Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 10043–10047, December 1990
Medical Sciences

Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells

(steroids/cytokines/endotoxin)

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Communicated by Pedro Cuatrecasas, September 17, 1990 (received for review July 30, 1990)

ABSTRACT **Vascular endothelial cells contain a constitutive nitric oxide (NO) synthase that is Ca^{2+}-dependent. In addition, we have found that these cells express, after activation with interferon-γ and lipopolysaccharide, an inducible Ca^{2+}-independent NO synthase that is distinct from the constitutive enzyme. The generation of NO by this enzyme was detectable after a lag period of 2 hr, reached a maximum between 6 and 12 hr, and was maintained for the duration of the experiment (48 hr). The expression of the inducible NO synthase was inhibited by the protein synthesis inhibitor cycloheximide, a compound that had no direct effect on the activity of either of the two enzymes. Furthermore, hydrocortisone and dexamethasone, but not progesterone, inhibited the expression of the inducible enzyme, without directly affecting the activity of either enzyme. The effect of these steroids was inhibited in a concentration-dependent manner by cortexolone, a partial agonist of glucocorticoid receptors. Thus, the inhibition of the induction of an NO synthase by glucocorticoids is a receptor-mediated event involving the inhibition of the synthesis of mRNA for *de novo* synthesis of this enzyme. The induction of this NO synthase may contribute to the pathophysiology of immunologically based conditions. Furthermore, the inhibition of this induction by anti-inflammatory steroids may explain some of the therapeutic and adverse effects of these compounds.**

The demonstration of nitric oxide (NO) biosynthesis from the terminal guanidino nitrogen atom(s) of L-arginine by vascular endothelial cells (1, 2) has been followed by the finding of NO synthesis in many other cells and tissues (3–11). There is increasing evidence that there are two distinct enzymes responsible for the biosynthesis of NO. One is constitutive, Ca^{2+}-, calmodulin-, and NADPH-dependent (6, 9, 12–14) and synthesizes NO as a transduction mechanism to regulate the activity of soluble guanylate cyclase. The other is induced in macrophages and other cells (3–5, 10, 15) by endotoxin and some cytokines. Furthermore, this latter enzyme is Ca^{2+}-independent and requires NADPH and tetrahydrobiopterin (4, 16, 17). The NO synthesized by this enzyme in macrophages contributes to their cytotoxic activity against tumor cells (18), bacteria (19), and protozoa (20, 21).

Other functions of NO released in response to these immunological stimuli have not yet been described. Recent evidence suggests that the hypotension induced by tumor necrosis factor (TNF) in dogs is NO-dependent (22), since it is reversed by N^G-monomethyl-L-arginine (L-MeArg), an inhibitor of the synthesis of NO from L-arginine (18, 23). Whether the hypotensive effect of TNF is due to rapid expression of an inducible enzyme or to an increase in activity of the constitutive enzyme responsible for the physiological regulation of blood flow and pressure (24, 25) has not been established. In addition, it is not clear whether the NO released by TNF comes from the vascular endothelium or from other sources. In this context, mouse brain microvascular endothelial cells produce NO_2^- 8 hr after exposure to interferon-γ (IFN-γ) in combination with TNF, interleukin 1β, or endotoxin (26). One possibility is that the vascular endothelium, besides possessing the constitutive NO synthase, has the potential to express an inducible NO synthase after immunological activation. This was investigated in the present experiments.

Since the expression of a number of proteins induced by cytokines in macrophages and endothelial cells is inhibited by glucocorticoids (27) and these compounds are used in the treatment of endotoxin shock mainly to prevent or reverse the hypotension (28), we have also investigated whether the induction of this NO synthase, if it occurred, could be inhibited by anti-inflammatory steroids.

MATERIALS AND METHODS

Porcine aortic endothelial cells were isolated and cultured on Cytodex 3 microcarriers (Pharmacia–LKB BioTechnology) for 7–8 days in Dulbecco's modified Eagle's medium containing 25 mM Hepes, 20% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml), as described (29). The cells (>98% endothelial, determined by factor VIII staining) were then incubated for a further 48 hr, unless otherwise stated, in the presence or absence of endotoxin lipopolysaccharide (LPS; 10 μg/ml) and IFN-γ (150 units/ml). The effects of cycloheximide or steroids on the constitutive or inducible enzyme were investigated in cells incubated with these compounds for 48 hr. The direct effects of these compounds on the activity of the NO synthases were determined by addition of these compounds to cytosols obtained from cells incubated for 48 hr in the presence or absence of LPS and IFN-γ.

Endothelial cells (0.3–1.0 ml of microcarriers containing $1.5–5.0 \times 10^7$ cells) were washed three times with Ca^{2+}-free Tyrode's solution and the release of NO was determined either by inhibition of platelet aggregation (30) or by the spectrophotometric determination of the quantitative oxidation of oxyhemoglobin to methemoglobin as described (31).

Measurement of NO by Inhibition of Platelet Aggregation. Endothelial cells (0.5 ml of microcarriers in a total volume of 1 ml) were incubated with bradykinin (100 nM) for 30 sec at 37°C in Tyrode's solution containing indomethacin (10 μM). After rapid filtration through a 0.22-μm (pore size) filter, the inhibition by the cell-free filtrates of collagen-induced aggregation was determined in suspensions of prostacyclin-washed human platelets (32) in the presence of a selective

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Abbreviations: L-MeArg, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TNF, tumor necrosis factor.

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inhibitor of the cGMP phosphodiesterase M&B 22948 (1 μM) as described (30). The Ca^{2+}-dependent release of NO was determined in Tyrode's solution in the presence of 0.2 mM Ca^{2+} and the Ca^{2+}-independent release of NO in Ca^{2+}-free Tyrode's solution containing 1 mM EGTA.

Spectrophotometric Determination of NO Synthase. Endothelial cells {1.0 ml of microcarriers in a total volume of 3 ml of homogenizing buffer [10 mM Hepes/0.32 M sucrose/0.1 mM EDTA/1 mM dithiothreitol/leupeptin (10 μg/ml)/aprotinin (2 μg/ml)/soybean trypsin inhibitor (10 μg/ml)/phenylmethylsulfonyl fluoride (10 μg/ml), pH 7.4] at 4°C} were homogenized twice for 3 sec with a Soniprep (MSE Scientific Instruments, Crawley, UK) homogenizer. The homogenate was centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was passed through a 2-ml column of cation-exchange resin (AG 50W-X8) to remove endogenous arginine. NO synthesis was measured in incubates containing 5 μM oxyhemoglobin and 50% (vol/vol) endothelial cytosol in 40 mM potassium phosphate (pH 7.2) containing $MgCl_2$ (1 mM) by using a dual wavelength spectrophotometer (Shimadzu UV-3000) as described (9). NO synthesis was initiated by addition of L-arginine (30 μM) and NADPH (300 μM). The rate of NO synthesis was linear for 10 min. The constitutive and inducible NO synthases were assayed by controlling the Ca^{2+} levels (0.2 mM or zero, respectively) in supernatants by Ca^{2+}/EGTA buffers (6).

Reagents. All reagents for cell culture except fetal calf serum (Flow Laboratories) were from GIBCO. *Salmonella typhosa* LPS (Difco); recombinant murine IFN-γ (Genzyme); prostacyclin and L-MeArg (Wellcome); L-arginine, indomethacin, dithiothreitol, soybean trypsin inhibitor, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, hydrocortisone, dexamethasone, progesterone, cortexolone, and cycloheximide (Sigma): NADPH (Boehringer Mannheim); propoxyphenyl-8-azapurine-6-one (M&B 22948, May & Baker, Dagenham, U.K.); collagen (Hormon-Chemie, Munich); and AG 50W-X8 resin (Bio-Rad) were obtained as indicated. Human hemoglobin was prepared as described (33).

Statistics. All values are expressed as mean ± SEM of *n* experiments and were compared by using Student's *t* test for unpaired data, and $P < 0.05$ was considered as statistically significant.

RESULTS

Release of NO from Endothelial Cells by a Constitutive and an Inducible NO Synthase. In the presence of 0.2 mM Ca^{2+}, unstimulated endothelial cells released a small, but significant, amount of NO that inhibited platelet aggregation by 10 ± 2% (Fig. 1*A*). This inhibition was significantly increased to 85 ± 5% when endothelial cells were stimulated with 100 nM bradykinin (Fig. 1*A*). After a further 48 hr of culture, the basal release and bradykinin-stimulated release of NO were not significantly different from these values, inhibiting platelet aggregation by 12 ± 3% ($n = 4$) and 81 ± 7% ($n = 4$), respectively.

In the absence of Ca^{2+} no basal or bradykinin-stimulated release of NO was detected either initially (Fig. 1*B*) or after a further 48 hr of culture in medium alone ($n = 3$). However, when cells were activated with LPS and IFN-γ for 48 hr, they spontaneously released NO, which inhibited platelet aggregation by 76 ± 8% (Figs. 1*C* and 2*A*). This release was consistently, but not significantly, enhanced by treatment with 100 nM bradykinin so that the inhibition of platelet aggregation was 88 ± 9% (Fig. 1*C*). The time course of this Ca^{2+}-independent release of NO was such that after a 2-hr incubation there was no significant release of NO. However, over the next few hours the release increased, reaching a maximum between 6 and 12 hr and persisting for the duration of the experiment (Fig. 2*A*).

Characteristics of the Cytosolic Constitutive and Inducible NO Synthases. In the presence of Ca^{2+} (0.2 mM) the rate of NO production by the constitutive NO synthase prepared from nonactivated cells was 87 ± 7 pmol per mg of protein per min ($n = 3$). This was not significantly changed when this enzyme was prepared from cells cultured for a further 48 hr (80 ± 9 pmol per mg of protein per min; $n = 3$). In the absence of Ca^{2+}, there was no detectable production of NO by the constitutive NO synthase either initially or after a further 48 hr in culture (Fig. 2*B*).

In cytosols obtained from endothelial cells activated with LPS and IFN-γ, for up to 2 hr, there was no detectable formation of NO in the absence of Ca^{2+} (<0.01 pmol per mg of protein per min). However, there was a significant Ca^{2+}-independent formation of NO in cytosols obtained from cells activated with LPS and IFN-γ for 48 hr (39 ± 2 pmol per mg of protein per min; Fig. 2*B*). The time course of the induction

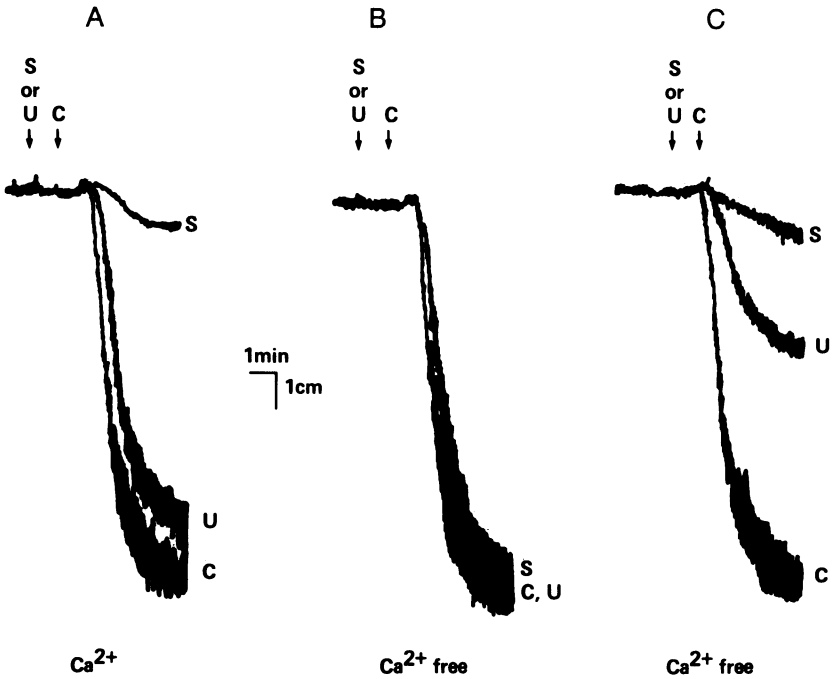

FIG. 1. Inhibition of collagen-induced platelet aggregation by NO released from endothelial cells. (*A*) In the presence of Ca^{2+}, unstimulated cells (U) released small amounts of NO. This was increased by stimulation with bradykinin (100 nM, S). Control aggregation (C) to collagen (4 μg/ml) is also shown. (*B*) In the absence of Ca^{2+}, the release of NO was not detected under these conditions. (*C*) NO released by endothelial cells activated with LPS and IFN-γ for 48-hr inhibited platelet aggregation in the absence of Ca^{2+} (U). This release was further increased by bradykinin (100 nM, S). Tracings are representative of three similar experiments.

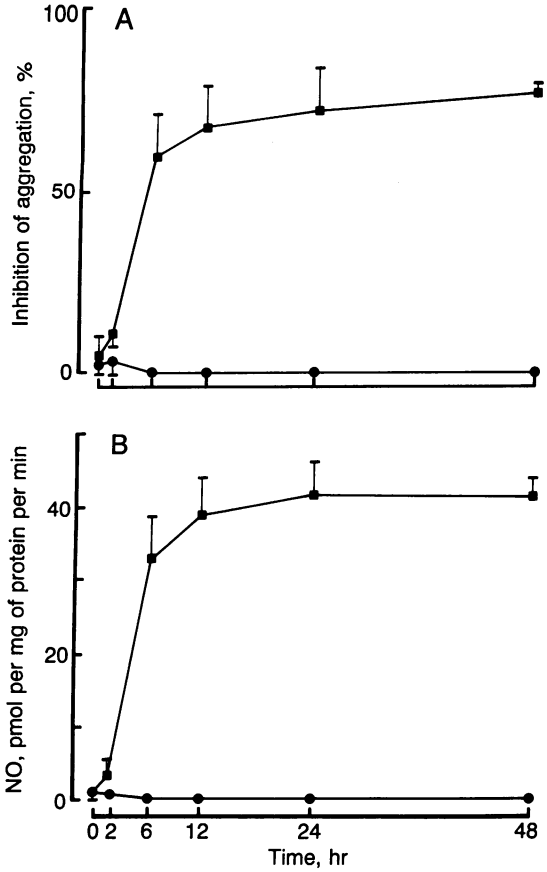

FIG. 2. Time-course of induction of Ca^{2+}-independent NO release and NO synthase after activation of endothelial cells by LPS and IFN-γ. (*A*) Release of NO from activated (■) and nonactivated (●) cells. (*B*) The activity of NO synthase from activated (■) and nonactivated (●) cells. Each point is the mean ± SEM of three experiments.

of this Ca^{2+}-independent NO formation was similar to that of the release of NO by intact endothelial cells (Fig. 2). Cytosols obtained from cells incubated for 48 hr with IFN-γ alone did not show any Ca^{2+}-independent NO synthesis (<0.01 pmol per mg of protein per min); however, the rate of synthesis of NO in cytosols obtained from cells incubated with LPS alone was 10 ± 2 pmol per mg of protein per min.

Both the Ca^{2+}-dependent constitutive enzyme and the Ca^{2+}-independent inducible enzyme were inhibited by L-MeArg (Fig. 3). Furthermore, L-MeArg (10 μM) abolished the Ca^{2+}-dependent and -independent release of NO from intact cells when measured by the inhibition of platelet aggregation (n = 3 for each).

Incubation of endothelial cells for 48 hr with cycloheximide (10 μM) had no effect on the Ca^{2+}-dependent NO synthase (n = 3); however, it inhibited, in a concentration-dependent manner, the induction of the Ca^{2+}-independent NO synthase (Fig. 4). Cycloheximide (10 μM) had no direct effect on the activity of the Ca^{2+}-independent NO synthase once it was expressed (48 hr, n = 3).

Inhibition by Glucocorticoids of the Induction of the NO Synthase. Incubation of cells either acutely or for 48 hr with hydrocortisone (3 μM), dexamethasone (1 μM), or progesterone (3 μM) did not affect the Ca^{2+}-dependent NO synthase (n = 3 for each compound). In contrast, hydrocortisone (0.1–3 μM) and dexamethasone (0.03–1 μM), but not progesterone (0.1–3 μM), inhibited in a concentration-dependent manner, the induction of the Ca^{2+}-independent NO synthase (Fig. 5). The inhibitory effect of hydrocortisone and dexamethasone was blocked in a concentration-dependent manner by cortexolone (Fig. 6). None of these compounds had any direct effect on the inducible enzyme once it was expressed (48 hr; n = 3 for each set of compounds).

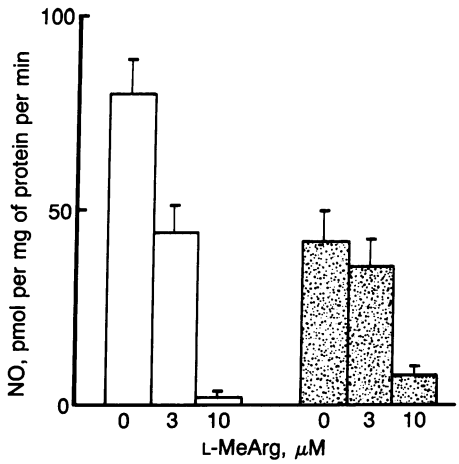

FIG. 3. Inhibition of the Ca^{2+}-dependent constitutive (open bars) and Ca^{2+}-independent inducible (stippled bars) NO synthases by L-MeArg. The results are the mean ± SEM of three experiments.

DISCUSSION

Vascular endothelial cells in culture release NO. This release, which can be enhanced by stimulation with bradykinin, is Ca^{2+}-dependent and inhibited by L-MeArg. These data, together with the finding of a Ca^{2+}-dependent synthesis of NO by the cytosol of these cells, confirm the existence of a constitutive NO synthase in the vascular endothelium (12–14).

Activation of endothelial cells with IFN-γ and LPS induces, after a 2-hr lag period, the Ca^{2+}-independent release of NO. This release, which is also inhibited by L-MeArg, is associated with the induction in the cytosol of a Ca^{2+}-independent NO synthase, which is distinct from the constitutive enzyme. This Ca^{2+}-independent enzyme may also be activated by bradykinin. These results suggest that the reported production of NO by mouse cerebral microvascular endothelial cells in response to immunomodulators (26) is not due to an increase in activity of the constitutive enzyme in these cells but to the induction of a different NO synthase.

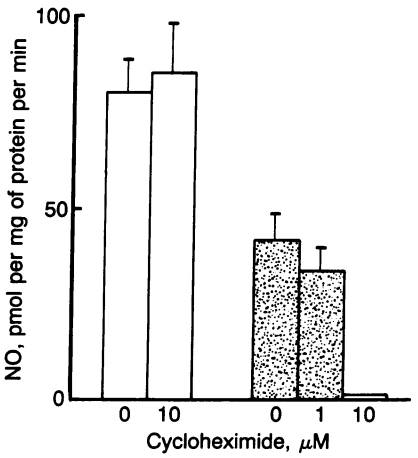

FIG. 4. Inhibition of the induction of the Ca^{2+}-independent NO synthase in endothelial cells by cycloheximide. Cycloheximide had no effect on the activity of the Ca^{2+}-dependent constitutive NO synthase (open bars) but caused concentration-dependent inhibition of the expression of the inducible enzyme (stippled bars). The results are the mean ± SEM of three experiments.

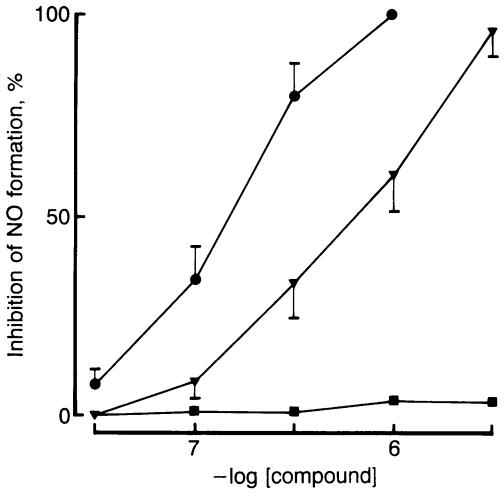

FIG. 5. Inhibition of the induction by the Ca^{2+}-independent NO synthase in endothelial cell cytosol by hydrocortisone or dexamethasone. Dexamethasone (●) and hydrocortisone (▼), but not progesterone (■), caused concentration-dependent inhibition of the induction of the Ca^{2+}-independent NO synthase. Control NO synthase activity was 44 ± 4 pmol per mg of protein per min. Each point is the mean ± SEM of three experiments.

The expression, but not the activity, of the inducible enzyme is abolished by the protein synthesis inhibitor cycloheximide, showing that this enzyme is synthesized *de novo* after activation with IFN-γ and LPS. In contrast, the activity of the Ca^{2+}-dependent NO synthase was not affected after a 48-hr incubation with this compound, indicating that these cells contain sufficient quantities of this enzyme at the beginning of the incubation period to last for the duration of the experiment.

The inducible NO release in the vascular endothelium is similar to that in macrophages (3–5), Kupffer cells (10), hepatocytes (11), and EMT-6 cells (15) for the enzymes responsible for the release of NO in these cells are Ca^{2+}-independent and are induced, over a similar time course, after activation of the cells. Furthermore, this enzyme, in contrast to the constitutive NO synthase, releases NO for long periods. Whether this endothelial NO synthase, like that of the macrophage, is also tetrahydrobiopterin-dependent (16, 17) now needs to be studied.

A further fundamental difference between these two enzymes is that steroids such as hydrocortisone and dexamethasone, but not progesterone, inhibit the expression of the inducible NO synthase without directly affecting the activity of either of them. This, together with the observation that cortexolone, a partial agonist of glucocorticoid receptors (34), antagonizes the action of these steroids, shows that the inhibition of the inducible enzyme is a receptor-mediated effect involving the formation of specific mRNA for the *de novo* synthesis of this enzyme.

The effect of glucocorticoids may be the result of their inhibition of endogenous cytokine synthesis (35–38). Alternatively, they may be acting by inhibition of the induction of the NO synthase or by the induction of a protein that inhibits the enzyme. All these possibilities remain to be investigated. Moreover, it is possible that longer term incubation with glucocorticoids may also affect the constitutive enzyme.

At present the only clearly established function of NO released during immunological reactions relates to the cytotoxic/cytostatic actions of activated macrophages (18–21). Immunologically generated NO from endothelial or other cells may, however, also bring about increases in blood flow and hypotension, modulation of leukocyte and platelet behavior, and/or other aspects of the immunological reaction. It is likely that the release of NO might act as a general modulatory mechanism that, when exaggerated, will contribute to the signs and symptoms of immunologically based conditions. This may be the case in endotoxin shock where NO, initially released as a defense mechanism, subsequently contributes to the final severe hypotension that characterizes this condition. Indeed, inhibitors of NO synthesis have been shown to enhance the liver (39) and intestinal (40) damage and also to reverse the hypotension (22) after administration of endotoxin or TNF. Because of this, it is difficult to predict at this stage what will be the net biological result of inhibiting NO formation in this and other pathological states.

In this context, it is possible that NO released for a prolonged period, after an immunological challenge, is also cytotoxic for the endothelial cells themselves, for the underlying vascular smooth muscle, and for other tissues. Indeed, NO-induced damage may contribute to the pathogenesis of

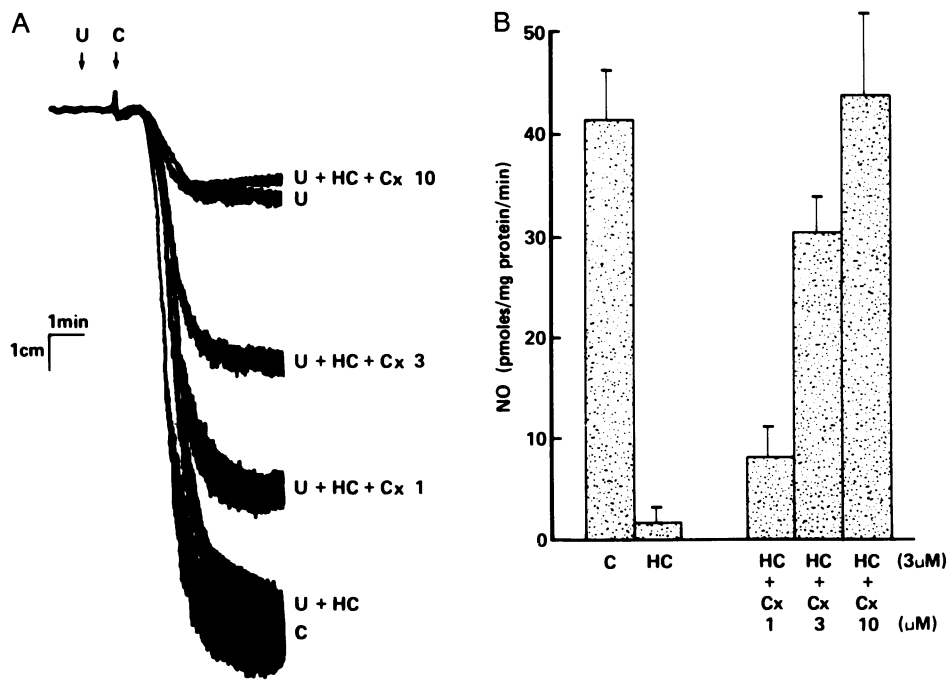

FIG. 6. Prevention by cortexolone of the inhibition by hydrocortisone of the induction of NO synthase. (*A*) In the absence of Ca^{2+}, unstimulated endothelial cells (U) released NO, which inhibited platelet aggregation. This activity was abolished when cells were incubated for 48 hr with hydrocortisone (3 μM, U+HC). The inhibitory activity of hydrocortisone was prevented in a concentration-dependent manner by cortexolone (Cx) [U + HC + Cx (number is concentration in μM)]. Tracings are representative of three experiments. (*B*) The formation of NO (C) was abolished by incubation of endothelial cells for 48 hr with hydrocortisone (HC). The inhibitory action of HC was prevented in a concentration-dependent manner by cortexolone (Cx). The results are the mean ± SEM of three experiments.

systemic necrotizing vasculitis, a syndrome that is treated with glucocorticoids (41). This possibility and the significance of the recent finding by two laboratories that the vascular smooth muscle may also release NO (42, 43) remain to be investigated.

The mechanism of the anti-inflammatory action of glucocorticoids is not fully understood. These compounds have a variety of acute and chronic actions that influence the inflammatory process (44). Our present results indicate that some of these can now be ascribed to inhibition of the induction of an NO synthase. It is likely that the antierythema and antiedema actions of glucocorticoids, as well as their beneficial effect on the hypotension of endotoxin shock, are due to inhibition of pathological vasodilatation induced by immunologically released NO. Moreover, glucocorticoids inhibit some of the cytotoxic actions of phagocytic cells. This may also be one of the mechanisms by which these compounds facilitate the spread of infections and prevent the consequences of delayed hypersensitivity reactions, as in graft rejection (27). Whether other actions of glucocorticoids are also the result of inhibition of immunologically induced NO generation remains to be investigated.

Finally, this Ca^{2+}-independent NO synthase can now be added to the growing list of inducible NO synthases and also to the list of proteins whose expression is induced in endothelial cells after activation by cytokines. It is remarkable that the endothelial cells, which release NO for the physiological regulation of blood flow and pressure, also release NO as a pathological entity with cytotoxic potential. For this, the vascular endothelium utilizes distinct biochemical pathways.

We now need to study the activation, distribution, substrate utilization, and interplay between these two pathways in physiology and pathophysiology. In this context, the finding that glucocorticoids inhibit the induction of one of these NO synthases will have a major impact on these investigations.

We thank Neale Foxwell and Tara Andrews for excellent technical assistance and Annie Higgs and Gillian Henderson for help in preparation of the manuscript.

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