General and Comparative Endocrinology 167 (2010) 317–325

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Grizzly bear corticosteroid binding globulin: Cloning and serum protein expression

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ARTICLE INFO

Article history:
Received 19 January 2010
Revised 18 March 2010
Accepted 22 March 2010
Available online 27 March 2010

Keywords:
Grizzly bears
Ursus arctos
Stress response
Cortisol
CBG
Protein expression

ABSTRACT

Serum corticosteroid levels are routinely measured as markers of stress in wild animals. However, corticosteroid levels rise rapidly in response to the acute stress of capture and restraint for sampling, limiting its use as an indicator of chronic stress. We hypothesized that serum corticosteroid binding globulin (CBG), the primary transport protein for corticosteroids in circulation, may be a better marker of the stress status prior to capture in grizzly bears (*Ursus arctos*). To test this, a full-length CBG cDNA was cloned and sequenced from grizzly bear testis and polyclonal antibodies were generated for detection of this protein in bear sera. The deduced nucleotide and protein sequences were 1218 bp and 405 amino acids, respectively. Multiple sequence alignments showed that grizzly bear CBG (gbCBG) was 90% and 83% identical to the dog CBG nucleotide and amino acid sequences, respectively. The affinity purified rabbit gbCBG antiserum detected grizzly bear but not human CBG. There were no sex differences in serum total cortisol concentration, while CBG expression was significantly higher in adult females compared to males. Serum cortisol levels were significantly higher in bears captured by leghold snare compared to those captured by remote drug delivery from helicopter. However, serum CBG expression between these two groups did not differ significantly. Overall, serum CBG levels may be a better marker of chronic stress, especially because this protein is not modulated by the stress of capture and restraint in grizzly bears.

1. Introduction

Grizzly bear (*Ursus arctos*) numbers are thought to be declining in Alberta, Canada, and populations are experiencing poor performance, including slower growth and reproductive dysfunction (Garshelis et al., 2005; Alberta Grizzly Bear Recovery Plan, 2008). Recent population inventories (2004–2008) have found fewer grizzly bears than expected in many of the population units sampled (Boulanger et al., 2005a,b; Alberta Grizzly Bear Inventory Team, 2007, 2008, 2009). Anthropogenic-related environmental change and the associated long-term stress (chronic stress) are thought to be major factors contributing to high mortality rates in some populations (Nielsen et al., 2008; Stenhouse et al., 2008). However, assessment of chronic stress in free-ranging mammals is difficult because animals are invariably subjected to capture and handling stressors for sampling that may confound interpretation of the stress status prior to capture (Kenagy and Place, 2000). For instance, elevation in serum cortisol levels is widely used as an indicator of stress in animals (Reeder and Kramer, 2005); however, levels of this steroid hormone are elevated in response to capture and handling of grizzly bears and are, therefore, unreliable as a marker of chronic stress (Cattet et al., 2003b; Hamilton, 2007).

The majority of corticosteroid in circulation is bound reversibly and with high affinity to a transport protein, corticosteroid binding globulin (CBG) (Rosner, 1991). Studies have shown that cortisol levels as high as 95% in circulation are bound to CBG in mammals, although serum albumin may also be an important cortisol binding protein with low affinity but high capacity. In addition, nearly 70% of CBG remain free of bound steroid and this protein is thought to influence cellular functions (Gayrard et al., 1996). The unbound fraction of cortisol ("free cortisol") is the biologically active form of the hormone and this is also rapidly eliminated by excretion from the kidneys (Rosner, 1990). Consequently, high CBG levels reduce free cortisol concentration and regulate the cellular actions of this hormone, including protecting tissues from potentially deleterious actions, including protein and lipid catabolism, and suppressed immune function (Rosner, 1990; Boonstra, 2005). Studies in mammals and birds have shown that exposure to chronic stressors elevates plasma cortisol levels while lowering the capacity of CBG to bind free cortisol (Boonstra, 2005; Breuner and Orchinik,

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doi:10.1016/j.ygcen.2010.03.027

2002). This will lead to an increase in free cortisol concentration, which if sustained, can lead to deleterious effects on the health of the animal (Boonstra, 2005). However, nothing is known about either serum levels of CBG or its role in serum cortisol regulation in bears.

Through initial trials, we confirmed that commercially available antibodies to mammalian CBG did not cross-react with bear serum. This necessitated the development of antibodies that specifically detects CBG in grizzly bear serum. The primary objective of this study, therefore, was to clone and sequence grizzly bear CBG and generate polyclonal antibodies to immunodetect this protein in bear sera. It has been shown that serum cortisol levels are modulated by the type of capture and restraint methods used for sampling grizzly bears (Cattet et al., 2003b). For instance, animals captured by leg-hold snares can be restrained for up to 2 hours before they are chemically-immobilized and sampled, whereas bears captured by remote drug delivery from helicopter are immobilized, sampled, and released over a shorter time frame (<1 h). Snared bears typically have higher serum cortisol levels when compared to levels in bears captured by helicopter darting (Cattet et al., 2003b). Consequently, using two different capture methods as a means to modulate serum cortisol levels, we also tested the hypothesis that serum CBG levels are less variable, unlike cortisol, and not altered by capture stress in these animals.

2. Methods

2.1. Animals

For molecular work, we used testis collected from a single adult grizzly bear killed by Alberta Government Fish and Wildlife Officers in defense of human life or property in September 2005. Sera collected from 46 grizzly bears captured between May 1999 and May 2006 for the Foothills Research Institute Grizzly Bear Project (research goals are summarized by Stenhouse and Graham (2007)) within a 150,000-km^2 area of western Alberta, Canada (49°00′–55°50′N, 113°50′–120°00′W), were used for cortisol and CBG determinations. Thirty-nine bears were captured using Aldrich leg-hold snares (Aldrich Snare Co., Clallam Bay, WA) and seven were captured using remote drug delivery from helicopter (helicopter darting). All animals used in our comparisons were sampled after emergence from winter dens (late April to early May) and prior to the end of the mating season (late June) to reduce seasonal variations. All bears were anesthetized by remote drug delivery (Paxarms NZ Ltd., Timaru, New Zealand and Dan-Inject Canada, Edmonton, Canada) using a combination of xylazine and zolazepam–tiletamine administered intramuscularly as xylazine (Cervizine 300; Wildlife Pharmaceuticals, Inc., Fort Collins, CO) at 2 mg/kg and Telazol (Fort Dodge Laboratories, Inc., Fort Dodge, IA) at 3 mg/kg estimated body weight (Cattet et al., 2003a).

Blood was collected by venipuncture from the jugular vein into sterile tubes, and samples were centrifuged within 8 h of collection to extract serum (and this was stored frozen at −20 °C) for cortisol and CBG analysis. At the conclusion of handling, atipamezole (Antisedan; Novartis Animal Health Canada, Inc., Mississauga, ON, Canada) was administered at 0.15–0.20 mg/kg, half-volume intramuscularly and half-volume intravenously, to reverse the effects of xylazine. The capture and sampling protocol was reviewed and approved by the University of Saskatchewan's Committee on Animal Care and Supply, and was in accordance with guidelines provided by the American Society of Mammalogists' Animal Care and Use Committee (Gannon et al., 2007) and the Canadian Council on Animal Care (2003) guidelines for the safe handling of wildlife.

2.2. Cloning and sequencing grizzly bear CBG cDNA

2.2.1. RNA extraction and cDNA synthesis

Approximately 50 mg of grizzly bear testis (kept frozen at −80 °C) was used for total RNA extraction using RNeasy Mini Kit (Qiagen; Mississauga, ON, Canada) and treated with DNase (Qiagen) to remove genomic DNA. RNA samples were quantified at 260/280 nm using a Nanodrop spectrophotometer (Wilmington, DE, USA), and 5 μL was loaded on an RNA denaturing gel to visually assess RNA integrity. First strand cDNA was synthesized using a commercial kit (MBI Fermentas; Burlington, ON, Canada), where 1 μg of total RNA was reverse transcribed using M-MuLV reverse transcriptase in a total volume of 20 μL according to the manufacturer's instruction.

2.2.2. PCR amplification and sequencing of CBG

Primers were designed to amplify two overlapping sections of grizzly bear CBG (Table 1) using Primer3 v0.4.0 software. Primers were designed by aligning dog (*Canis familiaris:* GenBank Accession No. XM_547960) and human (*Homo sapiens*: GenBank Accession No. NM_001756) CBG (hCBG) sequences. CBG RT-PCR amplification consisted of an initial denaturing period of 95 °C for 3 min, followed by 40 cycles of: (1) denaturing at 95 °C for 30 s, (2) annealing at 60 °C for 30 s, and (3) extension at 72 °C for 30 s. This was followed by a 10 min extension period at 72 °C. The PCR products were fractionated in 1.5% agarose gels along with DNA molecular weight standards (Fermentas Life Sciences, Glen Burnie, MD), stained with ethidium bromide, and images were captured under UV light. Amplified products were excised from the gel, purified and sequenced at the York University Core Molecular Biology and DNA Sequencing facility (Toronto, ON). The complete coding domain nucleotide and amino acid sequences for grizzly bear CBG was submitted to GenBank (Accession No. EU571738).

2.3. Multiple sequence alignment and phylogenetic tree

Primary structure sequences for grizzly bear, dog (XP_547960), chimpanzee (XP_510143), human (AAB59523), pig (NP_998977), rat (NP_001009663), sheep (P49920), and opossum (XP_001370999) CBG were aligned by ClustalX 2.0.12. The positions of the steroid-binding residues and conserved cysteine residues of rat CBG (Klieber et al., 2007) were used to determine conserved binding and cysteine residues in bears. A phylogenetic tree was calculated using PHYLIP 3.69 and CBG nucleotide sequences that were aligned by ClustalX. The tree was constructed using the maximum parismony method and bootstrap algorithm with 1000 bootstrap trials.

2.4. CBG peptide antibody synthesis

From the deduced amino acid sequence for grizzly bear CBG, an affinity-purified polyclonal antibody was generated in rabbits for the peptide sequence c-VQAKDPDTDVSPRTPHRDLAPNNVC-n (21st Century Biochemicals, Marlboro, MA, USA).

Table 1
Primers used for RT-PCR. Primers were designed against conserved nucleotide sequences found in dog (*Canis familiaris*) and human (*Homo sapiens*) CBG.

Set 1	Forward primer	5′-TCCCAGGTCACATAGCCAAT-3′
	Reverse primer	5′-CAAGTCTACAATTTTCCCTTGTGTC-3′
Set 2	Forward primer	5′-CCATGGCCTTAGCTATGCTG-3′
	Reverse primer	5′-TTAGGTCGGATTCACAACCTTT-3′