The Journal of Immunology

Local Amplification of Glucocorticoids by 11 β-Hydroxysteroid Dehydrogenase Type 1 Promotes Macrophage Phagocytosis of Apoptotic Leukocytes

James S. Gilmour, Agnes E. Coutinho, Jean-François Cailhier, Tak Yung Man, Michael Clay, Graham Thomas, Hayley J. Harris, John J. Mullins, Jonathan R. Seckl, John S. Savill and Karen E. Chapman

J Immunol 2006; 176:7605-7611; ;
doi: 10.4049/jimmunol.176.12.7605
http://www.jimmunol.org/content/176/12/7605

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Local Amplification of Glucocorticoids by 11β-Hydroxysteroid Dehydrogenase Type 1 Promotes Macrophage Phagocytosis of Apoptotic Leukocytes[1]

James S. Gilmour,*† Agnes E. Coutinho,*† Jean-François Cailhier,[2†] Tak Yung Man,* Michael Clay,† Graham Thomas,† Hayley J. Harris,* John J. Mullins,‡ Jonathan R. Seckl,* John S. Savill,[3†] and Karen E. Chapman[3,4]*

Glucocorticoids promote macrophage phagocytosis of leukocytes undergoing apoptosis. Prereceptor metabolism of glucocorticoids by 11β-hydroxysteroid dehydrogenases (11β-HSDs) modulates cellular steroid action. 11β-HSD type 1 amplifies intracellular levels of active glucocorticoids in mice by reactivating corticosterone from inert 11-dehydrocorticosterone in cells expressing the enzyme. In this study we describe the rapid (within 3 h) induction of 11β-HSD activity in cells elicited in the peritoneum by a single thioglycolate injection in mice. Levels remained high in peritoneal cells until resolution. In vitro experiments on mouse macrophages demonstrated that treatment with inert 11-dehydrocorticosterone for 24 h increased phagocytosis of apoptotic neutrophils to the same extent as corticosterone. This effect was dependent upon 11β-HSD1, as 11β-HSD1 mRNA, but not 11β-HSD2 mRNA, was expressed in these cells; 11-dehydrocorticosterone was ineffective in promoting phagocytosis by *Hsd11b1*$^{-/-}$ macrophages, and carbenoxolone, an 11β-HSD inhibitor, prevented the increase in phagocytosis elicited in wild-type macrophages by 11-dehydrocorticosterone. Importantly, as experimental peritonitis progressed, clearance of apoptotic neutrophils was delayed in *Hsd11b1*$^{-/-}$ mice. These data point to an early role for 11β-HSD1 in promoting the rapid clearance of apoptotic cells during the resolution of inflammation and indicate a novel target for therapy. ***The Journal of Immunology,*** **2006, 176: 7605–7611.**

Acute inflammation is normally self-resolving but may become persistent, leading to irreversible organ damage. It is crucial that mechanisms are engaged early in acute inflammation to promote the rapid and safe resolution of the response. A key event driving resolution of acute inflammation is macrophage clearance of leukocytes undergoing apoptosis at sites of inflammation (1–3). However, blood monocytes lack phagocytic capacity (4), and little is known of the mechanisms that operate early in the acute inflammatory response to promote the acquisition by maturing monocytes of phagocytic capacity for apoptotic leukocytes.

In previous work, synthetic glucocorticoids have been shown to exert proresolution effects on maturing monocytes, accelerating the acquisition of phagocytic capacity for apoptotic leukocytes and also increasing the capacity of individual macrophages to ingest multiple apoptotic cells (5–7). In vivo, control over physiological glucocorticoid action is exerted by 11β-hydroxysteroid dehydrogenase (11β-HSD),[5] which interconverts active glucocorticoids and their inert 11-keto forms within cells, thus regulating glucocorticoid access to receptors (reviewed in Refs. 8 and 9). Recent data have shown that 11β-HSD type 1 (11β-HSD1) provides an important amplification of glucocorticoid action in tissues by reducing inactive 11-ketosteroids (cortisone in humans, 11-dehydrocorticosterone in mice) derived from blood to yield active glucocorticoids (cortisol and corticosterone). Indeed, the physiological action of glucocorticoids in certain tissues may be dependent upon active glucocorticoids generated by the activity of 11β-HSD1, as mice deficient in 11β-HSD1 are protected against glucocorticoid-associated cognitive decline (10) and the adverse metabolic effects of glucocorticoids (11–13). Interestingly, *HSD11b1* (encoding 11β-HSD1) is not expressed in human monocytes but is induced upon differentiation to macrophages, where expression is further increased by the anti-inflammatory cytokines IL-4 and IL-13 (14).

However, whether 11β-HSD1-mediated activation of glucocorticoids has a role in the resolution of acute inflammatory responses remains unknown. Here we show in vivo that within the first 24 h of acute inflammation caused by direct injection of thioglycolate, 11β-HSD1 activity is dramatically induced in cells elicited to the peritoneum and remains high until resolution. In vitro, 11β-HSD1 is required to allow intrinsically inactive 11-keto glucocorticoids to promote macrophage phagocytic clearance of neutrophils undergoing apoptosis, and, crucially, mice deficient in 11β-HSD1

*Endocrinology Unit, Centre for Cardiovascular Sciences, †Medical Research Council Centre for Inflammation Research, and ‡Molecular Physiology Laboratory, Centre for Cardiovascular Sciences, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom

Received for publication June 8, 2005. Accepted for publication March 23, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Wellcome Trust Program Grants 065998 (to J.R.S. and J.J.M.) and 064487 (to J.S.S.) and by National Kidney Research Fund Grant R22/1/99 (to J.S.S.). J.S.G. was supported by a 4-year PhD award from the Wellcome Trust. J.-F.C. was funded by a fellowship award from the Canadian Institutes of Health Research.

[2] Current address: Division of Nephrology, Research Center of the University of Montréal Hospital Center (CRCHUM), 1560 Rue Sherbrooke Est, Laboratory M-9208, Pavillon Mailloux, Montréal, Québec H2L 4M1, Canada.

[3] J.S.S. and K.E.C. share joint authorship.

[4] Address correspondence and reprint requests to Dr. Karen E Chapman, Endocrinology Unit, Centre for Cardiovascular Sciences, The Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom. E-mail address: Karen.Chapman@ed.ac.uk

[5] Abbreviations used in this paper 11β-HSD, 11β-hydroxysteroid dehydrogenase; BMD, bone marrow derived; PMN, polymorphonuclear cell; RP, resident peritoneal TEP, thioglycolate-elicited peritoneal.

0022-1767/06/$02.00

show a delay in the clearance of apoptotic neutrophils as experimental peritonitis progresses. We propose that 11β-HSD1 is a component of hitherto poorly understood mechanisms that are engaged early in the acute inflammatory response to promote later resolution.

Materials and Methods

Animals

Mice homozygous for a targeted disruption of the *Hsd11b1* gene encoding 11β-HSD1 have been described previously (11). The disrupted *Hsd11b1* allele was backcrossed (>8 generations) to a C57BL/6J background (13). Male mice, 8–10 wk old, and age-matched C57BL/6J controls (obtained from Harlan Orlac), were used in all experiments and housed under controlled conditions (12 h of light at 21°C) with unrestricted access to water and standard chow. All experiments on animals were approved by the local ethics committee and were performed in accordance with the U.K. Home Office Animals (Scientific Procedures) Act of 1986.

Neutrophil isolation, macrophage preparation, cell culture, and cytokine assay

Human neutrophils (polymorphonuclear cells (PMN)) were isolated from fresh, citrated, peripheral blood of healthy volunteers as previously described (4). Briefly, erythrocyte sedimentation with 0.6% (w/v) dextran/PBS was followed by the fractionation of leukocytes by centrifugation on a discontinuous Percoll gradient (formed by overlaying solutions of 72, 68, and 55% Percoll/PBS (v/v)). PMN were aspirated from the appropriate gradient interface, washed in Ca^{2+}-free PBS, and suspended in Teflon pots (Roland Vetter Laborbedarf) at a density of 4×10^6/ml in Iscove's medium (Invitrogen Life Technologies) supplemented with 10% autologous plasma-derived serum. PMN were aged for 24 h at 37°C and 5% CO_2, which typically yielded >60% apoptotic PMN.

Murine bone marrow-derived (BMD) and thioglycolate-elicited peritoneal (TEP) macrophages were prepared as previously described (15). Briefly, bone marrow was flushed from femurs with a 25-gauge needle, and 4×10^5 cells were plated per well of 24-well plates in 1 ml of DMEM/F12 (Invitrogen Life Technologies) supplemented with 10% FCS, 500 U/ml penicillin, 500 U/ml streptomycin, and 10% monocyte-CSF-conditioned supplement from murine fibrosarcoma cell (L929) cultures (15). Cells were allowed to differentiate for 7 days with the medium changed every 3 days.

Resident peritoneal (RP) macrophages were washed from mouse peritoneum in 4 ml of sterile PBS through a 19-gauge needle. Exudate cells were washed in PBS, and 10^6 cells were seeded into each well of a 24-well plate in 1 ml of DMEM/F12. Following incubation for 1 h at 37°C and 5% CO_2, nonadherent cells were aspirated and medium was replaced with DMEM/F12 supplemented with 500 U/ml penicillin, 500 U/ml streptomycin, and 10% FCS. Inflammatory TEP macrophages were elicited into the peritoneum by sterile injection of 1.0 ml of 3% Brewer's thioglycolate and harvested at various time points as described above. In some experiments the peritoneum was lavaged at 48 h, and the number of free (nonphagocytosed) neutrophils with typical morphological features of apoptosis (4) was determined by microscopic counts of May-Giemsa-stained cytospin slides. To label resident peritoneal macrophages, 0.5 ml of 0.25 μM PKH-2 green fluorescent tracking dye (Sigma-Aldrich), was injected i.p. 24 h before thioglycolate injection. Peritoneal cells were lavaged 3 h following thioglycolate injection and sorted in a FACSDiva cell sorter (BD Biosciences) to separate unlabeled cells from PKH-2-labeled resident macrophages. Cell numbers were established after staining with PE-labeled Ab to F4/80 (Caltag Laboratories) followed by flow cytometry using a FACSCalibur instrument. Fluorescent Flow-Check Fluorospheres (Beckman Coulter) were added to each sample before analysis, and the ratio of cells to beads was used to calculate the absolute number of macrophages in peritoneal lavage fluid. Sorted cells were plated and allowed to adhere as described above before the assay of 11β-HSD1 activity.

IL-6 production was measured over 24 h. Peritoneal cells taken 24 h after thioglycolate injection were seeded at 5×10^5 cells per well of a 24-well plate. LPS (0.1 μg/ml) was added, and IL-6 levels in the medium were assayed by ELISA (R&D Systems) after 24 h of incubation at 37°C and 5% CO_2.

In vitro phagocytosis assay

The phagocytosis assay has been previously described (4). Briefly, macrophages were incubated with steroid or vehicle for 48 h, washed with PBS, and then overlaid with a 3-fold excess of apoptotic PMN in serum-free Iscove's medium. After incubation for 30 min at 37°C and 5% CO_2, cells were washed with cold (4°C) PBS to remove noningested PMN and then fixed with 2% Formalin solution. Ingestion was scored in one of two ways: 1) by selective staining of PMN myeloperoxidase using 0.03% (w/v) hydrogen peroxide and 0.1 mg/ml dimethoxybenzidine or 2) by loading PMN for 30 min with 1 μg/ml CellTracker Green CMFDA 5-chloromethylfluorescein diacetate (Invitrogen Life Technologies) before aging.

In vivo phagocytosis assay

In vivo phagocytosis assays were performed on day 1 or day 2 of thioglycolate-elicited peritonitis by i.p. injection of 30×10^6 apoptotic PMN (stained with CellTracker Green 5-chloromethylfluorescein diacetate) in 1 ml of PBS. Ten minutes later, peritoneal lavage was conducted with PBS as described above. Visualization of macrophages was aided by counterstaining the lavaged cells with anti-F4/80 Ab. Macrophages (0.5×10^6) were washed with PBS, collected, and incubated in 100 μl of blocking solution (nine parts 0.2% NaN_3, 0.5% BSA in PBS to one part murine serum) for 15 min at 4°C. PE-labeled Ab to F4/80 (Caltag Laboratories) was added at the manufacturer's suggested concentration and incubated for 45 min at 4°C. Macrophages were washed with PBS, fixed with 2% Formalin solution and examined using fluorescence microscopy.

11β-HSD assay

11β-HSD reductase and dehydrogenase activities in cell monolayers were determined as previously described (16). Briefly, 200 nM corticosterone or 11-dehydrocorticosterone containing trace amounts of [^{3}H]corticosterone (specific activity ~80 Ci/mmol; Amersham Biosciences) or [11-^{3}H]dehydrocorticosterone (made as previously described (16)), respectively, was added to the medium. At various times, steroids were extracted with ethyl acetate from aliquots of the medium, resuspended in ethanol, and separated by TLC. TLC plates were analyzed using a phosphorimaging device (Fuji FLA-2000) and Aida software (Raytek Scientific). Results were expressed either as the percentage of conversion of substrate into product (where the assay had exceeded the linear range) or as picomole product per hour per 10^5 cells (where the reaction remained linear with time).

RNA extraction and analysis

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Reverse transcription of RNA was conducted using a reverse transcriptase system (Promega) according to the manufacturer's protocol. Reactions (20 μl) contained 1 μg of total RNA, 5 mM $MgCl_2$, 1× reverse transcription buffer (supplied with the kit), 1 mM each dNTP, 1 U of RNasin (Promega), 15 U/μl reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$. Using a Hot Start bead kit (Promega), 5 μl of reverse transcription reaction was used in a 50 μl of PCR containing 1× DNA polymerase reaction buffer, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 0.1 μM each of primer, and 1.25 U of DNA polymerase. PCR primers and conditions were as previously described (17).

Results

11β-HSD1, but not 11β-HSD2, is expressed in murine macrophages

To determine whether 11β-HSD1 and/or 11β-HSD2 is expressed in murine macrophages, 11β-HSD assays were conducted on intact cells. Whereas 11β-HSD1 is predominantly an oxoreductase in intact cells, 11β-HSD2 activity is always oxidative with physiological substrates (18, 19). All cells contained 11β-oxoreductase activity, with the highest conversion of steroid substrate in BMD and TEP macrophages and lower conversion in RP macrophages and white blood cells. In contrast, 11β-dehydrogenase activity was undetectable in all intact cell types (Fig. 1*A*).

To confirm that the 11β-HSD activity detected in murine macrophages was due to 11β-HSD1 and not 11β-HSD2, RT-PCR was conducted on RNA isolated from BMD, RP, and TEP macrophages. RNA from all three types of macrophage produced a 461-bp RT-PCR product of identical size to the *Hsd11b1* product from mouse liver RNA (20) (Fig. 1*A*). In contrast, no *Hsd11b2* (encoding 11β-HSD2) RT-PCR product was produced from macrophage RNA, although it was clearly present as a 144-bp product when kidney RNA was used as a positive control (Fig. 1*B*).

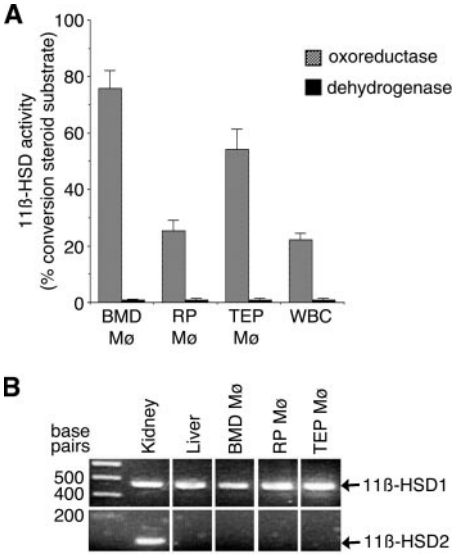

FIGURE 1. Expression of 11β-HSD1 in murine macrophages. *A*, 11β-HSD oxoreductase activity exclusively is detected in intact macrophages (Mϕ). Activity of 11β-HSD oxoreductase is reported as the percentage of the conversion of 200 nM 11-dehydrocorticosterone to corticosterone by 10^6 cells over 24 h; dehydrogenase activity is reported as the percentage of conversion of 200 nM corticosterone to 11-dehydrocorticosterone by 10^6 cells over 24 h. Values shown are mean ± SEM of four separate mice conducted in duplicate. *B*, mRNA encoding 11β-HSD1, but not 11β-HSD2, is detectable by RT-PCR of RNA (1 μg) from each of three murine macrophage types. Liver and kidney RNA (1 μg) were used as positive controls for 11β-HSD1 and 11β-HSD2, respectively (kidney expresses both). RT-PCR products of 461 bp (11β-HSD1) or 144 bp (11β-HSD2) are indicated. The image is of a single gel and is representative of RT-PCR conducted on at least three different mRNA samples of each cell type. WBC, white blood cells.

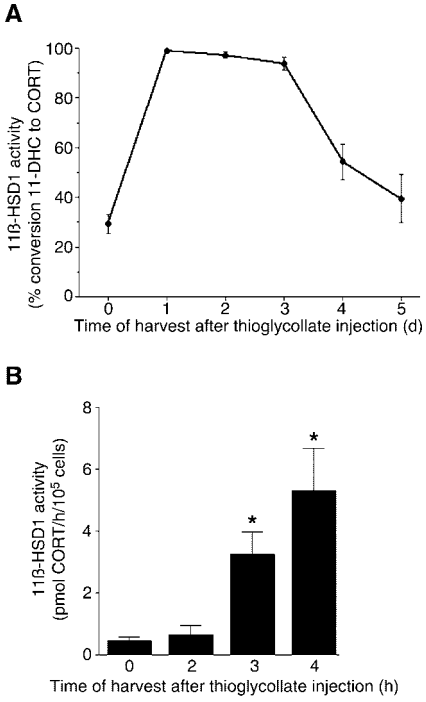

FIGURE 2. The activity of 11β-HSD1 is increased in murine peritoneal macrophages during thioglycolate-elicited peritonitis. *A*, Peritoneal cells were lavaged from mice at days 1–5 after i.p. injection of 3% thioglycolate and 11β-HSD1 (oxoreductase) activity assayed over 24 h in vitro. Activity of 11β-HSD1 was beyond the linear range and is thus reported as the percentage of the conversion of 200 nM 11-dehydrocorticosterone to corticosterone (CORT) by 10^6 cells in 24 h. Thioglycolate injection caused a significant increase in 11β-HSD1 activity at days 1–4 of peritonitis ($p < 0.001$; ANOVA), returning to basal levels by day 5. *B*, 11β-HSD1 (oxoreductase) activity in RP macrophages and adherent cells from lavages taken 2, 3, or 4 h after i.p. injection of 3% thioglycolate. Assays were conducted in vitro for up to 2 h and were within the linear range. There was a significant increase of 11β-HSD1 in peritoneal cells 3 h following thioglycolate ($p < 0.01$; ANOVA). Values shown are mean ± SEM of four to six separate mice at each time point, conducted in duplicate.

11β-HSD1 activity is rapidly and dramatically increased in cells elicited in the peritoneum during sterile peritonitis in vivo

The results shown in Fig. 1*A* indicated that TEP macrophages more efficiently reactivate glucocorticoids than do RP macrophages, suggesting that *Hsd11b1* expression is increased during the inflammatory response. Accordingly, 11β-HSD1 activity was assayed in peritoneal cells harvested at various times following i.p. thioglycolate injection. Whereas RP macrophages reduced ~30% of added inert 11-dehydrocorticosterone (200 nM) within the 24-h assay period, TEP macrophages taken 24 h after thioglycolate injection completely converted the added 11-dehydrocorticosterone to corticosterone in 24 h (Fig. 2*A*). Conversion levels remained close to 100% until day 3, after which 11β-oxoreductase activity decreased to a level comparable to that of RP macrophages (Fig. 2*A*). Macrophage 11β-HSD1 is therefore dramatically increased following the inflammatory stimulus.

To investigate further the initial time course of 11β-HSD1 induction, peritoneal cells were collected by lavage up to 4 h after thioglycolate injection, and 11β-HSD1 (reductase) activity was assayed over a further 1 h in vitro, a time at which steroid conversion was linear with time. A low level of activity was seen in adherent cells lavaged 2 h following thioglycolate injection that was similar to the level in resident cells unexposed to thioglycolate. There was a large increase in 11β-HSD1 activity in cells lavaged 3 or 4 h following thioglycolate injection (Fig. 2*B*). The increase between 2 and 3 h suggested either de novo induction in resident cells following a short lag period or the possibility that the increased activity is in cells elicited to the peritoneum between 2 and 3 h following thioglycolate injection. To distinguish between these possibilities, we labeled resident macrophages 24 h before thioglycolate by i.p. injection of PKH-2 green fluorescent tracking dye. Following lavage 3 h after thioglycolate injection, cells were sorted by flow cytometry into nonlabeled and PKH-2-labeled cells and assayed for 11β-HSD1 activity. Labeled and nonlabeled cells from mice that received dye but no thioglycolate had exactly the same level of 11β-HSD1 activity (Fig. 3). In contrast, unlabeled cells (nonresident) were clearly the population containing the high 11β-HSD1 activity in the mice that received thioglycolate (Fig. 3).

11-Dehydrocorticosterone increases phagocytosis by Hsd11b1$^{+/+}$ macrophages, but not Hsd11b1$^{-/-}$ macrophages, in vitro

The presence of 11β-HSD1 in macrophages and its rapid induction during acute inflammation suggest that it could have an important influence on the phagocytic capacity of macrophages. To investigate the possible functional relevance of macrophage *Hsd11b1* expression, we tested whether the inert 11β-HSD1 substrate, 11-dehydrocorticosterone, could mimic the effects of physiological corticosterone upon macrophage phagocytosis. BMD macrophages were incubated with 11-dehydrocorticosterone or corticosterone at concentrations within the physiological range (2–200 nM), and the effect upon phagocytosis was measured. Intrinsically inert 11-dehydrocorticosterone was equally as effective as corticosterone in stimulating phagocytosis by *Hsd11b1*$^{+/+}$ macrophages

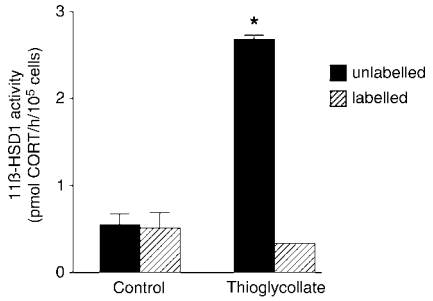

FIGURE 3. The increased 11β-HSD1 activity in the peritoneum is in elicited cells, not in resident macrophages. Twenty-four hours before thioglycolate injection, mice were injected with PKH-2 green fluorescent dye to label resident cells. Three hours following thioglycolate injection, cells were lavaged, sorted, and 11β-HSD1 activity (oxoreductase) was measured in adherent cells. Assays were conducted in vitro for up to 10 h and were within the linear range. The activity of 11β-HSD1 was significantly higher in nonresident cells following thioglycolate injection ($p < 0.001$; ANOVA). Values shown are mean ± SEM of four separate mice at each time point conducted in duplicate, except for labeled cells from thioglycolate injected mice, where cells from four mice were pooled and assayed in duplicate. CORT, Corticosterone.

(Fig. 4). This effect was dependent upon *Hsd11b1* expression, because 11-dehydrocorticosterone was completely without effect on macrophages from *Hsd11b1*$^{-/-}$ mice (Fig. 4). The lack of 11-dehydrocorticosterone effect upon *Hsd11b1*$^{-/-}$ macrophages was not due to an intrinsic (developmental) defect, because corticosterone caused an identical dose-dependent increase in phagocytosis by both *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ macrophages, with significant effects at low physiological concentrations (2 nM) of corticosterone and a 3-fold increase in phagocytic index with 200 nM corticosterone (Fig. 4), a level typically achieved during modest stress. Similar data were obtained when the 11β-HSD inhibitor, carbenoxolone, was added. Carbenoxolone had no effect on corticosterone-induced phagocytosis by *Hsd11b1*$^{+/+}$ macrophages but completely blocked the effect of 11-dehydrocorticosterone (data not shown). Importantly, *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ macrophages ingested saturating numbers of latex beads in a 30-min assay (data not shown), demonstrating that *Hsd11b1*$^{-/-}$ macrophages were able to phagocytose normally.

Phagocytic clearance of apoptotic leukocytes is delayed in the absence of 11β-HSD1

Sterile peritonitis induced in *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ mice by thioglycolate injection resolved within 4 days in both *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ mice (data not shown). In addition, there were no differences between genotypes in the ratio of macrophages to neutrophils in peritoneal lavages at any time point examined (Table I). Because 11β-HSD1 activity was induced in normal mice within a few hours of thioglycolate injection and remained very high until day 3, we reasoned that any phenotype should be apparent within the first 3 days following injection. To determine whether acquisition of phagocytic competence by macrophages is altered in *Hsd11b1*$^{-/-}$ mice, an in vivo phagocytosis assay was conducted. *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ mice were injected i.p. at day 1 or day 2 of sterile peritonitis with 30×10^6 apoptotic PMN, and phagocytosis was examined in peritoneal lavages 10 min later. Compared with controls, *Hsd11b1*$^{-/-}$ mice showed a very low level of phagocytosis after 1 day of peritonitis, which was increased to levels similar to *Hsd11b1*$^{+/+}$ mice after 2 days of peritonitis (Fig. 5*A*), suggesting that there is indeed a delay in acquisition of phagocytic competence in *Hsd11b1*$^{-/-}$ mice.

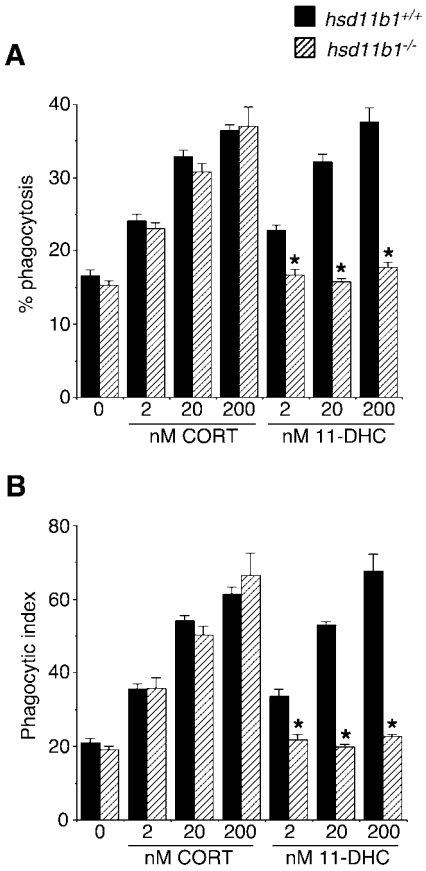

FIGURE 4. Phagocytosis by *Hsd11b1*$^{+/+}$ macrophages is increased by 11-dehydrocorticosterone, but phagocytosis by *Hsd11b1*$^{-/-}$ macrophages is not. BMD macrophages from *Hsd11b1*$^{+/+}$ or *Hsd11b1*$^{-/-}$ mice were cultured for 7 days with the addition of a steroid at day 5, either 0–200 nM corticosterone (CORT) or 2–200 nM 11-dehydrocorticosterone (11-DHC). On day 7, apoptotic PMN were overlaid onto macrophages (at a ratio of 4:1) for 30 min, and levels of phagocytosis were assessed by microscopy. *A*, the percentage of macrophages that had ingested one or more apoptotic PMN. *B*, the phagocytic index, i.e., the number of ingested apoptotic PMN per 100 macrophages. Corticosterone and 11-dehydrocorticosterone (11-DHC) significantly and equally increased phagocytosis and phagocytic index at doses ≥2 nM in *Hsd11b1*$^{+/+}$ macrophages ($p < 0.001$; ANOVA), whereas only corticosterone (CORT) was effective in *Hsd11b1*$^{-/-}$ macrophages ($p < 0.001$; ANOVA) to the same extent as in *Hsd11b1*$^{+/+}$ mice. Values shown are mean ± SEM of counts of at least 500 macrophages from four mice of each genotype conducted in duplicate. *, significantly different to *Hsd11b1*$^{+/+}$ mice; $p < 0.001$.

Consistent with this possibility, *Hsd11b1*$^{-/-}$ mice had increased free endogenous apoptotic neutrophils in peritoneal lavages 2 days after initiation of peritonitis compared with *Hsd11b1*$^{+/+}$ mice (Fig. 5*B*). Furthermore, production of IL-6 in stimulated peritoneal macrophages harvested 24 h following thioglycolate (when 11β-HSD1 activity is maximal) was increased in macrophages from *Hsd11b1*$^{-/-}$ mice (*Hsd11b1*$^{-/-}$ 678 ± 49 pg/ml vs *Hsd11b1*$^{+/+}$ 478 ± 72 pg/ml, $n = 5$/group; $p < 0.05$), suggesting a reduction in the normal glucocorticoid-mediated suppression of cytokine release.

Discussion

Our data define an early event in the acute inflammatory response, namely the rapid and dramatic increase in macrophage 11β-HSD1 activity that regulates the timing of a later key process favoring

Table I. *11β-HSD1-deficient mice have normal ratios of macrophages and PMN during thioglycolate-induced peritonitis*[a]

	Genotype	Mϕ (%)	PMN (%)
Control	*Hsd11b1*$^{+/+}$	91.7 ± 4.7	5.1 ± 5.0
	Hsd11b1$^{-/-}$	89.0 ± 1.6	3.2 ± 2.6
4 h	*Hsd11b1*$^{+/+}$	27.9 ± 4.0	67.1 ± 2.3
	Hsd11b1$^{-/-}$	23.5 ± 4.2	68.1 ± 5.0
1 day	*Hsd11b1*$^{+/+}$	51.8 ± 5.2	36.3 ± 6.2
	Hsd11b1$^{-/-}$	53.0 ± 4.2	32.7 ± 5.1
2 days	*Hsd11b1*$^{+/+}$	59.6 ± 10.0	16.8 ± 5.9
	Hsd11b1$^{-/-}$	62.6 ± 4.6	13.8 ± 1.7
3 days	*Hsd11b1*$^{+/+}$	92.1 ± 1.0	1.4 ± 1.5
	Hsd11b1$^{-/-}$	89.6 ± 2.6	0.4 ± 0.4
4 days	*Hsd11b1*$^{+/+}$	97.1 ± 1.8	0.1 ± 0.1
	Hsd11b1$^{-/-}$	96.2 ± 1.3	0.2 ± 0.3

[a] Macrophages (Mϕ) and PMN were scored as a percentage of the total in cytospins of cells harvested in peritoneal lavages at specified time points after the onset of peritonitis. Values represent mean ± SEM of counts of >2000 cells from each of four mice per time point. No significant differences between *Hsd11b1*$^{+/+}$ and *Hsd11b1*$^{-/-}$ mice were found.

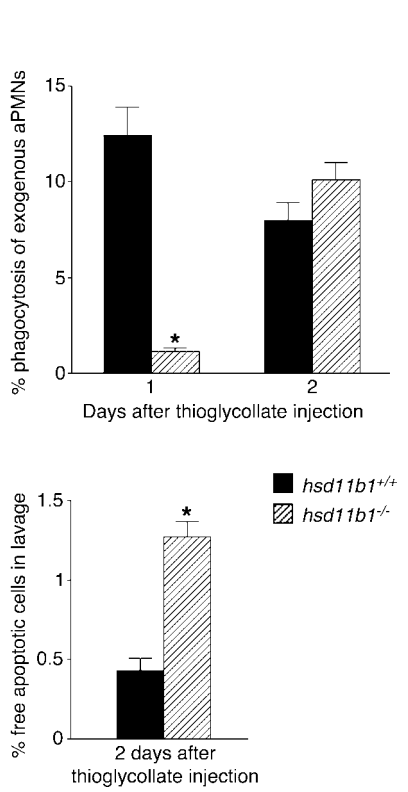

FIGURE 5. Mice deficient in 11β-HSD1 show a delay in acquisition of phagocytic competence with associated impairment of clearance of endogenous apoptotic PMN (aPMNs) during thioglycolate-elicited peritonitis in vivo. *A*, Exogenous apoptotic PMN were administered for 10 min by i.p. injection at day 1 or day 2 after the onset of thioglycolate-elicited peritonitis. Values shown are mean ± SEM of counts of at least 500 cells from each of four mice per genotype. *B*, The proportion of free endogenous apoptotic cells was scored as a percentage of the total cells on cytospins of peritoneal cells obtained by lavage on day 2 following the onset of thioglycolate-elicited peritonitis. Values shown are mean ± SEM for counts of at least 2×10^3 cells from each of four mice per genotype. *, significantly different to *Hsd11b1*$^{+/+}$ mice, $p < 0.001$; ANOVA.

resolution of inflammation, macrophage phagocytosis of neutrophils undergoing apoptosis. Activity of 11β-HSD1 was dramatically increased in cells elicited in the peritoneum within 3 h of thioglycolate injection, remaining markedly elevated in peritoneal macrophages up to 72 h following the onset of peritonitis. Crucially, mice lacking 11β-HSD1 had increased free endogenous apoptotic neutrophils 2 days after initiation of sterile peritonitis and showed a striking delay in the ability to clear exogenously administered apoptotic neutrophils with little evidence of "normal" clearance at 24 h. These results indicate a key physiological role for 11β-HSD1-mediated glucocorticoid reactivation in the early acquisition by macrophages of an increased capacity for clearance of apoptotic leukocytes.

Our in vivo data show that the induction of 11β-HSD1 early in the inflammatory response is physiologically important in determining the subsequent trajectory of the later response. At 24 h of sterile peritonitis, *Hsd11b1*$^{-/-}$ mice showed a marked reduction in the ability to clear exogenously administered apoptotic PMN compared with *Hsd11b1*$^{+/+}$ mice, whereas by day 2 full phagocytic competence was achieved in 11β-HSD1-deficient mice. Furthermore, *Hsd11b1*$^{-/-}$ mice had increased free endogenous apoptotic neutrophils after 2 d of sterile peritonitis, consistent with slower phagocytic clearance. However, *Hsd11b1*$^{-/-}$ macrophages were indistinguishable from *Hsd11b1*$^{+/+}$ macrophages in their phagocytic capacity in vitro, and physiologically relevant levels of corticosterone similarly promoted phagocytosis of apoptotic neutrophils by both genotypes, confirming that glucocorticoid-responsive pathways are intact in *Hsd11b1*$^{-/-}$ macrophages. These data show that 11β-HSD1 is not a prerequisite for macrophage differentiation and maturation to a phagocytic phenotype but rather suggest that it is required for the early attainment of phagocytic capacity for apoptotic cells in vivo. The delay in acquisition of phagocytic competence is thus likely to be a direct result of decreased glucocorticoid reactivation within the 11β-HSD1-deficient macrophage. Indeed, higher levels of IL-6 production (a cytokine potently suppressed by glucocorticoids (21)) by stimulated peritoneal macrophages are consistent with reduced glucocorticoid action within the peritoneal microenvironment of *Hsd11b1*$^{-/-}$ mice, despite their modestly elevated plasma glucocorticoid levels (11). Although IL-6 has no effect per se on the phagocytosis of apoptotic neutrophils by macrophages (22), glucocorticoids up-regulate components of the innate immune response in monocytes, including the scavenger receptor system (23). The involvement of such receptors in the recognition and phagocytosis of apoptotic cells has been postulated (24). Future work will examine whether the delayed clearance of apoptotic cells seen in *Hsd11b1*$^{-/-}$ mice is due to a delay in the expression of a glucocorticoid-inducible surface receptor normally expressed consequent to the rapid induction of 11β-HSD1.

Macrophage 11β-HSD activity was overwhelmingly reductive in intact cells due to exclusive expression of *Hsd11b1*, consistent with previous human data (14). The lower 11β-HSD1 activity in other blood cell types, including lymphocytes (25), suggests that macrophages are uniquely well placed to exploit the significant pool of intrinsically inactive 11-keto metabolites that circulate at levels equivalent to or exceeding basal free corticosterone or cortisol (26). The anti-inflammatory actions of 11-dehydrocorticosterone are evidenced by its equipotency with corticosterone in the ability to increase macrophage phagocytosis in vitro, dependent upon *Hsd11b1* expression. Escher et al. (27) have previously proposed an anti-inflammatory role for 11-keto glucocorticoids. Our data support this role and, in addition, illustrate the particular importance of 11β reduction of 11-keto glucocorticoids early in the inflammatory response in vivo. A similar anti-inflammatory role

for 11β-HSD1 in tissue repair of the ovary following ovulation has been proposed (28, 29) where 11β-HSD1 expression is induced in granulosa cells by the actions of luteinizing hormone and IL-1β (30) and in surface epithelial cells by IL-1α and TNF-α (31).

Induction of sterile inflammation within the peritoneum resulted in a rapid and dramatic increase in 11β-HSD1 activity in peritoneal cells, which declined to normal (resident) levels 4 days later. Although future work will be needed to substantiate the identity of the cell type highly expressing *Hsd11b1* at 3 h, by 24 h following thioglycolate injection, when 11β-HSD1 activity was extremely high, the majority of the peritoneal cells were adherent macrophages. Activity in macrophages remained high until the inflammation resolved. Resident macrophages clearly express 11β-HSD1 but at lower levels than thioglycolate-elicited macrophages. Following thioglycolate injection, resident macrophages rapidly leave the peritoneum, returning only once the inflammation is fully resolved (32). Consistent with this finding, our recovery of labeled resident macrophages was ~10-fold lower from thioglycolate-injected mice (our unpublished observations). Nevertheless, resident cells from injected mice had 11β-HSD1 activity similar to that of macrophages from control mice. The marked increase in 11β-HSD1 activity was in nonlabeled (nonresident recruited) cells, demonstrating clearly that the increase in 11β-HSD1 activity is in cells elicited in the peritoneum by thioglycolate injection and is not due to an increase de novo in the resident population. In support of this finding, we were unable to induce 11β-HSD1 activity in vitro in resident macrophages by the addition of thioglycolate, LPS, or supernatant from lavages of thioglycolate-injected mice (our unpublished data).

The mechanism underlying the rapid increase in 11β-HSD1 activity in inflammatory cells was likely to be distinct from the induction of 11β-HSD1 upon the differentiation of human monocytes to macrophages in which 11β-HSD1 was detectable only after 2 days in culture and was further increased by treatment with IL-4 or IL-13 (14). Classical "proinflammatory" mediators either have no effect upon 11β-HSD1 activity (TNF-α, IL-1β) or even decrease it (LPS) (14). The endogenous inflammatory mediator that effects the increase in 11β-HSD1 activity early in the inflammatory response to thioglycolate is currently unknown, but by virtue of the rapidity of the effect it is more likely to be a proinflammatory factor(s) rather than the "Th2" cytokines reported to induce 11β-HSD1 expression in human monocytes in vitro (14).

To conclude, we propose that local availability of inert 11-dehydrocorticosterone through conversion by macrophage 11β-HSD1 to active corticosterone contributes significantly to the early response to an inflammatory stimulus, promoting prompt resolution of inflammation in vivo. Deficiency in this enzyme results in the loss of this amplification system, resulting in lower local glucocorticoid action and a delay in achieving full phagocytic capacity. Safe phagocytic clearance of cells dying by apoptosis is critically important in the prevention of persistent inflammation and autoimmunity (2, 3). Cortisone, the substrate for 11β-HSD1 in humans, has classically been used to treat chronic inflammation, including rheumatoid arthritis. Orally administered cortisone, however, is a substrate for 11β-HSD1 in all tissues, and the metabolic side effects of the product (cortisol) have prompted the search for alternative "nonsteroidal" therapies. Our findings suggest that the anti-inflammatory properties of cortisone may reflect conversion to an active product by 11β-HSD1 within the macrophage rather than only by the liver as previously assumed. Further, the data suggest that modulation of macrophage 11β-HSD1 activity or targeted delivery of inactive glucocorticoid precursors to macrophages may be of use in the development of future therapies for chronic inflammatory diseases.

Acknowledgments

We are grateful to Dr. Janice Paterson for supplying the *Hsd11b1*$^{-/-}$ mice, Val Lyons for technical support, and Shona Johnston for flow cytometry. We also thank members of the Endocrine Unit and the Medical Research Council Centre for Inflammation Research, particularly Jeremy Hughes and Simon Brown, for helpful advice and discussion.

Disclosures

We have no financial conflict of interest.

References

1. Botto, M., C. Dell'Agnola, A. E. Bygrave, E. M. Thompson, H. T. Cook, F. Petry, M. Loos, P. P. Pandolfi, and M. J. Walport. 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. *Nat. Genet.* 19: 56–59.
2. Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J. Exp. Med.* 192: 359–366.
3. Savill, J., I. Dransfield, C. Gregory, and C. Haslett. 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat. Rev. Immunol.* 2: 965–975.
4. Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett. 1989. Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* 83: 865–875.
5. Liu, Y. Q., J. M. Cousin, J. Hughes, J. VanDamme, J. R. Seckl, C. Haslett, I. Dransfield, J. Savill, and A. G. Rossi. 1999. Glucocorticoids promote nonphlogistic phagocytosis of apoptotic leukocytes. *J. Immunol.* 162: 3639–3646.
6. Giles, K. M., K. Ross, A. G. Rossi, N. A. Hotchin, C. Haslett, and I. Dransfield. 2001. Glucocorticoid augmentation of macrophage capacity for phagocytosis of apoptotic cells is associated with reduced p130Cas expression, loss of paxillin/pyk2 phosphorylation, and high levels of active Rac. *J. Immunol.* 167: 976–986.
7. Heasman, S. J., K. M. Giles, C. Ward, A. G. Rossi, C. Haslett, and I. Dransfield. 2003. Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation. *J. Endocrinol.* 178: 29–36.
8. Seckl, J. R., and B. R. Walker. 2001. Minireview: 11β-hydroxysteroid dehydrogenase type 1: a tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142:1371–1376.
9. Tomlinson, J. W., and P. M. Stewart. 2001. Cortisol metabolism and the role of 11β-hydroxysteroid dehydrogenase. *Best Pract. Res. Clin. Endocrinol. Metab.* 15: 61–78.
10. Yau, J. L., J. Noble, C. J. Kenyon, C. Hibberd, Y. Kotelevtsev, J. J. Mullins, and J. R. Seckl. 2001. Lack of tissue glucocorticoid reactivation in 11β-hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. *Proc. Natl. Acad. Sci. USA* 98: 4716–4721.
11. Kotelevtsev, Y., M. C. Holmes, A. Burchell, P. M. Houston, D. Schmoll, P. Jamieson, R. Best, R. Brown, C. R. W. Edwards, J. R. Seckl, and J. J. Mullins. 1997. 11β-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity or stress. *Proc. Natl. Acad. Sci. USA* 94: 14924–14929.
12. Morton, N. M., M. C. Holmes, C. Fiévet, B. Staels, A. Tailleux, J. J. Mullins, and J. R. Seckl. 2001. Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxysteroid dehydrogenase type 1 null mice. *J. Biol. Chem.* 276: 41293–41300.
13. Morton, N. M., J. M. Paterson, H. Masuzaki, M. C. Holmes, B. Staels, C. Fievet, B. R. Walker, J. S. Flier, J. J. Mullins, and J. R. Seckl. 2004. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* 53: 931–938.
14. Thieringer, R., C. B. Le Grand, L. Carbin, T. Q. Cai, B. Wong, S. D. Wright, and A. Hermanowski-Vosatka. 2001. 11β-Hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. *J. Immunol.* 167: 30–35.
15. Fadok, V. A., J. S. Savill, C. Haslett, D. L. Bratton, D. E. Doherty, P. A. Campbell, and P. M. Henson. 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J. Immunol.* 149: 4029–4035.
16. Low, S. C., K. E. Chapman, C. R. W. Edwards, and J. R. Seckl. 1994. "Liver-type" 11β-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. *J. Mol. Endocrinol.* 13: 167–174.
17. Hadoke, P. W., C. Christy, Y. V. Kotelevtsev, B. C. Williams, C. J. Kenyon, J. R. Seckl, J. J. Mullins, and B. R. Walker. 2001. Endothelial cell dysfunction in mice after transgenic knockout of type 2, but not type 1, 11β-hydroxysteroid dehydrogenase. *Circulation* 104: 2832–2837.
18. Brown, R. W., K. E. Chapman, Y. Kotelevtsev, J. L. Yau, R. S. Lindsay, L. Brett, C. Leckie, P. Murad, V. Lyons, J. J. Mullins, C. R. W. Edwards, and J. R. Seckl. 1996. Cloning and production of antisera to human placental 11β-hydroxysteroid dehydrogenase type 2. *Biochem. J.* 313: 1007–1017.
19. Li, K. X., V. R. Obeyesekere, Z. S. Krozowski, and P. Ferrari. 1997. Oxoreductase and dehydrogenase activities of the human and rat 11β-hydroxysteroid dehydrogenase type 2 enzyme. *Endocrinology* 138: 2948–2952.

20. Rajan, V., K. E. Chapman, V. Lyons, P. Jamieson, J. J. Mullins, C. R. W. Edwards, and J. R. Seckl. 1995. Cloning sequencing and tissue-distribution of mouse 11β-hydroxysteroid dehydrogenase-1 cDNA. *J. Steroid Biochem. Mol. Biol.* 52: 141–147.
21. Schobitz, B., M. Van Den Dobbelsteen, F. Holsboer, W. Sutanto, and E. R. De Kloet. 1993. Regulation of interleukin 6 gene expression in rat. *Endocrinology* 132: 1569–1576.
22. Ren, Y., and J. Savill. 1995. Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. *J. Immunol.* 154: 2366–2374.
23. Galon, J., D. Franchimont, N. Hiroi, G. Frey, A. Boettner, M. Ehrhart-Bornstein, J. J. O'Shea, G. P. Chrousos, and S. R. Bornstein. 2002. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *FASEB J.* 16: 61–71.
24. Savill, J. 1998. Apoptosis: phagocytic docking without shocking. *Nature* 392: 442–443.
25. Zhang, T. Y., X. Ding, and R. A. Daynes. 2005. The expression of 11β-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. *J. Immunol.* 174: 879–889.
26. Harris, H. J., Y. Kotelevtsev, J. J. Mullins, J. R. Seckl, and M. C. Holmes. 2001. Intracellular regeneration of glucocorticoids by 11β-hydroxysteroid dehydrogenase (11β-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: analysis of 11β-HSD-1 deficient mice. *Endocrinology* 142: 114–120.
27. Escher, G., I. Galli, B. S. Vishwanath, B. M. Frey, and F. J. Frey. 1997. Tumor necrosis factor α and interleukin 1β enhance the cortisone/cortisol shuttle. *J. Exp. Med.* 186: 189–198.
28. Tetsuka, M., F. J. Thomas, M. J. Thomas, R. A. Anderson, J. I. Mason, and S. G. Hillier. 1997. Differential expression of messenger ribonucleic acids encoding 11β-hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells. *J. Clin. Endocrinol. Metab.* 82: 2006–2009.
29. Tetsuka, M., M. Milne, G. E. Simpson, and S. G. Hillier. 1999. Expression of 11β-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. *Biol. Reprod.* 60: 330–335.
30. Tetsuka, M., L. C. Haines, M. Milne, G. E. Simpson, and S. G. Hillier. 1999. Regulation of 11β-hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1β in cultured rat granulosa cells. *J. Endocrinol.* 163: 417–423.
31. Gubbay, O., W. Guo, M. T. Rae, D. Niven, A. F. Howie, A. S. McNeilly, L. Xu, and S. G. Hillier. 2004. Anti-inflammatory and proliferative responses in human and ovine ovarian surface epithelial cells. *Reproduction* 128: 607–614.
32. Melnicoff, M. J., P. K. Horan, and P. S. Morahan. 1989. Kinetics of changes in peritoneal cell populations following acute inflammation. *Cell. Immunol.* 118: 178–191. Vol. 176,