI (# = $p < 0.05$ vs Sham). G-CSF, MET and MET + G-CSF treated groups significantly reduced Bax/Bcl-2 ratio compared to Vehicle treated groups (* = $p < 0.05$). **D.** Cleaved caspase-3 levels are markedly increased by HI (# = $p < 0.05$ vs Sham), and relatively lowered by G-CSF and MET groups (no significance observed). MET + G-CSF significantly decreased caspase-3 levels (* = $p < 0.05$ vs Vehicle), and DEX + G-CSF group antagonized the effects (** = $p < 0.05$ vs MET + G-CSF).

Discussion

In the present study, we show that targeting the elevation of corticosterone in the blood with G-CSF and metyrapone can protect the neonatal brain from HI injury, increase development, and reduce apoptosis. We also show that administering dexamethasone after HI impairs development, and worsens apoptosis. These results support the hypothesis that elevated corticosteroids are detrimental to the functioning and recovery of neurons after an insult (Tombaugh and Sapolsky, 1992; McIntosh and Sapolsky, 1996).

G-CSF reduced infarct volume 24 hours after HI, and metyrapone similarly reduced infarct volume. When G-CSF was co-administered with metyrapone, no further reduction on infarct size was observed. As metyrapone could not potentiate the lowering effect of G-CSF on the infarct volume it may be probable that both G-CSF and metyrapone share a common mechanism of neuroprotective action. However this remains to be elucidated. We postulate that the combination of G-CSF+ metyrapone maximally reduced the progression of HI in the penumbra where milder injury is sustained as detected by TTC for that specific time point of 24 hours. Because HI injury is more severe and necrotic at its core (Nakajima et al, 2000; Pulera et al, 1998), the necrotic cells have been irreversibly damaged and are beyond rescue. The penumbra, the area surrounding the ischemic core, is not exposed to the same intensity of energy failure; these neurons are in a critical stage where they can be recovered. It is with that premise that combined therapy, may have maximally rescued the cells in the penumbra but could not reverse the damage caused at the core. Although no synergistic effect was observed in

the infarct volume analysis, the animals treated with G-CSF and metyrapone gained weight in comparison to all the other groups that were subjected to HI.

When the activity of corticosterone was agonized with dexamethasone no difference in infarct volume was detected when compared to the Vehicle treated group. The protection observed with G-CSF treatment was completely lost when combined with dexamethasone. This result suggests G-CSF may also exert its protection through lowering the corticosterone level. This data is of extreme importance since multiple reports suggest that dexamethasone treatment is neuroprotective in HI studies (Feng et al, 2011; Ikeda et al, 2005; Felszeghy et al, 2004; Ekert et al, 1997; Tuor et al, 1996). In those reports, dexamethasone was administered before inducing experimental HI. Since most incidences of HI can occur *in utero* thus making the time occurrence difficult to detect (Wörle et al, 1984; Perlman, 2006), the translatability of these findings are extremely difficult. Especially since pretreatment would imply indentifying children who are at risk for hypoxia ischemia, which is difficult to assess (Perlman, 2006; Butt et al, 2008). Additionally, a long-term study reports that preterm infants treated with dexamethasone for bronchopulmonary dysplasia or chronic lung disease (Halliday et al, 2009) were at increased risk for cerebral palsy and adverse neurological effects, all of which are outcomes caused by HI (Volpe, 2001; Vannucci et al, 2000). Although pre-treatment of dexamethasone does reduce HI injury and facilitates extubation, (Tuor et al, 1995; Davis et al, 2000), it could be detrimental for proper neurological development in preterm infants. Our results show that dexamethasone administration after HI does not reduce infarct volume, and further exacerbates development as indicated by weight loss. If G-CSF were to be administered clinically in a preterm child, the administration of

dexamethasone may antagonize its effects. This postulate would require human studies to be authenticated.

The occurrence of apoptosis prevails in the penumbra where milder injury has been sustained after the HI insult (Pulera et al, 1998). Apoptosis peaks from 24-72 hours; since its occurrence is delayed, it is an important and suitable target for treatment. Our results show that the expression of apoptotic markers are influenced by the hormones involved in the pituitary-adrenal response. The Bax/Bcl-2 ratio was significantly lowered by the administration of metyrapone alone and G-CSF. Additionally, the combined G-CSF + metyrapone treated group had significantly lower caspase-3 than G-CSF and metyrapone alone. These results suggest that the synergistic effect of both drugs could be attributed to other pathways all directed at lowering caspase-3. When dexamethasone was administered, higher expression of caspase-3 was observed, thus inferring that the progression of the disease is worsened by dexamethasone administration.

The pathophysiology of HI also involves an increased vascular permeability, which leads to brain edema. It is well documented clinically that the administration of corticosteroid can reduce brain edema (Betz et al, 1990 stroke; Heiss et al, 1996). The integrity of the blood brain barrier is usually compromised within hours of ischemic injuries (Zhang et al, 2000). Administering corticosteroid to target brain edema has been shown to reduce the pathological sequelae involved in brain swelling (Fishman, 1982). Our results however indicate that brain edema is significantly increased when dexamethasone 0.5 mg/kg is administered. The contradiction of our results to what is typically observed clinically could be due to the inverted-U shape dose effect curve of corticosteroids (Baldi and Bucherelli, 2005). The non-linear effect of corticosteroids

ought to be considered when interpreting our results. Understanding the exact dose response of dexamethasone and treatment in neonatal hypoxia ischemia therapy for brain edema is an area necessitating further studies. Also, whether targeting brain edema is sufficient to reduce neuronal cell death in neonates is another area granting further investigation. When it came to G-CSF and metyrapone, these drugs did not significantly reduce brain edema. The interpretation of this data suggests that their mechanism of action may not involve preserving blood brain barrier integrity.

It is clear that the pituitary-adrenal response is increased after HI, and that modulating its effects may prove beneficial in reducing infarct volume in the acute phase of injury. Considerable reports have shown that elevated plasma corticosterone can exacerbate neuronal damage in adult brain injury models of HI (Krugers et al, 2000, Stein and Sapolsky et al, 1988). Our results show that administering metyrapone reduced corticosterone levels in neonates after HI, however the administration of G-CSF reduced corticosterone levels more robustly as seen at the 4 hours time-point. At 24 hours, all the animals that had G-CSF in their treatment regimen had lower corticosterone levels than their control group. What is perplexing is that ACTH levels were not affected by the administration of G-CSF. This implies that G-CSF signaling is able to either directly affect steroidogenesis at the adrenal level, or decrease the free corticosterone in the blood by increasing corticosteroid-binding protein in the blood. It can be argued that G-CSF affects the pituitary-adrenal response as a by-standard effect of its capacity to reduce injury (Yata et al, 2007) and thus reducing the stress on the animal. However, we oppose this precept since G-CSF did not affect ACTH levels. Furthermore a previous report has shown that G-CSF can modulate the pituitary-adrenal response in naïve rats (Mucha et al,

2000). The mechanism by which G-CSF affects corticosterone level after HI should be a new area of interest to further understand its application clinically.

We conclude that reducing HI-induced corticosterone elevation with both G-CSF and metyrapone reduces infarct volume, and pro-apoptotic markers. Additionally, the nature of G-CSF neuroprotection may involve CORT suppression in the blood. We also demonstrate that administering dexamethasone after HI exacerbated neuronal damage, impaired development and apoptosis.

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CHAPTER THREE

GRANULOCYTE-COLONY STIMULATING FACTOR INHIBITS CORTICOSTERONE SYNTHESIS VIA THE JAK2/PI3K/PDE PATHWAY IN Y1 ADRENAL CORTICAL CELLS BY MODULATING CYCLIC ADENOSINE MONOSPHOSPHATE SIGNALING

Mélissa S. Charles[a,b], John H. Zhang[b,c,d], Jiping Tang[b,d].

[a] Department of Microbiology and Molecular Genetics, [b] Department of Physiology and Pharmacology, [c] Department of Neurosurgery, [d] Department of Anesthesiology, Loma Linda University School of Medicine, Loma Linda, CA, 92354, USA

Corresponding Author: Jiping Tang, MD
Department of Physiology and Pharmacology
Loma Linda University School of Medicine
Risley Hall, Room 214
Loma Linda, CA, 92350, USA
Phone: (909) 558-7693
Fax: (909) 558-0119
Email: jtang@llu.edu, jipingtang@yahoo.com

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Abstract

It has been reported that granulocyte-colony stimulating factor (G-CSF) influences the activity of the hypothalamic-pituitary-adrenal (HPA) axis primarily via the hormones adrecorticotropic hormone (ACTH) and glucocorticoids. Namely, G-CSF was shown to inhibit corticosterone in rodents after a hypoxic-ischemic event; the rodent specific glucocorticoid known to exacerbate injury after an insult. The manner in which G-CSF interacts with corticosterone biosynthesis remains to be examined. In this study, we investigate for the first time the underlying mechanism of G-CSF on corticosterone biosynthesis in a rodent Y1 adrenal cortical cell line. Cholera toxin was used to agonize corticosterone synthesis by constitutively increasing cyclic adenosine monophosphate (cAMP). Corticosterone and cAMP were quantitatively assayed using a commercial enzyme-linked immunosorbent assay (ELISA). Janus Kinase 2 (JAK2)/Phosphatidylinositol-3-kinases (PI3K)/Protein kinase B (Akt), the downstream signaling components of G-CSF receptor activation, their phosphorylated forms and Phosphodiesterase 3B (PDE3B) were detected by western blot. G-CSF at a low dose (30 ng/ml) inhibited corticosterone synthesis, but lost its inhibitory effect as you increased its concentration. The inhibitory effect of G-CSF was conferred by interfering with the cAMP signaling via the activation of JAK2/PI3K/PDE3B pathway, as verified with respective inhibitors. The degradation of cAMP by G-CSF signaling reduced corticosterone. We conclude that G-CSF mediates corticosterone synthesis inhibition at the adrenal level via JAK2 activation ultimately degrading intracellular cAMP in Y1 cells.

Introduction

Previous reports indicate that G-CSF, a neuroprotective and hematopoietic protein, may be involved in regulating hormones of the hypothalamic-pituitary-adrenal (HPA) axis, primarily adrenocorticotropic hormone (ACTH) and the rodent specific glucocorticoid, corticosterone (Mucha et al, 2000; Charles et al, 2012). The paucity of studies investigating the effect of hematopoietic growth factors on neuroendocrine activity highlights an important area that necessitates investigation (Zylińska et al, 1999; Tringali et al, 2007). Particularly in light of 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). Understanding how G-CSF influences HPA activity may become a beneficial area of study, particularly when it can downregulate the detrimental HPA activity reported in a rodent hypoxia-ischemia neuronal injury model (Charles et al, 2012).

The results from the previous reports suggest a probable relation and interaction between G-CSF and the organs of the HPA axis. Starting with the adrenal gland, no studies have demonstrated the direct effect of G-CSF on the adrenal cells involved in steroidogenesis. Furthermore, the expression of the G-CSF receptor on adrenal glands has not previously been reported. However, there are other reports illustrating that other ligands such as erythropoietin and leptin, that have similar downstream signaling pathways as G-CSF, interact with the HPA axis (Roubos et al, 2012; Hsu et al, 2006; Tringali et al, 2007; Tokgöz et al, 2002). The supposition that G-CSF may influence corticosterone synthesis is highly probable since its receptor belongs to a long chain

helical cytokine family whose ligands have shown to influence steroidogenesis (Hiroike et al, 2000; Mashburn and Atkinson, 2008; Hsu et al, 2006). The Janus Kinase 2 (JAK2)/ phosphatidylinositol-3-kinases (PI3K)/ protein kinase B (Akt) signaling pathway has been shown to inhibit steroidogenic products in cell culture models, and regulate steroidogenic proteins transcription and translation (Lefrancois-Martinez et al, 2011; Li et al, 2003; Hsu et al, 2006). In light of these observations, it is highly probable that G-CSF may exert its steroidogenic influences similarly.

At the adrenal level, steroidogenesis occurs in adrenal cortical cells, and is initiated by ACTH which increases the intracellular level of its second messenger cyclic adenosine monophosphate (cAMP) (Rainey et al, 2004; Cooke, 1999). 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 induction of cAMP production 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 its upstream regulator cAMP with phosphodiesterase 3B (PDE3B) (Hsu et al, 2006; Johnsen et al, 2009). Additionally, we have previously shown that G-CSF can reduce corticosterone after neonatal hypoxia–ischemia (Charles et al, 2012). Whether G-CSF influences the steroidogenic process initiated by cAMP through JAK2 activation in an adrenal cell culture model has yet to be explored.

In light of these observations, we propose to study for the first time the effects of G-CSF on steroidogenesis in Y1 adrenal cortical cells. We hypothesize that G-CSF mediates corticosterone synthesis inhibition on Y1 cells by activating the

JAK2/PI3K/Akt/PDE3B pathway. We will use cholera toxin to constitutively increase cAMP production, and test our hypothesis by inhibiting the JAK2/PI3K/PDE3B pathway with appropriate inhibitors. We will investigate the mechanistic effect of G-CSF on cAMP and corticosterone production.

Material and Methods

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), as a monolayer in a humidified atmosphere at 37^{o}C in 5% CO_2 in T75 flasks (BD Biosciences, San Jose, CA). Medium was changed every 4 days, and cells were sub-cultured after 8 days and split to a 1:3 ratio. The cells were 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^{TM} Automated Cell Counter (Bio-Rad Life Science, Hercules, CA) and seeded in 12 well plates at a concentration of 1 X 10^6 of live cells/well. The cells were grown in 2 ml of growth media/well for 48 hours. The cells were immediately serum starved for 8 hours as previously described (Calejman et al, 2011) and subsequently incubated with growth medium for 24 hours containing the appropriate chemicals for the respective groups.

Chemicals and Treatment

Cholera toxin, the JAK2 inhibitor Tyrphostin AG490 (AG490), the PI3K inhibitor LY-294002, and the PD3B inhibitor 3-isobutyl-1-methylxanthine (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, 5 mg/ml, and 0.5 M. G-CSF receptor chimera (Fc Chimera Active) was purchased from Abcam (Cambridge, MA) and G-CSF was obtained from Loma Linda University Pharmacy. The following concentrations were used: cholera toxin (50 ng/ml) (Hsu et al, 2006), AG490 (50 μM) (Chen et al, 2005), LY-294002 (20 μM) (Williams et al, 2010), IBMX (10 μM) (Montero-Hadjadje et al, 2006). A dose response for G-CSF (30, 100, 300 ng/ml) (Hsu et al, 2006) and the G-CSF receptor chimera (10, 30, 100 ng/ml) after cholera toxin treatment was conducted. At 24 hours, growth media were collected from each well for subsequent assay and analysis. G-CSF treated cells were collected at the following time points after the initiation of treatment: 0, 5, 15, 30, 60, 120 minutes to determine the activity of the JAK/PI3K/Akt/PDE3B pathway.

Cell Viability Assay

At 24 hours post-treatment, growth media were removed from each well and the cells were trypsinized. Trypan Blue Cell Exclusion was used to determine cell viability which was assayed and recorded using the TC10 Automated Cell Counter Cell Viability analysis and protocol (Bio-Rad).

Corticosterone and cAMP Quantitative Assay

Corticosterone was measured in the growth media 24 hours after treatment (Astort et al, 2009, Calejman et al, 2010) using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). 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 were removed and hydrochloric acid was added to the cells to stop the activity of phosphodiesterases. The levels of cAMP were quantitatively analyzed using a colorimetric competitive ELISA kit (Enzo Life Sciences) with a sensitivity of 0.30 pmol/ml.

Western Blot

Cells were washed with phosphate buffered saline (PBS) and incubated in 100 µl of RIPA cell lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 20 minutes. Cells were snap-frozen in liquid nitrogen, thawed, and centrifuged at 125g for 10 minutes. The supernatant was collected, and assayed for protein concentration using the spectroscopic 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), phosphor-JAK2 (Abcam), PI3K (Cell Signaling Technology, Danvers, MA), phosphor-PI3K (Cell

Signaling Technology), Akt (Cell Signaling Technology), phosphor-Akt (Cell Signaling Technology), PDE3B (Abcam), and actin (Santa Cruz Biotechnology). After washing the membranes three times, they were probed with a 1:1000 dilution secondary antibodies (Santa Cruz Biotechnology) for the specie of the primary antibody 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

The data are presented as the mean ± standard error 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 expression of the G-CSF receptor has not previously been reported in adrenal cells. Therefore, its expression was first investigated with western blot. Our results indicate that the G-CSF receptor protein is expressed in Y1 cells (Figure 3.1A) suggesting its function on adrenal cortical cells. The administration of cholera toxin significantly ($p<0.001$) increased the steroidogenic product of corticosterone 5 times the percentage of the vehicle group (Figure 3.1B). G-CSF administration with cholera toxin inhibited corticosterone synthesis at the low dose of 30 ng/ml ($p<0.05$ vs. cholera toxin)

but lost its inhibitory property as you increased the dose to 100 and 300 ng/ml (Figure 3.1B). The concentration of cholera toxin +G-CSF 300 ng/ml was significantly increased compared to cholera toxin + G-CSF 30 ng/ml ($p<0.05$). To determine if G-CSF receptor activation was responsible for the inhibitory effect observed on corticosterone synthesis, the cells were incubated with cholera toxin + G-CSF + G-CSF Receptor Chimera (10, 30, 100 ng/ml). The inhibition of corticosterone synthesis was lost when the G-CSF receptor chimera was added (Figure 3.1C). The inhibitory effect reached significance at 30 ng/mL. In light of the objective of this study, G-CSF 30 ng/mL was used for subsequent molecular assays and hormone analysis.

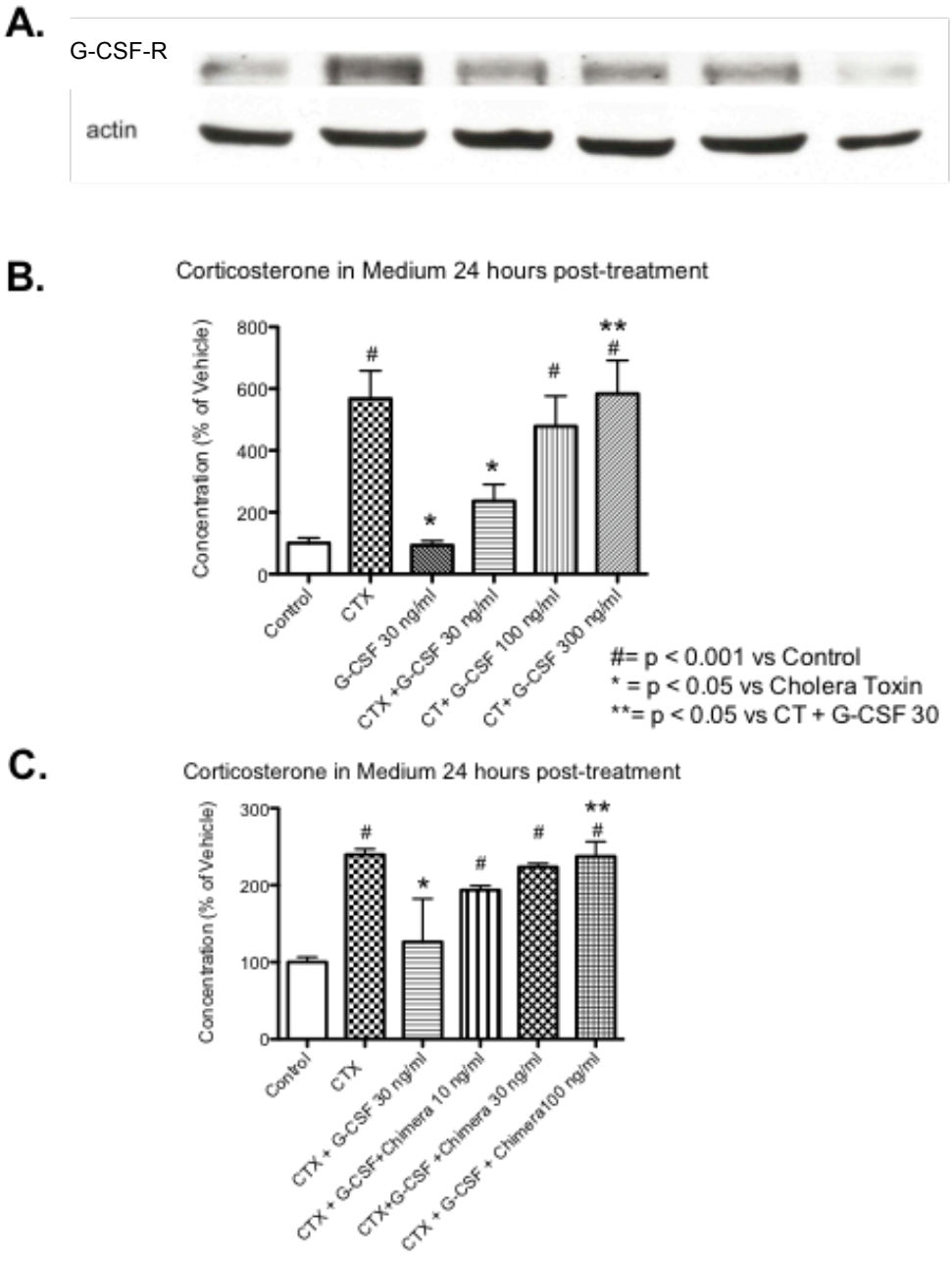

Figure 3.1. G-CSF influences cholera toxin-induced steroidogenesis via its own receptor on Y1 cells. A) G-CSF receptor is expressed in Y1 adrenal cortical cells. **B)** G-CSF significantly inhibits cholera toxin (CTX)-induced synthesis of corticosterone at low dose 30 ng/ml but loses its inhibitory property at 100 and 300 ng/ml. G-CSF 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 reach 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

G-CSF Activates the JAK2/PI3K/Akt Pathway in Y1 cells

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 has yet to be determined. Accordingly, G-CSF (30 ng/ml) was administered and protein expression of JAK2, PI3K and Akt as well as their phosphorylated active form were analyzed 0, 5, 15, 30, 60, 120 minutes after treatment initiation. JAK2 phosphorylation increased over the time course as well as the total JAK2 protein (Figure 3.2A). The ratio of phosphor-JAK2/total JAK2 peaked at 60 minutes but did not reach significance (Figure 3.2B). The increase of total JAK2 after G-CSF treatment significantly peaked 30 minutes after treatment ($p<0.05$ vs. 0 minutes). The protein expression of phosphor-PI3K increased over the time course and peaked 30 minutes after treatment (phosphor-PI3K/total PI3K) ($p<0.05$ vs. 0 minutes, Figure 3.2C). Total PI3K protein expression remained constant (Figure 3.2D). The ratio of phosphor-Akt/total Akt significantly increased and peaked 15 minutes post-treatment ($P<0.05$ vs. 0 minutes) (Figure 3.2E), while the total Akt protein expression remained constant (Figure 2F). PDE3B a downstream component of PI3K/Akt signaling was activated after G-CSF treatment and increased 5 minutes after treatment ($p<0.05$ vs 0 minutes) (Figure 3.3.)

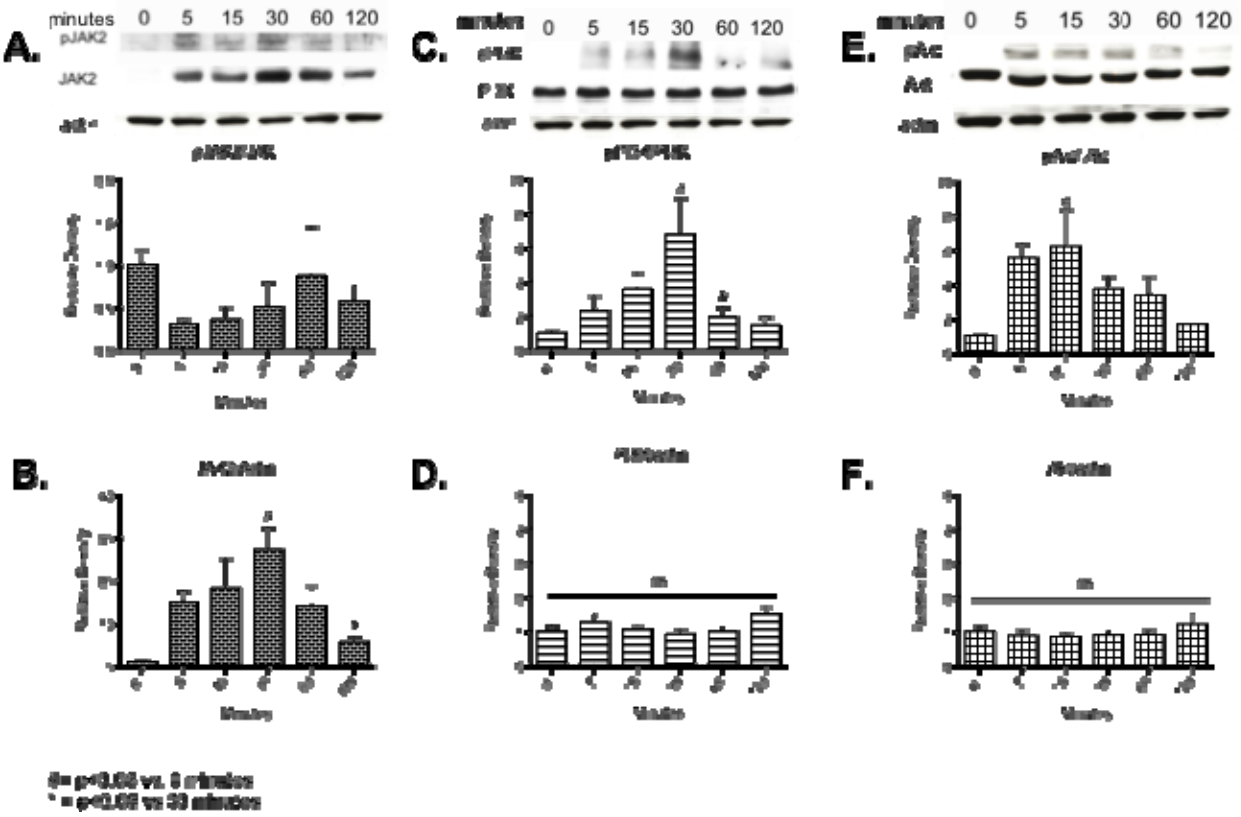

Figure 3.2. G-CSF activates the JAK2/PI3K/Akt pathway in Y1 cells. A) & B) Phophor-JAK2/total JAK2 in increased over the course of 120 minutes after G-CSF treatment and peaks at 60 minutes. The total JAK2 protein expression is increased over 120 minutes and significantly peaks at 30 minutes ($p<0.05$ vs 0 minutes). **C) & D)** Phosphor-PI3K/total PI3K peaks 30 minutes after G-CSF treatment ($p<0.05$ vs 0 minutes). Total PI3K expression is not affected by G-CSF treatment. **E) & F)** Phosphor-Akt/total Akt protein activation is increased after G-CSF treatment and peaks at 15 minutes ($p<0.05$ vs 0 minute). Total Akt remains unchanged. N=4/group

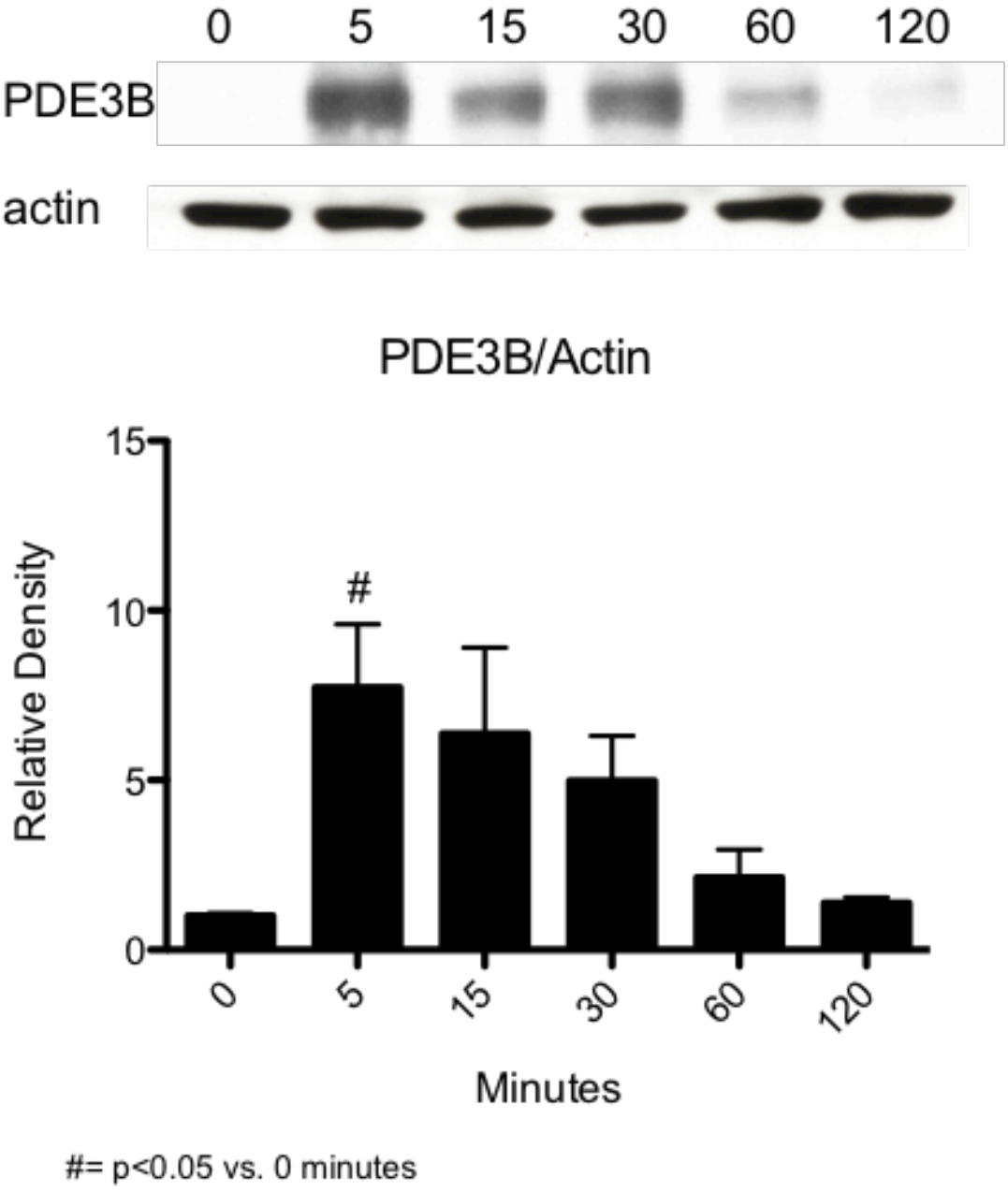

Figure 3.3. G-CSF activates PDE3B expression in Y1 cells. PDE3B is activated after G-CSF 30 ng/ml treatment and significantly peaks 5 minutes after treatment ($p<0.05$ vs 0 minute). PDE3B levels decrease over time. N=4/group

G-CSF Inhibits Corticosterone Synthesis via the JAK2/PI3K/PDE3B Pathway

To determine whether JAK2/PI3K and the downstream element PDE3B is involved in G-CSF inhibition of corticosterone synthesis, JAK2 inhibitor AG490, PI3K inhibitor LY-294002 and PDE3B inhibitor IBMX were added to cholera toxin + G-CSF 30 ng/mL treatment. Additionally, since the inhibitors were dissolved in DMSO, a control group DMSO (0.2%) was added for comparative analysis. DMSO 0.2% did not increase corticosterone synthesis compared to control group. 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 corticosterone synthesis ($p<0.05$ vs. cholera toxin + G-CSF 30 ng/mL) (Figure 3.4A). Corticosterone was significantly higher ($p<0.05$) in the media of cells in the following groups compared to cholera toxin + G-CSF 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. The administration of the drugs did not significantly affect the cell viability as assessed with Trypan Blue Cell Exclusion (Figure 3.4B)

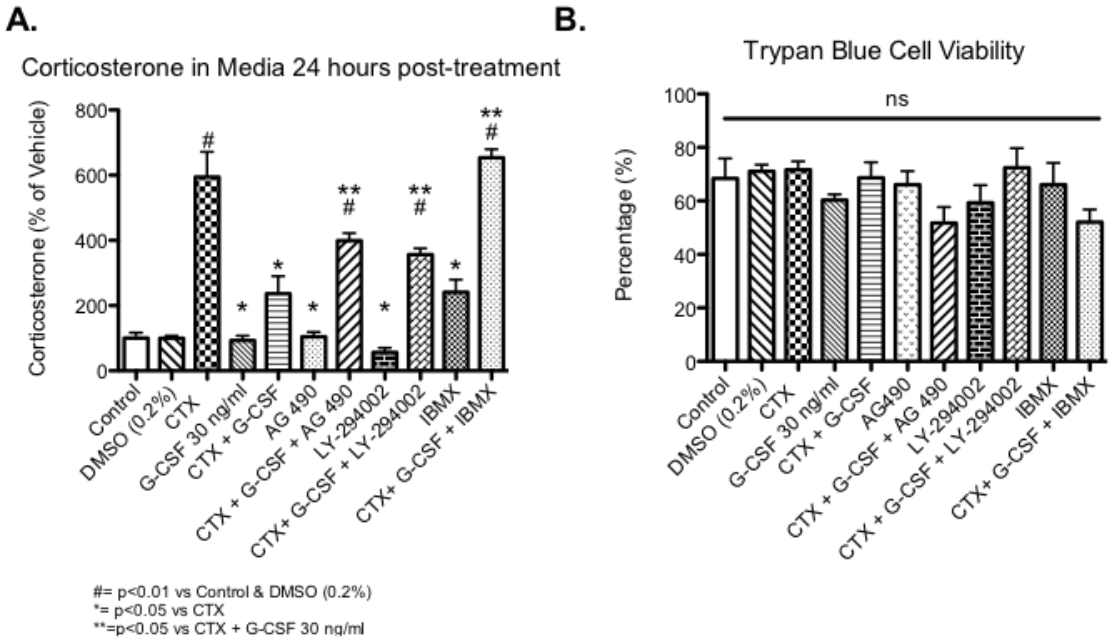

Figure 3.4. G-CSF inhibits corticosterone synthesis via the JAK2/PI3K/PDE pathway. A) The JAK2 inhibitor (AG490), PI3K inhibitor (LY-294002), and PDE inhibitor (IBMX) significantly reverse the corticosterone inhibition conferred by G-CSF on cholera toxin-induced steroidogenesis ($p<0.05$ vs CTX + G-CSF 30 ng/ml). The treatment with DMSO (0.2%), G-CSF 30 ng/ml, AG490, LY-294002, IBMX alone did not significantly affect basal corticosterone synthesis compared to Control group. **B)** Trypan Blue Cell Exclusion assay illustrates that the viability of each treated group is not significantly different. N=6/group.

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

The primary function of phosphodiesterases is to cleave the phosphodiester bond of cAMP, the primary messenger and mediator of steroidogenesis (Mehats et al, 2002). The measure of cAMP was assessed two hours after initiating treatment as previously described (Hsu et al, 2006). Cholera toxin treatment increased cAMP significantly compared to Control treated cells from 3.85 ± 0.20 pmol/L to 22.2 ± 2.84 pmol/ml ($p<0.01$ vs. Control and DMSO) (Figure 3.5). G-CSF administration alone did not significantly affect cAMP levels compared to Control and DMSO treated cells. The combination of cholera toxin + G-CSF 30 ng/ml significantly reduced the cholera toxin induced upregulation from 22.2 ± 2.84 pmol/mL to 14.47 ±0.60 pmol/mL (Figure 3.5). 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; 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 and significantly 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 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.

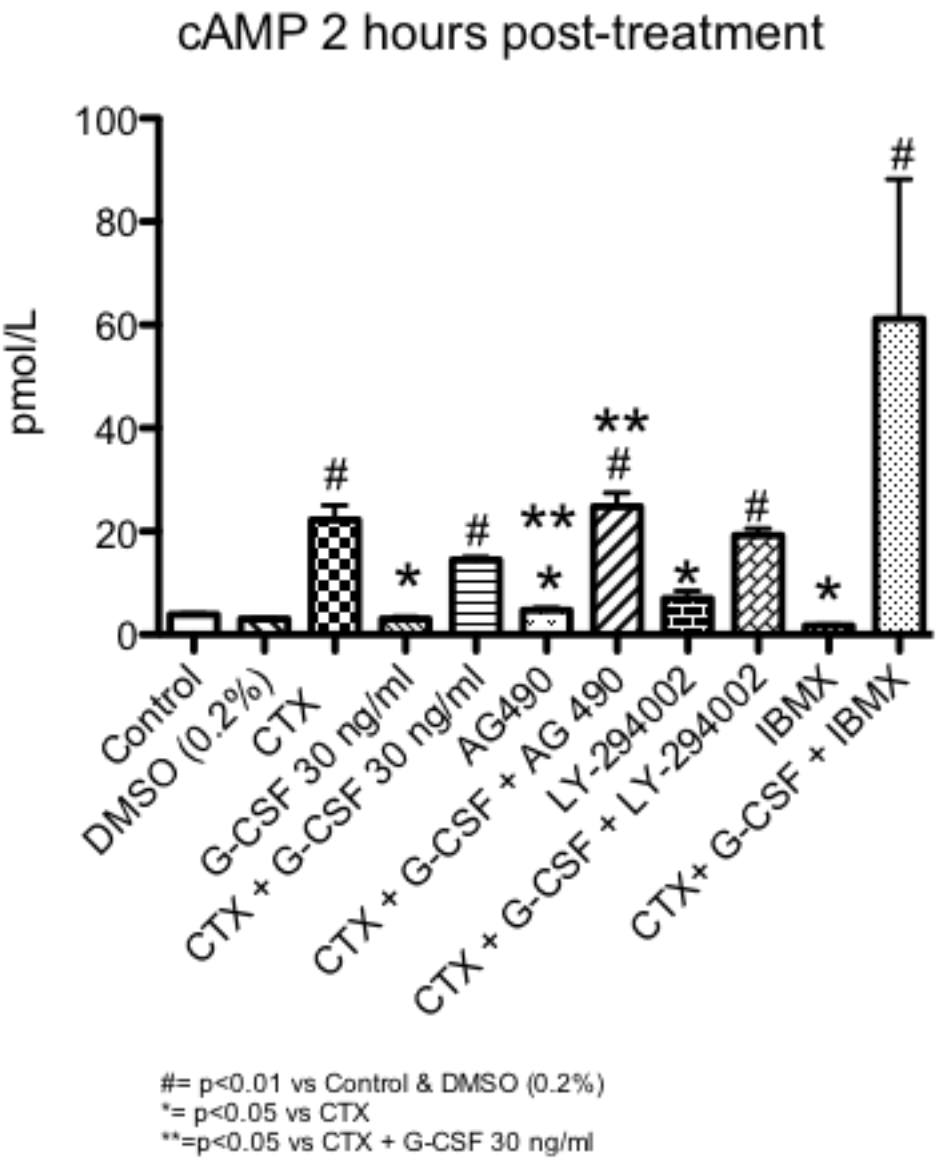

Figure 3.5. G-CSF abates cholera toxin-induced cAMP upregulation via the JAK2/PI3K/PDE pathway. CTX significantly increased cAMP levels from 3.85 ± 0.20 pmol/l to 22.20 ± 2.84 pmol/ml ($p<0.001$ vs Control and DMSO (0.2%)). G-CSF decreased the upregulation of cAMP to 14.47 ± 0.60 pmol/ml which was antagonized by adding to the treatment the JAK2, PI3K, and PDE inhibitor (AG490, LY-294002, IBMX). The treatment with DMSO (0.2%), G-CSF 30 ng/ml, AG490, LY-294002, and IBMX alone did not significantly affect basal cAMP. N= 6/group.

Discussion

In this current study we investigated for the first time the direct effect of G-CSF on adrenal steroidogenesis and explored the probable mechanism by which G-CSF-induced inhibition of steroidogenesis. Thereupon we examined the involvement of G-CSF in a well characterized rodent Y1 adrenal cortical cell line on steroidogenic products. We show that G-CSF at 30 ng/mL inhibits cholera toxin induced corticosterone synthesis and cAMP upregulation. We also report that the G-CSF receptor is expressed in adrenal cortical cells and by co-administering G-CSF with its G-CSF receptor chimera the inhibitory effect against cholera toxin is lost. Additionally, we demonstrate that G-CSF activates the JAK2/PI3K/Akt/PDE3B pathway in adrenal cells which is responsible for the inhibition of cAMP and steroidogenic signaling components and product hence blunting corticosterone biosynthesis.

We first determined whether G-CSF receptor protein was expressed in the adrenal cortical cells as no previous reports indicated the latter. Since our results show that the G-CSF receptor was expressed, a dose response using G-CSF was conducted to determine its influence on the glucocorticoid involved in HPA activity in rodent corticosterone. Our results show that G-CSF inhibits corticosterone synthesis at low dose (30 ng/mL), but as you increased the concentration the inhibitory effect was lost. We postulate that this phenomenon is probably a result of non-genomic effects due to the saturation of the G-CSF receptor. Hence other signaling pathways could be activated to oppose the inhibitory effect or 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 reconcile the results of Mucha and colleagues (2000) indicating that G-CSF increases corticosterone blood plasma level after chronic administration in naïve rats. However, this remains to be elucidated.

The inhibitory effect of G-CSF was modulated by the activation of its receptor as the addition of the G-CSF receptor chimera at various concentration resulted in an increased corticosterone product (Figure 3.1C). For the first time, we show that G-CSF has direct interaction with cells of an HPA organ. This is of critical importance primarily if the effect can potentially cause adverse or beneficial therapeutic effects in a clinical setting.

Since G-CSF is known to activate JAK2/PI3K/Akt in other tissues (Nakamae-Akahori et al, 2006) and that PI3K/Akt is upstream to PDE3B, we measured the total and activated forms of JAK2/PI3K/Akt and the expression of PDE3B over two hours after G-CSF treatment initiation. Our results indicate that JAK2 phosphorylation increased over two hours as did the total form of JAK2. Total JAK2 protein expression significantly increased and peaked 30 minutes after treatment initiation. The activated forms of PI3K, and Akt also increased over two hours peaking from 15-30 minutes, but unlike JAK2 the total forms of PI3K and Akt expression were not affected. G-CSF also increased PDE3B over the 120 minutes time course which peaked at 5 minutes. These results show that the JAK2/PI3K/Akt activation of G-CSF is preserved in rodent adrenal cortical cells.

The corticosterone measurements indicate that G-CSF inhibits cholera toxin via the JAK2/PI3K/PDE3B pathway since the addition of the inhibitors in the treatment reversed its inhibitory effect. Therefore, this suggests that the pathway may be involved in corticosterone synthesis inhibition. To verify that the differences reported amongst

each treated groups were not due to cell viability, Trypan Blue Exclusion was used. The results indicate that no significant difference was observed in cell viability amongst the groups. Therefore, the observed effects in corticosterone synthesis and other molecular events were attributed to the drugs influence on signaling pathways and not cell viability.

The elevation of cAMP observed two hours after the initiation of treatment with cholera toxin was inhibited by G-CSF. This report suggests that the activation of JAK2/PI3K/PD3B by G-CSF inhibits steroidogenesis by reducing cAMP levels. Furthermore, the inhibitors of JAK2/PI3K/PDE3B individually reverse the inhibition of cAMP levels. The levels of cAMP were further increased when treated with cholera toxin + G-CSF 30 ng/mL +IBMX compared to cells treated with cholera toxin alone. This occurrence may be a result of inhibiting the basal levels of PDEs, which are activated by steroidogenic signaling (Mehats et al, 2002). Steroidogenesis can initiate other signaling pathways such as calcium signaling whose downstream event includes the activation of PDEs (ibid). Therefore IBMX may inhibit the basal activity of PDEs, which would attribute to the higher cAMP levels.

Concerning the limited studies that look at G-CSF and HPA activity, one in naïve rats and one after hypoxia ischemia, one must understand the complexity of what is at hand. Markedly, the putative ability of G-CSF to influence HPA activity ought to be investigated also at the pituitary level and at the hypothalamic level. 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; Zylińska et al, 1999). In light of our previous report (Charles et al, 2012) indicating that ACTH was not increased in spite of

corticosterone inhibition, one must consider the delicate negative feedback and the probable interaction of G-CSF with the pituitary gland. This perplexing result puts in question the negative feedback which should have shown an increase of ACTH as a result of corticosterone inhibition. Therefore it is probable that G-CSF may have direct effects on each organ of the HPA axis. Determining the manner in which G-CSF influences HPA activity in a naïve model and after a diseased state merits further exploration.

In conclusion, we demonstrate that G-CSF has non-hematopoietic function 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 of corticosterone. We propose that a better understanding of the manner in which G-CSF has neuroendocrine properties may better translate its efficacy in a clinical setting, particularly when investigating diseases that involve the over-activation of the HPA axis.

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CHAPTER FOUR

DISCUSSION

How the activity of the HPA axis and its relation to G-CSF therapy reflect on pathophysiological outcomes of HI was not previously investigated. For that reason, the underlying foundation of this scientific initiative was to investigate the latter. We show for the first time that the neuroprotective properties of G-CSF depend on its inhibition of corticosterone synthesis. Furthermore, we demonstrate for the first time that G-CSF has direct effects on steroidogenesis by activating its receptor on adrenal cortical cells. These findings bifurcate our current knowledge of G-CSF function, similarly to when G-CSF was first discovered to be neuroprotective.

G-CSF was first reported almost a decade ago, to have neuroprotective properties in brain injury models such as stroke by activating its receptor in the central nervous system (CNS) (Schäbitz et al, 2003; Gibson et al, 2005). It was joining the ranks of another hematopoietic factor known to have modalities in the brain after injury, erythropoietin (Masuda et al, 1993; Digicaylioglu et al, 1995). Subsequent studies that emerged from this discovery have shed light on its multimodal properties that span across a multitude of brain insults (Park et al, 2005; Ren and Finkelstein, 2005; Schneider et al, 2005). The numerous reports have indicated G-CSF as an angiogenic, neurogenic, anti-inflammatory, and anti-apoptotic agent (Shyu et al, 2004; Gibson et al, 2005; Komine-Kobayashi et al, 2006; Schneider et al, 2005; Solaroglu et al, 2009, Strecker et al, 2010).

Currently it is in Phase II clinical trial for adult ischemic injury where it is well tolerated at high doses (Schäbitz et al, 2010). In addition to its promise in adult studies reflecting similar injury as that seen in neonates, age appropriate animal models of HI also indicate that G-CSF improves outcome after the insult (Yata et al, 2007, Fathali et al, 2010, Charles et al, 2012). G-CSF was once unrecognized for its function in the CNS, and we now show that its properties extend beyond its direct neuroprotective effects as illustrated in stroke studies. Unknowingly, G-CSF had properties that influenced a very dynamic and delicate neuroendocrine system, the HPA axis (Mucha et al, 2010).

There are numerous areas of HI pathophysiology that are still not investigated in neonates as it pertains to HPA activity after a brain insult. However, indices can be retrieved from other models indicating that high levels of glucocorticoids were associated with poor outcome (Feibel et al, 1977; Krugers et al, 1998; Stein-Sapolsky, 1988). In the adult HI injury model, results indicate that HPA overactivation in the acute phase was detrimental and its down-regulation led to improved neurological outcome (Krugers et al, 2000; Smith-Swintosky et al, 1996). We measured for the first time the hormones of ACTH and corticosterone in a neonatal HI model and indicated that G-CSF conferred its properties by significantly decreasing corticosterone. Using metyrapone, the inhibitor or corticosterone, and dexamethasone, a synthetic steroid that agonized the effects of corticosterone, we showed that regulation of pituitary-adrenal activity is of critical importance in G-CSF therapy. Especially since agonizing corticosterone led to impaired development, and increased pro-apoptotic biomarkers even when co-administered with G-CSF. Dexamethasone was administered after the insult, which was different from other studies administering it hours prior to HI in neonates (Tuor et al, 1993; Ikeda et al, 2002).

This difference yielded opposite effects. Pre-treatment with dexamethasone appears to be protective, while our data show that post-treatment exacerbates injury. Why post-treatment of dexamethasone is detrimental in contrast to pre-treatment is an area that remains to be elucidated; particularly when it comes to its co-administration with G-CSF. This difference in pre and post-treatment could be owed to the intact versus disrupted BBB, especially since dexamethasone does not cross an intact BBB. Hence its exacerbation of injury could be attributed to its effect in CNS, while the protective effects could be due to its impact in the periphery. This fact would need to be substantiated with adequate HI studies. With attention to the hormone results, metyrapone and dexamethasone show that the negative feedback in neonates in responsive. This finding suggests that elements of the HPA axis are functional in the neonatal rat after HI. For this reason, the suggestion that neonates have a hyporesponsive HPA axis needs to be examined, particularly in the context of a cerebrovascular event (Tu et al, 2006). Overall, the hormone data ultimately led to the question of how G-CSF inhibited corticosterone synthesis? The analysis of the data immediately pointed towards G-CSF having a probable direct interaction with the adrenal gland of the HPA axis.

The initial step was to determine whether the G-CSF receptor was expressed on adrenal cortical cells. We identified for the first time the expression of the G-CSF receptor on rodent adrenal cortical cells suggesting its direct effect on HPA activity. The results we reported were perplexing as they were interesting. It was interesting since G-CSF was shown to inhibit corticosterone synthesis. However this was true only at a low dose. It became perplexing when at higher doses G-CSF lost its inhibitory properties. The span of concentration that was utilized only covered so much ground. Therefore, one

must wonder, whether high doses of G-CSF could do the exact opposite? If this were the case then the study of Mucha and colleagues would be explained (2000). Particularly since they report that chronic administration of G-CSF increased ACTH and corticosterone in naïve rodent models. Whether this is a matter of alternate signaling pathways or saturation of G-CSF receptor activation remains to be investigated.

Understanding the mechanism of G-CSF signaling as it pertained to corticosterone inhibition is primordial to establish how the two components interrelate. Since the receptor was expressed on a different cell type, we verified whether the JAK/PI3K/Akt signaling was conserved. Our data indicate that the pathway is preserved in adrenal cortical cells, and that it is responsible for the inhibitory proponent of G-CSF activity on corticosterone synthesis.

Clinical Significance

The current therapeutic avenues of HI are extremely limited. Only hypothermic therapy has shown a confined amount of success (Koenisgberger et al, 2000). There is a plethora of determining factors including a paucity of adequate mechanistic animal studies that halt the progress of promising drugs in the clinical setting. The complexity of this issue contributes to the grim prognosis of HI patients. These patients are primarily premature infants, and since the development of premature babies differ from term babies, our study utilized a model reflective of that population. Can G-CSF uphold the promise that our animal studies suggest in the clinic? Perhaps. However one must consider the multiple physiological and molecular mechanisms that are activated by G-CSF. Particularly, when it comes to its inhibitory properties on steroidogenesis. Our

results show that co-administering G-CSF with dexamethasone does not protect the brain of the animal from injury (Charles et al, 2012). In fact, it worsened development and apoptosis (ibid). Scientifically this supported our hypothesis, but it challenges the translatability of G-CSF. Here is why. Preterm infants often have developmental, and lung maturation complications which are clinically treated with synthetic glucocorticoids (Davis et al, 2001). If G-CSF were studied in a human clinical trial, the co-administration of synthetic glucocorticoid would impair its neuroprotective ability as our data suggest. This devastating outcome could lead clinicians to prematurely overlook the promise of G-CSF. This could lead to a misguided conclusion, when ultimately the synthetic glucocorticoid commonly found in the treatment regimen of premature infants is what diverged the action of G-CSF. This study shows that in the acute phase, synthetic glucocorticoids are detrimental. This is substantiated by a longitudinal study in which preterm infants were at increased risk for cerebral palsy and adverse neurological effects when treated with synthetic glucocorticoids, all of which are outcomes caused by HI (Volpe, 2001; Vannucci et al, 2000). Nonetheless, because G-CSF potentially could increase glucocorticoid long-term, it could potentially facilitate extubation by endogenously increasing glucocorticoids (Mucha et al, 2000). This remains to be verified with animal and human studies.

G-CSF has an excellent safety record, and a well-known pharmacological profile due to is clinical use for neutropenia and bone marrow harvesting (Schneider et al, 2005; Neidhart et al, 1989; Morstyn et al, 1989). This advantage makes its translatability propitious. Additionally, its multimodal properties suggest that it can target the multiple facets of HI pathophysiological sequelae. Therefore, if it were to transition into clinical

trial for preterm infants, it could potentially be promising. The only caveat would be to consider the use of synthetic glucocorticoids to maximize the promise of G-CSF.

Future Directions

Our study focused on two general areas: the effect of G-CSF after HI on the synthesis of pituitary-adrenal axis hormones, and the mechanism by which it confers its effect. We approached the first part of our study by investigating the effect of G-CSF on pituitary-adrenal hormones as it pertained to apoptosis. Since glucocorticoids can also affect inflammation, and glutamate excitotoxicity after a neuronal insult, these areas merit further exploration (Dinkel et al, 2003; Goodman et al, 1996). Also, our study only investigated the effect of G-CSF in the acute phase. How chronic administration of G-CSF affects the pituitary-adrenal axis hormones long-term needs to be explored. The neurobehavioral outcomes, brain atrophy, angiogenesis, and neurogenesis should also be evaluated at long-term time points; particularly as in regards to dexamethasone, and metyrapone administration. Additionally, an adequate dose response for G-CSF and its effect on corticosterone should be investigated. Especially since the *in vitro* studies show that higher doses of G-CSF lost its inhibitory property. Not to mention, that a study using higher dose of G-CSF (200 μg/kg) did not protect the brain from neonatal HI injury, compared to our lower dose (50 μg/kg) (Schlager et al, 2011; Charles et al, 2012). Determining how this occurs and whether this effect is dependent on corticosterone is essential to harness the full therapeutic promise of G-CSF.

In addition to the neuroprotective effect of G-CSF, G-CSF influences the hormones of the neuroendocrine system. Our study focused on the direct interaction of G-

CSF on the adrenal gland of the HPA axis, but many other areas have yet to be investigated. There are cues from other studies showing growth factors or hormones with similar signaling cascade that exert effect of the HPA-axis. Therefore there is a high probability that the actions of G-CSF extend beyond the adrenal gland. Perhaps G-CSF controls the activity of the HPA axis at the level of the hypothalamus and/or the pituitary gland. The details of this possible interaction should be considered for subsequent studies.

Conclusion

G-CSF has pleiotropic physiological effects, and we demonstrate that its neuroprotection is dependent on its direct interaction with the HPA axis. We show that G-CSF inhibits corticosterone synthesis, and protect the neonatal rat brain from HI injury, by reducing infarct volume and reducing pro-apoptotic markers. Agonizing the effects of the HPA axis with a synthetic glucocorticoid exacerbated injury even when co-administered with G-CSF. This study highlighted a novel function of G-CSF, since it inhibited the HI-induced upregulation of corticosterone. We found that the G-CSF receptor was expressed on adrenal glands and conferred its inhibitory effect on corticosterone synthesis by activating the JAK2 signaling pathway. Future studies are needed to better understand the interaction with G-CSF and the HPA axis. Nevertheless, the promising therapeutic potential of G-CSF should continue to be pursued in subsequent preclinical stroke research.

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