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Inhibition of Glucocorticoid Receptor Binding by Nitric Oxide

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ABSTRACT

Septic shock is a dangerous condition with high mortality rates. In sepsis, the inducible form of nitric oxide (NO) synthase is induced, releasing high amounts of NO. Glucocorticoids have potent anti-inflammatory properties and are very effective in inhibiting the induction of this enzyme if administered before the shock onset. It is known that glucocorticoid receptor (GR) has critical cysteine residues for steroid binding in its hormone-binding and DNA-binding domains. It has also been reported that NO reacts with —SH groups, forming *S*-nitrosothiols. Therefore, we examined the potential effect of NO on the ligand-binding ability of GR. NO donors (*S*-nitroso-acetyl-DL-penicillamine, *S*-nitroso-DL-penicillamine, or *S*-nitroso-glutathione) decreased, in a time- and dose-dependent manner, the binding of [^{3}H]triamcinolone to immunoprecipitated GR from mouse L929 fibroblasts. The nonnitrosylated parent molecules, *N*-acetyl-DL-penicillamine, and reduced gluthatione were without effect. Scatchard plots revealed that the number of ligand binding sites and K_d were reduced (50%) by NO donors. Western blot analysis ruled out the possibility that dissociation of GR/heat shock protein 90 heterocomplex or decrease in GR protein would account for the inhibitory effect of NO. Decreased ligand binding to GR was found when NO donors were incubated with intact fibroblasts. Incubation with NO donors also decreased the steroid-induced reduction in [^{3}H]uridine incorporation into RNA. All of these NO effects were inhibited by the thiol-protecting agent dithiothreitol. Therefore, *S*-nitrosylation of critical —SH groups in GR by NO with consequent decreases in binding and affinity may be the mechanisms which explain the failure of glucocorticoids to exert their anti-inflammatory effects in septic shock.

Nitric oxide (NO) is a gas with low solubility in water at normal conditions but freely diffusible across biological membranes (Welch and Loscalzo, 1994). The L-arginine:NO pathway (Moncada et al. 1991) has been demonstrated to be involved in several biological processes in the cardiovascular and nervous systems. In these systems, NO is released by the action of constitutive Ca^{++}-dependent NO synthases (NOS) accounting for physiological vasodilation and neurotransmission (Bredt and Snyder, 1990; Moncada et al. 1991). Several L-arginine analogs inhibit these isoforms very efficiently although with low selectivity (Fukuto and Chaudhuri, 1995). Another isoform of NOS, the inducible Ca^{++}-independent NOS (iNOS) is expressed in phagocytic and other cell types after activation by endotoxin (lipopolysaccharide; LPS) and/or cytokines. Much larger amounts of NO are produced by this enzyme, accounting for the cytotoxicity of macrophages toward parasites and tumor cells (Moncada et al., 1991). The induction process of iNOS can be effectively prevented by glucocorticoids (Rees et al., 1990) and iNOS enzymatic activity is also inhibited by L-arginine analogs. NO can react with sulfhydryl groups to yield *S*-nitrosothiols (Arnelle and Stamler, 1995) and with superoxide anion, forming peroxynitrite, a powerful oxidant (Beckman et al., 1994). Formation of *S*-nitrosothiols is thought to have an intermediate role in cell metabolism or to serve as NO carriers (Welch and Loscalzo, 1994).

Septic shock is a state of inadequate tissue perfusion induced by microbial products and is characterized by low blood pressure and biochemical signals of oxygen deficit, accompanied by vascular damage, hyporeactivity to vasoconstrictors, and disseminated intravascular coagulation leading to multiple organ dysfunction and death (for a review, see Brandtzaeg, 1996). Septic shock is a serious condition with high mortality rates ranging from 20 to 80% (Parrillo, 1993). In spite of advances in recent years, the treatment of septic shock is still a challenge for physicians. It is generally accepted that adequate oxygenation, fluid replacement, and administration of inotropes, vasopressors, and antibiotics are important measures to be implemented in septic patients (Cohen and Glauser, 1991). NO is an important participant in septic shock. iNOS is induced in vascular smooth muscle

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ABBREVIATIONS: NO, nitric oxide (in this report, NO refers to either $NO^{\cdot}$, NO^{+} or NO^{-}); NOS, NO synthase; iNOS, inducible NOS; LPS, lipopolysaccharide; NFκB, nuclear factor κ-B system; GR, glucocorticoid receptor; HBD, hormone-binding domain; hsp, heat shock protein; DEX, dexamethasone; DTNB, dithionitrobenzoic acid; GSH, reduced gluthatione; NAP, *N*-acetyl-DL-penicillamine; SNAP, *S*-nitroso-DL-penicillamine; GSNO, *S*-nitroso-gluthatione; DTT, dithiothreitol; TA, triamcinolone acetonide.

by endotoxin/cytokines combination that leads to a profound vasodilation which is very difficult to counteract with vasoconstrictors. Ultimately, this hypotension leads to a diminished end organ perfusion with ensuing failure and death (Thijs et al., 1996).

Induction of iNOS and hypotension present in septic shock are completely prevented if animals are given glucocorticoids before shock onset (Rees et al., 1990). There are several mechanisms by which glucocorticoids inhibits inflammation, among them the interference with nuclear factor κ-B (NFκB) system. Some reports describe glucocorticoid-induced increases in NFκB-inhibitory protein levels (Auphan et al., 1995), but recent reports show that protein-protein interaction between glucocorticoid receptor (GR) and NFκB accounts for the steroid-mediated repression in gene induction (Scheinman et al., 1995; De Bosscher et al., 1997), whereas some reports indicate that both mechanisms are important (Wissink et al., 1998). In regard specifically to iNOS induction, corticoids have been shown to inhibit iNOS induction either through inhibition of NFκB-mediated transcription of iNOS gene by corticoid-induced increases in Iκ-B protein levels (Saura et al., 1998) or through protein-protein interaction between GR and NFκB, thereby inhibiting interaction of the latter with iNOS promoter and preventing the induction of iNOS transcription (Kleinert et al., 1996).

Owing to their potent anti-inflammatory properties and their ability to affect many of the mediator systems involved in septic shock, glucocorticoids were initially thought to be potentially beneficial in the treatment of this condition. Although some studies advocate that glucocorticoids can be beneficial in some types of sepsis (Thompson, 1993), most of the studies indicate that they are of no use in septic shock (Bone, 1991; Cohen and Glauser, 1991, for example). In fact, glucocorticoid therapy can lead to bacterial superinfections in some cases (Cohen and Glauser, 1991). Although there is a rise in cortisol levels during septic shock (Molijn et al., 1995) they fail to, for example, block induction of iNOS. Reasons for this failure may be a reduced binding of glucocorticoid to plasma transporter proteins (Pugeat et al., 1989) or a decreased GR binding (Huang et al., 1987; Li and Xu, 1988).

Steroid receptors are intracellular proteins with a domain called hormone-binding domain (HBD). For some types of steroid receptors, this domain must be associated with the chaperone 90-kDa heat shock protein (hsp90) for steroid binding to occur. In addition, the presence of a vicinally spaced pair of cysteine —SH groups in HBD is critical for adequate corticoid binding to the receptor. Addition of thiol reagents which form a disulfide bridge between the two —SH groups led to inactivated ligand binding to the receptor (Simons and Pratt, 1995).

Therefore, taking into account the high reactivity of NO toward —SH groups, their existence in GR and the failure of glucocorticoids in the treatment of septic shock, we performed experiments to study the effects of NO on glucocorticoid binding to their receptors.

Materials and Methods

Reagents. [6,7-^{3}H]triamcinolone acetonide (TA) (35.8 Ci/mmol), ^{125}I-conjugated goat anti-mouse immunoglobulin class G (IgG) and [5′-^{3}H]uridine (29 Ci/mmol) were purchased from DuPont NEN (Boston, MA). Nonimmune mouse IgG, protein A-Sepharose, goat anti-mouse IgG-horseradish peroxidase conjugate, dithionitrobenzoic acid (DTNB), reduced glutathione (GSH), *N*-acetyl-DL-penicillamine (NAP), and concanavalin A were purchased from Sigma Chemical Co. (St. Louis, MO). The BuGR2 monoclonal IgG antibody against the GR was obtained from Affinity Bioreagents (Golden, CO), and the AC88 monoclonal IgG antibody against hsp90 was obtained from StressGen (Victoria, British Columbia, Canada). *S*-nitroso-acetyl-DL-penicillamine (SNAP) was synthesized in house by the method of Field et al., (1978) and *S*-nitroso-glutathione (GSNO) was synthesized in house by the method of Hart (1985). Both reagents were >99% pure nitrosothiols as assessed by the method of Saville (1958).

Cell Culture and Cytosol Preparation. L929 mouse fibroblasts were grown in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were harvested by scraping into Earl's balanced salt solution and washed twice. The pellet was suspended in 1.5 volumes of HEM buffer (10 mM HEPES, 1 mM EDTA, and 20 mM sodium molybdate, pH 7.4) and ruptured by Dounce homogenization. Homogenates were centrifuged for 30 min at 100,000*g* with the supernatant from this step being referred to as cytosol.

Receptor Immunoadsorption and Western Blotting. The GR was immunoadsorbed from 200-μl aliquots of cytosol by rotation for 2 h at 4°C with 15 μl of BuGR2 antibody prebound to 70 μl of 20% (w/v) protein A-Sepharose. The immunopellets were washed three times with 1 ml of TEGM buffer (10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 50 mM NaCl, 4 mM EDTA, 10% glycerol, 20 mM molybdate, pH 7.6) and used as a source of GR heterocomplex. When Western blots were performed, the immunopellet was boiled in SDS sample buffer with 10% β-mercaptoethanol and the proteins were resolved in a 10% SDS-polyacrylamide gel. Proteins were electrotransferred to an Immobilon-P membrane and probed for the GR with 2 μg/ml BuGR2 and for hsp90 with 1 μg/ml AC88 antibodies. The immunoreactive bands were visualized after incubation with horseradish peroxidase-conjugated counter antibody. The bands were cut, reincubated with ^{125}I-labeled counter antibody, and autoradiographed.

Binding Assays and Treatment with Thiol-Reactive Reagents. The immunopellets were incubated with 100 nM [^{3}H]triamcinolone in HEM buffer for 4 h at 0°C. The final volume of reaction was 100 μl. The pellets were washed three times with 1 ml of TEGM buffer and were assayed for radioactivity. The nonspecific binding (10% of the total binding) was measured in the presence of 1 μM nonradioactive triamcinolone and substracted from the total binding. When incubations with thiol-reactive reagents were attempted, samples of steroid-free receptor or [^{3}H]steroid-bound receptor were incubated at 20°C with different concentrations of DTNB (for 30 min) or *S*-nitrosothiol reagents (for 1–3 h) in HEM buffer and the steroid binding capacity was measured as described above.

Scatchard Plots. L929 cytosol (10 mg of protein/ml) was treated with 1 mM GSNO or 1 mM SNAP for 3 h at 20°C. Afterward, aliquots of 100 μl were incubated for 5 h on ice with increasing concentrations of [^{3}H]triamcinolone in the range of 5×10^{-10} M to 1×10^{-7} M (the length of the plateau is from 2 to 20 h of incubation). Bound steroid was separated from free steroid by adding 1.5 volumes of 2% (w/v) charcoal-0.2% (w/v) dextran, shaking twice for 5 s, and then maintaining on ice for 5 min followed by centrifugation at 10,000 rpm for 5 min at 4°C. The radioactivity in 150 μl of supernatant was counted with 60% efficiency for tritium. The nonspecific binding measured in the presence of 1000-fold nonradioactive steroid was substracted to calculate the specific binding for each incubation. Kinetic parameters were calculated with Enzfitter program (Elsevier Biosoft, Amsterdam, The Netherlands).

Effect of *S*-Nitrosothiols on Whole Fibroblasts. L929 fibroblasts were plated onto 10-cm diameter Petri dishes and grown at 37°C (5% CO_2) in phenol red-free culture medium supplemented with 10% charcoal-stripped, delipidated bovine calf serum (Sigma, St. Louis MO). When cells reached 80% confluence, the following

incubations were performed: 1) cells were preincubated with 1 mM GSNO or 1 mM SNAP for 2 h; the medium was replaced by fresh medium, and the dishes were incubated with 100 nM [^{3}H]triamcinolone (±1 μM nonradioactive steroid) for 2 h on ice; and 2) cells were prelabeled with [^{3}H]steroid for 2 h at 0°C and then incubated with 1 mM GSNO or 1 mM SNAP for 2 h at 37°C. After these two treatments, cells were scraped and washed three times with phosphate-buffered saline/1% bovine serum albumin. The radioactivity was extracted from the resultant pellets with 0.5 ml of 100% ethanol for 30 min on ice. The ethanolic solution was transferred to scintillation vials and counted for tritium.

[^{3}H]Uridine Incorporation by L929 Fibroblasts. The effect of receptor nitrosylation on steroid-dependent inhibition of [^{3}H]uridine incorporation in L929 cells was investigated using a previously described method (Roldán et al., 1981; Hedger et al., 1989). Briefly, cells were grown up to 60% confluence in phenol red-free medium supplemented with 10% charcoal-stripped calf serum and 2 μg/ml concanavalin A was then added to the medium. After 10 h of incubation in the presence on concanavalin A, 1 mM GSNO or 1 mM SNAP was added to the medium and the incubation was continued for another 2 h. At this time, 0.1 μM dexamethasone (DEX) was added and the incubation was continued for an additional 2 h. Cells were labeled with 5 μCi of [^{3}H]uridine during the last 40 min of incubation. Fibroblasts were then harvested, centrifuged at 1000*g* for 2 min at 4°C, and the radioactive medium was aspirated. Cells were washed three times with fresh medium at 4°C and packed cells were then lysed (and RNA simultaneously precipitated) by adding cold 5% perchloric acid solution. After 30 min in ice, the acid-insoluble fraction was dissolved with 1 M NaOH and the radioactivity counted.

Statistics. Data are presented as means ± S.E.M. of the indicated number of experiments. Significant differences were evaluated by one-way analysis of variance followed by Student-Newman-Keuls test.

Results

Effect of DTNB on [^{3}H]Triamcinolone Binding to GR. This set of experiments was designed to confirm that the reactivity of sulfhydryl-modifying reagents with the steroid-bound GR is less than the reactivity observed in steroid-free GR (Stancato et al., 1996). As can be seen in Fig. 1, DTNB caused concentration-dependent reductions in tritiated TA binding when incubated with GR before the ligand (solid column). Noticeable effect was already seen at 100 μM DTNB (37% inhibition), whereas maximal reduction was seen with 10 mM DTNB (92% inhibition). This reduction in binding was completely reversed by the simultaneous presence of a —SH protecting agent, dithiothreitol (DTT). Once the binding between the ligand and the receptor was completed, the reactivity —SH groups of the GR toward DTNB decreased, as reflected by the almost total lack of effect of DTNB on binding (only 14% inhibition at 1 mM DTNB; open columns).

Time Course of NO Donors Effect on [^{3}H]TA Binding. When 1 mM of either NO donor GSNO or SNAP was incubated for 3 h with the GR before the addition of the ligand, a time-dependent inhibition in [^{3}H]TA binding was observed (Fig. 2). In addition, SNAP was more potent than GSNO in causing this inhibitory effect.

Effect of Increasing Concentrations of NO Donors on [^{3}H]TA Binding. Incubation of GR with SNAP or GSNO caused a progressive decrease in corticoid binding to its receptors (Fig. 3). The approximate IC_{50} values were 100 μM and 1 mM for SNAP and GSNO, respectively. The non-nitrosylated parent molecules (NAP and GSH) failed to affect [^{3}H]TA binding to the GR, hence confirming that GR-binding inhibition can be ascribed to NO (Fig. 3, open symbols). In addition, NO-induced decrease in binding indeed involved sulfhydryl groups as indicated by the protective effect of DTT

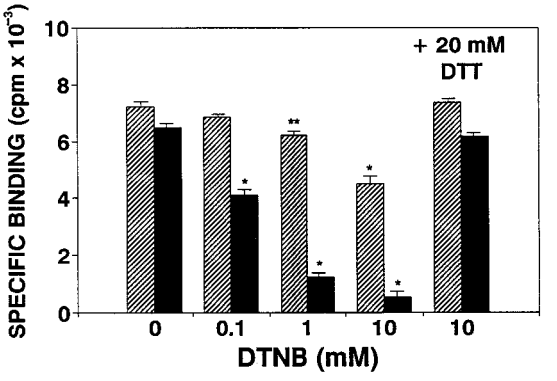

Fig. 1. Effect of DTNB on the steroid-binding capacity of the steroid-free and steroid-bound GR. Steroid-bound receptor (▨): L929 cytosol was immunoadsorbed with BuGR2 antibody to protein A-Sepharose and labeled for 4 h at 0°C with [^{3}H]triamcinolone in HEM buffer. After washing with TEGM buffer, the labeled immunopellets were incubated with the indicated concentrations of DTNB for 30 min at 20°C in HEM buffer. The free steroid was washed with TEGM buffer and the radioactivity was counted. Steroid-free receptor (■): The GR was immunoadsorbed with BuGR2 antibody as described above and incubated with the indicated concentrations of DTNB for 30 min at 20°C in HEM buffer. After washing with TEGM buffer, the immunopellets were labeled for 4 h at 0°C with [^{3}H]triamcinolone in HEM buffer, washed with TEGM, and the radioactivity was counted. DTT prevents the effects of DTNB (rightmost pair of columns): Treatments with 10 mM DTNB for 30 min at 20°C were also performed with the addition of 20 mM DTT to the incubation media. The immunopellets were washed with TEGM and counted (steroid-bound receptor), or labeled as described above with [^{3}H]triamcinolone (steroid-free receptor) and counted. Results represent the mean ± S.E.M. of three experiments. Statistical comparisons are with the corresponding DTNB control group (leftmost pair of columns): *$p < .001$; **$p < .005$.

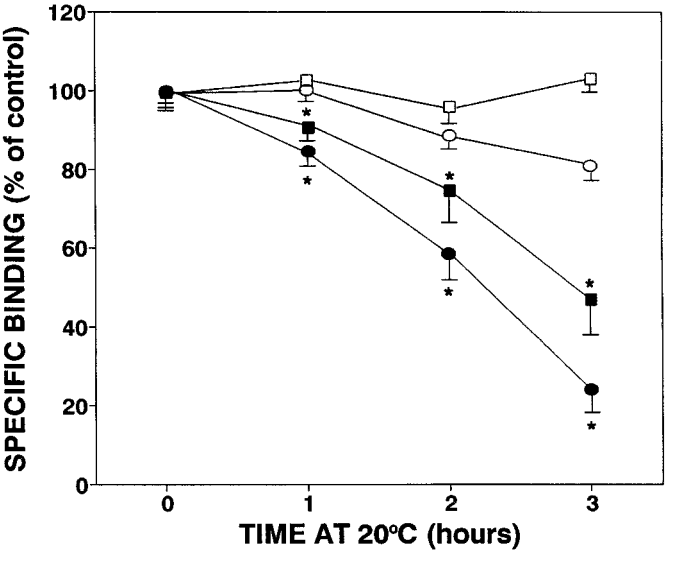

Fig. 2. Time-dependent inhibition of GR binding by NO donors. Immunoadsorbed GR was incubated in HEM buffer for different time periods at 20°C with 1 mM of the following compounds: GSH (□), NAP (○), GSNO (■), or SNAP (●). The samples were then placed on ice and incubated with 100 nM [^{3}H]triamcinolone for 4 h, washed three times with TEGM, and the radioactivity was counted. The results (mean ± S.E.M., $n = 3$) are expressed as percentage of the control incubated in the absence of compounds (63,361 ± 1508 cpm). Compared with the respective nonnitrosylated parent compound: *$p < .001$.

on the diminished binding caused by NO donors (Fig. 3, solid triangles).

Kinetic Parameters for [^{3}H]TA Binding in the Presence of NO Donors. To assess the type of effect NO would be causing to the GR, Scatchard plots were obtained from saturation experiments with the labeled corticoid. Results are shown in Fig. 4. Control cytosol has a number of specific binding sites of 56.3 ± 0.9 fmol/mg protein that were dramatically reduced to 36.3 ± 1.6 and 20.9 ± 1.2 fmol/mg protein after incubation with GSNO and SNAP, respectively ($p < .001$ compared to control). The K_d of control cytosol was 2.6 ± 0.2 nM and was significantly ($p < .01$) increased to 4.9 ± 0.8 and 5.3 ± 1.0 nM after incubation with GSNO and SNAP, respectively.

Lack of Effect of NO Donors on the Heterocomplex GR-hsp90. As depicted in Fig. 5A, incubation of fibroblast cytosol with either GSNO or SNAP did not change association of GR with hsp90, evidenced by identical coimmunoprecipitation of both proteins in samples treated with NO donors and untreated samples. The ratio of optical densities of GR and hsp90 was identical in all situations (0.165, 0.184, and 0.175 for control, GSNO, and SNAP, respectively). The lack of effect of SNAP and GSNO on in vitro disruption of the GR-hsp90 heterocomplex could also be observed when intact cells were tretated with both NO donors and GR and hsp90 were also coimmunoprecipitated (Fig. 5B). In addition, this experiment shows that the amount of receptor protein remained constant after the whole cell treatment. This observation was confirmed in Fig. 5C, where blots for GR were performed on aliquots of whole cytosol obtained from the cells used in Fig. 5B. This experiment excluded the possibility that the former results shown in Fig. 5 might be affected by differential efficiency of the GR immunoadsorption.

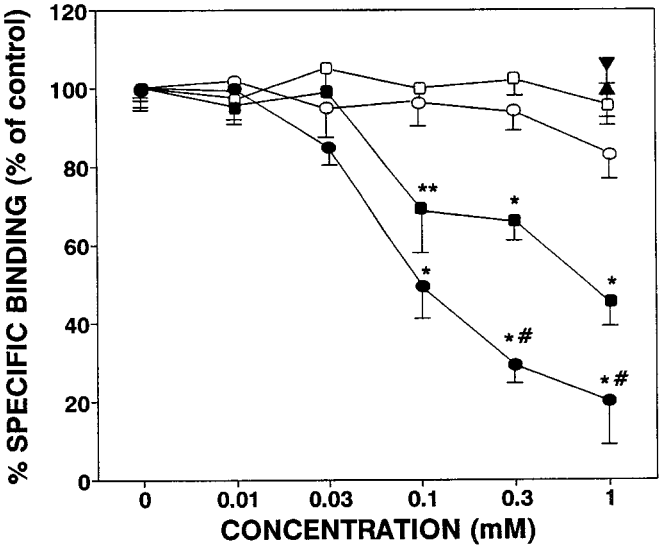

Fig. 3. Concentration-dependent inhibition of GR binding by NO donors. Immunoadsorbed GR was incubated in HEM buffer for 3 h at 20°C in the presence of different concentrations of GSH (□), NAP (○), GSNO (■), or SNAP (●). The samples were then placed on ice and incubated with 100 nM [^{3}H]triamcinolone for 4 h, washed three times with TEGM, and the radioactivity was counted. Treatments with 1 mM GSNO (▼) or 1 mM SNAP (▲) for 30 min at 20°C were also performed in the simultaneous presence of 2 mM DTT in the incubation medium. The results (mean ± S.E.M., $n = 3$) are expressed as percentage of the control incubated in the absence of compounds (70,732 ± 1514 cpm). Treatment with NO donor compound versus nonnitrosylated parent compound: *$p < .001$; **$p < .005$; SNAP versus GSNO: #$p < .001$.

Effect of NO Donors on [^{3}H]TA Binding in Intact L929 Cells. To exclude the possibility that the inhibitory effect of NO could be due to some experimental artifact brought about by GR immunopurification, [^{3}H]TA was added

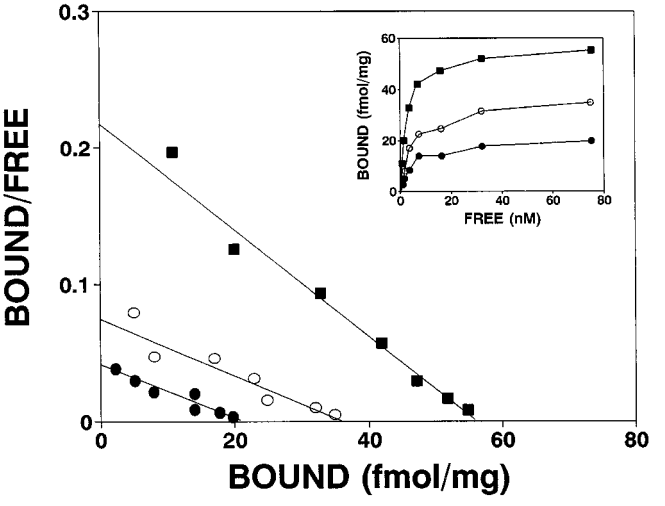

Fig. 4. Scatchard plots of cytosol treated with NO donors. L929 cells cytosol was preincubated for 3 h at 20°C without NO donors (■), and in the presence of 1 mM GSNO (○) or 1 mM SNAP (●). The saturation curve (inset) was performed with increasing concentrations of [^{3}H]triamcinolone for 5 h at 0°C as detailed in *Materials and Methods*.

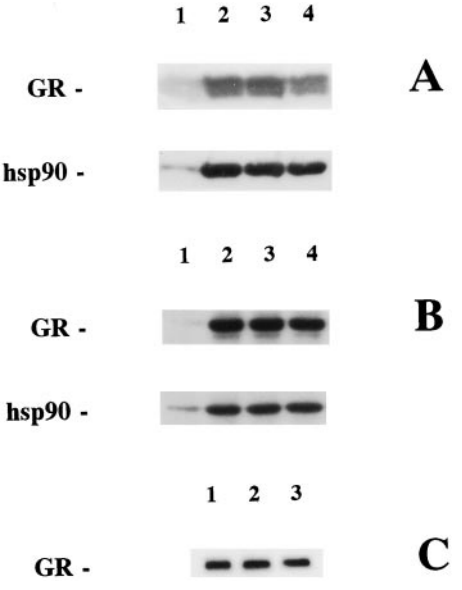

Fig. 5. Failure of NO donors to induce dissociation in GR-hsp90 heterocomplex. A, 200 μl of L929 cell cytosol in HEM buffer was incubated with 1 mM GSNO or 1 mM SNAP for 3 h at 20°C. The GR was immunoadsorbed with BuGR2 antibody or nonimmune mouse IgG, resolved in 10% SDS-polyacrylamide gel electrophoresis, electrotransferred to membranes, and blotted for the receptor and the coimmunoadsorbed hsp90 as detailed in *Materials and Methods*. Lane 1, nonimmune immunopellet; lane 2, immunopellet from untreated cytosol; lane 3, immunopellet from cytosol treated with GSNO; and lane 4, immunopellet from cytosol treated with SNAP. B, L929 fibroblasts were incubated with 1 mM GSNO or 1 mM SNAP for 2 h at 37°C. Cells were homogenized and centrifuged as in A. One hundred μl of cytosol was immunoadsorbed with 8 μl BuGR2 or 8 μl nonimmune IgG and blotted for GR and hsp90. Lane 1, nonimmune immunopellet from untreated cells; lane 2, immunopellet from untreated cells; lane 3, immunopellet from cells treated with GSNO; and lane 4, immunopellet from cells treated with SNAP. C, whole cell extracts were blotted for GR. Lane 1, 15 μl of whole cell extract obtained from untreated cells; lane 2, same for GSNO-treated cells, lane 3, same for SNAP-treated cells.

to the medium of intact L929 fibroblasts after cells were exposed to either SNAP or GSNO. As shown in Fig. 6, exposure to NO donors decreased [^{3}H]TA binding to GR in intact cells by 40 and 50% for GSNO and SNAP, respectively. The finding that —SH modification did not affect binding if performed after GR has interacted with [^{3}H]TA, as seen for DTNB in Fig. 1, was also observed for NO donors. For instance, binding was similar to control levels when SNAP or GSNO were added to cells after the ligand (Fig. 6). It is noteworthy that neither SNAP nor GSNO affected cell viability (assessed by trypan blue exclusion) under the conditions used in the assay (data not shown).

Effect of NO Donors on [^{3}H]uridine Uptake by Intact L929 Cells. To study the possibility that GR signaling may be affected by nitrosylation, we used a standard system for assaying glucocorticoid biological effect, namely, the inhibition of the incorporation of nucleotides into cells. Figure 7 shows that the incorporation of [^{3}H]uridine into fibroblasts treated with 0.1 μM DEX (column B) is significantly diminished (43%) compared to the incorporation observed in untreated cells (columns A). Treatment with GSNO or SNAP (columns C and D) alone had no effect on [^{3}H]uridine incorporation, demonstrating that NO donors do not affect per se the uptake of uridine. A partial inhibition of DEX-dependent effect (65% of incorporation) was observed when cells were treated with NO donors (columns E and F), whereas DEX was fully active when DTT was also present in the medium during incubation with GSNO and SNAP (columns G and H).

Discussion

The main findings of this report are that 1) NO decreases glucocorticoid binding to its receptor and 2) this effect is due to *S*-nitrosylation of critical —SH groups and cannot be attributable to dissociation of GR-hsp90 heterocomplex.

The HBD of the GR contains five cysteine residues, three of them closely spaced in the steroid-binding pocket (Stancato et al., 1996). This domain also has a binding region for hsp90 chaperone, the presence of which is essential for GR to display its binding conformation (Simons and Pratt, 1995) and, at the same time, to prevent GR binding to DNA through its DNA-binding domain. DNA-binding domain in GR also has a number of cysteines essential to its DNA-binding activity (Stancato et al., 1996). The sensitivity of the Cys residues to spontaneous oxidation or to chemical modification varies with the type of steroid receptor. For instance, glucocorticoid binding to its receptor is abolished by sulfhydryl-blocking agents such as *N*-ethylmaleimide and DTNB (Simons and Pratt, 1995), the same pattern being observed for renal mineralocorticoid receptor. However, the higher sensitivity exhibited to sulfhydryl reagents and iron by the mineralocorticoid receptor led to the hypothesis (Galigniana, 1996) that cysteine groups may be more protusive in mineralocorticoid receptor as compared to GR. The steroid binding ability of the GR present in mouse L929 fibroblasts was decreased by DTNB and prevented if triamcinolone was in the binding pocket as well as by the simultaneous presence of DTT in the reaction medium. The lack of effect of DTNB on GR binding after ligand is bound suggest that the presence of the ligand in the HBD may either sterically hinder access of the sulfhydryl reagents to critical —SH groups lying in the binding pocket and/or induce conformational changes in the protein which would prevent access to DTNB.

Incubation of GR with NO donors leads to both time- and concentration-dependent decreases in ligand binding. This effect can be entirely attributed to NO because nonnitrosylated parent molecules (NAP and GSH) were unable to affect ligand binding in all of the experiments described here. NO can interact with several intracellular targets, one of them

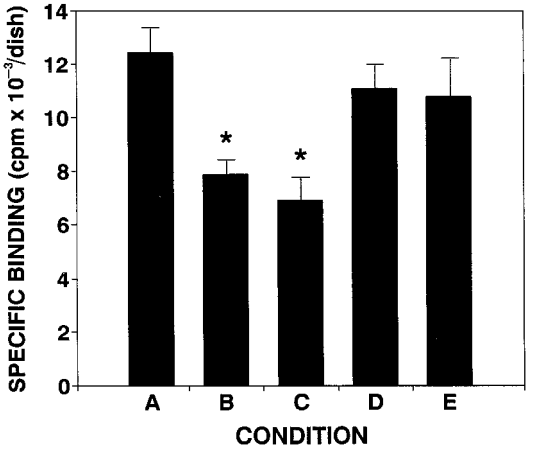

Fig. 6. Inhibition of GR binding by NO donors in intact cells. L929 fibroblasts were grown to 80% confluence in phenol red-free medium supplemented with 10% charcoal-stripped serum. The steroid binding capacity was measured as follows: cells without treatment (A), cells treated with 1 mM GSNO (B), or 1 mM SNAP (C) for 2 h at 37°C followed by incubation with 100 nM [^{3}H]triamcinolone for 2 h on ice; cells incubated with 100 nM [^{3}H]triamcinolone for 2 h on ice followed by further incubation for 2 h with 1 mM GSNO (D) or 1 mM SNAP (E). The intracellular radioactivity was extracted with ethanol and counted. Results are the mean ± S.E.M. of four experiments. Significantly different with respect to condition A (*$p < .005$).

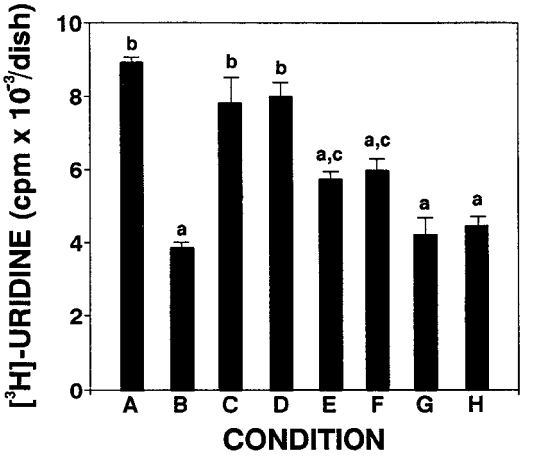

Fig. 7. Treatment of L929 cells with NO donors prevents DEX-dependent inhibition of uridine incorporation to RNA. L929 fibroblasts were incubated in the presence of 1 mM NO donor reagent (SNAP or GSNO). After 2 h, 0.1 μM DEX was added to the incubation mixture, and the incubation was continued for another 2 h. Cells were labeled with 5 μCi of [^{3}H]uridine during the last 40 min of incubation. Cells were then washed, lysed, and the radioactivity recovered from the perchloric acid-insoluble fraction was counted. Columns represent the following treatments (mean ± S.E.M., $n = 5$): A, untreated cells; B, 0.1 μM DEX for 2 h; C, 1 mM GSNO for 4 h; D, 1 mM SNAP for 4 h; E, 1 mM GSNO for 4 h, and 0.1 μM DEX was added during the last 2 h of incubation; F, 1 mM SNAP for 4 h, and 0.1 μM DEX was added during the last 2 h of incubation; G, 1 mM GSNO and 2 mM DTT for 4 h, and 0.1 μM DEX was added during the last 2 h of incubation; H) 1 mM SNAP and 2 mM DTT for 4 h, and 0.1 μM DEX was added during the last 2 h of incubation. Different from condition A: $p < .001$ (a). Different from condition B: $p < .001$ (b) and $p < .010$ (c).

being —SH groups (Fang, 1997). Reaction of NO with —SH groups yields *S*-nitrosothiols which are able to release NO back into solution. In at least some circumstances, two vicinal *S*-nitrosothiols can form a disulfide bridge upon NO release (Arnelle and Stamler, 1995). If this occurs in a protein or an enzyme, the loss of free —SH groups could cause conformational changes or restraints that would affect its physiological role. Because simultaneous incubation of NO donors and sulfhydryl-protecting agents (such as DTT) abolished the effect of NO on ligand binding to GR, it is suggested that the nitrosylation of critical cysteine(s) residue(s) of the GR may be the reason for the loss of the steroid binding capacity. However, it is likely that the changes caused by NO in GR are either too discrete or are occuring in a domain that is not essential for hsp90 binding. At least under the conditions used in our experiments, no dissociation of hsp90 from GR was observed. This observation is valid for cytosols (Fig. 5A) and for whole cells (Fig. 5B) incubated with NO donors, therefore showing that GR remains associated with hsp90 in the presence of NO. Moreover, as the amount of GR remains the same after NO donor incubation (Fig. 5C), dissociation of GR-hsp90 heterocomplex and reduction in GR protein amount can be ruled out as explanations for diminished binding induced by NO. In addition, it seems that the nitrosylation of critical cysteine(s) residue(s) in the GR by NO is irreversible or slowly reversible because *S*-nitrosothiol removal did not reverse the decreased ligand binding. Our data do not offer evidence on what Cys residue is being nitrosylated by NO but is clear that at least one residue in HBD is being affected. Kinetics of NO release from *S*-nitrosothiols varies considerably depending on the lateral chains in the molecule of *S*-nitrosothiol (Arnelle and Stamler, 1995). This may explain why SNAP seems to be more effective in inhibiting ligand binding to GR than GSNO, as the SNAP releases NO faster than the GSNO (Arnelle and Stamler, 1995). For instance, SNAP and GSNO, both at 1 mM, releases 57.7 and 36.6 μM nitrite at 37°C and 17.1 and 4.1 μM nitrite at 20°C after 1 h incubation in Dulbecco's modified Eagle's medium, respectively (data not shown). This is more clearly seen in shorter times of incubation (for example, compare Figs. 2 and 3). On the other hand, when *S*-nitrosothiols were incubated with GR either in immunopurified or intracellular form after the ligand has been added, no decrease in binding was observed (see Fig. 6). This observation parallels with the experiments performed with DTNB as shown in Fig. 1. This suggests that once the ligand is into its pocket, the Cys residue(s) being nitrosylated is no longer in a form amenable to NO attack. Although our results do not provide evidence of the molecular details, this would mean that once in the presence of the ligand, GR is protected from *S*-nitrosylation and that steroid binding is not reversed by NO. Nitric oxide effect on ligand binding to GR seems to be related to both a loss of binding sites and to a decrease in the affinity of GR for the steroid, as evidenced from the Scatchard plots in the presence of NO donors. Finally, the decrease in ligand binding to GR caused by NO is likely to be a relevant finding with functional consequences. First, it can be shown to occur in intact cells (Fig. 6). Second, it caused a decrease in a well known corticoid effect, namely, the incorporation of uridine into RNA (Roldán et al., 1981; Hedger et al., 1989). Incubation of whole L929 cells with NO donors inhibited the steroid-induced uridine incorporation which was partially restored to control levels by the simultaneous presence of a —SH protecting agent, DTT (Fig. 7), indicating that *S*-nitrosylation of GR by NO decreases receptor signaling.

High amounts of NO (assessed as plasma nitrate + nitrite, an index of NO production in vivo) are released during sepsis in animals (50 and 1000 μM, in control and LPS-injected mice, respectively; Rees, 1995) and in humans (35 and 127 μM in normal and septic patients, respectively; Spack et al., 1997). These values are greater than the amount released by NO donors in the conditions used in the present report. In addition, one has to take into account that in vivo, local concentrations (such as in vascular smooth muscle, where iNOS is induced by LPS) must be much higher. Therefore, the inhibition of binding and signaling of steroids caused by NO must be of greater magnitude in septic shock than in our in vitro system.

In experimental models, injection of glucocorticoids before or in combination with bacterial endotoxin effectively prevents induction of iNOS (Rees et al., 1990; Wright et al., 1992). Notwithstanding other effects, this action of glucocorticoids is able to render animals almost oblivious to LPS effects. This indicates that NO may indeed play a prominent role in septic shock. However, as described before, administration of glucocorticoids after septic shock onset completely fails to improve patient or animal condition. Therefore, based on our results, we would like to suggest that the reason why glucocorticoids fail to exert their powerful actions in septic shock is due to the fact that the high amounts of NO being produced in this pathological condition are decreasing GR number and affinity, possibly by *S*-nitrosylation of critical —SH groups.

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