

## Proinflammatory cytokines inhibit human placental 11 $\beta$ -hydroxysteroid dehydrogenase type 2 activity through Ca<sup>2+</sup> and cAMP pathways

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**Kossintseva, Iren, Susan Wong, Ed Johnstone, Larry Guilbert, David M. Olson, and B. F. Mitchell.** Proinflammatory cytokines inhibit human placental 11 $\beta$ -hydroxysteroid dehydrogenase type 2 activity through Ca<sup>2+</sup> and cAMP pathways. *Am J Physiol Endocrinol Metab* 290: E282–E288, 2006. First published September 20, 2005; doi:10.1152/ajpendo.00328.2005.—Excessive fetal exposure to glucocorticoids has been implicated in the etiology of adult metabolic and cardiovascular disease. Placental 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ -HSD2) may protect the fetus from excessive glucocorticoid exposure. Maternal stress may be accompanied by elevated levels of cortisol and increased proinflammatory cytokines [interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )]. We hypothesize that proinflammatory cytokines inhibit human placental 11 $\beta$ -HSD activity. We incubated explant cultures of term human placental villi in the presence or absence of 10 ng/ml IL-1 $\beta$ , IL-6, or TNF- $\alpha$ , with or without agonists or antagonists of intracellular Ca<sup>2+</sup> and adenylyl cyclase. Activity for 11 $\beta$ -HSD2 was estimated using a radioisotope assay, and mRNA was measured using quantitative RT-PCR. All cytokines significantly ( $P \leq 0.05$ ) reduced 11 $\beta$ -HSD2 activity (>75% suppression); maximal inhibition occurred within 2 h and was maintained for at least 24 h. The IL-1 $\beta$ -induced inhibitory activity was attenuated using a Ca<sup>2+</sup> channel blocker (nifedipine), an intracellular Ca<sup>2+</sup> antagonist [8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate], or the adenylyl cyclase stimulant forskolin. Conversely, 11 $\beta$ -HSD2 activity was diminished in the presence of the Ca<sup>2+</sup> ionophore A-23187 or the adenylyl cyclase inhibitor SQ-22536. mRNA levels for 11 $\beta$ -HSD2 were not changed by any of the treatments. Proinflammatory cytokines inhibit human placental 11 $\beta$ -HSD2 activity through a mechanism that involves increased intracellular Ca<sup>2+</sup> and inhibition of adenylyl cyclase. This could result in excessive fetal exposure to maternal cortisol. This mechanism might mediate part of the increased risk of metabolic and cardiovascular disease in adult offspring.

interleukin-1 $\beta$ ; interleukin-6; tumor necrosis factor- $\alpha$ ; cortisol; metabolic and vascular disease

INTERCONVERSION OF THE ACTIVE GLUCOCORTICOID cortisol and its inactive metabolite cortisone is catalyzed by 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD). There are two isoforms of this enzyme as follows(1, 2): 11 $\beta$ -HSD1, which has low affinity for cortisol, is widely distributed, operates bidirectionally, and requires NADP(H) as a cofactor, and 11 $\beta$ -HSD2, which is a high-affinity, unidirectional oxidase and is NAD<sup>+</sup> dependent. Human placental 11 $\beta$ -HSD activity is almost entirely the result of the type 2 isozyme (49). Cortisol concentrations in pregnant women are approximately fourfold higher than fetal concentrations throughout gestation. This gradient is maintained largely by oxidation of cortisol by placental 11 $\beta$ -

HSD2. Recent studies have suggested that excessive glucocorticoid activity in the fetus may lead to several adverse consequences, including dysregulation of the fetal hypothalamic-pituitary-adrenal axis (33, 42), impairment of fetal growth (37), and a higher incidence of metabolic and cardiovascular disease in the adult (9, 46).

Maternal complications during pregnancy, such as infections, intrauterine growth restriction, and preeclampsia, often are accompanied by elevated levels of proinflammatory cytokines, including interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (6, 21, 48). These complications often result in low-birth-weight babies, either from restricted fetal growth or early birth. Low birth weight also is associated with a higher incidence of metabolic and cardiovascular disease in the adult (5). Human placental 11 $\beta$ -HSD mRNA is significantly reduced in pregnancies that give rise to growth-restricted babies (23, 34). The link between the increased incidence of adult disease and an adverse fetal environment has been suggested to be excessive fetal exposure to maternal cortisol (12, 28).

A recent report demonstrates that proinflammatory cytokines may inhibit the activity of 11 $\beta$ -HSD2 in human chondrocytes (11). Furthermore, both mRNA and the enzyme activity level for 11 $\beta$ -HSD2 can be increased by stimulation of the adenylyl cyclase (AC) pathway (16, 41). Conversely, increased intracellular Ca<sup>2+</sup> inhibits human placental 11 $\beta$ -HSD2 activity (19). We hypothesized that increases in proinflammatory cytokines would decrease activity of human placental 11 $\beta$ -HSD2 through mechanisms involving these pathways. This would lead to increased fetal exposure to the elevated maternal cortisol concentrations that occur in maternal conditions associated with low birth weight. The increased fetal glucocorticoid exposure may provide short-term benefit by increasing differentiation and maturation of organ systems such as the lung and liver (9). However, it may also play a role in the mechanism resulting in the observed increased incidence of adult disease. Interference with this mechanism could potentially modulate the long-term adverse consequences of this process. Our objectives in this study were to determine the effect of proinflammatory cytokines on human placental 11 $\beta$ -HSD2 expression and activity and to investigate the roles of intracellular Ca<sup>2+</sup> and AC in these effects.

### MATERIALS AND METHODS

*Explant incubations and treatments.* With approval of the Human Research Ethics Board of the University of Alberta, placentas were collected after spontaneous vaginal delivery or elective cesarean

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section at term from 20 women with uncomplicated pregnancies. Placental villous explants were performed as described previously (35). The placental tissue was kept in cold, sterile PBS until the explant cultures were established within 2 h from delivery. Small explants of villous material ( $\sim 1$  mm<sup>3</sup>, weighing  $\sim 2$  mg) were dissected from randomly selected healthy appearing sites, and four explants were incubated in each well at 37°C containing 1 ml CMRL-1066 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS, 1  $\mu$ g/ml insulin, 100  $\mu$ g/ml streptomycin sulfate, and 1,000 IU/ml penicillin G. As an index of villous explant condition, human chorionic gonadotrophin (hCG) concentrations were measured in the medium daily using an enzyme immunoassay kit (DRG International, Mountainside, NJ) according to the manufacturer's instructions. To confirm viability of the explant tissue, lactate dehydrogenase (LDH) activity in the medium was assessed daily (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's instructions. Based on previous extensive validation of this technique (35) and our own preliminary experiments (Fig. 1A), a 5-day incubation was conducted before treatment with cytokines or other pharmacological test agents. At this time, hCG levels were stable at peak levels, and there was no evidence of cell death as indicated by media levels of LDH (Fig. 1B).

After 5 days in culture, the explants were treated with proinflammatory cytokines IL-1 $\beta$ , IL-6, or TNF- $\alpha$  (Biosource International, Camarillo, CA) at a concentration of 10 ng/ml, based on previous literature (10), for up to 24 h. Appropriate time controls without treatment were performed in each experiment. The role of Ca<sup>2+</sup> was assessed ( $n = 3$  experiments) using the Ca<sup>2+</sup> channel blocker nifedipine (100  $\mu$ M; see Ref. 9), the intracellular Ca<sup>2+</sup> antagonist 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate [TMB-8 (100  $\mu$ M); see Ref. 39], or the Ca<sup>2+</sup> ionophore A-23187 (10  $\mu$ M). To assess the involvement of AC, we used ( $n = 3$  experiments) the AC stimulant forskolin (5  $\mu$ M) or the AC inhibitor SQ-22563 (10  $\mu$ M; see Ref. 14). At the end of each experiment, explants were frozen in liquid N<sub>2</sub> and stored at  $-80^{\circ}$ C until assay. All Ca<sup>2+</sup> and AC modulators were obtained from Sigma-Aldrich.

**11 $\beta$ -HSD enzyme assay.** At the time of enzyme assay, the frozen tissues were homogenized in 3 ml Tris-EDTA (50 mM) buffer using a Polytron (Brinkmann Instruments, Mississauga, ON, Canada), three bursts of 20 s. After centrifugation at 1,000 rpm for 15 min, the supernatant was obtained, and protein concentration was measured using the Bradford technique. Each assay tube contained  $\sim 100,000$  dpm [1,2,6,7-<sup>3</sup>H(N)]cortisol (2.59–3.7 TBq/mmol; PerkinElmer Life Sciences, Boston, MA) in addition to cold cortisol (Sigma-Aldrich) and 10 mM NAD<sup>+</sup> in a total reaction volume of 500  $\mu$ l. At the end of the reaction, 40  $\mu$ g of carrier cortisol and cortisone were added in 4 ml of ethyl acetate, and the tubes were placed on ice. Steroids were extracted two times using 4 ml of ethyl acetate, and, after centrifugation at 1,000 rpm for 10 min, the aqueous layer was frozen using dry ice. The organic phase was removed and evaporated at room temperature. The residue was reconstituted in 100  $\mu$ l of ethyl acetate, applied to TLC plates (Whatman, Clifton, NJ), and separated using the

solvent system chloroform-ethanol 89:11. Bands were visualized using I<sub>2</sub> vapor and scraped into scintillation fluid, and the radioactivity was measured using a Beckman model LS5000TD liquid scintillation counter (Beckman Coulter, Fullerton, CA).

Preliminary experiments were performed to determine the optimal time, protein concentration, and substrate concentration to be used in the assay. On the basis of these experiments, the following standard assay conditions were derived:  $\sim 100$   $\mu$ g of homogenate protein were incubated with 3  $\mu$ M substrate cortisol for 30 min. The experimental results were calculated by expressing the conversion rates of cortisol to cortisone in the presence of a cytokine as a percentage of that in the corresponding control in the absence of cytokine.

**RT-PCR.** RNA was extracted using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). Samples were treated with amplification-grade DNase I (Sigma-Aldrich). The purity of the extracted RNA was assessed by spectrophotometry, and the concentration of RNA was calculated. The sample was diluted to 50 ng mRNA/ $\mu$ l.

Reverse transcription was performed using the Taqman Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). A mixture of random hexamers (50  $\mu$ M) and 7  $\mu$ l of RNA-free water was incubated with 100 ng of RNA at 65°C for 5 min to denature the secondary structures of mRNA and for annealing of the hexamer primers. After this incubation, the following reagents were added: 2  $\mu$ l 10 $\times$  RT buffer, 4.4  $\mu$ l 25 mM MgCl<sub>2</sub>, 4  $\mu$ l deoxy-NTP mixture (2.5 mM of each), 0.4  $\mu$ l RNase inhibitor (20 U/ml), 0.5  $\mu$ l Multi Scribe RT (50 U/ $\mu$ l), and 0.7  $\mu$ l H<sub>2</sub>O. The total mixture (20  $\mu$ l) was incubated at 25°C for 10 min for equilibration, then at 48°C for 60 min to allow cDNA synthesis, and then at 95°C for 5 min to inactivate the RT enzyme. The resultant mixture was stored at  $-20^{\circ}$ C until ready for PCR. As a negative control, the reaction was also performed in randomly chosen samples in the absence of the Multi Scribe RT.

Real-time PCR was performed in triplicate, using a total reaction volume of 25  $\mu$ l/well. The primer pairs (36) were as follows: for 11 $\beta$ -HSD2, forward TCAAGACAGAGTCAGTGAGAGAAACG and reverse GGAAGTCCCATGCAAGTG, which produced a 129-nt amplicon; and for cyclophilin, forward CACCGTGTCTTCGACATCAC and reverse CCAGTGCTCAGAGCTCGAAAG, which produced a 114-nt amplicon. Each protocol was optimized individually. All reagents for the PCR reactions were from the SYBR Green PCR Core Reagents Kit (Applied Biosystems). The master mix included the following: 2.5  $\mu$ l 10 $\times$  SYBR Green buffer, 3.0  $\mu$ l of 25 mM MgCl<sub>2</sub>, 2.0  $\mu$ l dNTP mix (dATP, dCTP, dGTP: 2.5 mM each; dUTP: 5.0 mM), 0.13  $\mu$ l Ampli Taq Gold (5 U/ $\mu$ l), 0.25  $\mu$ l Amp Erase UNG (1 U/ $\mu$ l), 0.5  $\mu$ l forward primer, 0.5  $\mu$ l reverse primer, 1  $\mu$ l cDNA template, and 15.12  $\mu$ l autoclaved H<sub>2</sub>O. For the blank control, autoclaved H<sub>2</sub>O replaced the cDNA template. The PCR reaction was initiated for 2 min at 50°C and then 10 min at 95°C. The fluorescence was measured at the end of each cycle and plotted against the threshold cycle (C<sub>T</sub>) number to determine the C<sub>T</sub>. After amplification, the purity of the amplified cDNA was checked by assessing a melt curve of the amplified products.

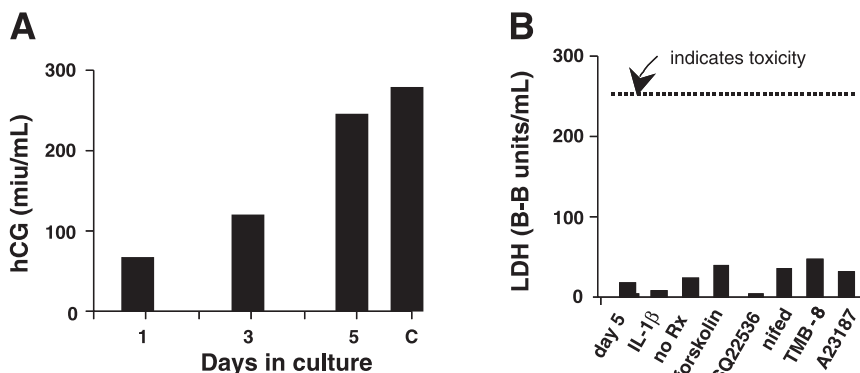


Fig. 1. Viability of the human placental explant culture system. A: explants produced increasing quantities of human chorionic gonadotrophin (hCG) over the 5-day pretreatment period. Treatment with IL-1 $\beta$  (C; 10 ng/ml) had no effect on hCG secretion. B: treatment over 24 h, beginning after day 5 pretreatment incubation, did not cause significant effect on the concentrations of lactate dehydrogenase (LDH) in the medium. The dotted line indicates the concentration at which significant cell toxicity is present. TMB-8, 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate.

For quantification, we use the “ $\Delta$ - $\Delta$ ” method to determine the relative expression between groups (43).  $C_T$  was determined for the gene of interest (GOI) and a housekeeping gene (HKG; cyclophilin). The data were normalized by subtracting the  $C_T$  of the GOI from the  $C_T$  of the HKG to determine the  $\Delta C_T$ . The relative expression level of the GOI in an experimental sample was compared with that of a control or calibrator sample by subtracting their  $\Delta C_T$  values and using the following formula: relative degree of change =  $2^{-x}$ , where  $x$  is the difference between the  $\Delta C_T$  of the experimental sample and the calibrator samples.

**Statistical analyses.** The analyses of the 11 $\beta$ -HSD activity and mRNA over time and the effects of treatment were performed using one-way ANOVA followed by post hoc Tukey-Kramer Multiple Comparisons test. The data are presented in Figs. 1–5 as means  $\pm$  SE. Statistical significance was defined as  $P \leq 0.05$ .

## RESULTS

After 5 days in culture, the hCG concentrations were at peak levels (Fig. 1A), and there was no increase in LDH in the medium to suggest cell death (Fig. 1B). Similarly, the cytokine treatment had no influence on the health of the explants, as assessed by the ability to synthesize and secrete hCG and the presence of LDH in the media. Human placenta contains abundant 11 $\beta$ -HSD2 activity. In our validation experiments, we observed activity for conversion of cortisol to cortisone by untreated explants in the range 10–25 pmol·mg protein<sup>-1</sup>·min<sup>-1</sup>.

During the day of treatment (*day 6* in culture), the 11 $\beta$ -HSD2 activity did not change significantly over the 24 h in the absence of treatment (Fig. 2). Treatment with IL-1 $\beta$ , TNF- $\alpha$ , or IL-6 caused a significant reduction in 11 $\beta$ -HSD2 activity. The effects were near maximal after 2 h of incubation and persisted for at least 24 h. In some experiments with each of the three cytokines, incubations were performed for intervals <2 h, and 11 $\beta$ -HSD2 activity was suppressed by >50% by 0.5 h (data not shown). At 2 h, 11 $\beta$ -HSD2 activity was reduced to  $25.2 \pm 5.5$ ,  $24.8 \pm 7.0$  and  $22.7 \pm 4.1\%$  of control activity by IL-1 $\beta$ , TNF- $\alpha$  and IL-6, respectively. By 24 h, the activity was reduced to  $11.7 \pm 3.1$ ,  $0.8 \pm 0.4$ , and  $5.5 \pm 2.9\%$  of control values respectively.

We assessed the role of Ca<sup>2+</sup> as a mediator of the suppressive effects of IL-1 $\beta$  on placental 11 $\beta$ -HSD2 activity at 2 and 24 h using several pharmacological tools (Figs. 3A and 4A). The patterns were the same at 2 and 24 h. Inhibition of extracellular Ca<sup>2+</sup> entry in the cells using nifedipine prevented the inhibitory effects of IL-1 $\beta$ . A similar result was obtained by prevention of release of Ca<sup>2+</sup> from intracellular stores using TMB-8. Neither nifedipine nor TMB-8 had any effect alone.

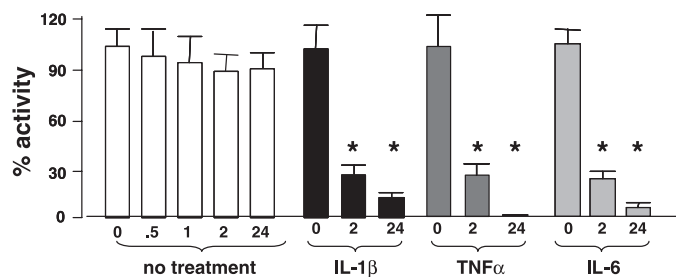


Fig. 2. Enzyme activity for 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (HSD2) expressed as a percentage of *time 0* levels. There was no significant effect of 24 h in culture without treatment. However, each of the cytokines (10 ng/ml) caused significant inhibition of 11 $\beta$ -HSD activity by 2 h, and this was further inhibited by 24 h. \* $P < 0.05$ .

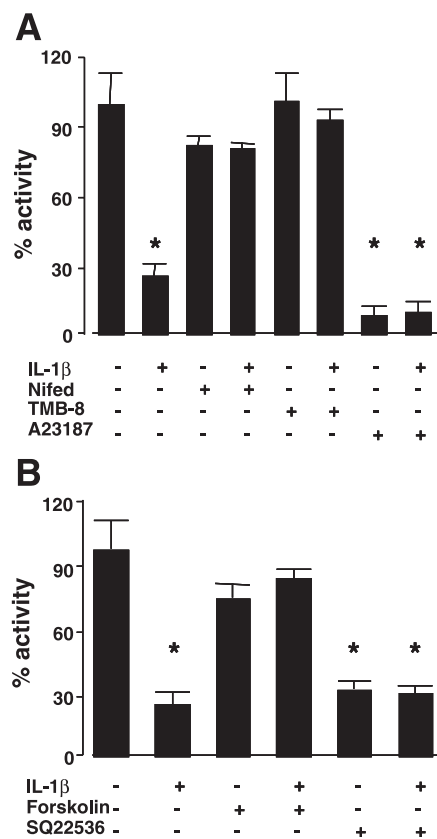


Fig. 3. Effects of agonists and antagonists of Ca<sup>2+</sup> and cAMP on the inhibitory action of IL-1 $\beta$  on 11 $\beta$ -HSD2 activity after 2-h incubation. A: Ca<sup>2+</sup> antagonists nifedipine (nifed) and TMB-8 had no significant effect on basal 11 $\beta$ -HSD2 activity, but either antagonist significantly prevented the IL-1 $\beta$ -induced inhibitory effects. The Ca<sup>2+</sup> ionophore A-23187 significantly inhibited 11 $\beta$ -HSD2 activity, and there was no additive effect when combined with IL-1 $\beta$ . B: adenylyl cyclase stimulant forskolin had no effect on basal 11 $\beta$ -HSD2 activity but prevented the IL-1 $\beta$ -induced inhibition. Conversely, the adenylyl cyclase inhibitor SQ-22536 significantly inhibited the enzyme activity without apparent additive effects to IL-1 $\beta$ . \*Significant difference ( $P \leq 0.05$ ) from the untreated control incubations.

Conversely, increasing intracellular Ca<sup>2+</sup> with the Ca<sup>2+</sup> ionophore A-23187 caused suppression of 11 $\beta$ -HSD activity to an extent similar to IL-1 $\beta$  treatment.

The potential role of AC in mediation of the suppressive effect of IL-1 $\beta$  was assessed using both an agonist and an antagonist (Figs. 3B and 4B). The AC stimulant forskolin was without effect by itself but markedly abrogated the inhibitory effect of IL-1 $\beta$  at both 2 and 24 h. Conversely, the AC inhibitor SQ-22536 had a suppressive effect on 11 $\beta$ -HSD2 activity similar to IL-1 $\beta$  at both time points. There was no apparent synergistic effect of SQ-22536 plus IL-1 $\beta$ .

To determine whether the decreased enzyme activity might be because of inhibition of transcription, we used RT-PCR to assess mRNA levels for 11 $\beta$ -HSD2 in human placental explants treated with the factors noted to cause significant inhibition in enzyme activity (Fig. 5). None of these factors was associated with decreased expression of mRNA for 11 $\beta$ -HSD2.

## DISCUSSION

In the milieu of maternal stress accompanied by excessive maternal cortisol and proinflammatory cytokine concentrations, it appears intuitive that the placenta would increase

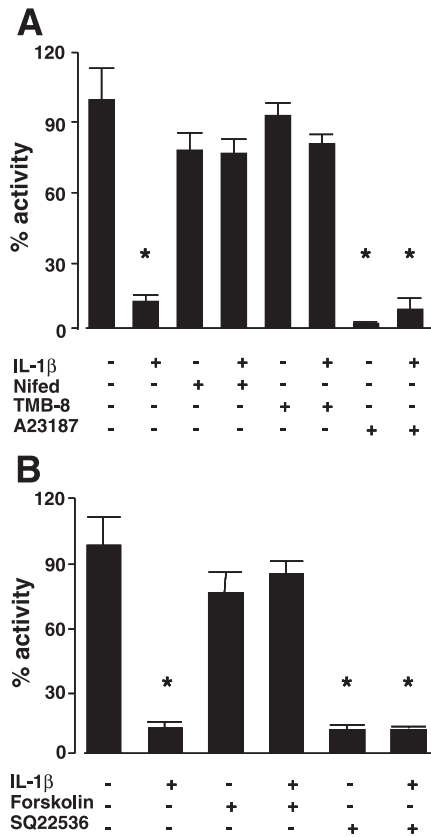


Fig. 4. Effects of agonists and antagonists of  $\text{Ca}^{2+}$  and cAMP on the inhibitory action of IL-1 $\beta$  on 11 $\beta$ -HSD2 activity after 24-h incubation. The effects were qualitatively similar to those demonstrated after 2 h incubation (see legend for Fig. 3).

11 $\beta$ -HSD2 activity to protect the fetus from excessive cortisol exposure. However, the current results indicate a very potent inhibitory effect of proinflammatory cytokines on 11 $\beta$ -HSD2 activity. Our basal enzyme activity rates were similar to those measured by Rogerson et al. (44) and slightly lower than the estimates of Murphy and Clifton (36). The inhibitory activity was similar for all three cytokines tested. The inhibition was near maximal by 2 h of exposure and, in some instances, almost completely inhibited by 24 h. These findings are similar to a previous study showing marked suppression of 11 $\beta$ -HSD2 activity by IL-1 $\beta$  and TNF- $\alpha$  in the human osteosarcoma cell line MG-63 (11). However, these latter results were noted at 48 h and were not measured earlier. Cytokine treatment had no effect on 11 $\beta$ -HSD2 expression in JEG-3 cells (22). In contrast, 11 $\beta$ -HSD1 expression is increased by treatment with IL-1 $\beta$  or TNF- $\alpha$  in glomerular mesangial cells (13) and JEG-3 cells (22).

The clinical implications of these effects are not clear but could be very important. In pregnancies complicated by histological chorioamnionitis, placental 11 $\beta$ -HSD2 mRNA and activity were reduced significantly (22). Our data could provide a mechanism through which this reduced activity occurs. In normal pregnancy, placental 11 $\beta$ -HSD mRNA (45) and activity (47) increase through pregnancy. However, at the end of human (36) and baboon (31) gestation, there is a significant reduction in 11 $\beta$ -HSD2 activity, which may play a role in fetal organ maturation and the timing of parturition (9). Hence,

abnormal regulation of 11 $\beta$ -HSD2 activity could have adverse consequences regarding the timing of birth or fetal maturation. Furthermore, low placental 11 $\beta$ -HSD2 activity has been associated with low birth weight in some studies (34, 49) but not in others (44). Intrauterine growth restriction is often accompanied by elevated maternal serum concentrations of proinflammatory cytokines (6, 21, 48). Thus it is possible that the elevated cytokines that accompany the placental pathology leading to fetal growth restriction also inhibit placental 11 $\beta$ -HSD2 activity and increase fetal exposure to maternal cortisol. This may represent a mechanism leading to the observed increased incidence of adult metabolic and cardiovascular disease in these circumstances.

The 11 $\beta$ -HSD2 isoform was cloned from the kidney (1) and placenta (7). It differs from 11 $\beta$ -HSD1 in that it requires  $\text{NAD}^+$  as cofactor, catalyzes only the oxidative reaction, and has a lower  $K_m$  (10–100 nM). The tissue distribution of the enzyme is similar to that for the mineralocorticoid receptor (MR), and it is thought that the enzyme, by oxidizing cortisol, protects against excessive activation of MR by cortisol. Indeed, abnormal expression of this gene causes the syndrome of apparent mineralocorticoid excess (51). In the human placenta, the type 2 isoform accounts for essentially all of the 11 $\beta$ -HSD activity in the human placenta (49). Unlike other sites, the enzyme in this tissue appears not to serve a protective function for the MR. It has been suggested that the principal function of placental 11 $\beta$ -HSD2 is to protect the developing fetus against excessive exposure to glucocorticoid. Failure of this function could result in permanent dysregulation of the hypothalamic-pituitary-adrenal axis of the developing fetus (33, 42) and predispose to an increased incidence of cardiovascular and metabolic disease in the adult (46). It is possible that, in the face of maternal pregnancy complications, the short-term benefits of excessive fetal cortisol (perhaps accelerated maturation and earlier birth) outweigh the potential long-term adverse consequences of excessive fetal cortisol exposure.

Because of the rapid action of the inhibitory effects, we first investigated nongenomic signaling pathways. The data demonstrate clearly that intracellular  $\text{Ca}^{2+}$  is an important mediator of the rapid inhibitory effects of IL-1 $\beta$  on 11 $\beta$ -HSD activity. The inhibitory effects were reproduced using a  $\text{Ca}^{2+}$  ionophore. The IL-1 $\beta$ -induced inhibition was attenuated by blocking the influx of extracellular  $\text{Ca}^{2+}$  using nifedipine or by blocking inositol trisphosphate-induced  $\text{Ca}^{2+}$  release from in-

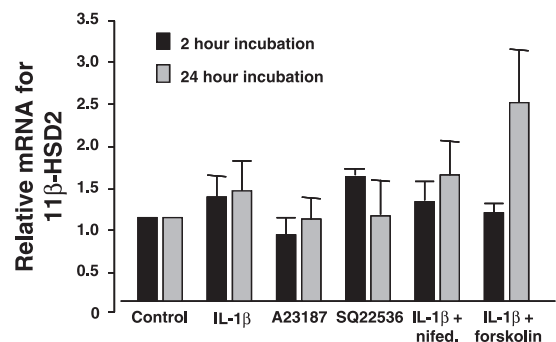


Fig. 5. Relative expression of mRNA for 11 $\beta$ -HSD2 after 2- or 24-h treatments with IL-1 $\beta$  or the factors noted to reduce the enzyme activity. None of these treatments caused a significant change in the concentrations of mRNA for 11 $\beta$ -HSD2 ( $n = 3$ ).

tracellular stores using TMB-8 (39). These results confirm and extend the findings of Hardy et al. (19) who noted a 50% decrease in 11 $\beta$ -HSD2 activity with a PGF<sub>2 $\alpha$</sub> -induced increase in Ca<sup>2+</sup>. As in the present study, the inhibitory effect was blocked in the presence of the intracellular chelator BAPTA. These findings suggest the involvement of a G $\alpha_q$ -coupled receptor stimulation of phospholipase C.

Signal transduction mechanisms underlying the effects of IL-1 $\beta$  are numerous and diverse and appear to be very dependent on the cell type studied as well as the cell culture conditions (4). Most studies have focused on the long-term genomic effects of IL-1 $\beta$  on regulation of genes involved with cell death or replication. Relatively little is known regarding acute, rapid signal transduction pathways linked to proinflammatory cytokines and their receptors. However, in synovial fibroblasts, IL-1 $\beta$  causes rapid membrane depolarization that can be blocked by Ca<sup>2+</sup> channel inhibitors or protein kinase C antagonists (26). As in our study, the effects of IL-1 $\beta$  could be reproduced using a Ca<sup>2+</sup> ionophore, although it was administered in addition to a phorbol ester to stimulate protein kinase C. IL-1 $\beta$  also causes an acute increase in intracellular Ca<sup>2+</sup> in human fibroblasts (3) and chondrocytes (30). The latter study demonstrated that the source of Ca<sup>2+</sup> was from both intracellular and extracellular stores. The rapid pyrogenic response to injected IL-1 $\beta$  also appears to be mediated through a pathway involving increased intracellular Ca<sup>2+</sup> arising mainly from the intracellular stores (40). TNF- $\alpha$  may signal through the TNF receptor isoform TNFR1 to stimulate rapid transient waves of Ca<sup>2+</sup>, mostly arising from intracellular stores (32). This pathway appears to be mediated by a mechanism that involves ceramide intermediates that are converted to sphingosine 1-phosphate, which can activate a G $\alpha_q$ -linked membrane receptor. For IL-6, studies again have focused on genomic mechanisms, but there is evidence that this cytokine can induce a transient increase in phosphoinositide turnover with increased intracellular Ca<sup>2+</sup> concentration in rat mesangial cells (15). In pheochromocytoma cells, IL-6 caused direct activation of phospholipase C- $\gamma$  (27). Conversely, IL-6 caused no increase in intracellular Ca<sup>2+</sup> in the salivary cell line SMG-C6 (29). Overall, these data support the concept that the inhibitory effects of proinflammatory cytokines on 11 $\beta$ -HSD2 may be mediated in part through regulation of intracellular Ca<sup>2+</sup>.

The responses to agonists and antagonists of AC also provided interesting results. The AC stimulant forskolin almost completely abrogated the inhibitory response of the cytokines on 11 $\beta$ -HSD2 activity. Conversely, the AC inhibitor SQ-22536 had effects similar to those of the cytokines. Our data differ slightly from those of Gibb and Sun (16) in that we measured no effect of forskolin alone, whereas they noted an increase in both mRNA and enzyme activity for 11 $\beta$ -HSD2 in cultured human trophoblast cells. The discrepancy may be because of differences in the concentration of forskolin (their use of 100  $\mu$ M, whereas we used only 5  $\mu$ M). However, both studies suggest that the inhibitory effects of the cytokines could be mediated by reducing cAMP levels. This pattern of effects is similar to that seen in rat hepatocytes where IL-1 $\beta$  treatment greatly increases expression of inducible nitric oxide synthase (iNOS), an effect that is significantly diminished by forskolin and enhanced by SQ-22536 (18). In rat cardiac fibroblasts, IL-1 $\beta$  attenuates cAMP accumulation by a mechanism that is mediated by increased iNOS activity, resulting in increased

phosphodiesterase (PDE) activity, thus increasing cAMP degradation (17). In human myometrial cells, prolonged IL-1 $\beta$  treatment causes an increase in PDE activity that appears to be dependent on an increase in PGE<sub>2</sub> (38). Intracellular signaling pathways for TNF- $\alpha$  also may include cAMP. In porcine renal epithelial cells, TNF- $\alpha$  treatment caused decreased 11 $\beta$ -HSD2 activity, and this was reversed by cotreatment with forskolin (20). Similarly to IL-1 $\beta$ , TNF- $\alpha$  also may increase PDE activity, resulting in lower cAMP levels (24). There has been little, if any, research into the acute effects of IL-6 on cAMP accumulation.

The mechanisms through which Ca<sup>2+</sup> and cAMP affect 11 $\beta$ -HSD2 activity remain to be determined, although it appears from our data that both operate through translational or posttranslational mechanisms. Increased Ca<sup>2+</sup> appears to have a direct inhibitory action on the catalytic site of human placental 11 $\beta$ -HSD2 without interfering with the substrate-binding or cofactor-binding sites (19). It will be interesting to determine if increased Ca<sup>2+</sup> or lowered cAMP directly affects the process of translation and whether there are interactions between Ca<sup>2+</sup> and cAMP. Similarly, it will be interesting to determine if cAMP-stimulated protein kinase A affects 11 $\beta$ -HSD2 activity.

The present data demonstrating significant inhibition of 11 $\beta$ -HSD2 activity confirm the recent findings of Chisaka et al. (10). In their study, there was a modest decline in 11 $\beta$ -HSD2 activity at 6 h, but, in contrast to our data, this was accompanied by a similar decline in mRNA. At 24 h, there was no difference from controls for either activity or mRNA. The reason for the differences between these two studies may be related to the technique of tissue preparation. In their study, placental tissue was digested with trypsin and cytotrophoblast cells, separated using density gradient centrifugation, and cultured for 3 days before treatment. In our study, an explant of syncytialized villi was cultured for 5 days before treatment.

In general, levels of mRNA for 11 $\beta$ -HSD2 tend to correlate well with enzyme activity. The mRNA levels increase during pregnancy and then decline slightly before parturition (31, 36). There is some conflict in the literature regarding mRNA levels for placental 11 $\beta$ -HSD2 in intrauterine growth restriction. Some studies show decreased levels (34), and others show no change (36, 44). The regulation of 11 $\beta$ -HSD2 gene expression appears to be complex. It can be increased by estrogen and decreased by progesterone (8, 42). Treatment with glucocorticoids increased mRNA for 11 $\beta$ -HSD2 in human trophoblast cells (50) and in baboon placenta (31) but decreased 11 $\beta$ -HSD2 expression in ovine placenta (25). Interestingly, 24-h treatment with IL-1 $\beta$  of JEG-3 cells derived from human choriocarcinoma caused an increase in 11 $\beta$ -HSD2 mRNA and protein (41). In the present studies, because there was no change in mRNA for 11 $\beta$ -HSD2 after treatment with the factors that inhibited the enzyme activity, we conclude that the effects of the proinflammatory cytokines as well as Ca<sup>2+</sup> and the inhibition of AC occurred at the posttranscriptional level.

In summary, these data clearly demonstrate inhibitory effects of proinflammatory cytokines on human term placental 11 $\beta$ -HSD2 activity levels without concomitant changes in mRNA. We noted a consistent and highly significant rapid reduction in enzyme activity. This inhibition of enzyme activity appeared to be mediated by increased intracellular Ca<sup>2+</sup> concentrations and by reduced AC activity acting through a

posttranscriptional mechanism. These findings could have important clinical implications in that this may lead to excessive fetal cortisol exposure, which has been associated with an increased incidence of adult metabolic, cardiovascular, and neurological disease. Thus the data may provide a causal link between these adult diseases and inflammatory conditions in pregnancy, including but not limited to growth restriction. Relatively little is known about the acute, nongenomic effects of the proinflammatory cytokines. Increased understanding of the pathways involved may be useful in preventing some of the long-term consequences of an adverse fetal environment.

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