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Plasma pregnenolone levels in cynomolgus monkeys following pharmacological challenges of the hypothalamic–pituitary–adrenal axis

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Abstract

Pregnenolone (PREG) is an endogenous neuroactive steroid that is increased in rodent brain and plasma after hypothalamic–pituitary–adrenal (HPA) activation by acute stress or ethanol administration. Plasma levels of PREG metabolites are altered by pharmacological challenges of the HPA axis, however little is known about HPA regulation of PREG levels in monkeys. PREG concentrations were determined by radioimmunoassay in plasma samples from cynomolgus monkeys, following challenge with naloxone (125 and 375 μg/kg), corticotropin-releasing factor (CRF; 1 μg/kg), dexamethasone (130 μg/kg), adrenocorticotropic hormone (ACTH; 10 ng/kg; 4–6 h after 0.5 mg/kg dexamethasone) and ethanol (1.0 and 1.5 g/kg). Naloxone increased PREG levels, while CRF appeared to increase metabolism of PREG to deoxycorticosterone (DOC). ACTH, administered after dexamethasone, reduced PREG levels, despite an increase in plasma cortisol. Ethanol did not alter PREG levels. Changes in PREG levels were correlated with changes in DOC levels after naloxone 125 μg/kg, CRF, ethanol 1.5 g/kg, and dexamethasone challenges. Furthermore, dexamethasone-induced changes in PREG levels were correlated with subsequent alcohol intake. These data suggest that PREG responses to dexamethasone challenge may represent a trait marker of alcohol drinking. The lack of effect of ethanol on PREG levels suggests differential regulation in non-human primates vs. rodents.

Keywords: Pregnenolone (PREG); Deoxycorticosterone (DOC); Hypothalamic–pituitary–adrenal axis; Cynomolgus monkeys; Ethanol

1. Introduction

Pregnenolone (PREG) is a steroid hormone synthesized from cholesterol in a reaction catalyzed by the mitochondrial enzyme CYP11A (cholesterol side-chain cleavage or P450scc). The conversion of cholesterol into PREG is the first and rate limiting step of steroidogenesis, hence PREG is the precursor of numerous different steroid hormones, including glucocorticoids (cortisol in humans and non-human primates and corticosterone in rodents) and mineralocorticoids (aldosterone) at the level of the adrenal gland, or the 5α/5β-reduced neuroactive steroids 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP or allopregnanolone), 3α-hydroxy-5β-pregnan-20-one (3α,5β-THP or pregnanolone), 3α,21-dihydroxy-5α-pregnan-20-one (3α,5α-THDOC or allotetrahydrodeoxycorticosterone) and 3α,5β-THDOC.

PREG has been suggested to have effects on both cognition and mood. PREG and its sulfated derivative enhance learning and memory in rodent models and pregnenolone sulfate administration to aged rats reverses age-related cognitive decline (Flood et al., 1992, 1995; Vallée et al., 1997, 2001). PREG induces anxiogenic effects in mice (Melchior and Ritzman, 1994), and it has recently been shown to attenuate diazepam-induced sedation (Meieran et al., 2004). PREG levels are altered in patients with anxiety-depressive disorder (George et al., 1994) and PMS (Wang et al., 1996), and they are elevated

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in postmortem brain of subjects with schizophrenia and bipolar disorder (Marx et al., 2006).

The brain and plasma concentrations of PREG and its neuroactive metabolites are increased by acute stress in rodents (Barbaccia et al., 1996; Purdy et al., 1991). Similarly, stress elevates levels of 3α,5α-THP in human subjects (Droogleever Fortuyn et al., 2004; Girdler et al., 2001). The increase in neuroactive steroid levels elicited by stress appears to be mediated by activation of the hypothalamus–pituitary–adrenal (HPA) axis, since it is no longer apparent in adrenalectomized animals (Purdy et al., 1991).

Activation of the HPA axis under acute stress triggers the release of corticotropin releasing factor (CRF) from the hypothalamus. CRF induces the synthesis of adrenocorticotropic hormone (ACTH) from the pituitary, which stimulates the adrenal cortex to release glucocorticoids, their precursors progesterone and deoxycorticosterone (DOC) and also neuroactive steroids. Glucocorticoids, as well as 3α,5α-THP and 3α,5α-THDOC, have a negative feedback upon the hypothalamus and pituitary, thus inhibiting CRF production, release, ACTH release and subsequent corticosterone levels in rodents (Owens et al., 1992; Patchev et al., 1994, 1996).

We have recently demonstrated that DOC levels in plasma from cynomolgus monkeys are regulated by naloxone, CRF and dexamethasone challenges to the HPA axis. However, circulating DOC levels did not change in response to acute challenges of 1.0 g/kg or 1.5 g/kg ethanol (i.g.), even though average blood ethanol levels were as high as 147 mg/dl (Porcu et al., 2006). In contrast, DOC levels are markedly increased in rat brain and plasma following acute ethanol administration (Khisti et al., 2005), suggesting that higher doses of alcohol are necessary to activate the HPA axis in monkeys, or perhaps that DOC synthesis is differentially regulated in non-human primates compared to rodents (Porcu et al., 2006).

It is well known that systemic administration of ethanol increases both plasma and brain levels of PREG and its neuroactive metabolites 3α,5α-THP and 3α,5α-THDOC in rodents (Barbaccia et al., 1999; Korneyev et al., 1993; Morrow et al., 1999; O'Dell et al., 2004; VanDoren et al., 2000). Laboratory administration of low doses of ethanol has recently been reported to increase PREG levels in healthy human subjects (Pierucci-Lagha et al., 2006), while 3α,5α-THP levels are decreased in the same subjects. In contrast, other studies have reported increased plasma 3α,5α-THP levels in human adolescent emergency room patients with signs of ethanol intoxication (Torres and Ortega, 2003, 2004). The increase in PREG and its neuroactive metabolites, induced by acute ethanol in rats, is prevented by adrenalectomy/orchiectomy, thus, suggesting that ethanol activates the HPA axis to modulate neuroactive steroids synthesis (Korneyev et al., 1993; O'Dell et al., 2004).

Chronic ethanol consumption results in adaptation of the HPA axis causing decreased levels of corticosterone (Spencer and McEwen, 1990) and blunted elevation of cerebral cortical 3α,5α-THP (Morrow et al., 2001) and plasma and brain DOC levels (Khisti et al., 2005) in rats. Similar alterations in the HPA axis are observed in actively drinking or alcohol dependent human subjects. These patients show attenuated responsiveness of the HPA axis following stimulation with naloxone, CRF, exogenous ACTH administered after dexamethasone, and they exhibit a greater suppression of cortisol and ACTH levels following dexamethasone suppression (Adinoff et al., 2005a,b; Inder et al., 1995; Wand and Dobs, 1991).

The aim of this study was to characterize plasma PREG levels following pharmacological challenges of the HPA axis, since PREG has effects on cognition and mood and is the precursor of other neuroactive steroids and hormones that are regulated by HPA axis modulation. Furthermore, PREG and its metabolites can penetrate the blood brain barrier and thus influence the overall concentration of brain neuroactive steroids; therefore changes in peripheral PREG levels might reflect changes in its brain content. Given that both stress and ethanol modulate neuroactive steroids and given the role played by the HPA axis in the etiology of alcoholism, the regulation of plasma PREG levels by the HPA axis was studied in eleven male cynomolgus monkeys before they were enrolled in a protocol of ethanol self-administration (Vivian et al., 2001). We hypothesized that PREG levels in monkey plasma would be regulated by pharmacological challenge of the HPA axis.

2. Materials and methods

2.1. Animals

Eleven adult (5–6 years old at the time the pharmacological challenges took place) male cynomolgus monkeys (*Macaca fascicularis*) were individually housed in 76×60×70 cm stainless steel cages in an environment maintained at 21± 1 °C, with 30–50% humidity and a 11:13 h light:dark cycle. Monkeys were maintained in a positive caloric and fluid balance throughout the experiments. The study was conducted in accordance with the Wake Forest University Animal Care and Use Committee and the guidelines for the care and use of laboratory animal resources (Commission on Life Sciences, National Research Council, 1996; NIH Guide for the Care and Use of Laboratory Animals Publication No. 85-23, revised 1985).

The monkeys were trained to comply with awake venipuncture to collect blood for the steroid assays as described elsewhere (Porcu et al., 2006). Each step in the behavioral training was considered complete when the animals performed the behavior readily and with minimal observable distress. Briefly, twice a day each monkey was trained with positive reinforcement to move to the front of the cage and present its leg through an opening in the cage (10×10 cm). Blood draws from the femoral vein were performed multiple times per week continuing throughout the experiment and all sera were frozen for future use. To administer the pharmacological challenges to the monkeys and collect the necessary blood samples following these challenges under non-stressful conditions, the monkeys were trained to sit in a primate restraining chair. For each challenge test, the order in which the animals were handled was randomly assigned although each animal was assigned a specific chair for the entire endocrine profile to ensure proper

fit. As the animals became comfortable sitting in the chairs, blood draws were obtained via the femoral vein to simulate the blood sampling during the pharmacological challenges. The next phase in training was to train the animals to accept a nasal-gastric feeding tube for eventual ethanol administration as part of the pharmacological challenge. During this training, only tap water was administered in volumes approximate to testing volumes. The final stage of behavioral training was to train the animals to sit quietly while an intravenous line was inserted in the saphenous vein for the eventual administration of cortrosyn (ACTH) and CRF. The endocrine profiling was conducted using the same procedures. Each week during endocrine profiling, a blood sample was obtained for a hematocrit value to monitor for anemia.

2.2. Blood sampling

Femoral blood samples were obtained with a 22 g×1 in. Vacutainer needle and a 3 ml Vacutainer hematology tube (Becton Dickinson, Franklin Lakes, NJ, USA). All blood samples were stored on ice until centrifuged (approximately 5 min). Samples were spun at 3000 rpm for 15 min at 4 °C in a Beckman Coulter refrigerated centrifuge (Model Allegra 21R, Beckman Coulter, Fullerton, CA, USA). The plasma was pipetted into 2 ml microtubes in 100 μl aliquots. Plasma samples for PREG and DOC analysis were frozen at −80 °C and stored until processing. From the ethanol challenge samples, blood ethanol concentrations (BEC) were determined in whole blood (20 μl) samples obtained from the Vacutainer tube prior to centrifugation. Blood samples were sealed in air-tight vials containing 500 μl of distilled water and 20 μl of isopropanol (10% internal standard) and stored at −4 °C until assay using gas chromatography (Hewlett Packard 5890 Series II, Avondale, PA, USA).

2.3. Pharmacological profiling

An extensive series of assessments of the HPA axis were made prior to studies of ethanol self-administration. These tests were designed to reflect clinical assessments commonly available in human research protocols. There were a total of eight pharmacological challenges [naloxone (2 doses), CRF, ACTH, dexamethasone, ethanol (2 doses) and saline]. Each pharmacological challenge was conducted between 8:00 am and 2:00 pm (lights on at 7:00 am), except for the dexamethasone challenge. We conducted two challenges per week, allowing 3–4 days of washout between challenges and requiring four weeks to complete the endocrine profile. The order of pharmacological challenges for all monkeys was as follows: Week 1 Dexamethasone on Monday, ACTH on Thursday; Week 2 Naloxone 125 μg/kg on Monday, Naloxone 375 μg/kg on Friday; Week 3 Saline on Tuesday, Ethanol 1.0 g/kg on Friday; Week 4 Ethanol 1.5 g/kg on Tuesday, CRF on Friday (see Table 1).

2.3.1. Saline challenge

Saline was administered and blood samples (3 ml) were drawn at 15, 30, 60, 90 and 120 min and assayed for steroids to use as baseline comparisons to measure the effects of pharmacological challenges of the HPA axis.

Table 1
Endocrine profile schedule

Week (day)	Treatment	Blood sampling	
		Pre-drug	Post-drug
1 (Monday)	Dexamethasone	−24 h	10 h
1 (Thursday)	ACTH	−15 min	15, 30 min
2 (Monday)	Naloxone (125 μg/kg)	None	15, 30, 60, 90, 120 min
2 (Friday)	Naloxone (375 μg/kg)	None	15, 30, 60, 90, 120 min
3 (Tuesday)	Saline	None	15, 30, 60, 90, 120 min
3 (Friday)	Ethanol (1.0 g/kg)	None	15, 60, 90, 120 min
4 (Tuesday)	Ethanol (1.5 g/kg)	None	15, 60, 90, 120 min
4 (Friday)	CRF	None	15, 30, 60 min

2.3.2. Naloxone challenge

Naloxone results in elevated HPA response by blocking inhibitory opioid input to CRF neurons in the hypothalamus (Wand et al., 1998). We tested 125 and 375 μg/kg naloxone individually, with each dose administration separated by at least 72 h. Blood samples (3 ml) were drawn at 15, 30, 60, 90 and 120 min and assayed for PREG.

2.3.3. CRF challenge

Pituitary response to CRF was assessed using the CRF challenge test (Gold et al., 1984; Sapolsky, 1989, Waltman et al., 1994). Monkeys were fasted overnight. The next morning a 3 ml blood sample was taken followed by administration of 1 μg/kg ovine CRF into the saphenous vein. Blood samples (3 ml each) were obtained at 15, 30 and 60 min and assayed for PREG.

2.3.4. ACTH challenge

This test assessed adrenocortical secretion of PREG following exogenous ACTH after endogenous HPA axis activity has been suppressed by a large dose of dexamethasone. Specifically, following an overnight fast, the animals were administered dexamethasone (0.5 mg/kg, intramuscularly, i.m.). Four to six hours later, during the maximum dexamethasone suppression of adrenal activity, a blood sample was taken and then animals were administered the ACTH challenge (Cortrosyn, 10 ng/kg, intravenously i.v.). Blood samples were then taken at 15 and 30 min following ACTH infusion and assayed for PREG.

2.3.5. Dexamethasone suppression test

The purpose of this test was to assess the sensitivity of the hypothalamus and pituitary to negative feedback from circulating levels of cortisol (Davidson et al., 1984; Mossman and Somoza, 1989). Since dexamethasone binds with great affinity to the cortisol receptor it was used in relatively small amounts to test the sensitivity to negative feedback (Kalin and Shelton, 1984). A morning (8:00 am) blood sample was taken for a baseline measure of steroids. That evening (10:00 pm) a low dose (130 μg/kg, i.m.) of dexamethasone was administered. The next morning (8:00 am) another blood sample was taken and assayed for PREG.

2.3.6. Ethanol challenge

Ethanol produces changes in circulating cortisol (Schuckit et al., 1987; Gianoulakis et al., 1996). We first tested a dose of 1.0 g/kg ethanol; five days later we tested a higher dose of 1.5 g/kg ethanol. Blood samples (3 ml) were drawn at 15, 60, 90 and 120 min following intragastric administration of ethanol and assayed for PREG. In addition, BEC at 60, 90 and 120 min were determined from the same blood samples.

2.4. Drugs

Naloxone hydrochloride dihydrate and ovine CRF were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. ACTH (Cortrosyn) (0.25 mg/vial reconstituted with sterile saline; Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA, USA) and dexamethasone (10 and 4 mg/ml for the ACTH and dexamethasone profiles, respectively; Baxter, Deerfield, IL, USA) were obtained in a commercial formulation from the Wake Forest University Baptist Medical Center pharmacy. All drugs were diluted in sterile saline to make the appropriate concentrations. Ethanol 95% was purchased from Warner-Graham, Cockeysville, MD, USA and was diluted in tap water to a concentration of 20% (w/v) for the 1.0 g/kg dose and 30% (w/v) for the 1.5 g/kg dose to keep the volume delivered constant across both tests.

2.5. Pregnenolone assay

PREG levels were determined by radioimmunoassay (RIA) as follows. Plasma samples (250 µl) were extracted three times with 2 ml diethyl ether; 1000 cpm of [^{3}H]PREG (SpA = 14 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) were added to each sample for recovery estimation. The dried extracts were reconstituted in 2 ml RIA buffer of which 0.5 ml were used for the assay (run in duplicate) and for recovery determination. Average recovery of PREG was 81 ± 0.4%. The antiserum for PREG was purchased from MP Biomedicals (Orangeburg, NY, USA) and diluted according to manufacturer's instructions. This antiserum cross-reacts 100% with PREG and PREG-sulfate, however the recoveries of [^{3}H]PREG-sulfate after diethyl ether extraction were only 4.8 ±0.7%. The antiserum cross-reacts with 3α,5α-THP 16%, 3α,5β-THP 5.9%, progesterone 3.1%, 3α,5α-THDOC 1.1%. Less than 1% cross-reactivity was observed for 5α-dihydroprogesterone, 17α-hydroxyprogesterone, 20α-dihydroprogesterone, 17α-hydroxypregnenolone, DOC, cortisol, 11-deoxycortisol, corticosterone, androsterone, 5α-dihydrotestosterone, cholesterol, 17β-estradiol, estrone and estriol. Unknown samples were compared to concurrently run standards using a one-site competition model and adjusted for extraction efficiency. PREG values are expressed as ng/ml plasma. The sensitivity of the assay is 25 pg/ml. Intra-assay and inter-assay coefficients of variation were 7.71% and 5.93%, respectively.

2.6. Data analysis

Data were analyzed using a commercially available statistical program (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA). Two-way repeated measures ANOVAs, considering the factors treatment and time, and repeated for time, were performed for the naloxone, CRF and ethanol challenges. The ACTH challenge was analyzed by one-way repeated measures ANOVA. Post-hoc comparisons were performed by the Bonferroni test. The dexamethasone challenge was analyzed by the Student's *t* test. ANOVAs were performed on both raw values and log-transformed values to control for potential heterogeneity of variance. The detection of statistical significance was not altered by log transformation of the data and *p* values following analysis of log-transformed data are shown. Values are expressed as mean ± SEM of the raw data and *p* values less than 0.05 were considered statistically significant.

PREG responses were measured repeatedly across time and therefore comparisons versus the saline baseline serve as the control for each animal's response to repeated blood sampling over time. Correlations between percent changes in PREG and previously reported DOC responses (Porcu et al., 2006) were determined within each test. In addition, correlations between hormone responses and the subsequent average daily ethanol intake over twelve months of voluntary ethanol self-administration were calculated using the Pearson's coefficient. The values used in the correlation matrix for subsequent average daily ethanol intake were previously published in relation to DOC responses (Porcu et al., 2006).

3. Results

3.1. PREG levels following HPA axis pharmacological challenges

PREG levels in monkey plasma are significantly decreased over time, following administration of saline; the decrease is apparent at 60 min (−36%, $p<0.01$ vs 15 min), and is maximal at 120 min after injection (−54%, $p<0.001$ vs 15 min). In contrast, the saline challenge did not alter cortisol levels across

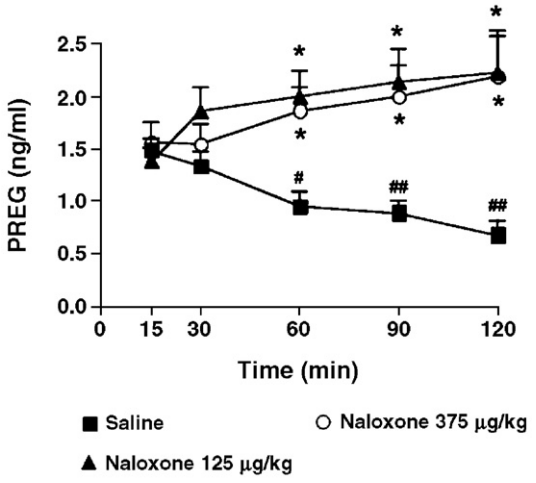

Fig. 1. Time course of PREG plasma levels after administration of naloxone 125 and 375 µg/kg (i.m.) and saline. Data are expressed as ng/ml and are mean ± SEM of values obtained from ten monkeys. *$p<0.001$ vs the respective saline value; $^{\#}p<0.01$, $^{\#\#}p<0.001$ vs saline at 15 min (two way repeated measures ANOVA followed by Bonferroni post-hoc test).

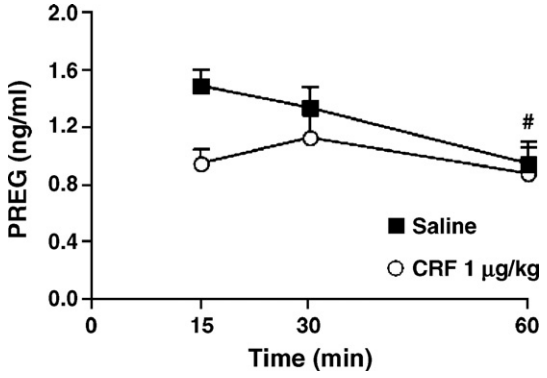

Fig. 2. Time course of PREG plasma levels after administration of CRF 1 µg/kg (i.v.) and saline. Data are expressed as mean±SEM of values obtained from ten monkeys. $^{\#}p<0.01$ vs saline at 15 min (two way repeated measures ANOVA followed by Bonferroni post-hoc test).

time (26±1, 25±1, 25±2, 25±2, 23±2 µg/dl, respectively for 15, 30, 60, 90 and 120 min).

The intramuscular (i.m.) administration of naloxone significantly increases plasma PREG levels in monkeys (Fig. 1). Two-way ANOVA revealed a significant main effect of treatment [$F(2, 27)=11.39$; $p=0.0003$], no significant effect of time [$F(4, 27)=0.49$; $p=0.74$] and a significant interaction between factors [$F(8, 27)=11.75$; $p<0.0001$]. Both doses of naloxone tested, 125 µg/kg and 375 µg/kg, induced a time-dependent increase in PREG levels, compared to the respective saline time points; the effect was apparent at 60 min (+109% and +96%, $p<0.001$, respectively), the levels remained significantly elevated at 90 min and were maximally increased at 120 min (+222 and +216%, $p<0.001$, respectively).

The intravenous (i.v.) administration of CRF (1 µg/kg), which stimulates the HPA axis at the pituitary level, failed to

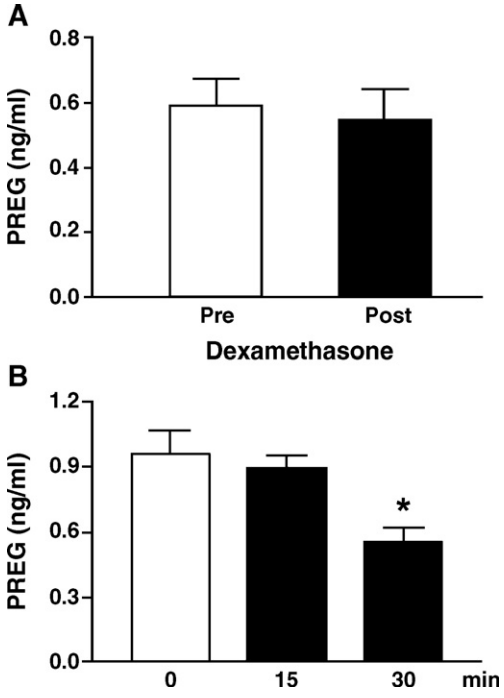

Fig. 3. (A) Effect of dexamethasone (130 µg/kg, i.m.) administration on PREG plasma levels. (B) Effect of ACTH administration on PREG plasma levels. ACTH (10 ng/kg, i.v.) was administered 4 to 6 h following dexamethasone suppression (0.5 mg/kg, i.m.). Data are expressed as ng/ml and are mean±SEM of values obtained from eleven monkeys. *$p<0.01$ vs the pre-ACTH (0) value (one-way repeated measures ANOVA followed by Bonferroni post-hoc test).

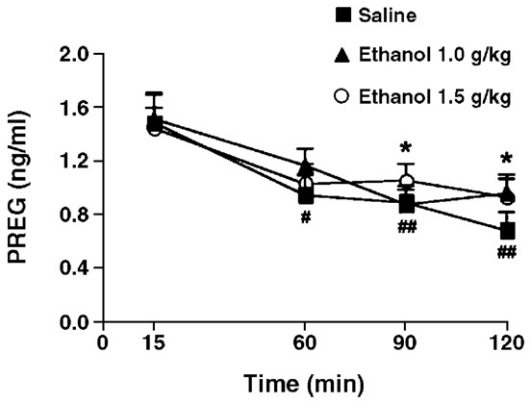

Fig. 4. Time course of PREG plasma levels after administration of ethanol 1.0 and 1.5 g/kg (i.g.) and saline. Data are expressed as ng/ml and are mean±SEM of values obtained from ten monkeys. *$p<0.05$ vs ethanol 1.0 and 1.5 g/kg treatment at the respective 15 min time point; $^{\#}p<0.01$, $^{\#\#}p<0.001$ vs saline at 15 min (two way repeated measures ANOVA followed by Bonferroni post-hoc test).

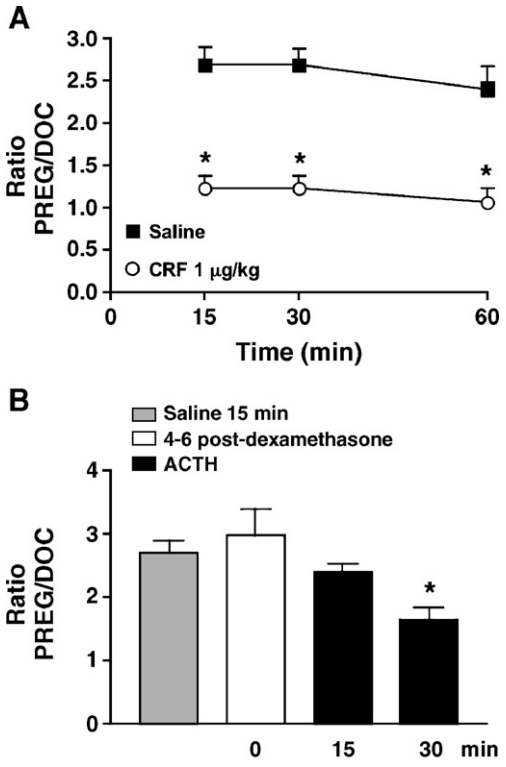

Fig. 5. (A) Ratio between PREG and DOC levels measured following CRF challenge. Data are expressed as mean±SEM of values obtained from ten monkeys. *$p<0.001$ vs the respective saline time point (two way repeated measures ANOVA followed by Bonferroni post-hoc test). (B) Ratio between PREG and DOC levels measured following the ACTH challenge and the saline challenge at 15 min. Data are expressed as mean±SEM of values obtained from ten monkeys. *$p<0.05$ vs pre-ACTH (post-dexamethasone) and saline treatments (one-way repeated measures ANOVA followed by Bonferroni post-hoc test).

Table 2
Ratios between PREG and DOC levels following HPA axis challenges

Pharmacological challenge	Time (min)				
	15	30	60	90	120
Saline	2.70±0.21[b]	2.70±0.18[b]	2.41±0.27[a]	2.05±0.20	1.61±0.19
Naloxone 125 μg/kg	2.31±0.20	3.01±0.37	2.84±0.30	2.90±0.24	3.03±0.47[c]
Naloxone 375 μg/kg	2.38±0.35	2.32±0.28	2.44±0.23	2.33±0.28	2.69±0.35[b]
Ethanol 1.0 g/kg	2.79±0.29	–	3.09±0.43	2.61±0.22	3.18±0.37[b]
Ethanol 1.5 g/kg	3.24±0.37	–	2.93±0.20	3.13±0.37	3.11±0.28[c]
	Pre-dexamethasone			Post-dexamethasone	
Dexamethasone 130 μg/kg	2.20±0.36			3.58±0.65	

Data are expressed as ratio between PREG and DOC levels measured in plasma and are mean±SEM of values obtained from ten–eleven monkeys. [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ vs saline at 120 min (two way repeated measures ANOVA followed by Bonferroni post-hoc test).

increase PREG concentrations in monkey plasma, as shown in Fig. 2. Two-way ANOVA revealed a significant main effect of time [$F(2, 18)=10.16$; $p=0.0003$], no significant effect of treatment [$F(1, 18)=1.58$; $p=0.22$] and no significant interaction between factors [$F(2, 18)=1.74$; $p=0.19$]. In contrast, CRF challenge increased cortisol levels from 24.6±1.7 μg/dl to 33.9±1.5 μg/dl ($p<0.001$).

Partial suppression of the HPA axis with dexamethasone (130 μg/kg, i.m.) did not modify PREG levels in plasma samples from monkeys (Fig. 3A), despite an 83% decrease in cortisol levels ($p<0.0001$). To determine the effect of exogenous ACTH under conditions where endogenous HPA activity was suppressed, ACTH (10 ng/kg, i.v.) was administered 4–6 h after injection of 0.5 mg/kg dexamethasone. ACTH reduced PREG levels by 43% ($p<0.01$) at 30 min (Fig. 3B) compared to pre-ACTH (post-dexamethasone) levels.

3.2. PREG levels following acute ethanol administration

Acute ethanol administration did not modify circulating DOC levels in monkeys (Porcu et al., 2006); however acute ethanol stimulates the HPA axis response (Rivier, 1996) and dramatically increases neuroactive steroids and their precursors in rat brain and plasma (Korneyev et al., 1993; Barbaccia et al., 1999; Morrow et al., 1999; Khisti et al., 2005). We thus evaluated the effect of ethanol administration on PREG levels.

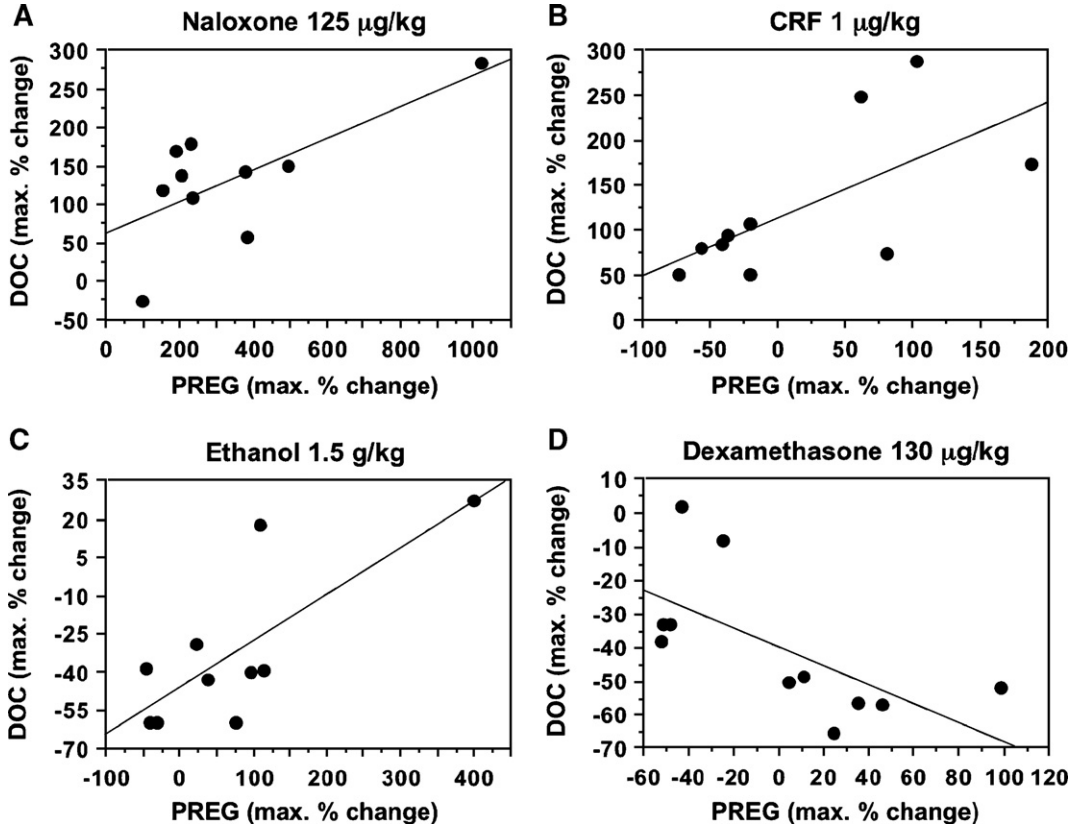

Fig. 6. Maximal percent PREG changes are positively correlated with maximal percent DOC changes after HPA axis challenges. (A) Naloxone 125 μg/kg challenge: Pearson $r=0.69$, $p=0.024$, $n=10$. (B) CRF challenge: Pearson $r=0.66$, $p=0.038$, $n=10$. (C) Ethanol 1.5 g/kg challenge: Pearson $r=0.77$, $p=0.008$, $n=10$. (D) Dexamethasone challenge: Pearson $r=-0.65$, $p=0.027$, $n=11$.

As shown in Fig. 4, intragastric administration of ethanol (1.0 and 1.5 g/kg) failed to change plasma PREG levels in comparison to saline. Two-way ANOVA revealed a significant main effect of time [$F(3, 27)=18.36$; $p<0.0001$], no significant effect of treatment [$F(2, 27)=0.27$; $p=0.76$] and no significant interaction between factors [$F(6, 27)=1.02$; $p=0.42$]. As previously reported, the average±SEM of blood ethanol concentration values were 76±6, 87±4 and 89±3 mg/dl of blood after 60, 90 and 120 min, respectively, from administration of ethanol 1.0 g/kg. Following ethanol 1.5 g/kg, blood ethanol concentrations were 125±8, 142±6 and 147±5 mg/dl of blood at 60, 90 and 120 min, respectively (Porcu et al., 2006).

3.3. PREG to DOC ratios

To test the hypothesis that a rapid conversion of PREG into its metabolites might account for the lack of effect of some of the challenges on PREG concentrations, we calculated the ratio between PREG and DOC levels, previously measured in the same plasma samples (Porcu et al., 2006).

CRF infusion significantly decreased the ratio (−54%, $p<0.001$) when compared to saline, suggesting that PREG was indeed metabolized into DOC or other metabolites (Fig. 5A). The ratio between PREG and DOC 30 min following ACTH was significantly reduced when compared to both pre-ACTH (post-dexamethasone) and 15 min after saline challenge conditions (−44% and −38%, respectively; $p<0.05$) (Fig. 5B).

No changes in the ratio between PREG and DOC following naloxone, dexamethasone or ethanol administration were observed at 120 min, where the ratios were increased compared to saline administration (Table 2).

3.4. Correlation between changes in PREG and DOC levels

PREG levels were measured in the same plasma samples previously used for the determination of DOC levels (Porcu et al., 2006). We analyzed potential correlations between the percent maximal changes in PREG levels and the corresponding percent change in DOC levels following various neuroendocrine challenges. Fig. 6 shows a significant positive correlation between PREG and DOC (A) following challenge with naloxone 125 μg/kg (Pearson $r=0.69$, $p=0.024$, $n=10$) and between PREG and DOC (B) following challenge with CRF 1 μg/kg (Pearson $r=0.66$, $p=0.038$, $n=10$). Percent maximal changes in PREG plasma levels were also correlated with percent maximal changes in DOC levels following administration of ethanol 1.5 g/kg (Fig. 6C) (Pearson $r=0.77$, $p=0.008$, $n=10$). In contrast, the changes observed in PREG levels vs. DOC levels after dexamethasone challenge were negatively correlated (Fig. 6D) (Pearson $r=-0.65$, $p=0.027$, $n=11$).

3.5. Correlation between changes in PREG levels and voluntary ethanol drinking

After evaluation of HPA axis responses, the monkeys were induced to drink ethanol and allowed twelve months of free ethanol self-administration 22 h a day, using a procedure previously described (Vivian et al., 2001). We determined correlations between PREG levels in plasma with the average daily ethanol (g/kg) self-administered over twelve months of alcohol access. The mean PREG levels following saline administration were not correlated with subsequent ethanol intake as shown in Fig. 7A (Pearson $r=0.11$, $p=0.79$, $n=9$). Similar results were found at each time point following saline administration (data not shown).

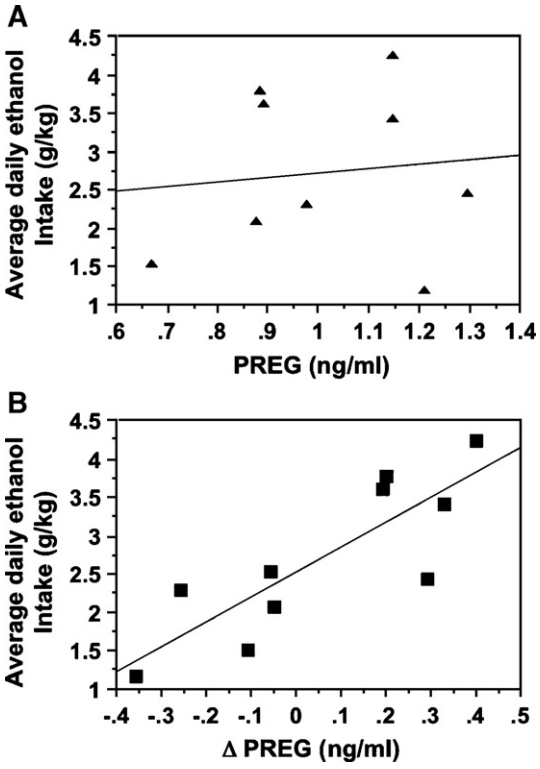

Fig. 7. Pearson correlation between PREG levels and subsequent average daily ethanol intake (g/kg) over 12 months of alcohol self-administration. (A) Basal PREG levels are expressed as ng/ml and are mean±SEM of five determinations for each monkey under saline control. Pearson $r=0.11$, $p=0.79$, $n=9$. (B) PREG levels are expressed as ng/ml of the absolute change (Δ) between pre- and post-dexamethasone challenge. Pearson $r=0.84$, $p=0.001$, $n=10$.

In contrast, we observed a significant correlation between the degree of dexamethasone modulation of plasma PREG concentrations and subsequent alcohol intake (Fig. 7B). The change in PREG levels observed following dexamethasone administration was positively correlated with the average daily alcohol consumption (Pearson $r=0.84$, $p=0.001$, $n=10$), that is, a greater suppression of PREG levels was predictive of higher subsequent alcohol consumption. This effect was unique to dexamethasone challenge, since no correlation was observed between PREG responses and subsequent alcohol intake under the other challenges of the HPA axis.

4. Discussion

The present data show that administration of 125 and 375 μg/kg naloxone increases PREG levels in monkey plasma samples. However, circulating PREG levels are not elevated by exogenous CRF (1 μg/kg, i.v.) or ACTH infusion (10 ng/kg),

despite elevations of plasma cortisol (Porcu et al., 2006; Grant et al., in preparation). Indeed, administration of exogenous ACTH 4 to 6 h after dexamethasone administration (0.5 mg/kg) lowered PREG levels compared to pre-ACTH (post-dexamethasone) values.

Endogenous opioids induce an inhibitory modulation of CRF release from the hypothalamus, thus suppressing circulating HPA hormonal levels (Wand et al., 1998). The opioid antagonist naloxone releases the inhibitory opiate input to the hypothalamus, stimulates CRF secretion, and consequently the release of ACTH from the pituitary and cortisol from the adrenals. The increase in plasma PREG levels observed in monkeys after the naloxone challenge could involve factors other than CRF or ACTH levels since infusion of these regulators did not increase PREG levels in monkey plasma. However the lack of effect of CRF or ACTH on PREG levels appears to be related to enhanced metabolism of PREG since both CRF and ACTH decreased the PREG/DOC ratio. Moreover, CRF release from the hypothalamus due to naloxone administration may produce higher CRF and ACTH levels than the concentrations administered to the monkeys. Therefore, it appears that HPA modulation influences PREG levels and metabolism.

The CRF challenge stimulates the secretion of ACTH from the pituitary and subsequently increases DOC and cortisol release from the adrenals (Porcu et al., 2006). The dose of CRF used in our experiment (1 μg/kg) is similar to that used in human studies (Waltman et al., 1994). This dose, while able to increase cortisol and DOC levels in plasma samples from monkeys (Porcu et al., 2006), did not change circulating PREG levels, suggesting that PREG and DOC levels might be differentially regulated. PREG may be less likely to accumulate in plasma after HPA axis activation due to rapid metabolism to other steroids, including DOC.

Dexamethasone (130 μg/kg) was ineffective in regulating PREG levels measured 10 h after administration. Unfortunately, few data are available on PREG levels following a dexamethasone suppression test. Aedo et al. (1981) have reported a decrease in PREG levels 9 h after administration of 1 mg of dexamethasone. The dose of dexamethasone used in our experiments is considered a low, but effective, dose (130 μg/kg) for HPA axis suppression (Kalin and Shelton, 1984), and this dose suppressed the synthesis of the PREG metabolite DOC (Porcu et al., 2006). When ACTH was administered 4 to 6 h after a higher dose of dexamethasone (0.5 mg/kg), PREG levels were decreased at 30 min, compared to pre-ACTH levels. This could be due to rapid metabolism of PREG. Furthermore, we cannot exclude the possibility that ACTH elevates PREG levels at earlier time points.

These present results might suggest that PREG levels are subject to complex regulation involving factors other than HPA axis modulation. The ability of naloxone to increase PREG levels may be independent of HPA axis activation since CRF and ACTH infusion did not produce similar effects on PREG levels. Opioid receptors are also present in peripheral tissue such as the adrenals (Witter et al., 1996) and a direct action of naloxone on these receptors cannot be ruled out. Moreover, since opioidergic neurons regulate the hypothalamic secretion of gonadotropin releasing hormone (GnRH) (Kalra et al., 1997), it is possible that the increase in circulating PREG following naloxone is due to a diminution in the opioid inhibition of GnRH, leading to increased gonadal steroidogenesis. Another hypothesis could involve a direct action of naloxone upon the enzymes involved in steroid biosynthesis. For example, both fluoxetine and ethanol have been shown to alter biosynthesis of steroids by direct actions on the 3α-hydroxysteroid oxidoreductase enzyme and the steroidogenic acute regulatory protein (Griffin and Mellon, 1999; Khisti et al., 2003). Further studies are needed to investigate these possibilities.

Plasma PREG levels do not change following acute administration of ethanol (1.0–1.5 g/kg) in monkeys. This observation agrees with our previous data showing that DOC levels are not influenced by acute ethanol administration in the same monkeys (Porcu et al., 2006). Recent data in humans has shown an increase in circulating PREG levels in healthy subjects receiving three standard drinks (0.7–0.8 g/kg ethanol) (Pierucci-Lagha et al., 2006). In addition, DHEA, a direct metabolite of PREG, was significantly increased in the same subjects, while 3α,5α-THP levels were reduced (Pierucci-Lagha et al., 2006) or unchanged (Holdstock et al., 2006) in humans who have been administered moderate doses of ethanol under laboratory conditions.

The present data contrast with rodent studies showing that acute ethanol increases both plasma and brain PREG levels. An anesthetic dose of ethanol (4.6 g/kg, p.o.) but also the dose of 2 g/kg (i.p.) induced an increase in PREG levels within 30 min of administration. This effect is likely mediated by the HPA axis since it is absent in adrenalectomized/orchiectomized rats (Korneyev et al., 1993; O'Dell et al., 2004). Acute ethanol administration in rodents also results in a dramatic increase in neuroactive steroid levels. Both cortical and plasma 3α,5α-THP are increased following intraperitoneal injection of 1.5 g/kg ethanol, and the effect is greater at the dose of 2.0 g/kg (Morrow et al., 1999; VanDoren et al., 2000). Others have reported an increase in brain and plasma levels of 3α,5α-THP and 3α,5α-THDOC following the administration of 1.0 g/kg in Sardinian alcohol-preferring rats (Barbaccia et al., 1999). These results suggest that higher doses of ethanol might be necessary to stimulate the HPA axis and thus increase the levels of PREG and its metabolites in non-human primates. However, Williams et al. (2004) have shown that intravenous administration of ethanol up to 1.9 g/kg failed to increase plasma ACTH levels in rhesus monkeys. Higher doses of ethanol, such as 2.0 g/kg, have been reported to increase cortisol levels in monkeys, under conditions where monkeys were restrained on a flat surface while receiving ethanol, which may contribute to HPA axis activation (Barr et al., 2004). The possibility that PREG and its neuroactive metabolites might be differentially regulated in non-human primates compared to rodents cannot be ruled out; future studies will be necessary to further address this question.

The use of multiple pharmacological challenges over the course of a month could produce secondary effects that cannot be ruled out. Since 3–4 day washout periods were incorporated into the experimental design, it is unlikely that prior challenges influenced PREG levels, however this remains a possibility.

Basal PREG levels measured in Week 1 were similar to PREG levels 2 h following saline administration in Week 3, suggesting that secondary effects of multiple challenges were minimal.

We have shown a positive correlation between dexamethasone-induced changes in PREG levels and the average daily ethanol consumption during a twelve-month period following the endocrine profiling. The degree of dexamethasone-induced suppression in PREG levels was positively correlated with subsequent alcohol intake. A similar relationship has been observed for DOC under the same experimental challenge: dexamethasone-induced suppression of DOC formation was negatively correlated with ethanol self-administration (Porcu et al., 2006). This might suggest that steroid sensitivity to dexamethasone suppression is somehow predictive of subsequent alcohol consumption in the monkeys. Since the monkeys had no alcohol exposure prior to the HPA axis challenges, the correlation between subsequent alcohol drinking and suppression of the PREG (and/or DOC) response to dexamethasone may indicate a trait marker of propensity to drink alcohol. It is worth noting that the responses of PREG and DOC to dexamethasone treatment are inversely related to alcohol intake levels. The explanation for the opposite relationship between the effects of dexamethasone on PREG vs. DOC levels is unclear at this time, but may be related to the differential metabolism of these steroids. PREG can be converted to PREG-sulfate as well as DOC and this difference may have physiological relevance.

In conclusion, we have shown that PREG levels are increased by naloxone activation of HPA axis, but not by CRF and ACTH pharmacological challenges that elevate cortisol levels in monkey plasma. CRF and ACTH administration decrease the ratio of plasma PREG/DOC suggesting that these hormones increase PREG metabolism. Ethanol (1.0–1.5 g/kg) has no effect on PREG levels in monkey plasma, thus suggesting that regulation of its synthesis in monkeys differs from rodents. The evidence that the changes in PREG levels observed after dexamethasone challenge are positively correlated with subsequent daily ethanol consumption over twelve months, might suggest that PREG responses to dexamethasone challenge are indicative of a propensity to drink alcohol.

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