Ex vivo stimulation of whole blood as a means to determine glucocorticoid sensitivity

Christopher Burnsides1,*
Jacqueline Corry1,*
Jacob Alexander1
Catherine Balint1
David Cosmar1
Gary Phillips2
Jeanette I Webster Marketon1,3

1Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Department of Internal Medicine, 2Center for Biostatistics, 3Institute for Behavioral Medicine Research, Wexner Medical Center at The Ohio State University, Columbus, OH, USA

*JC and CB have equally contributed to this work

Purpose: Glucocorticoids are commonly prescribed to treat a number of diseases including the majority of inflammatory diseases. Despite considerable interpersonal variability in response to glucocorticoids, an insensitivity rate of about 30%, and the risk of adverse side effects of glucocorticoid therapy, currently no assay is performed to determine sensitivity.

Patients and methods: Here we propose a whole blood ex vivo stimulation assay to interrogate known glucocorticoid receptor (GR) up- and downregulated genes to indicate glucocorticoid sensitivity. We have chosen to employ real-time PCR in order to provide a relatively fast and inexpensive assay.

Results: We show that the GR-regulated genes, GILZ and FKBP51, are upregulated in whole blood by treatment with dexamethasone and that LPS-induction of cytokines (IL-6 and TNFα) are repressed by dexamethasone in a dose responsive manner. There is considerable interpersonal variability in the maximum induction of these genes but little variation in the EC50 and IC50 concentrations. The regulation of the GR-induced genes differs throughout the day whereas the suppression of LPS-induced cytokines is not as sensitive to time of day.

Conclusion: In all, this assay would provide a method to determine glucocorticoid receptor responsiveness in whole blood.

Keywords: glucocorticoid responsiveness, gene regulation, nuclear receptor, GILZ, FKBP51, cytokines

Introduction
Glucocorticoids have been used in the treatment of many inflammatory diseases, such as rheumatoid arthritis, asthma, multiple sclerosis, and inflammatory bowel disease since the 1940s. Glucocorticoids are one of the most widely prescribed drugs. It has been suggested that 1%-3% of the adult population currently uses glucocorticoids.1

Glucocorticoids are potent regulators of various aspects of immunity.2 They function through a cytosolic receptor, the glucocorticoid receptor (GR), which is a member of the nuclear hormone receptor superfamily. GR binds to DNA to modulate gene transcription.3 Upon ligand binding, GR dissociates from a protein complex that includes heat shock proteins and immunophilins. It then dimerizes and translocates to the nucleus where it binds to specific DNA sequences called glucocorticoid response elements to activate gene transcription.4 GR also interferes with other signaling pathways, such as activator protein-1 and nuclear factor kappa B (NFκB)5 to repress gene transcription. It is by this repression of these pro-inflammatory pathways that many of the anti-inflammatory actions of glucocorticoids are mediated.
Despite their usefulness, glucocorticoid treatment is not without risk. Serious side effects include osteoporosis, adrenal insufficiency, glaucoma, cataracts, and hypertension. Glucocorticoid use has also been associated with increased risk of some cancers, cardiovascular disease, fractures, and infections. Glucocorticoid resistance, inherited or acquired, is a major problem in the treatment of many diseases including asthma, ulcerative colitis, systemic lupus erythematosus, and rheumatoid arthritis, with patients exhibiting glucocorticoid resistance being reported as high as 30%. In addition, it is also estimated that 30% of a normal healthy population is glucocorticoid nonresponsive.

Clinical tests are typically not performed prior to prescription of glucocorticoids. Most clinical decisions are based on cortisol measurements as is used in the treatment of sepsis. However, studies have suggested that steroid bioavailability does not predict clinical outcomes. Assays to determine GR function have been suggested in the literature. These include the dexamethasone suppression test, inhibition of peripheral blood mononuclear cell (PBMC) proliferation, quantification of number and affinity of GRs in PBMCs, glucocorticoid suppression of lipopolysaccharide (LPS)-induced genes (primarily cytokines) in alveolar macrophages and PBMCs or blood, genome-wide promoter-based bioinformatics analysis of transcriptional activity, glucocorticoid-induced cutaneous vasoconstrictor (skin blanching), and glucocorticoid-induced gene expression.

Here we propose an assay to test GR function particularly for the use of glucocorticoids in the treatment of inflammatory diseases. We propose interrogation of both known GR upregulated as well as downregulated genes. Although many anti-inflammatory actions of glucocorticoids are mediated through their ability to downregulate immune factors, they also upregulate genes that are involved in the regulation of inflammation such as glucocorticoid inducible leucine zipper (GILZ), which inhibits the NFκB pathway, and mitogen activated protein kinase phosphatase 1 (MKP-1). We have proposed to use whole nonseparated blood and real-time polymerase chain reaction to make the assay relatively quick and affordable.

Material and methods
Ex vivo stimulation of whole blood

Normal healthy human blood samples were obtained in lavender Hemogard stopper venous blood collection tubes with spray-dried K2 EDTA (10.8 mg) (ThermoFisher Scientific, Pittsburgh, PA) after informed written consent was obtained, as approved by the Institutional Review Board. The demographics of the subjects are shown in Table 1. Three milliliters blood was diluted 1:1 with phosphate buffered saline and triplicates of 500 μL aliquots were either untreated or treated with 100 nM dexamethasone (Sigma-Aldrich, St Louis, MO), 1 ng/mL LPS (Sigma-Aldrich), or a combination of dexamethasone and LPS for 2 hours at 37°C. Following incubation, red blood cells (RBC) were lysed by addition of 3 mL of RBC lysis buffer (5 M NH₄Cl, 5 M KHCO₃, 1 mM EDTA) for 15 minutes at room temperature and centrifugation at 1400 rpm for 10 minutes. The pellet was washed twice more with 500 μL RBC lysis buffer and centrifuged for 2 minutes at 3000 rpm to ensure removal of all hemes. The pellets were resuspended in 400 μL of Ribozol RNA extraction reagent (ISC BioExpress, Kaysville, UT).

Quantification of GR-regulated genes by real-time PCR

Total RNA was extracted using the Ribozol RNA extraction reagent following the manufacturer’s instructions with the following modifications. Volumes were adjusted to fit the 400 μL volume of Ribozol, and the isopropanol precipitation step was allowed to proceed overnight at ~20°C. In addition, 75% ethanol was chilled before washing the pellet and an additional 1 minute centrifugation at 7500 × g was added to remove excess ethanol. Five hundred nanograms RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Twenty microliters of cDNA was diluted by addition of 100 μL DEPC-treated water. Real-time PCR was performed on 8 μL diluted cDNA in a 20 μL reaction volume containing 1X Power SYBR® Green PCR master mix (Applied Biosystems) and 0.125 μM of each primer (Table 2). The following protocol was used on the ABI 7300 real-time PCR system (Applied Biosystems): heated to 50°C for 2 minutes, denatured for 10 minutes at 50°C, and then subjected to 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Duplicate cycle threshold (Ct) values of triplicate samples were analyzed using the comparative Ct (ΔΔCt) method (Applied Biosystems). The fold induction (2-ΔΔCt) was obtained by normalizing to two endogenous genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclic AMP-accessory protein (CAP-1), and expressed relative to

Table 1 Demographics of normal human subjects (n = 12)

<table>
<thead>
<tr>
<th>Age, mean ± 1 SD</th>
<th>32.7 ± 7.3 years</th>
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<tbody>
<tr>
<td>Sex</td>
<td>8 female/4 male</td>
</tr>
<tr>
<td>Race</td>
<td>10 Caucasian/1 Asian/1 other</td>
</tr>
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</table>

**Abbreviation:** SD, standard deviation.
the amount in nontreated cells (GR-induced genes) or LPS only treated cells (GR repressed genes).

Interleukin 6 (IL-6) enzyme-linked immunosorbent assay (ELISA) methods

Whole blood was stimulated as above for 24 hours. Serum was then separated by centrifugation at 10,000 × g for 10 minutes. Serum was stored at −80°C until assayed. IL-6 was assayed by ELISA using the Human IL-6 ELISA MAX Set Deluxe Kit (Biolegend, San Diego, CA) according to manufacturer’s instructions.

Statistical methods

In this descriptive study summary statistics (coefficient of variation [CV], median, and the interquartile range [IQR]) are presented as box plots in Figures 1 and 2 (Table 3). A CV greater than one is considered to have a high degree of variability relative to its mean. For Figures 3–5 the median and the IQR are presented as summary statistics (Table 4). Random-effect linear regression was used to estimate the slope over time or over pre- and post-treatment. Random effects were used since observations are nested within a particular subject. This method takes into account the variability within and between patients and uses this inherent correlation when estimating the standard errors that are used to test slope coefficients. As this study was descriptive in nature, no inferential test comparing different groups were run. All analyses were run using Stata software (v 12.0; Stata Corporation, College Station, TX).

### Results

Interpersonal variations in GR function in whole blood

Whole blood, drawn from 12 normal healthy volunteers at 8:30 am ± 1 hour who had not eaten for 1 hour prior to the blood draw, was stimulated ex vivo with 100 nM dexamethasone (Figure 1A) or with 1 ng/mL LPS in the presence and absence of 100 nM dexamethasone (Figure 1B) for 2 hours. RNA was isolated and the expression of the known GR upregulated genes — GILZ, FK506 binding protein 51 (FKBP51), arachidonate 5-lipoxygenase-activating protein (FLAP); also known as ALOX5AP), and MKP-1 — and the known GR downregulated genes — IL-6, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) — were determined by real-time PCR. As can be seen in Figure 1A, 100 nM dexamethasone induced GILZ, FKBP51, FLAP, and MKP-1 with considerable interpersonal variation. GILZ was induced 3–6-fold (coefficient of variance [CV] of 0.149) against LPS only. Dexamethasone repressed genes IL-6, tumor necrosis factor α, and interferon γ — were determined by real-time PCR. As can be seen in Figure 1A, 100 nM dexamethasone induced GILZ, FKBP51, FLAP, and MKP-1 with considerable interpersonal variation. GILZ was induced 3–6-fold (coefficient of variance [CV] of 0.149) against LPS only.

### Table 2 Real-time PCR primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer (5'−3')</th>
<th>Reverse primer (5'−3')</th>
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<tbody>
<tr>
<td>GILZ</td>
<td>gcacaetttctcactcctccc</td>
<td>tccagactcctcactcctccc</td>
</tr>
<tr>
<td>FKBP51</td>
<td>cccctgacgcttcctccc</td>
<td>aaacactcctcactcctccc</td>
</tr>
<tr>
<td>FLAP</td>
<td>gcgggtgacgcttcctccc</td>
<td>aataactcctcactcctccc</td>
</tr>
<tr>
<td>MKP-1</td>
<td>gcgcgggtgcctcctccc</td>
<td>aaacactcctcactcctccc</td>
</tr>
</tbody>
</table>

Abbreviations: GILZ, glucocorticoid inducible leucine zipper; FKBP51, FK506 binding protein 51; FLAP, arachidonate 5-lipoxygenase-activating protein; MKP-1, mitogen activated protein kinase phosphatase 1.
Diurnal variation of GR function

Given the natural diurnal cortisol cycle, we next tested the effect of time of day on this assay. Whole blood from 10 individuals (Figure 3) was drawn at 8:30 am, 12:30 pm, 4:30 pm, and 8:30 pm ± 1 hour and was stimulated with 100 nM dexamethasone in the presence or absence of 1 ng/mL LPS for 2 hours. RNA was isolated and the expression of the GR upregulated genes GILZ and FKBP51 and the GR downregulated genes IL-6 and TNFα (Figure 3B) determined by real-time PCR. The GR upregulated genes showed a significant increase in their induction (decrease in ΔΔCt) by 100 nM dexamethasone across the duration of the day (GILZ ΔΔCt slope −0.04, P-value 0.002; FKBP51 ΔΔCt slope −0.06, P-value < 0.001). Dexamethasone suppression of LPS-induced IL-6 significantly increased from 8:30 am to 12:30 pm (ΔΔCt slope 1.69, P-value 0.019) but then did not alter for the remainder of the day. Dexamethasone suppression of LPS-induced TNFα was not significantly altered during the day.

Practicality of the assay

In order to test the practicality of the assay, given that the assay may not be able to be performed immediately, we tested the effect of storing the blood (in the EDTA tube) in a refrigerator for 24 hours prior to performing the assay. Again, two upregulated and two downregulated genes were tested in 10 individuals (Figure 4). Whole blood was drawn and either the assay was performed immediately (closed squares) or the EDTA tubes were stored at 4°C for 24 hours before performing the assay (open squares). As can be seen in Figure 4A and B dexamethasone induction of GILZ and
FKBP51 and dexamethasone suppression of LPS-induced IL-6 and TNFα was considerably different in the sample that had been stored for 24 hours compared to the sample that was analyzed fresh.

Finally, we compared this assay to the dexamethasone suppression of LPS-induced cytokines analyzed by ELISA, which has been previously used. Whole blood was stimulated with 100 nM dexamethasone (A and B) or 1 ng/ml LPS with and without 100 nM dexamethasone (C and D) for 2 hours. RNA was isolated and cDNA transcribed. Real-time PCR was performed to determine expression of the GR upregulated genes GILZ (A) and FKBP51 (B) and the GR downregulated genes IL-6 (C) and TNFα (D).

Notes: The expression of these genes was normalized to the housekeeping genes GAPDH and CAP-1. $\Delta\Delta$Ct was calculated for GILZ and FKBP51 as treatment (dexamethasone) against no treatment and for IL-6 and TNFα as treatment (dexamethasone + LPS) against LPS only. The slope of the $\Delta\Delta$Ct value was calculated and tested for significance using a random-effects linear regression analysis.

Abbreviations: LPS, lipopolysaccharide; PCR, polymerase chain reaction; GR, glucocorticoid receptor; GILZ, glucocorticoid inducible leucine zipper; FKBP51, FK506 binding protein 51; IL-6, interleukin 6; TNFα, tumor necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAP-1, cyclic AMP-accessory protein; $\Delta\Delta$Ct, comparative cycle threshold.

Figure 3 Whole blood was drawn from 10 individuals at 8:30 am, 12:30 pm, 4:30 pm, and 8:30 pm and was stimulated with 100 nM dexamethasone (A and B) or 1 ng/mL LPS with and without 100 nM dexamethasone (C and D) for 2 hours. RNA was isolated and cDNA transcribed. Real-time PCR was performed to determine expression of the GR upregulated genes GILZ (A) and FKBP51 (B) and the GR downregulated genes IL-6 (C) and TNFα (D).

Measure of glucocorticoid responsiveness

Finally, we compared the glucocorticoid suppression of LPS-induced cytokines analyzed by ELISA, which has been previously used. Whole blood was stimulated with 100 nM dexamethasone in the presence or absence of 1 ng/mL LPS for either 2 or 24 hours. After 2 hours RNA was isolated, cDNA prepared, and the expression of IL-6 mRNA determined by real-time PCR (circles). After 24 hours, serum was separated and IL-6 protein levels were determined by ELISA (squares). As can be seen in Figure 5 the extent of repression of LPS-induced IL-6 production is comparable between these two different assays.

Discussion

We have shown that ex vivo stimulation of whole blood can be used to assess GR function by interrogating known GR up- and downregulated genes. Figure 1 shows that there is considerable interpersonal variation in the maximal induction or repression of these genes. However, there is less variability in the EC$_{50}$ and IC$_{50}$ values (Figure 2) suggesting that the relative efficacy for dexamethasone is similar between individuals but the extent of the response is varied. These data also indicate that glucocorticoid induction of GR upregulated genes increases throughout the course of the day, when serum cortisol levels are decreasing, but that glucocorticoid suppression of LPS-induced cytokines is relatively unchanged during the day (Figure 3). In our studies, the subject had not eaten for 1 hour before the blood draw (self-reported) but food intake, as well as other factors, could affect the outcome of this assay. Therefore, it is necessary to control that as many factors as possible (such as time of day and food intake) remain constant when making comparisons either between different individuals or within the same person.

In an attempt to address the practicability of this assay, we assessed if blood could be stored in the collection tubes prior to performing the assay. The data in Figure 4 would suggest that it is not possible and further work would be needed to identify conditions, which would allow for prolonged storage prior to the assay. Indeed, another study suggested that delayed processing decreased glucocorticoid sensitivity.$^{46}$ Finally, we compared the glucocorticoid suppression of LPS-induced cytokines measured by real-time PCR and ELISA (Figure 5). Comparable results were found with...
that it would be more appropriate to assess a combination of both up- and downregulated genes as some GR upregulated genes, such as GILZ\(^{40-43}\) and MKP-1,\(^{44}\) also play a role in the anti-inflammatory properties of glucocorticoids.

Other assays that have been used include the dexamethasone suppression test,\(^{20-25}\) which measures the response of the adrenal glands to adrenocorticotropic hormone; inhibition of stimulated PBMC proliferation,\(^{20,27-30}\) which correlates with the response to glucocorticoid therapy in some diseases;\(^{12,13,48,49}\) quantification of the number and affinity of GR in the PBMCs,\(^{20,30,31}\) bioinformatics profiling,\(^{37,38}\) and cutaneous vasoconstrictor (skin blanching),\(^{21,39}\) which determines topical potency of glucocorticoids. Attempts to obtain concordance between many of these assays have failed. Chriguer et al compared the dexamethasone suppression test, PBMC proliferation assay, and the numbers of GR in normal healthy volunteers. They found concordance between these assays in the majority of people but in a few there was no concordance suggesting possible tissue-specific glucocorticoid sensitivity.\(^{20}\) Ebrecht et al found no concordance between the dexamethasone suppression of LPS-induced cytokines, skin blanching, and the dexamethasone suppression test in healthy volunteers, again suggesting target tissue specificity.\(^{21}\) Vasiliadi et al also found no concordance between the dexamethasone suppression test
and dexamethasone inhibition of LPS-induced TNFα and Smit et al found no concordance between the dexamethasone suppression test and expression of GR up- and downregulated genes. Concordance between the cutaneous vasoconstrictor assay and glucocorticoid sensitivity in asthma has been varied.

Others have shown that there is considerable interpersonal variability in glucocorticoid responses. Interestingly, serum cortisol did not correlate with genomewide transcriptional analysis of GR and NFκB-regulated genes. A few studies have investigated the effect of the diurnal cortisol cycle on these assays but with conflicting results. No difference in dexamethasone suppression of LPS-induced cytokines in the evening or morning was seen in one study. In another, increased glucocorticoid inhibition of LPS-induced TNFα was seen in the morning compared to the evening hours. However, both these studies pooled several individuals and are confounded by possible interpersonal variations. Recently, a study has described differences in glucocorticoid sensitivity in morning and afternoon samples but comparison within the same individual was not made. Again, confounding factors could also affect the outcome of this and other GR functional assays, including stress (social isolation stress), depression, exercise, and disease state.

### Conclusion
We suggest that this assay could be used to determine glucocorticoid sensitivity. One caveat is that this assay will not be able to determine differences in glucocorticoid sensitivity in a person if it is tissue specific. However, if it is possible for viable cells to be obtained from the tissue of interest, for example by bronchoalveolar lavage, then this assay may prove useful in analyzing tissue specific responsiveness.

### Acknowledgments/disclosure
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### References


