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Expression of Cyclooxygenase Enzymes in Rat Hypothalamo-Pituitary-Adrenal Axis

Effects of Endotoxin and Glucocorticoids

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Prostaglandins play a key role in mediating the hypothalamo-pituitary-adrenocortical (HPA) responses to immune insults. This study aimed to provide some insight into the relative contributions of the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2) to the generation of these prostanoids by examining the effects of (1) endotoxin treatment on the expression of COX-1 and COX-2 mRNAs in the various components of the HPA axis in control and glucocorticoid pretreated rats, and (2) selective inhibition of COX-2 on the production of corticosterone by adrenal tissue in vitro. Endotoxin caused a marked rise in COX-2 mRNA in the adrenal gland that was evident 3 and 6 h after the injection and was prevented by pretreatment with dexamethasone. It also induced a modest increase in COX-2 mRNA in the hypothalamus but not in the hippocampus or anterior pituitary gland. By contrast, COX-1 mRNA was largely unaffected by the drug treatments in all tissues studied. In vitro the selective COX-2 inhibitor SC-236 caused a marked reduction in adrenocorticotropic hormone–driven corticosterone release, as did the nonselective COX inhibitor, indomethacin. These results support a role of COX-2 in the manifestation of the HPA responses to endotoxin, particularly within the adrenal gland.

Key Words: Cyclooxygenase; dexamethasone; endotoxin; hypothalamo-pituitary-adrenocortical axis; cyclooxygenase inhibitors.

Introduction

Reciprocal communication between the brain-neuroendocrine and immune systems is fundamental to host defense because it provides a means whereby the central nervous system can detect alterations in immune status and initiate responses (behavioral, physiologic, and immunoregulatory) that are designed to protect the host and, thus, to restore homeostasis. The hypothalamo-pituitary-adrenocortical (HPA) axis is particularly important in this regard because its end products, the glucocorticoids, are key regulators of immune/inflammatory cell function and other homeostatic processes. The HPA axis itself is readily activated by acute immune insults. Thus, factors such as viral antigens and bacterial cell wall-derived endotoxin (lipopolysaccharide [LPS] *(1,2)* provoke marked increases in circulating glucocorticoids in experimental animals and in humans.

There is now unequivocal evidence that the HPA responses to endotoxin are driven primarily by cytokines, and in particular by tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, which are released sequentially from endotoxin-activated macrophages. The mechanisms by which the cytokines activate the HPA axis are complex (for reviews see refs. *1* and *2*). Early studies focused on the hypothalamus and provided evidence for increased release of corticotropin-releasing hormone (CRH) *(3,4)* and arginine vasopressin (AVP) *(5)*. However, while data from in vitro experiments and studies involving intrahypothalamic injections of cytokines favored an action on or close to the CRH/AVP neurons, questions about the ability of cytokines to penetrate the blood-brain barrier pointed to a more complex response, as did other findings. Current evidence suggests that, depening on the route of endotoxin/cytokine administration, the hypothalamic response may involve activation of afferent pathways to the paraventricular nucleus (PVN), e.g., via vagal afferents or brain stem nuclei *(6,7)* and actions of the cytokines on the endothelial cells of the blood-brain barrier *(8,9)*. These, in turn, facilitate the release of CRH/AVP via mechanisms involving local generation of cytokines within the hypothalamus *(2,10)* and/or the release of other mediators that activate the hypophysiotrophic neurons *(11,12)*. Several lines of evidence suggest that prostaglandins are particularly important in this regard. For example, the increases in CRH/AVP release induced in vivo and in vitro by IL-1β and IL-6 are associated with marked increases in the generation

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of prostaglandin E_2 (PGE_2) within the hypothalamus *(13–15)*. Furthermore, central injections of antibodies against PGE_1, PGE_2, and $PGF_{2\alpha}$ abrogate the hypersecretion of adrenocorticotropic hormone (ACTH) induced by IL-1β *(16)*. Similarly, inhibitors of prostaglandin synthesis attenuate the corticotropic responses to intra–median eminence injections of IL-1β *(13,17)* and suppress the expression of c-*fos* in the PVN induced by central or peripheral administration of IL-1β or endotoxin *(18)*. These drugs also effectively inhibit the release of CRH/AVP from hypothalamic tissue induced in vitro by IL-1β, TNF-α, or IL-6 *(10,15)*.

While there can be little doubt about the importance of the hypothalamus in effecting the HPA responses to immune insults, increasing evidence suggests that cytokines exert further actions at the levels of the anterior pituitary gland and adrenal cortex that serve to augment and sustain the adrenocortical response. IL-1β and IL-6 are readily detectable in both pituitary and adrenal tissue, where their expression is enhanced by endotoxin treatment *(2,19–21)*. They also stimulate the release of ACTH and corticosterone in vitro from pituitary and adrenal tissue, respectively, albeit after a relatively long contact time *(10,22–24)*. The potential significance of cytokine actions at the adrenal level is further exemplified by reports that IL-1β provokes the release of corticosterone in hypophysectomized rats and from evidence that the hypersecretion of corticosterone elicited in vivo by local inflammatory lesions is sustained for some hours after the plasma ACTH has declined to the resting level *(25)*. It seems unlikely that prostanoids contribute to the regulatory actions of IL-1β and IL-6 on the corticotrophs since data from a variety of in vivo and in vitro studies have shown that prostanoids exert a marked inhibitory influence on the evoked release of ACTH *(26–28)*. They may, however, fulfill an important role in the adrenal gland because prostaglandins have been reported to have ACTH-like effects on adrenal tissue *(29)* and to have a second-messenger role in corticosteroidogenesis *(30,31)* that is dependent on prostanoid generation *(32,33)*.

The synthesis of prostaglandins requires the enzyme cyclooxygenase (COX) (prostaglandin G/H synthase), which catalyzes the conversion of arachidonic acid to PGH. COX exists in at least two isoforms (COX-1 and COX-2), which are coded on separate genes *(34,35)* and subject to differential regulation *(34–37)*. COX-1 is constitutively expressed and appears to be responsible for the generation of prostanoids under physiologic conditions. By contrast, COX-2 is normally expressed in high levels only after induction by, e.g., endotoxin, cytokines, and growth factors *(35)*. It is thus largely responsible for generation of the prostanoids that fulfill a significant role in the manifestation of pathophysiologic responses, such as inflammation, and of critically timed physiologic events such as ovulation and parturition *(35)*. Studies in peripheral cells and tissues have shown that the expression of COX-2 is subject to glucocorticoid regulation. It is thus sensitive to alterations in adrenal status and depressed by exogenous glucocorticoids *(36)*.

Despite the evidence that prostaglandins play a key role in mediating the HPA responses to immune insults, little is known of the relative importance of COX-1 and COX-2 in this context. Accordingly, the present study was designed to investigate the effect of an immune/inflammatory insult (ip injection of endotoxin) on the expression of COX-1 and COX-2 mRNAs in the HPA axis of control and glucocorticoid-treated rats using reverse transcriptase polymerase chain reaction (RT-PCR). It also examined the effects of a selective COX-2 inhibitor on the steroidogenic activity of rat adrenal tissue in vitro.

Results

In Vivo Studies

Endotoxin (1.0 mg/kg intraperitoneally) produced the anticipated increase in serum corticosterone levels. Thus, 6 h after the injection, the serum corticosterone concentration in the endotoxin-treated group (133.6 ± 12.5 ng/mL) was significantly higher ($p < 0.01$) than that in vehicle-treated controls (53.4 ± 3.2 ng/mL) or in untreated animals (57.3 ± 3.0 ng/mL).

Figures 1 and 2 illustrate the effects of endotoxin treatment (1.0 mg/kg intraperitoneally) on the expression of COX-2 and COX-1 mRNA, respectively, in the hippocampus, hypothalamus, anterior pituitary gland, and adrenal gland of the rat and show how it is modified by pretreatment with dexamethasone. The data shown are normalized to the housekeeping gene, GAPDH, and expressed as a percentage of the saline-treated control. COX-1 and COX-2 mRNAs were detected in all tissues (cycle profiles on tissue pools showed that both genes were present in reasonable abundance); their expression was unaffected by administration of the sterile saline endotoxin vehicle (data not shown). Endotoxin caused a significant increase in COX-2 mRNA in the adrenal gland that was evident 3 and 6 h after injection ($p < 0.05$ vs vehicle control; Fig. 1D). An increase in COX-2 mRNA expression was also evident in the hypothalamus 6 h after administration of endotoxin ($p < 0.05$ vs vehicle control; Fig. 1B), but the apparent rise at 3 h did not reach significance. By contrast, endotoxin failed to influence COX-2 expression in either the hippocampus (Fig. 1A) or the anterior pituitary gland (Fig. 1C). The significant increases in COX-2 mRNA expression observed in both the adrenal gland (Fig. 1D) and the hypothalamus (Fig. 1B) were prevented by pretreatment with dexamethasone. Paradoxically, however, in the dexamethasone-treated group, COX-2 mRNA expression was augmented in the hypothalamus 3 h after the injection of endotoxin. The steroid treatment did not affect the expression of COX-2 mRNA in the pituitary gland in control or endotoxin-treated rats (Fig. 1C) but caused a small reduction in hippocampal

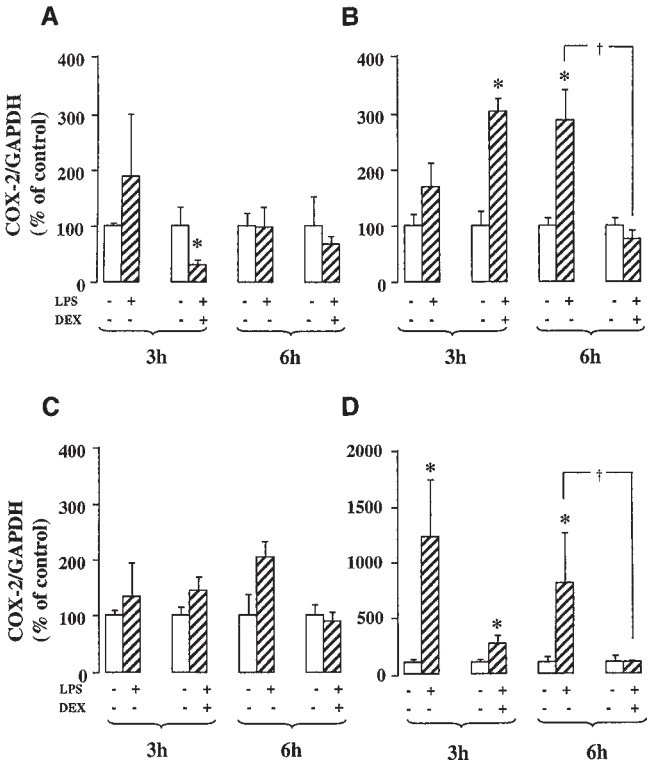

Fig. 1. Effects of endotoxin (LPS) and dexamethasone (DEX) on COX-2 mRNA expression in (**A**) hippocampus, (**B**) hypothalamus, (**C**) pituitary gland, (**D**) and adrenal gland of rat. Male rats were treated with dexamethasone sodium phosphate (2 mg/L of drinking water for 48 h; controls received normal drinking water) before treatment with endotoxin (LPS, *E. coli* K-235, 1.0 mg/kg intraperitoneally; ▨) or an equal volume of saline (1.0 mL/kg intraperitoneally; □). Tissue was collected 3 and 6 h later. Each point represents the mean ± SEM. *$p < 0.05$ vs vehicle control; †$p = 0.05$ vs vehicle pretreatment (Mann-Whitney U test, $n = 3$ or 4).

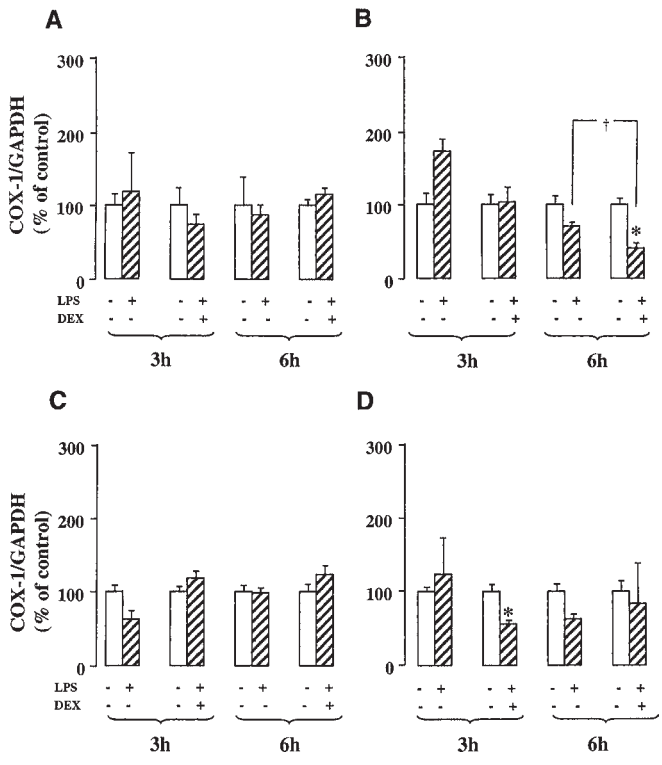

Fig. 2. Effects of endotoxin (LPS) and dexamethasone (DEX) on COX-1 mRNA expression in (**A**) hippocampus, (**B**) hypothalamus, (**C**) pituitary gland, and (**D**) adrenal gland of rat. Male rats were treated with dexamethasone sodium phosphate (2.4 mg/L of drinking water for 48 h; controls received normal drinking water) before treatment with endotoxin (LPS, *E. coli* K-235, 1.0 mg/kg intraperitoneally; ▨) or an equal volume of saline (1.0 ml/kg intraperitoneally; □).Tissue was collected 3 and 6 h after injection. Each point represents the mean ± SEM. *$p < 0.05$ vs vehicle control; †$p = 0.05$ vs vehicle pretreatment (Mann-Whitney U test, $n = 3$ or 4).

COX-2 mRNA 3 h after the injection of endotoxin ($p < 0.05$ vs saline control).

By contrast, endotoxin did not affect the expression of COX-1 mRNA in any of the tissues studied (Fig. 2 A-D). Dexamethasone also failed to influence COX-1 mRNA expression in the hippocampus and pituitary gland ($p > 0.05$ vs vehicle or endotoxin). However, in the adrenal gland and the hypothalamus, it caused a small but significant reduction in COX-1 mRNA 3 and 6 h, respectively, after the injection of endotoxin ($p = 0.05$ vs vehicle).

In Vitro Studies

Figure 3 shows the effects of indomethacin (nonselective COX inhibitor; IC_{50} COX-1: 0.028 µmol; IC_{50} COX-2: 1.68 µmol) *(38)* and SC-236 (COX-2 selective inhibitor; IC_{50} COX-1: 17.6 µmol; IC_{50} COX-2: 0.009 µmol) *(39,40)* on the secretion of immunoreactive (ir)-corticosterone by acutely dispersed adrenal cells. A submaximal dose of ACTH (10^{-9} *M*) caused a significant ($p < 0.01$ vs basal) increase in release of ir-corticosterone. This effect was reduced by indomethacin (10^{-5} *M*, $p < 0.05$; Fig. 3A) and SC-236 (10^{-5} *M*, $p < 0.001$; Fig. 3B). Neither indomethacin nor SC-236 alone influenced basal ir-corticosterone release ($p > 0.05$ vs vehicle; Fig. 3).

Discussion

Our study exploited a semiquantitative RT-PCR method *(41)* to examine the impact of endotoxin on the expression of the COX-1 and COX-2 mRNAs in the rat hippocampus, hypothalamus, pituitary gland, and adrenal gland. Although this is an established, highly sensitive method of detecting mRNA, quantification of the data requires careful control. We therefore undertook preliminary cycle profiling to ensure that the amplification of each gene product fell within the exponential phase where reaction product yield is proportional to the amount of starting material. In addition, we normalized all values to GAPDH, a housekeeping gene that

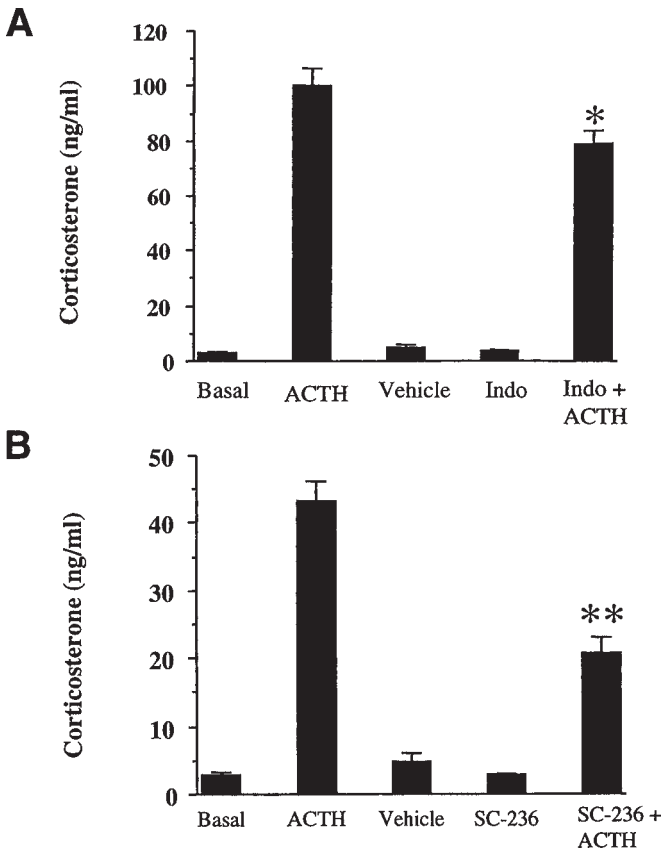

Fig. 3. Effects of (**A**) ACTH (10^{-9} M) and indomethacin (Indo; 10^{-5} *M*) and (**B**) ACTH (10^{-9} *M*) and SC-236 (10^{-5} *M*) on release of ir-corticosterone from acutely dispersed rat adrenal cells in vitro. Columns represent the mean ± SEM ($n = 6$). *$p < 0.05$ vs ACTH; **$p < 0.01$ vs ACTH (analysis of variance [ANOVA] and Fisher test).

is constitutively expressed at a low to moderate level in all tissues studied and that was amplified in parallel during the assay. Working within these boundaries, we were able to detect two- to threefold changes in relative gene expression. Clearly, direct measures of the protein product would be required to confirm that newly expressed mRNA is effectively translated. However, many studies in other tissues have shown a good correlation between levels of COX-2 mRNA and COX protein and/or COX enzymatic activity after endotoxin or cytokine treatment *(42,43)*. Our data confirm reports that COX-2 mRNA is expressed constitutively in various brain areas, such as in the hippocampus, where it has been localized mainly to neuronal cells *(44–47)*. They also provide novel evidence that endotoxin induces a glucocorticoid-reversible increase in COX-2 expression in the hypothalamus and adrenal gland without affecting expression of the enzyme in either the hippocampus or anterior pituitary gland. In addition, the data show that blockade of COX-2 inhibits adrenal corticosteroidogenesis in vitro. Taken together, these finding suggest that COX-2 contributes to the mechanisms that effect the HPA responses to immune/inflammatory insults. Additional roles may also be played by COX-1, the mRNA that was readily detectable in all tissues studies but largely unaffected by the various experimental manipulations employed.

Our finding that endotoxin produces an increase in COX-2 mRNA expression within the hypothalamus accords with data from a previous study based on *in situ* hybridization in which a rise in COX-2 mRNA was observed in the PVN of LPS-treated rats *(46)*. They are also in line with the many studies that advocate a role for COX and its prostanoid products within the hypothalamus in the manifestation of the HPA response to immune insults (see Introduction). Much further work, however, is necessary to delineate the roles of COX-1 and COX-2 in this regard. Previous studies have shown that the ACTH response to an ip injection of endotoxin is maximal within 2 h *(2)*, yet we observed an increase in COX-2 mRNA in the hypothalamus only 6 h after treatment with endotoxin. It is thus possible that COX-1, which was readily detected in the hypothalamus at all time points studied but whose expression was unaffected by the various drug treatments, is the key player in driving the early phase of the response. On the other hand, it is possible that functionally important changes in COX-2 mRNA expression that would not have been detectable by our method occurred in discrete loci within the hypothalamus and/or at earlier time points. Such arguments are supported by evidence that endotoxin causes small increases in COX-2 mRNA expression in neuronal tissue but much more pronounced changes in the microvasculature, most notably in the endothelial and leptomeningeal cells *(45–47)*, which may provide an important route of cytokine signaling to the brain *(48)*.

Surprisingly, the most striking changes in COX-2 mRNA were observed in the adrenal gland, which raises the possibility that the isozyme fulfills a significant role in the manifestation of the adrenal response to endotoxin. Although we have not yet examined steroidogenesis in endotoxin-treated adrenal tissue, further evidence of this effect was derived from our in vitro study that demonstrated the ability of the type 2–selective COX inhibitor, SC-236, to mimic the inhibitory effects of the nonselective COX inhibitor, indomethacin, on ACTH-induced corticosterone secretion by acutely dispersed adrenal cells. These findings fit well with other data suggesting that endotoxin targets the adrenal gland and demonstrate local roles for both cytokines and prostanoids in the control of steroidogenesis. Thus, IL-1β, IL-6, and TNF-α are produced by the adrenal gland *(20, 49)*, where their expression is greatly enhanced by endotoxin *(20,21,50)*. Histologic studies have revealed that a resident macrophage population, found mainly at the corticomedullary junction, provides a significant source of the three cytokines *(51–53)*. In addition, IL-1 is produced by chromaffin cells in rodents while IL-6 and TNF-α are expressed in the zona glomerulosa *(54)*. IL-1β and IL-6 have been reported to act directly on the adrenal gland to increase corticosterone secretion *(23,24)* via a prostanoid-dependent mechanism *(32,33,55)*. Furthermore, their effects, which

are synergistic with those of ACTH *(24)*, follow a time course that approximates the endotoxin-induced increase in COX-2 mRNA reported herein *(32,33,55)*. Thus, while the source of COX-2 mRNA, and hence prostanoids within the adrenal, remains to be determined, it seems likely that the increased expression of the enzyme provoked by endotoxin is dependent on the release of cytokine either from chromaffin cells *(49,56,57)* or from the local macrophage population.

The failure of endotoxin to induce COX-2 mRNA in the pituitary gland and hippocampus is difficult to explain since the expression of IL-1β and IL-6 in both tissues is greatly enhanced by endotoxin treatment *(20,50)*. However, in line with these findings, Lacroix and Rivest *(46)* also found COX-2 mRNA expression in the hippocampus to be unaffected by endotoxin treatment. Moreover, although prostanoids are implicated in the signal transduction mechanisms that control pituitary function, their effects on ACTH secretion are inhibitory *(28)* and would therefore be unlikely to contribute to the modest positive effects of locally produced cytokines on the release of ACTH.

Glucocorticoids are well known to suppress the induction of COX-2 mRNA in conditions of inflammation *(58)*, acting via mechanisms that may involve both the inhibition of transcription *(36)* and destabilization of the mature mRNA transcript *(59)*. In accord with this premise, our results show that the increases in COX-2 mRNA observed in the adrenal gland 3 and 6 h after the injection of endotoxin are prevented by pretreatment with dexamethasone. The steroid treatment also suppressed the induction of COX-2 mRNA evident 6 h after the administration of endotoxin in the hypothalamus but paradoxically increased COX-2 mRNA 3 h after the toxin treatment. The reason for this is uncertain but may be related to the complex pharmacokinetics of the steroid. Unlike several other glucocorticoids, dexamethasone normally has only limited access to central tissues because it is rapidly exported from the brain by the drug-exporting P-glycoprotein encoded by the multiple drug resistance gene *(66)*; it is therefore purported to produce a "chemical adrenalectomy" of the brain. Administration of the steroid would, however, be expected to suppress the endotoxin-induced secretion of corticosterone through actions at the pituitary level *(10)*. Thus, at the 3-h time point, the hypothalamus would be exposed to relatively low levels of corticosterone, which may permit an exaggerated COX-2 mRNA response to endotoxin. In this event, how could the marked inhibitory action of dexamethasone on the endotoxin-induced induction of hypothalamic COX-2 mRNA at 6 h be explained? One possibility is through a change in permeability of the blood-brain barrier that increases the bioavailability of dexamethasone within the brain. Such a mechanism is supported by evidence that as the pathologic effects of endotoxin manifest themselves, the effectiveness of the blood-brain barrier is reduced *(61)*.

In conclusion, our observations suggest that the hyperactivity of the HPA axis observed in conditions of inflammatory stress is dependent, at least in part, on the generation of COX-2 within both the hypothalamus and the adrenal gland. While more detailed analysis of the time- and dose-dependent changes in COX mRNA and protein/enzymic activity are now warranted, the data thus raise the possibility that COX-2 inhibitors may compromise the HPA response to certain stressors, an area that merits investigation. The marked changes in COX-2 mRNA observed in the adrenal gland in endotoxin-treated animals are particularly interesting and may be indicative of a mechanism through which the adrenocortical response to immune insults is maintained or supplemented. They therefore support the premise that the adrenal gland is an important additional locus of the interplay between the endocrine and host defense systems that is critical to homeostasis.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 200 ± 10 g and bred in-house from a closed, specific pathogen-free colony were used. They were housed four per cage in a room with controlled lighting (lights on from 8:00 AM to 8:00 PM), humidity (50%), and temperature (21–23ºC) for at least 7 d prior to the experiment and were weighed daily thereafter by the individual who undertook the subsequent experimental procedures. Food and water were available ad libitum. Experiments were started between 8:00 AM and 10:00 AM to avoid changes associated with the circadian rhythm. The study was carried out under license in accordance with the UK Scientific Procedures (Animals) Act (1986).

Drugs

Endotoxin (*E. coli* K235; Sigma), dexamethasone sodium phosphate (David Bull, Warwick, UK), indomethacin (Sigma), SC-236 (Pharmacia), and $ACTH_{1-24}$ (tetracosactrin B.P.; Alliance, Chippenham, UK) were used. Indomethacin and SC-236 were each initially dissolved in small amounts of ethanol and subsequently diluted in EBSS, the final concentration of ethanol never exceeded 0.5%, and appropriate vehicle controls were included in all experiments.

Administration of Drugs

Endotoxin (*E. coli* K-235, 1.0 mg/kg), or a corresponding volume of the sterile saline vehicle (0.9% NaCl solution, 0.2 mL/rat), was administered by ip injection, and tissues were collected 3 or 6 h later. When used, dexamethasone sodium phosphate (2.4 mg/L of drinking water) was administered for 48 h before the administration of endotoxin and saline.

Collection of Blood and Tissues

Treated and untreated control animals were killed by decapitation, and the hippocampus, hypothalamus, pituitary gland, and adrenal gland were removed and frozen immediately

on dry ice until RNA extraction. Blood was collected from the trunk into plastic tubes. The serum was separated by centrifugation (2000g, 10 min, 4ºC) and stored at –20ºC for subsequent determination of corticosterone.

Static Incubation of Dispersed Adrenal Cells

The protocol used was based on the method of Rafferty et al. *(62)*. Briefly, adrenal glands obtained postmortem from decapitated rats were decapsulated and the cells dissociated by incubation (40 min, 37ºC) with collagenase (2 mg/mL) (Boehringer Mannheim, Sussex, UK) in Earle's balanced salt solution (EBSS) pH 7.4, phenol red free; (Sigma, Poole, Dorset, UK). The dispersion was aided by gentle trituration (30 s every 10 min). The resulting cell suspension was filtered through a 100-µm nylon mesh and centrifuged (100*g*, 15 min). The cells in the pellet formed were resuspended in EBSS and the centrifugation was repeated. After further resuspension in EBSS (1 mL), cell viability was assessed by the trypan blue exclusion test (always >85%). The cells were then plated out to a density of 2×10^5 cells/well in 96-well cell culture plates (Costar, Cambridge, MA) and preincubated for 1 h in EBSS at 37ºC in a humid atmosphere saturated with 95% O_2/5% CO_2 gas. The plates were centrifuged (2000*g*, 10 s), the supernatants discarded, and the cells incubated for a further 2 h in the presence or absence of a submaximal concentration of ACTH ($ACTH_{1–24}$, $10^{–9}$ *M*, in EBSS). After centrifugation (2000*g*, 10 s), the supernatant fluid was collected and either assayed immediately for ir- corticosterone or stored at –20ºC for subsequent corticosterone measurement. When appropriate, COX inhibitors or vehicle was included during both the preincubation and the final incubation period.

Reverse-Transcriptase Polymerase Chain Reaction

The expression of COX-1 and COX-2 mRNAs was determined by semiquantitative RT-PCR using a modification of the method of Newton et al. *(41)* and GAPDH as a housekeeping gene.

Reverse Transcription and Amplification

Total RNA was isolated by the method of Chomczynski and Sacchi *(63)* and reverse transcribed into cDNA for use as the template for PCR. RNA samples (1 µg) were denatured at 70ºC for 5 min. Reverse transcription was performed at 37ºC for 60 min in a reaction volume of 20 µL containing random hexanucleotide primers (0.2 µg) (Pharmacia UK), RT buffer, 0.1 mol/L dithiothreitol, 1 mmol/L of each dNTP, and 40 U of mouse myeloma leukemia virus RT (Invitrogen, Paisley, UK). The RT reaction was stopped by heating at 90ºC for 5 min and made up to 100 µL with H_2O. PCR was performed in a total volume of 25 µL containing 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTPs, 125 ng of each sense and antisense primer and 1 U of Biotaq polymerase (Bioline, UK). The primers sequences (5'-3') used were as follows: COX-1: ATA CCG AAA GAG GTT TGG CC (sense), GTT AGC AAA GGA TTC AGC ATC (antisense); COX-2: CAT CAG TTT TTC AAG ACA GAT CA (sense), CCC ACT CAG GAT GCT CCT G (antisense); GAPDH: TCC CTC AAG ATT GTC AGC AA (sense), AGA TCC ACA ACG GAT ACA TT (antisense) *(64)*. An annealing temperature of 55ºC was used for all primers, and product sizes generated were 591 (COX-1), 351 (COX-2), and 309 bp (GAPDH). The cycling parameters were as follows: denaturing: 94ºC, 30 s; annealing: 30 s; extension: 72ºC, 30 s; an appropriate number of PCR cycles was run for each gene (for details of determination, see next section) and followed by a 72ºC, 5-min extension. The RT-PCR products for COX-1, COX-2, and GAPDH were then verified by double-stranded sequencing.

Establishment of Exponential Phase of Reaction by PCR Cycle Profiling

Cycle profiles for COX-1, COX-2, and cytoplasmic GAPDH were performed to determine the exponential phase of amplification in which product formation was proportional to starting template. Following reverse transcription, equal aliquots from each sample were mixed to create a pooled cDNA sample comprising adrenal gland, hippocampus, hypothalamus, or pituitary gland. The pooled material, which represented an "average" sample, was used as a template for PCR cycle profiling, using the following numbers of cycle: COX-1 and COX-2: 15, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36; GAPDH: 10, 15, 18, 20, 22, 24, 26, 28, 30, 35. Aliquots (10 µL) of the amplified products were run on 1.2% agarose gels, stained with ethidium bromide, and analyzed visually. Additional aliquots (5 µL) of the amplified products were also dot blotted onto Hybond-N filters (Amersham Pharmacia Biotech UK) using standard techniques. The filters were then hybridized to radiolabeled ^{32}PdCTP COX-1, COX-2, and cytoplasmic GAPDH cDNA probes, generated by subcloning the relevant RT-PCR products into a pGEM-T vector and verified by sequencing and washed to high-stringency conditions. Expression of COX-1, COX-2, and GAPDH mRNA was assessed by scintillation counting of dot blots. Counts were plotted against cycle number to establish the exponential phase of amplification. Figure 4 illustrates a typical PCR cycle profile for COX-2 cDNA. On the basis of the data, 29 (which fell within the exponential phase) was the cycle number selected for amplification in the subsequent semiquantitative studies. The cycle profile for COX-1 was similar (data not shown), and the cycle number 29 also appeared to be optimal for this gene product and was used subsequently. For GAPDH, the linear range of amplification was assessed to be 18–24 cycles, and a cycle number of 21 was chosen for amplification (data not shown). Figure 5 demonstrates Southern blot analysis of the RT-PCR products in each of the tissues studied and shows that each product was of the expected

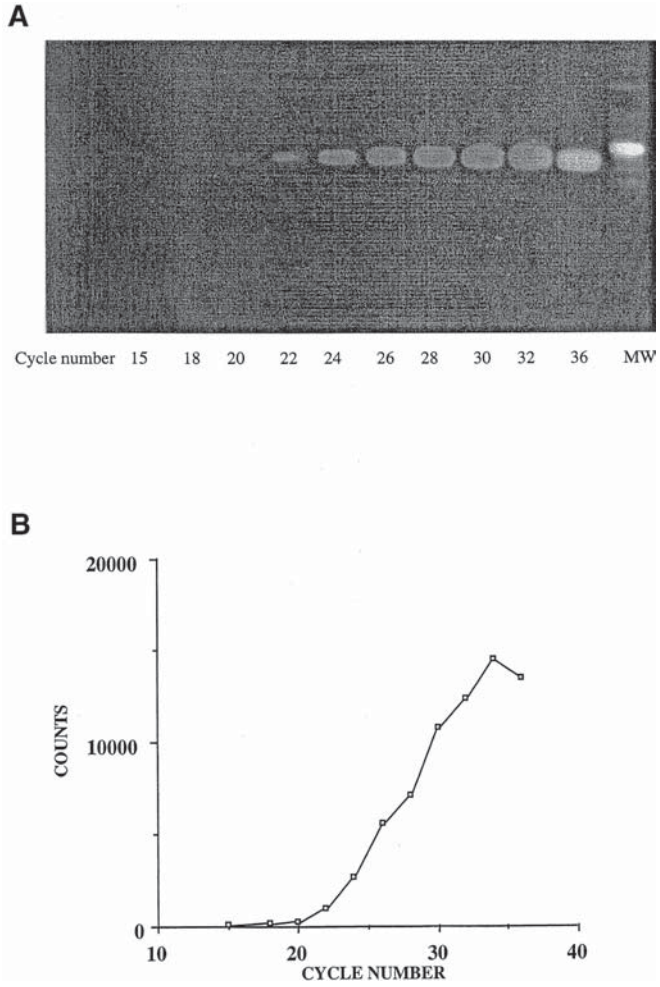

Fig. 4. PCR cycle profile to establish exponential phase of reaction. (**A**) Ethidium bromide–stained gel showing increasing COX-2 PCR product with increasing cycle number using an "average" sample as template; (**B**) expression of COX-2 assessed by scintillation counting of dot blots. Counts were plotted against cycle number to establish the exponential phase of amplification.

size (i.e., COX-1, 591 bp; COX-2, 351 bp; GAPDH, 309 bp). In addition, Northern analysis of mRNA from hypothalamic tissue confirmed the presence of the 4.4-kb COX-2 transcript (results not shown).

Analysis and Semiquantitation of Samples by Southern Blotting and Cerenkov Counting

By using the cycle numbers determined in the previous section, we determined that 2.5 µL of cDNA from each sample (prepared as already described) had to be added to a total PCR reaction volume of 25 µL and amplified by PCR. Aliquots (10 µL) of the product were size fractionated on 1.2% agarose gels. Southern hybridization was used to confirm identity of products and, since all primer pairs cross at least one intron, to check for possible genomic contamination. Figure 5 presents a typical Southern blot showing that the RT-PCR products generated in each

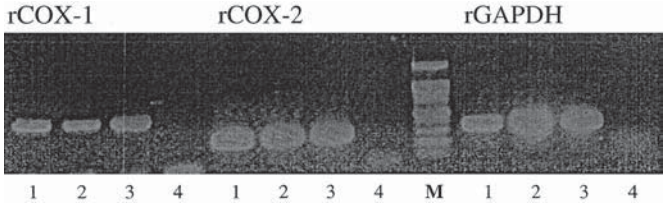

Fig. 5. Detection of COX-1, COX-2, and GAPDH in rat hippocampus, pituitary gland, and adrenal gland by RT-PCR. Ethidium bromide staining of RT-PCR products with primers COX-1, COX-2, and GAPDH is shown. Lane 1, adrenal; lane 2, hippocampus; lane 3, pituitary; lane 4, blank; lane M, 1-kb ladder.

of the tissues studied were of the expected size (i.e., COX-1, 591 bp; COX-2, 351 bp; GAPDH, 309 bp). In addition to Southern analysis, 5 µL of each PCR reaction was dot blotted onto a Hybond-N filter and hybridized overnight at 65ºC to the appropriate radiolabeled 32PdCTP cDNA probe. Membranes were washed to high-stringency conditions with 0.1X sodium citrate buffer at 65ºC. The dot blots were excised and the radioactivity was measured by Cerenkov counting *(65)*. Expression levels of COX-1 and COX-2 in the adrenal gland, hippocampus, hypothalamus, and pituitary gland were normalized with respect to GAPDH and expressed as a percentage of control.

Radioimmunoassay of Corticosterone

Corticosterone released in vitro was measured by the radioimmunoassay method of Newsome et al. *(66)*, using a well-characterized antiserum (Sigma) and ^{3}H-labeled corticosterone (Amersham International PLC, UK). Separation of bound and free steroid was achieved by the addition of dextran-coated charcoal. The intra- and interassay coefficients of variation (CVs) were 4.2 and 5.7%, respectively. Because this assay is not suitable for the measurement of corticosterone in serum, a kit purchased from Immunodiagnostic Systems (Boldon, Tyne and Wear, UK) that uses a ^{121}I-labeled ligand was used to determine corticosterone in the serum samples. The sensitivity of this assay was 0.39 ng/mL, and the antiserum supplied showed negligible crossreactivity with dexamethasone. The intraassay CV was 4.2%, and dilution curves of the samples were parallel with those of the standard curve. Samples were run in the same assay to avoid interassay variation.

Statistical Analyses

Measurements of COX-1 and COX-2 mRNAs (corrected for GAPDH) are presented as the means ± SEM for each group. The differences between experimental groups were analyzed by the Mann-Whitney U test. Data from the in vitro experiments and measurements of serum corticosterone were analyzed by one-way ANOVA followed by Fisher test of least difference. Data were considered to be significant when $p \leq 0.05$.

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