

Circulating Glucocorticoid Bioactivity in the Preterm Newborn after Antenatal Betamethasone Treatment

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Antenatal glucocorticoid treatment of mothers at risk of premature delivery is highly cost-effective in reducing neonatal mortality and morbidity. However, there is only limited information on the actual glucocorticoid bioactivity (GBA) reaching the fetus. By employing a recently developed recombinant cell bioassay, we studied circulating GBA in preterm newborns exposed to the standard antenatal betamethasone regimen (12 mg betamethasone twice, 24-h interval, for the mother; repeated in 7–10 d if required). Plasma GBA and cortisol concentrations were measured in cord blood of 71 infants (mean gestational age, 28.9 wk; range, 24.6–32 wk; mean birth weight, 1208 g; range, 480–2010 g). The median time between the last administered betamethasone dose and birth was 2.0 d. Cord GBA ranged from less than 15.6 to 170 nmol/liter cortisol equivalents. The level was highly dependent on the time between the last betamethasone dose and birth, *i.e.* infants born

shortly (<12 h) after the last steroid dose displayed on average 4-fold higher GBA than that in the reference group (infants with >7 d since the last betamethasone dose before birth or without treatment; 74 vs. 21 nmol/liter cortisol equivalents; $P < 0.0001$). By contrast, if more than 72 h had elapsed between the last steroid dose and birth, circulating GBA was strongly dependent on cord cortisol ($r = 0.85$; $P < 0.0001$; $n = 30$). In multiple regression analysis adjusted for cord cortisol concentration and the time since the last steroid dose, increased umbilical artery resistance, a sign of severe fetal distress, was associated with lower cord GBA ($P = 0.01$). In conclusion, antenatal exposure of preterm fetuses to betamethasone causes a sizeable, but brief, peak of supraphysiological GBA, and approximately 3 d after the last betamethasone dose, circulating GBA derives from cord cortisol concentration. (*J Clin Endocrinol Metab* 89: 3999–4003, 2004)

A SINGLE COURSE of antenatal glucocorticoid for mothers at risk of preterm delivery is well established in reducing neonatal morbidity and mortality (1, 2). Recent evidence, however, has suggested that repeated treatment courses may be associated with marked side-effects that outweigh any benefits (1–3). This subject is of further interest because subtle variations in the perinatal glucocorticoid milieu have been suggested to exert life-long programming effects on an individual's glucocorticoid metabolism, affecting the risk of common late-life disorders such as cardiovascular disease (4).

As yet, there is poor understanding of the net effect of routine antenatal glucocorticoid treatment on circulating glucocorticoid bioactivity (GBA). Betamethasone, a widely used antenatal glucocorticoid, is transferred in effective amounts through the placenta (5) and is not bound by serum corticosteroid-binding globulin (6). This could be expected to result in high glucocorticoid bioactivity in the fetus, but this idea has not been characterized in detail. Moreover, it is not known whether the reduced cord vein (7) and postnatal (8) serum cortisol concentrations after antenatal betamethasone exposure are a sign of adrenal suppression, *i.e.* whether they are accompanied by reduced circulating GBA.

Abbreviations: GBA, Glucocorticoid bioactivity; excess GBA, glucocorticoid bioactivity not caused by cortisol; GR, glucocorticoid receptor.

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To provide data essential for optimizing the effect of perinatal glucocorticoid therapy in the fetal circulation, we employed a recently developed recombinant cell bioassay (9) after routinely administered antenatal betamethasone treatment. The bioassay is based on the expression of human glucocorticoid receptor (GR) together with an appropriate reporter gene in mammalian cells, and it allows assessment of circulating GBA in a small volume of patient sample, even in preterm newborns.

Subjects and Methods

Study population

After the exclusion of the infants of mothers receiving inhaled ($n = 5$) or systemic ($n = 2$) glucocorticoids other than betamethasone, the study population consisted of 71 preterm infants born before 32 wk gestation at Helsinki University Central Hospital (Helsinki, Finland). Table 1 shows their clinical data.

Gestational age was confirmed by ultrasonography before 20 wk gestation. The infants were weighed immediately after birth. To describe intrauterine growth in units adjusted for gestational age, relative birth weight, expressed in sd units, was determined separately for both sexes with reference to current Finnish standards (10). Maternal hypertension during pregnancy was defined as systolic blood pressure of 140 mm Hg or more, a diastolic blood pressure of 90 mm Hg or more, or a 30-mm Hg or greater increase in systolic blood pressure or a 15-mm Hg or greater increase in diastolic blood pressure. Preeclampsia was diagnosed when proteinuria of 0.3 g/d or more was present together with hypertension. Increased umbilical artery resistance was defined as Doppler flow velocitometry showing an umbilical artery resistance index of 2 sd or more above the mean for gestational age (11). Diagnosis of gestational diabetes was based on the oral glucose tolerance test, with a venous plasma glucose level exceeding 4.8 mmol/liter (baseline), 10.0 mmol/

liter (1 h), or 8.7 mmol/liter (2 h). Neonatal respiratory distress syndrome was diagnosed when an infant less than 24 h of age required treatment with a ventilator with either an inspired air oxygen fraction over 40% or a mean airway pressure over 7 cm H₂O or an arterial/alveolar oxygen ratio less than 0.22. Persistent ductus arteriosus was recorded when its closure required treatment by means of indomethacin or surgery.

Betamethasone (12 mg, im, twice, 24-h interval; treatment repeated in 7–10 d if necessary) was administered as antenatal glucocorticoid treatment when a preterm delivery was imminent. The time between the last betamethasone dose and birth, and the number of betamethasone treatments were both considered as variables in the data analysis.

The study protocol was approved by the institutional review board of Helsinki University Central Hospital.

Biochemical assays

Cord vein blood was drawn into EDTA tubes (Vacuette, Greiner Bio-One, Kremsmunster, Austria), with plasma separated immediately and stored at –20 C until analyzed. Plasma cortisol concentrations were

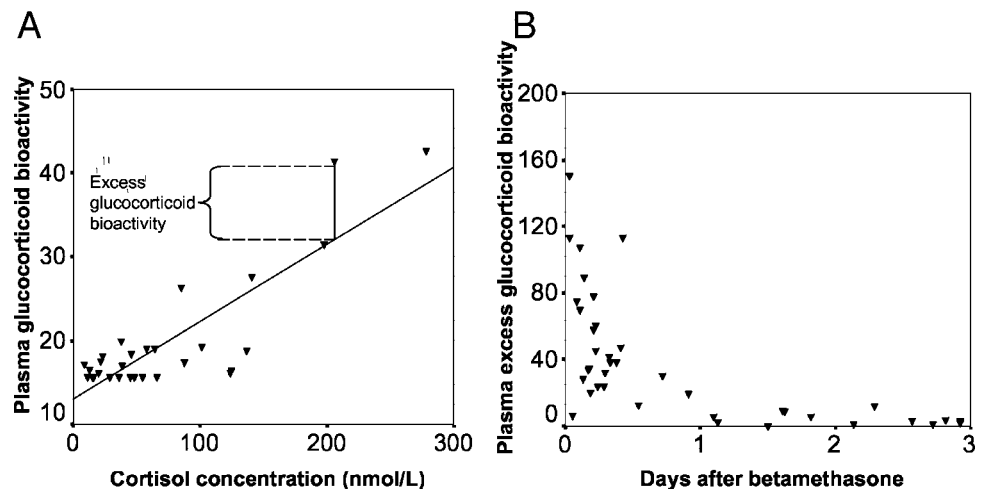
TABLE 1. Clinical data

Gestational age (wk)	28.9 ± 2.1 (24.6–32.0) ^a
Birth weight (g)	1208 ± 379 (480–2010) ^a
Relative birth weight (SD units)	–0.89 ± 1.49 (–4.3 to –3.0) ^a
Male/female	37/34
Twins	21
Preeclampsia	15
Maternal hypertension other than preeclampsia	13
Increased umbilical artery resistance	19 ^b
Diabetes	
Type 1	4
Gestational	5
Cesarean section	42
No. of betamethasone courses	
0	3
1	54
2	8
3	6
Time since last betamethasone	
<12 h	24
12–72 h	17
72 h to 7 d	17
>7 d	10

^a Mean ± SD (range).

^b Of the 19 subjects with increased umbilical artery resistance, 13 were from a preeclamptic pregnancy, and 4 were from a pregnancy with maternal hypertension not fulfilling the criteria of preeclampsia.

FIG. 1. A, Calculation of excess plasma glucocorticoid bioactivity. The figure shows plasma cortisol concentration (nanomoles per liter) and plasma glucocorticoid bioactivity (nanomoles per liter cortisol equivalents) in 30 preterm infants in samples taken a minimum of 72 h after the last betamethasone dose or in infants whose mothers received no betamethasone, *i.e.* with no betamethasone expected in the circulation (7). Excess glucocorticoid bioactivity, in relation to cortisol concentration, is denoted by the residual to the regression slope. B, Relationship between excess glucocorticoid bioactivity (nanomoles per liter cortisol equivalents) and time since last betamethasone dose (days) in infants at a maximum of 72 h after the last betamethasone treatment.



measured using a direct method with Guildhay antiserum HPS631/1G and a cortisol-3 carboxymethylxime-histamine-[¹²⁵I] tracer as described previously (12). The cross-reactivity of this assay with cortisone is 1.2% (12).

GBA was measured directly from 10- μ l plasma samples using a recombinant cell bioassay in which COS-1 cells are transfected with expression vectors encoding human GR and a nuclear receptor coregulator, ARIP3, together with an appropriate reporter gene (9). In the current work, GBA values less than 15.6 nmol/liter cortisol equivalents were considered undetectable. In data analyses, values below this limit were set at 15.6 nmol/liter cortisol equivalents. The GBA assay was originally validated by use of human serum (9), but samples of EDTA plasma were used in the current study. EDTA inhibits the blood clotting cascade by chelating calcium. Even small differences in blood sample volume may influence the final EDTA concentration in plasma, and subsequently, the cells in the bioassay are potentially exposed to a low free calcium concentration. In accordance with this hypothesis, samples from nine subjects not included in the study caused a cell viability problem during the bioassay, which was abolished by supplementing the culture medium with 4.1 mM CaCl₂ before dilution of the plasma samples 1:10. The plasma samples in this study did not, however, display problems with regard to cell viability.

Due to the small amount of plasma available from each newborn, we were unable to estimate assay precision in a conventional fashion. However, plasma glucocorticoid bioactivity in seven pairs of twins correlated strongly ($r = 0.99$), indicating high precision of the bioassay. In addition, in samples expected to contain no betamethasone, plasma glucocorticoid bioactivity levels correlated strongly with results obtained in a conventional RIA ($r = 0.85$; $P < 0.0001$; Fig. 1A); the strength of this correlation was similar to that after previous measurements carried out in human serum (9).

Data analysis

Right-skewed variables (cortisol concentration and total and excess GBA) were log-transformed to normal distributions. Simple and multiple linear or logistic regression was used to assess correlation between variables. Because of the logarithmic transformation of the independent variables, each regression coefficient indicates the percent change in the independent variable (GBA or cortisol) caused by one unit change in the dependent variable. When two groups were compared, a *t* test was used. To allow for possible nonlinear effects, reference cell dummy coding was employed to account for the time between the last betamethasone dose and birth. Subjects were divided into four groups according to the time between the last betamethasone dose and birth: 1) less than 12 h, 2) 12–72 h, 3) 72 h to 7 d, and 4) more than 7 d or no betamethasone. A dummy variable was created for each of groups 1, 2, and 3 and coded 1 if the subject belonged to that group and 0 otherwise. Based on the results of previous studies (7, 13), subjects in group 4 were no longer expected to show any effect of administered betamethasone. They had each dummy variable coded as 0. This coding allowed group 4 to serve as a reference

group; the regression coefficient of each dummy variable in groups 1, 2, and 3 denotes difference from the reference group (14).

Results

The clinical characteristics of the 71 preterm infants are shown in Table 1. In the 30 infants who had received betamethasone more than 72 h before birth or no betamethasone, cord vein GBA was highly dependent on the cord plasma cortisol concentration ($r = 0.85$; $P < 0.0001$; Fig. 1A). Based on this strong correlation, excess GBA, *i.e.* GBA not caused by cortisol, was calculated as the residual to the regression slope in this group, as shown in Fig. 1A. In infants who had received betamethasone within 12 h before birth, most (85%) of the mean total GBA consisted of excess GBA (Fig. 1B). However, excess GBA as well as total GBA had decreased to a stable level when 24–48 h had elapsed between the last steroid dose and birth (Fig. 1B).

Figure 2 summarizes cord plasma GBA, excess GBA, and cortisol concentrations at different time intervals after antenatal betamethasone administration. The infants with at least 7 d between the last betamethasone dose and birth together with infants not exposed to betamethasone served as a reference group ($n = 13$). In comparison with the reference group, cord vein cortisol concentrations were lower in all other groups of infants who had received betamethasone less than 7 d before birth (Fig. 2). However, there was no evidence of suppression of plasma GBA or excess GBA (GBA not caused by cortisol) after betamethasone treatment (Fig. 2). We further adjusted for possible confounding factors by calculating regression equations explaining the contribution of each independent variable to GBA (Table 2). Again, there was no evidence of suppression of GBA at any time after the last betamethasone dose. When the regression model was not adjusted for cord vein cortisol concentration (not shown), the total number of betamethasone doses given showed a negative association with GBA, suggesting that betamethasone suppressed maternal and/or fetal adrenal function and cortisol production. Accordingly, this association faded away when cortisol concentration was entered into the equation (Table 2). Therefore, we calculated an additional regression equation with plasma cortisol concentration as the dependent variable. Adjusting for gestational age, mode of delivery, and the time since the last betamethasone dose, one additional course of betamethasone appeared to be associated with a 42% decrease (95% confidence interval, 24–56%; $P = 0.0002$) in cord vein cortisol concentration.

Increased umbilical artery resistance was associated with lower plasma GBA. This relationship remained significant even after adjustment for potential confounding factors (Table 2). Similar, albeit weaker, correlations were seen for pre-eclampsia and maternal hypertension. Plasma GBA was not associated with gestational age at birth, relative birth weight, or the presence or absence of maternal diabetes, neonatal respiratory distress syndrome, or patent ductus arteriosus. However, higher cord vein GBA was associated with a higher minimum mean arterial pressure ($P = 0.04$) during the first 24 h after birth.

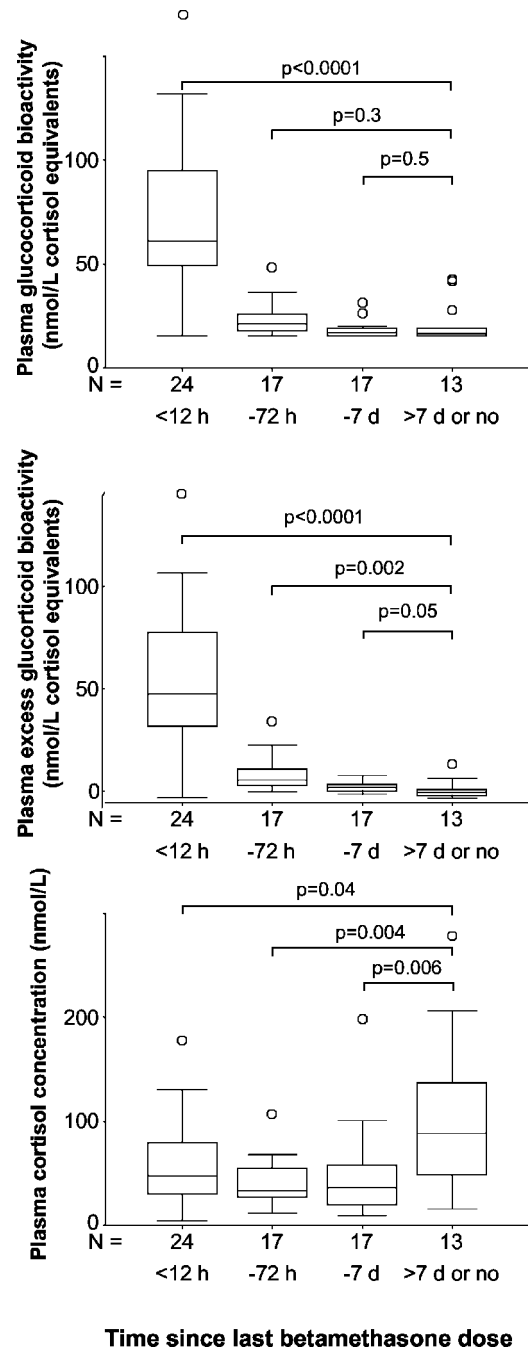


FIG. 2. Box plots (median, range, 25th and 75th percentiles, and extreme values) of plasma glucocorticoid bioactivity, excess glucocorticoid bioactivity, and cortisol concentration in relation to the time between the last betamethasone dose and birth. Values of P indicate two-way comparisons with the reference group (infants unexposed to betamethasone or with at least 7 d between the last betamethasone exposure and birth).

Discussion

The efficacy of antenatal glucocorticoid treatment was recognized 3 decades ago (15). Thereafter, this therapy been established to bring about a reduction in neonatal mortality, respiratory distress syndrome, and intraventricular hemorrhage (1). Soon after the therapy was introduced into clinical

TABLE 2. Multiple regression model demonstrating the contribution of each independent variable to variation in circulating glucocorticoid bioactivity

	Time since last betamethasone			No. of betamethasone courses	Increased umbilical artery resistance	Cord vein cortisol (nmol/liter)
	0–12 h	12–72 h	72 h to 7 d			
% Change	280 (190–500)	43 (7–90)	23 (–8 to 63)	–3.1 (–17.0 to 13.2)	–29 (–45 to –8)	22 (7–38)
<i>P</i> value	<0.0001	0.02	0.2	0.7	0.01	0.003

Each regression coefficient is expressed as the percent change (95% confidence interval in *parentheses*) in GBA produced by a one-unit change in the dependent variable. For the effect of the time between last betamethasone dose and birth, the percentages refer to comparison with the reference group, *i.e.* infants with at least 7 d between the last betamethasone dose and birth and those who received no betamethasone. The regression equation is adjusted for gestational age at birth, relative birth weight, and mode of delivery. Adjusted r^2 of the model = 0.73.

practice, there were reports on its effects on the circulating glucocorticoid milieu of the fetus (7, 13). The methodology, however, was indirect and cumbersome, relying on a GR binding assay that involved use of a rat hepatoma cell line, after which the effects of cortisol and betamethasone were distinguished by an assay based on the specific binding of cortisol to corticosteroid-binding globulin. The GR binding assay requires an extraction procedure (16), and thus the potential effect of circulating steroid-binding proteins on the bioavailable glucocorticoids is ignored. In addition, an *in vitro* receptor binding assay is unable to distinguish between different receptor agonists and antagonists that may both bind to GR. The 4-fold higher mean cord plasma GBA we found in preterm infants at less than 12 h since the last betamethasone dose before birth corresponds well with the previously observed 4-fold increase in peak GR binding during a similar betamethasone regimen (7). Moreover, our results are essentially in accord with a previous observation of the fairly short duration of excess GBA after betamethasone treatment (7).

One of the concerns regarding antenatal glucocorticoid treatment has been the possibility of adrenal suppression. Obviously, in this and previous studies (7, 13, 17–19), maternal and fetal cortisol levels are decreased by means of negative central feedback as long as the synthetic glucocorticoid remains in the circulation. Nevertheless, whether a single course of betamethasone could be associated with decreased GBA attributable to hypothalamic-pituitary-adrenal axis suppression has been doubtful. Previous radio-receptor assay studies have suggested a period of slightly decreased receptor binding between 2.5 and 7 d after a single betamethasone course (7). However, we did not find a corresponding period of decreased GBA, arguing against any major suppressive effect of a single betamethasone course on cord vein cortisol. It must be emphasized that we assessed conditions at birth, not the ability of the hypothalamic-pituitary-adrenal axis of the infant to respond to stressful events during postnatal life. This is a major question in present day neonatology, in which the possible role of antenatal glucocorticoids in postnatal adrenal insufficiency remains unresolved.

We found a weak association between reduced GBA and a higher number of glucocorticoid courses. As expected, this association was attenuated when adjusted for cortisol concentration, which, in turn, was clearly negatively associated with the number of glucocorticoid treatments given. This suggests that at the least, repeated betamethasone doses are associated with suppression of maternal or fetal cortisol synthesis, or both. Repeating a course of antenatal glucocorti-

coids may be harmful and is no longer recommended outside clinical trials (2). Thus, our findings add to observations indicating possible adverse effects of multiple betamethasone treatment courses.

Increased umbilical artery resistance is an end-stage feature of severe intrauterine growth restriction, preeclampsia, and other disorders of impaired placental function and as such is probably the most specific marker of severe fetal distress. Perhaps surprisingly (19), in the present study it appeared to be associated with reduced GBA, even after adjustment for potential confounding factors and cortisol concentration. It is of note that infants with intrauterine growth restriction and those born after preeclamptic pregnancies have an increased risk of respiratory distress syndrome (20). Although these infants do benefit from antenatal glucocorticoids (2), there are reports suggesting that the effect may be reduced compared with that in infants with other etiologies of prematurity (20, 21). However, it must be emphasized that our findings need to be verified in prospective work before any conclusions can be drawn.

Although the benefits of a single course of antenatal glucocorticoids are well established, indications for postnatal glucocorticoids in small preterm infants remain much more controversial (22). A number of small preterm infants exhibit cortisol concentrations that seem disproportionately low in relation to the severity of their illness, and some appear to benefit from low dose cortisol replacement (23). On the other hand, much longer and more intense dosage regimens, such as a 42-d dexamethasone course (24), have been used, for example, to facilitate the weaning of an infant from a respirator. Such long regimens have, however, recently been associated with major side-effects, including cerebral palsy (25) and impaired neuromotor and cognitive function at school age (26). The current transactivation bioassay is not capable of measuring all of the biological effects of glucocorticoids, which also include repression of gene expression and/or nongenomic effects possibly mediated by steroid-selective membrane receptors or direct interactions with cell membranes (27, 28). However, considering the above-mentioned discrepancies in the clinical use of glucocorticoids, a bioassay such as ours should be valuable in future trials in terms of enabling comparisons between bioactivities brought about by different steroids and their relationships to desired and undesired treatment effects.

In conclusion, a routinely used betamethasone regimen given to mothers with an imminent premature delivery results in high cord vein GBA that returns to the reference level 1–2 d after the last steroid dose. This regimen was not associated with a subsequent period of subnormal cord plasma

GBA, although repeated treatment courses suppressed cord vein cortisol and GBA levels. Given the large number of glucocorticoid-related controversies in present day peri- and neonatology, the recombinant cell bioassay should have wide implications in terms of evaluation of the effects of exogenous or physiological glucocorticoids under different clinical conditions.

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