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Evidence for a glucocorticoid receptor beta splice variant in the rat and its physiological regulation in liver

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ABSTRACT

Glucocorticoids are important regulators of metabolism and immune function. Synthetic glucocorticoids are extensively used for immunosuppression/anti-inflammatory therapy. Since the glucocorticoid receptor (GR) is central to most hormone effects; its *in vivo* regulation will influence hormone/drug action. An alternative splice variant, GRβ, is present in humans and may function as a dominant negative regulator of GR transcriptional activity. Recently, a similar splice variant was reported in mouse, although the mechanism of alternative splicing differs from that in humans. We present evidence that a splice variant of GR with an alternative C-terminus also occurs in the rat by a mechanism of intron inclusion. A highly quantitative qRT-PCR assay for the simultaneous measurement of both splice variants in a single sample was developed in order to accurately measure their regulation. We used this assay to assess the tissue specific expression of both mRNAs, and demonstrate that GRα is predominant in all tissues. In addition, the regulation of both GRα and GRβ mRNA by various physiological factors in rat liver was assessed. GRα showed a robust circadian rhythm, which was entrained with the circadian oscillation of the endogenous hormone. Time series experiments showed that both corticosteroids and LPS but not insulin dosing resulted in the transient down-regulation of GRα mRNA. LPS treatment also resulted in down-regulation of GRβ expression. A modest up-regulation in GRβ expression was observed only in animals having chronically elevated plasma insulin concentrations. However the expression of GRβ was significantly lower than that of GRα in all cases.

1. Introduction

Glucocorticoids, a class of steroid hormones which are produced in a distinct circadian pattern by the adrenal cortex, have wide-ranging regulatory effects on both development and metabolism. The regulation of glucose metabolism/energy flux via systemic control of gluconeogenesis is a prominent homeostatic effect. Regulation of energy flux by glucocorticoids involves skeletal muscle and adipose tissue as well as liver. In addition, glucocorticoids have immune modulatory effects which are clinically exploited in the extensive pharmacologic use of synthetic glucocorticoids (corticosteroids) for anti-inflammatory therapy. However, their clinical use is often limited by adverse effects with long term and/or high dose treatment, which primarily stem from a magnification of normal metabolic effects [1,2].

The driving force mediating most glucocorticoid/corticosteroid effects is the glucocorticoid receptor (GR) [3,4]. GR is localized to the cytoplasm in an inactive form complexed with heat shock and associated proteins in the absence of hormone. High affinity binding releases the receptor from the complex when hormone is present and the hormone-receptor complex translocates into the nucleus. Most metabolic effects are mediated by homodimer binding of hormone-bound receptor to specific DNA regulatory sites (GREs) with a subsequent alteration in that gene's rate of transcription, a process generally referred to as transactivation. In contrast, many immune related effects appear to be mediated by a different molecular mechanism, termed transrepression, whereby hormone-bound receptor interacts with and modulates the action of other transcription factors such as NfκB and AP-1. GR is central to most of the molecular actions of glucocorticoids in either case. GR has been documented to be present in most mammalian tissues and cell types consistent with the wide-ranging effects of glucocorticoids.

In humans, an alternative form of the receptor, which data suggests acts as a dominant negative regulator of GR-mediated effects on gene transcription, has been characterized [3,4]. This alternative

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isoform was first predicted in 1985 by Hollenberg and colleagues in conjunction with cDNA cloning of human GR [5]. Subsequently, genomic cloning by Encio et al. demonstrated that this alternative form arises by differential splicing of the same gene [6]. In humans, exons 1–8 are common to both the major receptor isoform (GRα) and the alternative form (GRβ), while the two isoforms diverge in the use of different regions of exon 9. For several years it was generally accepted that the beta isoform of GR did not exist in rodents based on observations by Otto and colleagues [7]. They demonstrated that exon 9 of mouse did not contain alternative splice sites, and RT-PCR with primers specific for the regions of mouse exon 9 comparable to the human GRβ isoform produced no product. However, Hinds et al. more recently demonstrated that an alternative form of GR (termed GRβ) is expressed in mouse both *in vivo* and *in vitro* [8]. In contrast to humans, alternative splicing in the mouse occurs by inclusion of intron 8 rather than differential splicing of exon 9. As with the human isoform, *in vitro* transfection experiments with GRβ expressing plasmids suggest that the beta isoform may function as a negative regulator of GRα transcriptional regulation. Evidence was presented that expressions of the two murine isoforms may be regulated by glucocorticoids, cytokines, and insulin. However, since GRα and GRβ mRNAs were measured separately and reported as relative expression, more quantitative measurements may be useful in assessing the physiological relevance of splice variant expression.

Here we demonstrate alternative splicing of GR occurs in the rat by a mechanism attributed to intron inclusion. A TAQMAN-based real-time qRTPCR assay was developed for the simultaneous and specific measurement of both isoforms in the same sample, quantitated against a chimeric cRNA standard containing both alpha-specific and beta-specific as well as common sequences. *In vivo* expression was measured in a panel of tissues taken from control animals, and physiologically relevant dynamics of regulation in rat liver were examined.

2. Experimental

2.1. Animal experimentation

Tissues used in these experiments were taken from population-based animal studies previously conducted in our laboratories. All protocols adhered to the 'Principles of Laboratory Animal Care' (NIH publication 85–23, revised in 1985) and were approved by the University at Buffalo Institutional Animal Care and Use Committee. Details of MPL administration [9], circadian regulation [10], and diabetic animals [11] have been published. For LPS administration, male Lewis rats (Harlan, Indianapolis, IN) were injected (ip) with a single dose of *Escherichia coli* LPS (serotype 111:B8) purchased from Sigma Chemicals and prepared in sterile saline. Animals received a single sub-lethal dose (50 μg/kg) and were sacrificed under ketamine:xylazine anesthesia at various times after dosing. For acute insulin studies, male Wistar rats (Harlan) were injected with a single dose of human insulin (Humulin R, Eli Lilly, Indianapolis, IN) administered through the penile vein under Nembutal anesthesia. Tissues from all studies were rapidly dissected, frozen in liquid nitrogen, and were stored at −80 °C.

2.2. RNA preparation

Frozen tissue samples were ground into a fine powder in a mortar cooled by liquid nitrogen. Frozen tissue was extracted with Trizol Reagent (InVitrogen, Carlsbad CA) and further purified by passage through RNeasy mini-columns (QIAGEN, Valencia, CA) as previously described [10,11]. All samples exhibited 260/280 absorbance ratios of approximately 2.0, and all showed intact ribosomal 28S and 18S RNA bands in an approximate ratio of 2:1 as visualized by ethidium bromide staining.

2.3. In silico comparison of rat and mouse GR sequences

cDNA RefSeq data were downloaded for GR (nuclear receptor subfamily 3, group C, member 1; Nr3C1) for mouse (NM_008173.3) and rat (NM_012576), and aligned using the BLAST algorithm, NCBI. Alignment of full sequence data for mouse (6345 nts) and rat (6327 nts) indicated 93% homology in the full sequences and 95% homology in identified coding regions. Exon boundary information and sequence data were retrieved from ENSEMBL, with 9 exons delineated in mouse and 8 exons in rat. Intron sequence datum was then obtained from NCBI using this information. Alignment of exon sequences from rat and mouse demonstrated that ENSEMBL exon 1 in mouse (which contains exclusively non-coding sequence) is not present in rat, but otherwise exon boundaries show perfect alignment (Supplementary Table S1 and Fig. S1); therefore the exon designated as 2 by ENSEMBL in mouse is equivalent to exon 1 in rat and so on. Corresponding introns in rat and mouse are roughly similar in size, but show less size similarity and less homology than exons; intron 7 in rat and intron 8 in mouse have 73% identity.

2.4. Conventional RT-PCR

Reverse transcription of rat spleen RNA was carried out using oligo(dT) primer and Superscript III (InVitrogen) in a 20 μl reaction volume containing 1 μg total rat spleen RNA according to manufacturer's instructions. PCR amplification was carried out with Platinum TAQ (InVitrogen) in a 50 μl reaction volume using 1–2 μl of the RT reaction as template. PCR was carried out in a GeneAmp 9700 thermo cycler (Perkin-Elmer) with the following conditions: 94° – 5 min followed by 40 cycles of: 94° – 1 min, 60° – 1 min, 72° – 1 min, and an extension step of 72° – 7 min. PCR products were visualized by agarose gel electrophoresis. Where indicated, PCR products were extracted and purified from agarose gels for sequencing using Illustra GFX PCR DNA and Gel Band Purification kits (GE Healthcare). Automated Sanger sequencing was performed at the Roswell Park Memorial Institute DNA Facility (Buffalo, NY).

2.5. Cloning and cRNA production

A cRNA was used to construct standard curves for subsequent qRTPCR analysis. The cRNA standard was synthesized from a cloned chimeric construct containing sequences included in exons 5–7 (common to alpha and beta) and intron 7 (beta specific) linked to sequences contained in exon 8 (alpha specific) as depicted in Fig. 1. PCR product 1 consisted of a 5-prime Sac I site, positions 1832–2245 of the reference sequence (NM_0012576), positions 1–230 of Intron 7, and a 3-prime Xho I site. PCR product 2 consisted of a 5-prime Xho I site, positions 2259–2661 of the reference sequence, and a 3-prime Bam H1 site. Both PCR products were simultaneously cloned into pGEM 3Z using T4 ligase of restriction digested PCR products. Purified cloned DNA (Qiaprep Spin Miniprep, Qiagen) was verified by sequencing, linearized with Xba I, and a cRNA standard was *in vitro* transcribed from the linear construct (Megascript T7 Kit, Ambion, Austin TX). Size, purity and integrity of the cRNA were assessed by formaldehyde agarose gel electrophoresis and spectrophotometry.

2.6. Development of qRTPCR Assay

A TAQMAN based real-time qRTPCR assay was developed and validated according to MIQE standards [12]. The developed assay allows for the simultaneous measurement of alpha and beta splice