Steroid Hormones Inhibit Induction of Spontaneous Nitric Oxide Production in Cultured Hepatocytes without Changes in Arginase Activity or Urea Production (43566)

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Abstract. **The spontaneous formation of nitrites was examined in the medium of cultured rat hepatocytes and taken as a measure of nitric oxide generation. The rate of nitrite formation increased after 8–12 hr in culture which was blocked by the addition of dexamethasone, actinomycin D, or cycloheximide. Various glucocorticoids, mineralocorticoids, and sex steroids also inhibited nitrite formation by varying degrees, without affecting arginase activity or urea production. The inhibition of nitric oxide formation appears, therefore, not to be due to changes in the availability of arginine. The results suggest that nitric-oxide synthase is induced in hepatocytes in culture and show that anti-inflammatory glucocorticoids are not the only steroids that inhibit nitric oxide formation.** [P.S.E.B.M. 1993, Vol 202]

A large number of cell types have the ability to synthesize nitric oxide (NO) from arginine, by the enzyme nitric-oxide synthase (for review, see Ref. 1), including liver parenchymal cells (2–5) and Kupffer cells (resident macrophages) (2, 6). Administration of endotoxin to rats induces nitric-oxide synthase in the liver (2, 4). Knowles *et al.* (4) reported this to occur in the parenchymal cells of the liver, whereas Billiar *et al.* (2, 6) have reported this to take place only in the nonparenchymal cells (Kupffer/endothelial). While NO production has been implicated in mediating the cytotoxic actions of activated macrophages (1), the role of liver parenchymal NO production is not clear.

Anti-inflammatory glucocorticoids have been shown to inhibit lipopolysaccharide (LPS)-stimulated NO production in liver parenchymal cells (5) and other cells (1, 5, 7–9) that can be mimicked by protein synthesis inhibitors, but not by progesterone (7–9). Little attention has been focused on the mechanism of action of glucocorticoids, or on the spontaneous production of nitric oxides in cultured parenchymal cells.

Recently it has been shown that the availability of arginine is highly regulated in endothelial cells and may be an important factor in the regulation of NO formation (10–12). Nitric-oxide synthase in the liver would compete with arginase for available arginine. In view of the fact that glucocorticoids have been shown to increase the activity of arginase (13–15), with an increase in urea cycle activity and urea production, the availability of arginine may be decreased, which could account for the inhibition of observed NO formation.

In this report, we examined the relationship between NO formation, arginase activity, and urea production. We show that the glucocorticoids, mineralocorticoids, and sex steroids can all inhibit spontaneous nitrite release to varying degrees without affecting arginase activity or urea production.

Materials and Methods

Preparation and Culture of Hepatocytes. Hepatocytes were prepared by collagenase digestion as described (16, 17) and parenchymal cells were attached to 23-mm, 12-well culture plates at a density of 5×10^5 cells/well in 0.75 ml of modified Liebowitz L15 medium containing 10% newborn calf serum (18, 19).

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Received March 31, 1992. [P.S.E.B.M. 1993, Vol 202]
Accepted November 24, 1992.

0037-9727/93/2024-0499$3.00/0

Additions constituted no more than 0.3% of the final volume of the medium. Steroid hormones were dissolved in dimethyl sulfoxide, which was also added as a control. Cells were maintained for 20 hr before use in the presence or absence of steroid hormones. The steroid hormones did not affect the plating efficiency of the cells, or the viability of the hepatocytes as judged by retention of lactate dehydrogenase activity, cell protein content, and trypan blue exclusion (results not shown). The contamination of steroid preparations was <0.3% for aldosterone and <0.1% for all the others.

Assay for Nitrites. NO is rapidly converted to NO_2- and NO_3- (in the presence of oxygen and water) which are relatively stable, inactive end products (1). The presence of nitrites is indicative of NO formation. The assay for nitrites was based on the Griess reaction (20). Protein was determined by the method of Bradford (21). Results are expressed as nmol nitrite released/mg cell protein.

Arginase Activity and Urea Production. Urea was determined as described (22), where urea is hydrolyzed by urease to ammonia and converted to indophenol. Medium (25 μl) was added to 250 μl of urease buffer containing: 15 units/ml urease in 100 m*M* sodium acetate (pH 5) and incubated for 15 min at 37°C. Phenol reagent (0.5 ml) (50 g of phenol and 0.5 g of sodium nitroprusside/liter) and 0.5 ml of hypochlorite reagent (25 g of NaOH and 42 ml of sodium hypochlorite, 4–6%/liter) were added. Samples were mixed and left for 2 hr and the absorbance was measured at 645 nm. The assay was linear up to 100 nmol. Results are expressed as μmol urea produced/mg cell protein.

The assay for arginase was based on that of Garganta and Bond (22). Briefly, cells were scraped in 1 ml of 50 m*M* KPi (pH 7.4) containing 150 m*M* KCl. Samples were sonicated for 6 × 1 sec and were diluted 1/20 with the same buffer. Arginase activity was measured in the presence or absence of manganese, a known activator of arginase (22, 23). Sample (40 μl) was preincubated for 30 min at 37°C with either 10 μl of H_2O or 5 m*M* $MnCl_2$ (1 m*M* final). Reagent buffer was then added containing: 12.5 m*M* $NaHCO_3$ (pH 9.5) and 12.5 m*M* arginine in a final volume of 250 μl and incubated for up to 30 min at 37°C. Urease buffer (250 μl) (described above) was then added and tubes were further incubated for 15 min. The acidic pH of the urease buffer inhibits arginase activity, which is determined by the presence of urea. Reaction was terminated by the addition of 1 ml of both phenol and hypochlorite reagents. Standard curves for urea were performed in KPi buffer containing arginine and sodium acetate. Activity is expressed as nmol/min/mg cell protein. The assay was linear for at least 1 hr (results not shown) and manganese stimulated arginase activity 2.4-fold, in close agreement with reported values (23).

Materials. Tissue culture plates were from Costar. Collagenase, $NaNO_2$, tissue culture reagents, and steroids were from Sigma.

Results and Discussion

Incubation of parenchymal cells for up to 28 hr in primary culture led to an increase in the accumulation of nitrites in the medium. Between 0–8 hr, there was little appearance of nitrite in the medium. At 3 hr, nitrite was barely detectable with this assay (0.025–0.030 OD units). Typical readings at 8 hr of culture ranged between 0.075 and 0.100 OD units and greatly increased thereafter, resulting in OD units between 0.25 and 0.58 at 20 hr. Dexamethasone (3 n*M*), when added between 0–4 hr in culture, virtually abolished the increase in nitrite formation (82 ± 7% for $n = 5$; Fig. 1), as expected (1, 5, 7–9). However, when added after 8–12 hr in culture, dexamethasone had only a limited effect (21 ± 5% for $n = 5$; Fig. 1). Very similar results were obtained with actinomycin D and cycloheximide, as shown in an earlier publication (24). This suggests that dexamethasone blocks the synthesis of nitric-oxide synthase, or some other factor involved in the regulation of the enzyme, which occurs with a lag time of 8–12 hr, and that once induced, dexamethasone (or the protein synthesis inhibitors) showed little effect.

The following experiments were performed to determine whether steroids other than glucocorticoids could inhibit nitric-oxide synthase. For these studies, all agents were added at the time of cell plating and nitrite formation was measured after 20 hr in culture.

Dexamethasone potently inhibited the spontaneous release of nitrite with an estimated median effective concentration (EC_{50}) of 0.5 n*M* (Fig. 2A and Table I). Other natural anti-inflammatory glucocorticoids, corticosterone, cortisone, and hydrocortisone, also inhibited nitrite formation with EC_{50} values in the 0.1–1

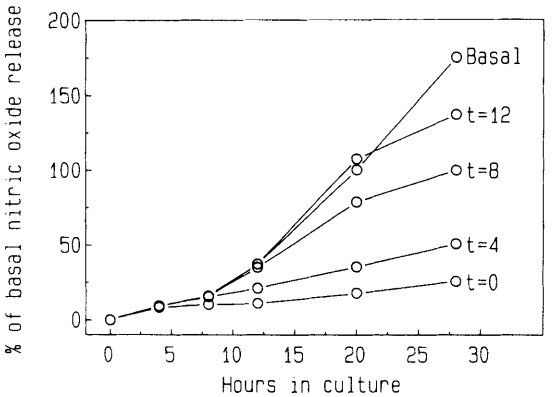

Figure 1. Time course for the accumulation of nitrites in the medium of cultured hepatocytes. Hepatocytes were cultured for the times indicated and the nitrite concentration was determined in the medium. Dexamethasone (3 n*M*) was added to the medium at the times indicated. Results are expressed as the percentage of nitrite in the medium of control incubations relative to that seen after 20 hr and are means ± SE from five paired independent experiments.

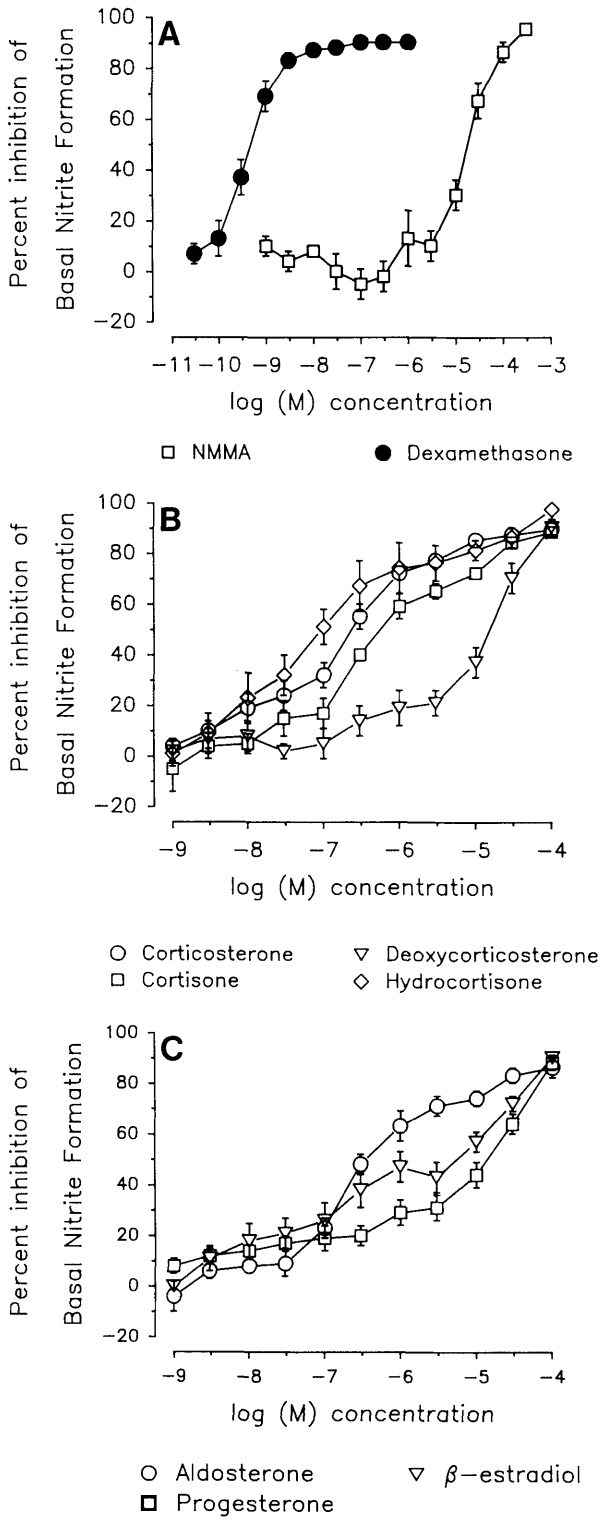

Figure 2. Effects of steroids and N^G-monomethyl-L-arginine (NMMA) on nitrite formation. Hepatocytes were incubated for 20 hr in the presence of agents as indicated. Results are means ± SE from three to seven independent experiments as indicated in Table I and are expressed as percentage of inhibition of basal release of nitrite, which was 33.7 ± 5.8 nmol released/mg protein.

Table I. Relative Potencies of Various Steroids on Inhibition of Nitrite Formation

Additions	Independent experiments (*n*)	EC_{50} values[a]
Dexamethasone	7	0.51 ± 0.04 n*M*
Hydrocortisone	7	0.11 ± 0.01 μ*M*
Corticosterone	7	0.25 ± 0.04 μ*M*
Aldosterone	5	0.67 ± 0.17 μ*M*
Cortisone	5	0.97 ± 0.19 μ*M*
β-Estradiol	7	1.53 ± 0.50 μ*M*
Deoxycorticosterone	5	10.71 ± 1.44 μ*M*
Progesterone	7	11.87 ± 2.33 μ*M*
NMMA	3	18.76 ± 1.82 μ*M*

[a] The EC_{50} values for the inhibition of nitrite formation were calculated from data shown in Figure 2 using the Enzfitter computer program. The correlation of the best curve fit in cultured rat hepatocytes was >0.95 for all additions.

μ*M* range (Fig. 2B). Deoxycorticosterone, which is derived from progesterone and is a precursor of corticosterone and aldosterone, was 10- to 100-fold less effective then the anti-inflammatory glucocorticoids (Fig. 2B). Deoxycorticosterone has no glucocorticoid potency, but has approximately 5% of the potency of aldosterone as a mineralocorticoid. Aldosterone was found to be a potent inhibitor of nitrite accumulation, with an EC_{50} of less than 1 μ*M* (Fig. 2C and Table I), which is less than that of cortisone. The potency of aldosterone was approximately 20-fold higher than that of deoxycorticosterone, which agrees with the relative potencies of these two steroids with regard to mineralocorticoid activity. These results suggest that glucocorticoids and mineralocorticoids are both equally effective at inhibiting spontaneous nitrite production.

The sex steroids progesterone and β-estradiol also inhibited nitrite formation (Fig. 2C). Progesterone was fairly weak (EC_{50} of 12 μ*M*), having a similar potency to that of deoxycorticosterone. However, Di Rosa *et al.* (7), Radomski *et al.* (8), and Pfeilschifter (9) saw no effects of progesterone at 5–10 μ*M* in macrophages, endothelial cells, or renal mesangial cells on inhibiting the induction of nitric oxide synthase by LPS, interferon-τ or interleukin-1β. In cultured rat hepatocytes, 10 μ*M* progesterone inhibited spontaneous nitrite formation by 44 ± 5% (n = 7; Fig. 2C). β-Estradiol, on the other hand, was quite potent at inhibiting nitrite formation (Fig. 2C), with an EC_{50} value similar to that of cortisone.

The appearance of nitrite in the medium was inhibited by the nitric oxide synthase inhibitor N^G-monomethylarginine (NMMA), with an EC_{50} of approximately 20 μ*M* (Fig. 2A). The high EC_{50} is probably a reflection of the fact that the L15 medium contains 2.4 m*M* arginine. However, the results show that nitrite formation in the medium is dependent upon nitric oxide synthase activity.

A certain degree of cell-type selective inhibition of nitric oxide synthesis can be demonstrated by using different arginine analogs. The arginine analog N^G-monomethylarginine (NMMA) inhibits nitric oxide synthesis by both the inducible, tetrahydrobiopterin- and flavin-dependent activity exemplified by the macrophage enzyme and a constitutive, Ca^{2+}-dependent activity exemplified by the endothelial cell enzyme. In contrast, N^G-nitro-L-arginine is much less potent with macrophages and more effectively inhibits constitutive nitric-oxide synthase (25). In our hands, NMMA and N^G-nitro-L-arginine methyl ester used at a 30-μM concentration were equipotent in inhibiting nitrite accumulation by cultured rat hepatocytes. The respective values expressed as percentage of basal nitrite release were as follows: NMMA, 30 μM in hepatocytes of 30- and 3-hr saline-infused rats: $10 \pm 1\%$ ($n = 5$) and $26 \pm 15\%$ ($n = 3$), respectively (24); NAME, 30 μM in hepatocytes of 30-hr saline-infused rats: $15.8 \pm 0.7\%$ (mean ± range from two independent experiments). In two experiments, N^G-nitro-L-arginine inhibition of nitric oxide synthesis was contrasted with that of NMMA in rat hepatocytes, and it was clear that more nitroarginine is needed than NMMA to see the same inhibition. The values were $10.4 \pm 1.0\%$ of basal release with 0.1 mM NMMA versus $15.0 \pm 6.5\%$ with 0.25 mM nitroarginine. These results are consistent with the presence of an inducible, or primarily inducible enzyme, with minimal contribution of constitutive nitric oxide synthase activity.

The bile acids cholate and deoxycholate, which like steroids are derived from cholesterol, did not inhibit nitrite formation at concentrations up to 10 μM, nor did they affect hepatocyte viability as determined by trypan blue exclusion and protein content of the cells. Similarly indomethacin, a cyclooxygenase inhibitor, had no significant effect.

The activity of arginase was also measured in cell homogenates from the same cultures. Trace metal accumulation, including Mn^{2+}, has been reported in the livers of diabetic rats (23, 26). As arginase activity is dependent upon Mn^{2+}, changes in arginase activity may be due to increased arginase protein or increased Mn^{2+} concentration/availability in the cytosol (23, 26). To overcome this problem, arginase activity was measured in the presence or absence of added Mn^{2+}. Changes in arginase activity in the presence of Mn^{2+} would be coincident with an increase in the amount of arginase protein. As described in Materials and Methods, preincubation of cell extracts with 1 mM $MnCl_2$ increased arginase activity by 2.4-fold, in close agreement with the 2.6-fold increase reported in liver cytosol (23). As shown in Table II, none of the steroid hormones tested had any significant effect on arginase activity either in the presence or absence of Mn^{2+}.

Arginase activity has been reported to be induced by glucocorticoids in fetal (13) and adult (14) rat hepatocytes in culture as well as in rat hepatoma cell lines (15). With H4 hepatomas, an increase in arginase was detected after 24 hr and continued to rise for 72 hr (15). In fetal hepatocytes, arginase activity was increased only after 72 hr of treatment with glucocorticoids (13). In adult hepatocytes, slight increases were seen after 48 hr, which were greatly potentiated by the simultaneous addition of glucagon (14). The lack of effect of steroid hormones on arginase activity after 20 hr would, therefore, not be inconsistent with the reported effects. We have shown, however, that steroid hormones can inhibit nitrite formation as early as 8–12 hr (Fig. 1). Increased flow of arginine through arginase and the urea cycle may occur without large changes in arginase activity and would lower cytosolic arginine concentrations, thereby inhibiting nitrite formation. The production of urea may be a more sensitive indicator of increased arginase activity or urea cycle activity. Therefore, urea formation in the medium of cultured hepatocytes was also measured along with nitrites. Again, we found that none of the steroid hormones has any significant effect on the accumulation of urea in the medium after 20 hr. This, again, is presumably due to the length of time required for the induction of urea cycle enzymes. Recently it has been reported that the synthesis of arginine from citrulline has an important role in the maintenance of the availability of arginine for the production of nitric oxide in cultured endothelial cells (10–12). Although we cannot rule out that such mechanisms exist in the liver, the results presented here suggest that removal of intracellular arginine through increased arginase activity or urea cycle activity is not a factor in steroid-mediated inhibition of nitric oxide formation.

The function of NO production by the parenchymal cells of the liver remains unclear. Billiar *et al.* (27) reported that inhibition of nitric oxide synthase enhanced sepsis-induced liver damage *in vivo*, whereas *in vitro*, NO production had a cytotoxic effect on parenchymal cells, by inhibiting protein synthesis (3, 28).

The results presented here show that nitric oxide synthase is probably induced in parenchymal cells in culture with a lag time of 8–12 hr. A potential mechanism for the induction of NO synthase in hepatocytes may involve the membrane receptors on rat hepatocytes for the inner core region of LPS (29). The binding of LPS to these membrane receptors may then be followed by induction of gene transcription and protein translation resulting in the expression of an isoform of nitric-oxide synthase.

The production of nitrites in the medium is inhibited not only by the anti-inflammatory glucocorticoids but also by mineralocorticoids and sex steroids. The results also suggest that once induced, steroid hormones show limited effect on nitric oxide synthase activity.

Table II. Effects of Various Steroids on Formation of Nitrites and Urea and Arginase Activity[a]

Additions		Nitrite formation	Urea formation	Arginase	
				$-Mn^{2+}$	$+Mn^{2+}$
Basal		(33.7 ± 5.8 nmol/mg protein)	(2.43 ± 0.31 μmol/mg protein)	(0.51 ± 0.09 nmol/min/mg protein)	(1.22 ± 0.27 nmol/min/mg protein)
			% of basal values		
Dexamethasone	1 n*M*	31 ± 6[b]	97 ± 10	98 ± 6	97 ± 2
Corticosterone	1 μ*M*	28 ± 2[b]	95 ± 7	112 ± 9	98 ± 11
Cortisone	1 μ*M*	41 ± 5[b]	88 ± 3	99 ± 5	101 ± 3
Hydrocortisone	1 μ*M*	26 ± 9[b]	97 ± 7	97 ± 6	85 ± 6
Aldosterone	1 μ*M*	37 ± 6[b]	102 ± 4	91 ± 2	93 ± 8
β-Estradiol	1 μ*M*	53 ± 6[b]	93 ± 9	90 ± 10	98 ± 4
Progesterone	10 μ*M*	60 ± 1[b]	86 ± 7	90 ± 4	95 ± 4
Deoxycorticosterone	10 μ*M*	63 ± 6[b]	95 ± 9	97 ± 10	97 ± 5

[a]Hepatocytes were incubated for 20 hr in the presence of steroids. Nitrite and urea formation in the medium and arginase activity in cell extracts were determined as described in Materials and Methods. Results are means ± SE from three to seven independent experiments and are expressed as percentage of basal values which are shown.
[b] The significance of difference between the groups was determined by paired *t* test, $P < 0.001$.

The steroid hormones probably inhibit the *de novo* synthesis of nitric oxide synthase, since the effects were mimicked by protein synthesis inhibitors.

This work was supported by NIH Grant GM32654. The use of animals conformed to NIH guidelines. We thank Dr. Zoltan Spolarics for helpful discussion and Curtis vande Stouwe for the preparation of hepatocytes.

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