JCI The Journal of Clinical Investigation

Amendment history:
- Erratum (March 2003)

Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids

Florian P. Limbourg, … , Michael A. Moskowitz, James K. Liao

J Clin Invest. 2002;110(11):1729-1738. https://doi.org/10.1172/JCI15481.

Article **Vascular biology**

Many cellular responses to corticosteroids involve the transcriptional modulation of target genes by the glucocorticoid receptor (GR). A rapid, non-nuclear effect of GR was found to mediate neuroprotection. High-dose corticosteroids (20 mg/kg intraperitoneally), given within 2 hours of transient cerebral ischemia, acutely increased endothelial nitric oxide synthase (eNOS) activity, augmented regional cerebral blood flow (CBF) by 40% to 50%, and reduced cerebral infarct size by 32%. These neuroprotective effects of corticosteroids were abolished by the GR antagonist RU486 and by inhibition of phosphatidylinositol 3-kinase (PI3K), and were absent in *eNOS*$^{-/-}$ mice. To determine the mechanism by which GR activated eNOS, we measured the effect of corticosteroids on PI3K and the protein kinase Akt. In a ligand-dependent manner, GR activated PI3K and Akt in vitro and in vivo caused NO-dependent vasodilation, which was blocked by cotreatment with RU486 or the PI3K inhibitor LY294002 but not by transcriptional inhibitors. Indeed, a mutant GR, which cannot dimerize and bind to DNA, still activated PI3K and Akt in response to corticosteroids. These findings indicate that non-nuclear GR rapidly activates eNOS through the PI3K/Akt pathway and suggest that this mechanism mediates the acute neuroprotective effects […]

Rapid nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids

Florian P. Limbourg,[1] Zhihong Huang,[2] Jean-Christophe Plumier,[2] Tommaso Simoncini,[3] Masayuki Fujioka,[2] Jan Tuckermann,[4] Günther Schütz,[4] Michael A. Moskowitz,[2] and James K. Liao[1]

[1]Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA
[2]Laboratory of Stroke and Neurovascular Regulation, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA
[3]Department of Reproductive Medicine and Child Development, University of Pisa, Pisa, Italy
[4]Department of Molecular Biology of the Cell I, German Cancer Research Center, Heidelberg, Germany

Many cellular responses to corticosteroids involve the transcriptional modulation of target genes by the glucocorticoid receptor (GR). A rapid, non-nuclear effect of GR was found to mediate neuroprotection. High-dose corticosteroids (20 mg/kg intraperitoneally), given within 2 hours of transient cerebral ischemia, acutely increased endothelial nitric oxide synthase (eNOS) activity, augmented regional cerebral blood flow (CBF) by 40% to 50%, and reduced cerebral infarct size by 32%. These neuroprotective effects of corticosteroids were abolished by the GR antagonist RU486 and by inhibition of phosphatidylinositol 3-kinase (PI3K), and were absent in *eNOS*$^{-/-}$ mice. To determine the mechanism by which GR activated eNOS, we measured the effect of corticosteroids on PI3K and the protein kinase Akt. In a ligand-dependent manner, GR activated PI3K and Akt in vitro and in vivo caused NO-dependent vasodilation, which was blocked by cotreatment with RU486 or the PI3K inhibitor LY294002 but not by transcriptional inhibitors. Indeed, a mutant GR, which cannot dimerize and bind to DNA, still activated PI3K and Akt in response to corticosteroids. These findings indicate that non-nuclear GR rapidly activates eNOS through the PI3K/Akt pathway and suggest that this mechanism mediates the acute neuroprotective effects of corticosteroids through augmentation of CBF.

J. Clin. Invest. **110**:1729–1738 (2002). doi:10.1172/JCI200215481.

Introduction

Steroid hormones are essential for normal development and stress responses. Corticosteroids bind to and activate the cytoplasmic glucocorticoid receptor (GR), a widely expressed steroid hormone receptor (1). In the classic model of steroid hormone action, GR acts as a ligand-dependent transcription factor by either activating or repressing gene expression through direct interactions with DNA or other transcription factors (2). The actions of GR are essential for survival. Mice lacking GR (*GR*$^{-/-}$) die at birth (3); in contrast, overexpression of GR renders mice resistant to stress and endotoxic shock (4). Although the absence of GR is incompatible with life, DNA binding and transactivation of target genes by GR is not essential for development or survival, since mice with a targeted mutation in the dimerization domain of GR (*GR*^{dim}), which impairs cooperative DNA binding and transactivation by GR homodimers, lack GR-dependent gene expression but survive to adulthood (5).

Some effects of corticosteroids on the brain and vascular system are very rapid, which makes transcriptional mechanisms of action unlikely. For example, behavioral changes in amphibians and increases of inositol trisphosphate in vascular smooth-muscle cells occur within minutes of corticosteroid administration (6, 7). Furthermore, the acute, antianaphylactic effects of high-dose corticosteroids in mice are unaltered by the transcriptional inhibitor actinomycin D (8). Thus, GR may exert important nontranscriptional functions.

Received for publication March 19, 2002, and accepted in revised form October 8, 2002.

Address correspondence to: James K. Liao, Vascular Medicine Research, Brigham and Women's Hospital, 65 Landsdowne Street, Room 275, Cambridge, Massachusetts 02139, USA. Phone: (617) 768-8424; Fax: (617) 768-8425; E-mail: jliao@rics.bwh.harvard.edu.
Florian P. Limbourg and Zhihong Huang contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: glucocorticoid receptor (GR); endothelial nitric oxide synthase (eNOS); neuronal nitric oxide synthase (nNOS); cerebral blood flow (CBF); phosphatidylinositol 3-kinase (PI3K); mitogen-activated protein kinase (MAPK); estrogen receptor α (ERα); N^{ω}-nitro-L-arginine methyl ester (L-NAME); bovine aortic endothelial cells (BAECs); 2,3,5-triphenyltetrazolium (TTC); middle-cerebral-artery occlusion (MCAo); phosphatidylinositol 3,4,5-trisphosphate (PIP_3); acetylcholine (ACh); endothelial cells (ECs); human umbilical-vein ECs (HUVECs); human aortic ECs (HAECs); glucocorticoid-responsive element (GRE); mouse embryonic fibroblasts (MEFs); mineralocorticoid receptor (MR); serum- and glucocorticoid-regulated kinase (SGK).

There is growing evidence that nontranscriptional actions of steroid hormone receptors regulate physiologically important processes. For example, osteocyte apoptosis is regulated by the androgen and estrogen steroid receptors through nontranscriptional activation of Src and mitogen-activated protein kinase (MAPK) (9). Vascular nitric oxide (NO) production and vasodilation by estrogen depend on nontranscriptional activation of endothelial NO synthase (eNOS) and are mediated by estrogen receptor α–dependent (ERα)-dependent activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (10–12). It is important to note that the vascular protective effects of estrogen are dependent on the nontranscriptional activation of eNOS through the PI3K/Akt pathway (11).

Endothelial-derived NO is a critical mediator of vascular integrity. Vascular NO production regulates cerebrovascular perfusion and protects against stroke by increasing collateral flow to the ischemic area (13). Mice lacking eNOS exhibit larger cerebral infarctions (14), and further inhibition of NOS activity by N^{ω}-nitro-L-arginine methyl ester (L-NAME) decreases cerebral blood flow (CBF) and increases infarct size after ischemia in mice lacking neuronal nitric oxide synthase (nNOS) (15). In contrast, enhanced NO production by administration of the eNOS substrate, L-arginine, or upregulation of eNOS by statins confer protection from stroke (16, 17). Therefore, conditions that enhance eNOS activity could have beneficial effects on cerebrovascular disease.

Corticosteroids have been used in the treatment of stroke with conflicting results (18). Some clinical and experimental data, however, suggest a beneficial role for these steroids in reducing ischemic tissue injury. Corticosteroids reduce ischemic injury in myocardial infarction (19) and substantially reduce stroke size in focal cerebral ischemia (20–22). Indeed, we have recently shown that the anti-inflammatory and myocardial protective effects of acute steroid therapy are mediated by nontranscriptional activation of eNOS through the PI3K/Akt pathway (23). However, long-term corticosteroid therapy may be associated with an increased risk of stroke, due in part to transcriptional downregulation of eNOS expression and an increase in systemic blood pressure (24). The mechanism of stroke protection by steroids, therefore, is essentially unknown. Here, we examine whether non-nuclear signaling by GR could contribute to neuroprotection by a novel mechanism involving an increase in CBF.

Methods

Cells and reagents. Human endothelial cells were maintained in EGM-2 growth medium (Clonetics, San Diego, California, USA) and used at the third passage. Bovine aortic endothelial cells (BAECs) and COS7 cells were grown in DMEM (Life Technologies Gibco BRL, Grand Island, New York, USA) with 10% FCS. For experiments, cells were cultured for 48 hours in phenol red–free medium with charcoal-stripped FCS, and this procedure was followed by serum-starvation. Cells were incubated with dexamethasone (Sigma-Aldrich, St. Louis, Missouri, USA), RU486 (Biomol, Plymouth Meeting, Pennsylvania, USA), actinomycin D, LY294002 (Calbiochem, San Diego, California), or vehicle (DMSO). Phosphatidylinositol 4,5-bisphosphate was purchased from Biomol and [^{32}P]γATP from New England Nuclear Life Science Products (Boston, Massachusetts, USA). The following antibodies were used: GR (rabbit, Santa Cruz Biotechnology, Santa Cruz, California, USA; mouse, Transduction Laboratories, Lexington, Kentucky, USA); MR (rabbit and goat, Santa Cruz Biotech); ERα (rabbit, Santa Cruz Biotech; mouse, Neomarkers, Freemont, California, USA); p85α (rabbit, Upstate Biotechnology, Lake Placid, New York, USA; mouse, Transduction Laboratories); P-Akt and Akt (rabbit, Cell Signaling Technology, Beverly, Massachusetts, USA); P-Tyr 4G10 (mouse, Upstate Biotechnology); eNOS (mouse, Transduction Labs); actin (rabbit, Sigma-Aldrich). All other reagents were purchased from Sigma-Aldrich.

PI3K assay. Approximately 500 μg of protein in buffer A (1% NP-40, 20 mM Tris [pH 7.4], 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM Na_3VO_4) were incubated with 1 μg of GR antibody and immunoprecipitated with 40 μl of 1:1 protein A/agarose. PI3K activity in the immunoprecipitate was assayed as described (25). The phospholipids were extracted with 160 μl of chloroform/methanol (1:1, v/v) and separated by borate thin-layer chromatography.

Akt kinase assay and Western blotting. Cells or tissues were homogenized in lysis buffer (1% Triton, 20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4), and Akt kinase activity was determined by detection of phosphorylated GSK-3 fusion protein using the Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer's guidelines. For Western blotting of cell extracts, typically 25–40 μg of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes (Osmonics, Westborough, Massachusetts, USA), and probed with antibodies according to the manufacturer's instructions.

Transient transfection and reporter assays. BAECs were transfected for 6 hours with 8 μg of pGRE-Luc (Clontech, Palo Alto, California, USA) and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) at a ratio of 3:1. COS7 cells were transfected with 0.1 μg of expression plasmid and 0.4 μg of GRE-Luc using the Fugene 6 Transfection Reagent (Roche Molecular Diagnostics, Mannheim, Germany) at a ratio of 3:1. In all conditions, pCMV β-galactosidase was cotransfected, luciferase and β-galactosidase activity was determined with the Dual-Light kit (Tropix, Bedford, Massachusetts, USA), and luciferase activity was normalized to β-galactosidase activity.

eNOS activity assay and cGMP measurement. eNOS activity was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in the presence or

absence of the competitive NOS inhibitor L-NAME (1 mM) using an NOS assay kit (Calbiochem-Novabiochem). Mouse aortae were homogenized in ice-cold PBS containing 1 mM EDTA, the homogenates were pelleted, and 5 μg of protein extracts from the supernatant were used for the eNOS assay as described (17). Unlabeled L-arginine was added to [^{3}H]L-arginine (specific activity, 60 Ci/mmol) at a ratio of 3:1. For measurement of intracellular cGMP (26), cells were pretreated with 0.5 mM isobutylmethylxanthine for 15 minutes, and proteins were extracted with 0.1 N HCl and analyzed with a cGMP radioimmunoassay kit (Amersham International, Little Chalfont, United Kingdom).

Real-time PCR. Total RNA isolation and reverse transcription from ischemic regions were performed as described (27). Real-time PCR was conducted using Biorad iCycler (iCycler iQ Multi-Color Real Time PCR Detection System; Biorad, Hercules, California, USA). The following primer pairs were used: mouse eNOS (5′-TTCCGGCTGCCACCTGATCCTAA-3′ forward and 5′-AACATGTGTCCTTGCTCGAGGCA-3′ reverse) and mouse GAPDH (5′-GAGAATGGGAAGCTTGTCATC-3′ forward and 5′-GTCCACCACCCTGTTGCTGTA-3′ reverse). The fluorescence of Cyber green (PE Biosystems, Foster City, California, USA) was used for quantitation of amplicons. The PCR conditions for eNOS were 95°C (13:30 minutes), 94°C (5:00 minutes), and then 34 cycles of 94°C (40 seconds), 60°C (1:20 minutes), and 72°C (1:20 minutes), followed by 88°C (15 seconds) for data collection. The PCR conditions for GAPDH were 95°C (13:30 minutes), 94°C (5:00 minutes), and then 23 cycles of 94°C (1 minute), 62°C (2:00 minutes), and 72°C (2:30 minutes), followed by 83°C (15 seconds) for data collection. The relative amount of eNOS cDNA was calculated by reference to the GAPDH cDNA in the sample using the Ct method (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, California, USA) and then compared with the amount at the 0 time point. Ct is the point at which the exponential increase in signal (fluorescence) crosses a somewhat arbitrary signal level (usually 10 times background) and is related to the copy number by 2^{-Ct}.

Vascular ring bioassay. Wall tension was measured as described (28). Briefly, isolated aortae were cut into rings (1.5–2 mm) and mounted on an isometric myograph (610M, Danish Myo Technology, Aarhus, Denmark) in physiological solution (118 mM NaCl, 4.6 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 10 mM glucose, 0.025 mM EDTA) at 37°C with or without dexamethasone for 60 minutes. After equilibration, contractile responses were recorded using a data-acquisition and —recording software (Myodaq and Myodata, Danish Myo Technology).

Transient middle-cerebral-artery occlusion. All experiments were conducted in accordance with National Institute of Health and Massachusetts General Hospital institutional guidelines. The left middle cerebral artery of male SV-129 (Taconic, Germantown, New York, USA) or *eNOS*$^{-/-}$ mice was occluded for 2 hours by advancing an 8-0 nylon silicone-coated monofilament 10 mm distal to the carotid bifurcation as described (14). The infarcted areas were measured on brain sections stained with 2,3,5-triphenyltetrazolium (TTC; 24 hours) or on hematoxylin and eosin–stained cryostat sections (72 hours) with an image-analysis system (Bioquant IV, R&M Biometrics, Nashville, Tennessee, USA) and were quantitated by summing the volumes of each section. In some experiments, infarct volume was corrected for edema (29). Regional CBF was determined by laser Doppler flowmetry (Perimed PF2B, Stockholm, Sweden) and recorded on a MacLab/8 data acquisition system (AD Instruments, Milford, Massachusetts, USA) as described previously. Serum dexamethasone levels were determined by Quest Diagnostics Inc (San Juan Capistrano, California, USA).

CBF measurement. Regional CBF was quantified using the [^{14}C]iodoantipyrine autoradiography technique as described previously (30). Isoflurane-anesthetized mice received [^{14}C]iodoantipyrine (5 μCi in 100 μl saline) for 1 minute by infusion. Arterial blood samples were collected at 5-second intervals onto preweighed filter paper disks and measured by liquid scintillation spectrometry (RackBeta 1209; Pharmacia-Wallac, Gaithersburg, Maryland, USA). Immediately after decapitation, the whole head was immersed in chilled isopentane over dry ice (–45°C), and then the frozen brains were sectioned coronally (20 μm). The sections were thaw-mounted on glass coverslips, dried (60°C), and exposed to autoradiography along with [^{14}C]polymer standards (Amersham Life Sciences Inc.). In some experiments, CBF was determined using the indicator fractionation technique with N-isopropyl-[methyl 1,3-^{14}C]-p-iodoamphetamine as described previously (17).

Statistics. The difference in cerebral infarct volume was analyzed by one-way ANOVA followed by Duncan's test. CBF and ring experiments were analyzed by two-way ANOVA followed by *t*-test or Tukey test, respectively. Student's *t* test was used for all other analyses. All values are expressed as means ± SEM unless otherwise indicated.

Results

Acute neuroprotective effects of corticosteroids mediated by eNOS. To determine whether acute administration of corticosteroids protects against ischemic stroke, mice were treated with the synthetic corticosteroid dexamethasone and subjected to transient middle cerebral-artery occlusion (MCAo). In a dose-dependent manner, dexamethasone treatment 1 hour before and 2 hours after MCAo decreased cerebral infarct volume by 32% as compared with vehicle treatment (74 ± 8 mm3 vs. 108 ± 9 mm^3, $P < 0.05$). The reduction in stroke volume by dexamethasone was evident up to 3 days after ischemia, suggesting sustained neuroprotection by corticosteroids (Figure 1a).

To evaluate neuroprotection by corticosteroids *after* the onset of ischemia, we compared the efficacy of different treatment regimens to reduce stroke size (schema in Figure 1b). The standard regimen was com-

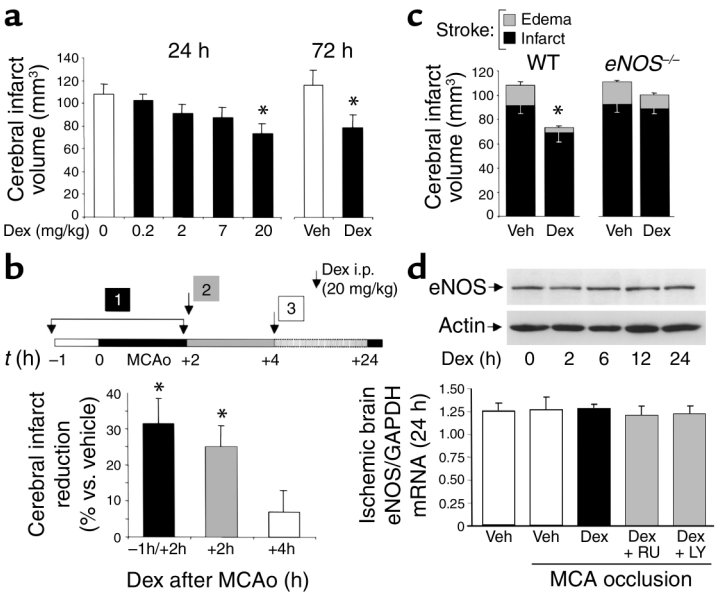

Figure 1

Acute neuroprotection by corticosteroids requires eNOS. (**a**) Dose–dependent effects of dexamethasone (Dex) on stroke volume derived from TTC-stained (24 hours) or hematoxylin and eosin–stained (72 hours) brain sections after MCAo (n = 8–11). *P < 0.05 vs. vehicle. (**b**) Effects of delayed steroid treatment on neuroprotection. Shown is a schema of treatment regimens and corresponding cerebral infarct reduction by dexamethasone relative to the vehicle (Veh) group (n = 7–11). *P < 0.05 vs. vehicle. (**c**) Infarct volume corrected for edema in wild-type and *eNOS*$^{-/-}$ mice treated with vehicle or dexamethasone (20 mg/kg bolus administered intraperitoneally [i.p.]) 24 hours after MCAo (n = 5–11). Wild-type mice are identical to those in (**a**). *P < 0.05 vs. all other groups; WT, wild-type. (**d**) The eNOS protein and mRNA levels after steroid treatment. Equal amounts of aortic tissue homogenates (40 μg) were immunoblotted against eNOS followed by stripping and reprobing against actin (top panels). A representative experiment is shown. Three separate experiments yielded similar results. Brain eNOS mRNA expression from mice treated with vehicle or dexamethasone with or without RU486 (RU) or LY294002 (LY) for 24 hours is shown in the lower panel. The corrected eNOS mRNA levels (eNOS/GAPDH) were determined by real time PCR ($n \geq 3$ for each condition). *t*, time.

pared to a single bolus injection of dexamethasone 2 or 4 hours after the onset of ischemia. Administration of dexamethasone up to 2 hours after the onset of ischemia reduced stroke volume by 25% as compared with the vehicle group (84 ± 7 mm3 vs. 112 ± 8 mm^3, P < 0.05), an effect that was lost when treatment was initiated 4 hours after MCAo (Figure 1b). Thus, corticosteroids rapidly confer neuroprotection when administered early after the onset of ischemia.

Reduction in myocardial infarct size by steroids involves the nontranscriptional activation of eNOS (23). To test whether eNOS mediates the acute cerebrovascular protective effect of dexamethasone, cerebral infarct volume was evaluated in mice with a targeted disruption of the *eNOS* gene (*eNOS*$^{-/-}$) (31). In contrast to the wild-type mice treated with high-dose steroids, *eNOS*$^{-/-}$ mice treated with dexamethasone showed no reduction in stroke size (reduction, 4% ± 10%; P > 0.05) after transient ischemia (Figure 1c), whereas physiological parameters were comparable (data not shown). These findings indicate that eNOS mediates the acute neuroprotective effects of corticosteroids. Although eNOS is required for neuroprotection by steroids, eNOS mRNA and protein levels in brain tissues were unchanged in vivo, suggesting a nontranscriptional mechanism of eNOS enhancement (Figure 1d).

Corticosteroids have been thought to ameliorate ischemic tissue damage by reducing associated brain edema (18). Nevertheless, when stroke volume was corrected for brain edema (29), the protective effect of dexamethasone was sustained (65 ± 8 mm3 vs. 92 ± 7 mm^3, n = 11, P < 0.05), indicating that corticosteroids protected against cerebral injury (Figure 1c). It is interesting to note that we also found an eNOS-dependent effect of steroids on edema formation. Dexamethasone reduced cerebral edema by 37% in the *eNOS*$^{-/-}$ mice as compared with 72% in the wild-type strain (17± 3 mm^3 [n = 10] vs. 5 ±1 mm^3 [n = 11], P < 0.05).

Corticosteroids enhance NO-dependent CBF. Because cerebral infarct size is inversely related to CBF, and endothelium-derived NO regulates cerebral vascular tone and CBF (32), we examined whether corticosteroids can enhance regional CBF to ischemic areas of the brain. Mice were subjected to transient MCAo and regional intraischemic CBF was determined using [^{14}C]iodoantipyrine autoradiography. After MCAo, there was little or no blood flow in the cortical and subcortical parietal lobe of vehicle-treated mice. In mice treated with dexamethasone, the entire core infarct zone appeared smaller, and blood flow was substantially higher in the core region, particularly in the caudate putamen and in the adjacent peri-ischemic areas (Figure 2a). These findings demonstrate that corticosteroids can enhance intraischemic CBF.

To confirm that corticosteroids augment regional CBF, absolute CBF was quantified by [^{14}C]iodoantipyrine measurement. Within 1 hour, treatment with dexamethasone increased absolute CBF in the core ischemic zone (caudate putamen and thalamus) and the penumbral areas (Figure 2b). It is interesting to note that corticosteroids also enhanced CBF in nonischemic areas of the brain. Since equal percentages of ischemia between treatment groups were confirmed by laser Doppler flowmetry (Table 1 and Methods), this demonstrates a beneficial effect of corticosteroids on absolute ischemic CBF. In addition, it provides evidence that enhancement of cerebral perfusion by steroids occurs rapidly, suggesting a nongenomic mechanism of action. Indeed, the increase in CBF by corticosteroids was mediated by NO, since no augmentation of CBF was observed in either the ischemic or nonischemic hemispheres after MCAo in *eNOS*$^{-/-}$ mice (Figure 2c).

Nontranscriptional activation of PI3K mediates stroke protection by corticosteroids. Endothelial NO release is enhanced

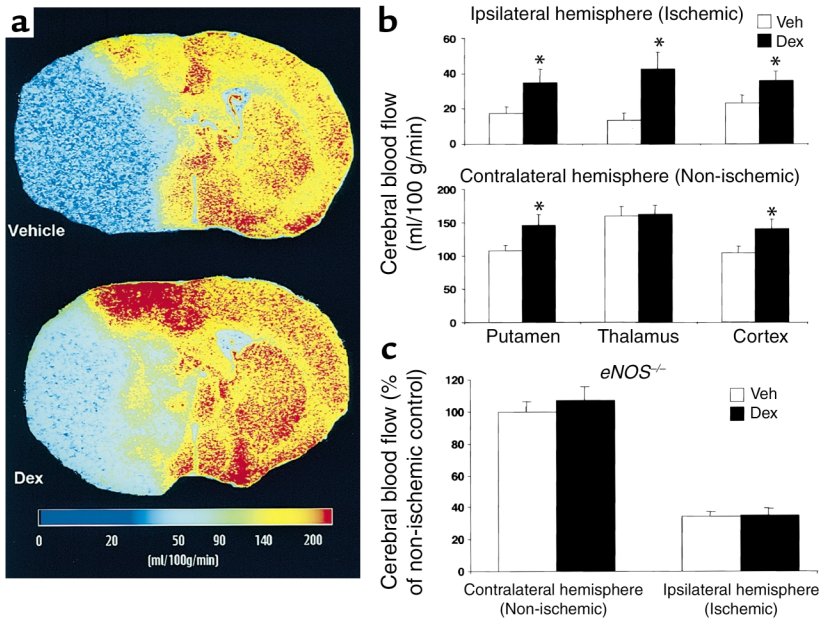

Figure 2
Regulation of CBF by corticosteroids.(**a**) Representative pseudocolor image of CBF ([^{14}C]iodoantipyrine autoradiography) from coronal brain sections 1 hour after MCAo in mice treated with dexamethasone (20 mg/kg) or vehicle. (**b**) Regional changes in absolute CBF ([^{14}C]iodoantipyrine) in ipsilateral and contralateral brain areas of mice treated with dexamethasone (20 mg/kg administered intraperitoneally) or vehicle (*n* = 10 per group). (**c**) Changes in absolute CBF in *eNOS*$^{-/-}$ mice were determined by the [^{14}C]iodoamphetamine technique and expressed as percentages of contralateral CBF of vehicle-treated mice. **P* < 0.05 vs. vehicle.

through direct phosphorylation of eNOS by the protein kinase Akt downstream of PI3K (33, 34). We have previously shown that GR activates eNOS through the PI3K/Akt pathway in a nontranscriptional fashion (23). To determine whether rapid GR signaling to PI3K mediates neuroprotection by steroids, we evaluated cerebral infarct volume with the PI3K inhibitor, LY294002, and the GR antagonist RU486. As compared with the high-dose treatment group, LY294002 and RU486 both reversed the beneficial effects of dexamethasone on cerebral infarct volume without altering baseline values, suggesting that neuroprotection is mediated by PI3K and GR (Figure 3a). Comparison of physiological parameters, mortality, and serum dexamethasone levels after treatment revealed no significant difference between treatment groups (Table 1).

Class I PI3K catalyzes the formation of 3′-phosphorylated phosphatidylinositols (e.g., phosphatidylinositol 3,4,5-trisphosphate [PIP_3]) from phosphatidylinositol phosphate precursors (35). This leads to the subsequent recruitment and activation of protein kinase Akt (36). To evaluate the rapid signaling mechanisms initiated by GR in vivo, we measured GR-associated PI3K activity from brains of dexamethasone-treated mice. Dexamethasone treatment leads to a rapid increase in PI3K activity, as measured by the generation of PIP_3. GR-asso-

Table 1
Effects of high-dose corticosteroids on SV-129 mice

		Vehicle	Dex	Dex + RU	Dex + LY
MABP (mm Hg)	Pre	106 ± 112	112 ± 12	109 ± 11	102 ± 10
	MCAo	104 ± 11	107 ± 11	105 ± 9	98 ± 12
	Post	99 ± 8	100 ± 10	98 ± 10	101 ± 18
CBF (%)	Pre	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	MCAo	10 ± 4	8 ± 4	12 ± 5	15 ± 8
	Post	103 ± 17	107 ± 24	102 ± 19	97 ± 18
pH (arterial)	Pre	7.32 ± 0.04	7.32 ± 0.02	7.37 ± 0.03	7.31 ± 0.02
	MCAo	7.33 ± 0.04	7.32 ± 0.03	7.35 ± 0.04	7.35 ± 0.02
	Post	7.33 ± 0.02	7.32 ± 0.03	7.36 ± 0.04	7.32 ± 0.03
$paCO_2$ (mm Hg)	Pre	39.6 ± 5.1	41.4 ± 4.2	42.3 ± 6.1	42.3 ± 5.6
	MCAo	40.4 ± 6.9	41.7 ± 5.6	41.5 ± 4.9	41.7 ± 4.6
	Post	41.0 ± 4.1	43.4 ± 2.7	43.6 ± 3.0	39.4 ± 4.5
paO_2 (mm Hg)	Pre	136 ± 16	136 ± 19	129 ± 17	124 ± 13
	MCAo	143 ± 12	137 ± 12	145 ± 15	125 ± 14
	Post	137 ± 8	128 ± 10	135 ± 11	128 ± 15
RT (°C)	Pre	36.9 ± 0.2	36.7 ± 0.4	37.2 ± 0.3	36.7 ± 0.2
	MCAo	36.7 ± 0.4	36.8 ± 0.4	36.8 ± 0.4	37.1 ± 0.4
	Post	36.5 ± 0.3	36.8 ± 0.5	36.8 ± 0.4	37.1 ± 0.4
Mortality (%)		10	8	10	9
Dex levels (μg/dl, 24 hours)	NA	0.9 ± 0.2	0.7 ± 0.1	0.6 ± 0.5	

SV-129 mice were treated with dexamethasone (20 mg/kg), RU486 (200 mg/kg), and LY294002 (5 mg/kg). Data are means ± SD (*n* = 5–12). Dex, dexamethasone; RU, RU486; LY, LY294002; MABP, mean arterial blood pressure; Pre, before MCAo; Post, after MCAo; RT, rectal temperature; $PaCO_2$, partial pressure arterial CO_2; PaO_2, partial pressure arterial pressure O_2; NA, not available.

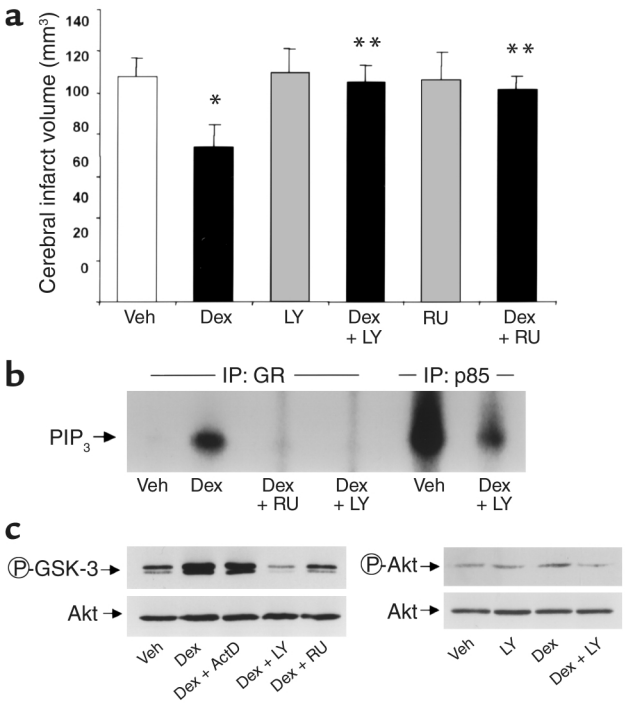

Figure 3
Activation of PI3K/Akt mediates neuroprotection. (**a**) Neuroprotection by dexamethasone is mediated by GR and PI3K. Cerebral infarct volume after MCAo in response to dexamethasone (20 mg/kg), RU486 (200 mg/kg) and LY294002 (5 mg/kg, n = 7–11). Vehicle and dexamethasone groups are identical to those in figure 1a. *P < 0.05 vs. vehicle, **P < 0.05 vs. dexamethasone. RU, RU486; LY, LY294002. (**b**) In vivo activation of PI3K by dexamethasone. PI3K activity from mouse brain tissue immunoprecipitated with GR or p85 antibody. The product of the kinase reaction, PIP_3, was visualized by autoradiography. (**c**) The left panel shows Akt activity and total Akt from mouse brain tissue. Mice were treated with vehicle or dexamethasone, with or without actinomycin D (ActD) (5 mg/kg), RU486, or LY294002. The right panel shows P-Akt (S473) and total Akt from mouse aortae. P, phosphorylation form of the protein. Representative experiments are shown.

ciated PI3K activity was blocked by cotreatment with the PI3K inhibitor LY294002, and the GR antagonist RU486, suggesting that the effect of dexamethasone was mediated by GR (Figure 3b).

Furthermore, steroid treatment leads to in vivo activation of Akt downstream of GR and PI3K, as determined by Akt kinase activity (upper panels in Figure 3c) and Akt phosphorylation (lower panels in Figure 3c). Of note, the transcriptional inhibitor actinomycin D, in doses reported to inhibit transcriptional responses to thyroid hormone and dexamethasone in vivo (8, 37), did not inhibit activation of Akt by corticosteroids. Thus, activation of the PI3K/Akt axis in vivo does not require induction of gene expression by GR. These findings suggest that activation of the PI3K/Akt axis mediates acute neuroprotection by steroids.

Regulation of vascular tone by corticosteroids. Blood flow is partially determined by peripheral vascular resistance, which is negatively regulated by endothelium-derived NO. To determine the effects of acute administration of corticosteroids on vascular tone, we measured wall tension of vascular rings. In vitro, the α-adrenergic agonist phenylephrine caused dose-dependent contraction of aortic rings, which can be reversed by acetylcholine (ACh) in an NO-dependent manner. Cotreatment of aortic rings with relatively high doses of dexamethasone reduced aortic-ring wall tension (Figure 4, a and b), which was reversed by RU486 and LY294002 (Figure 4c). Furthermore, in the absence of eNOS activity, due to either inhibition by L-NAME or lack of enzyme in *eNOS*$^{-/-}$ tissue, baseline contraction was markedly increased and remained unchanged by dexamethasone or ACh. These data demonstrate that dexamethasone exerts rapid, NO-dependent effects and suggest that vasorelaxation by dexamethasone requires activation of eNOS through GR and PI3K.

To determine whether corticosteroids actually stimulate eNOS in vivo, mice were treated with dexamethasone, and aortic NOS activity was measured by the enzymatic conversion of the eNOS substrate L-arginine to L-citrulline (Figure 4d). Dexamethasone treatment induced a threefold increase in eNOS activity, which was completely blocked by cotreatment with LY294002 and RU486. In contrast, baseline NOS activity was virtually absent in *eNOS*$^{-/-}$ mice and remained unresponsive to dexamethasone stimulation (data not shown), indicating that GR specifically enhances eNOS activity through a PI3K-dependent mechanism in vivo.

Nontranscriptional actions of GR require high-dose corticosteroids. Dexamethasone activates eNOS in human endothelial cells (ECs) in a nontranscriptional fashion, which is mediated by the PI3K/Akt pathway (23). Generation of NO increases intracellular cGMP through stimulation of guanylate cyclase. To confirm that dexamethasone stimulates NO production in ECs from different vascular domains, we measured cGMP levels (Figure 5a). Dexamethasone increased cGMP levels about twofold in human umbilical-vein ECs (HUVECs), an effect which was reversed by LY294002 and the NOS inhibitor L-NAME. Similar effects were observed in human aortic ECs (HAECs). We also compared the ability of different corticosteroids to activate eNOS (Figure 5b). All of the tested corticosteroids, including the endogenous steroid hydrocortisone, were found to activate eNOS, demonstrating a corticosteroid class effect on eNOS activity.

Nontranscriptional activation of PI3K in ECs requires high doses of dexamethasone (23). Furthermore, only high-dose steroid treatment provides neuroprotection. This is in contrast to the genomic responses to steroids. To further characterize the transcriptional and nontranscriptional actions of GR, we compared the dose–response data for eNOS activity with results from a glucocorticoid-responsive element–dependent (GRE-dependent) luciferase reporter. Although maximal induction of eNOS activity required doses of 100 nM to 1 μM of dexamethasone, much lower doses of dexamethasone (10 nM) readily induced maximal GRE-

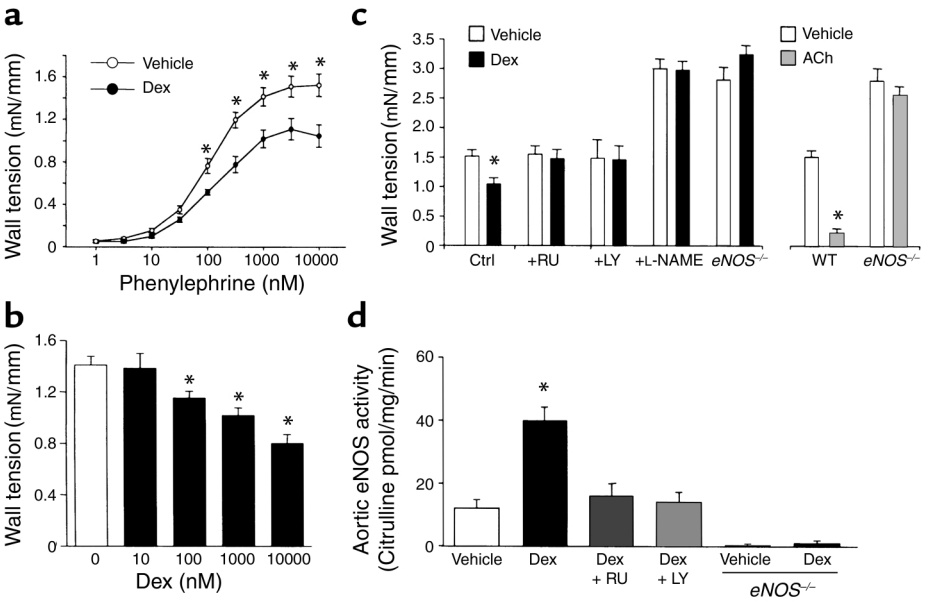

Figure 4
NO-dependent vasodilation by corticosteroids. (**a**) Wall tension to cumulative concentrations of phenylephrine in aortic rings incubated with 1 μM dexamethasone ($n \geq 6$). (**b**) Dose-dependent effects of dexamethasone on wall tension in aortic rings pretreated with 1 μM phenylephrine ($n \geq 4$). (**c**) Wall tension in aortic rings from wild-type or *eNOS*$^{-/-}$ mice treated with phenylephrine and dexamethasone or ACh (3μM) with or without RU486, LY294002, or L-NAME ($n \geq 4$). (**d**) Aortic eNOS activity from wild-type or *eNOS*$^{-/-}$ mice treated with vehicle or dexamethasone with or without RU486 or LY294002 for 2 hours. Three separate experiments yielded similar results. *$P < 0.05$.

mediated transcriptional activity. This was reflected in an approximately 10-fold difference in EC_{50} values (1.1 nM for GRE-reporter vs. 13 nM for eNOS activity) and EC_{max} values (10 nM for GRE-reporter vs. 100 nM for eNOS activity), as derived from logarithmically transformed curve-fit analysis (Figure 5c).

Consistent with our in vitro findings, the peak serum dexamethasone level in mice after administration of neuroprotective doses (20 mg/kg bolus administered intraperitoneally) was approximately 140 nM (5.5 μg/dl) (Figure 5d). No neuroprotection was observed when mice were given 0.2 mg/kg of intraperitoneal dexamethasone, which gave a peak dexamethasone level of 33 nM (1.3 μg/dl). Together, these findings indicate that pharmacologic concentrations of corticosteroids are required for nontranscriptional activation of eNOS and NO-mediated neuroprotection. Despite such high concentrations of corticosteroids, the observed effects are relatively specific since the neuroprotective effects of corticosteroids are absent in *eNOS*$^{-/-}$ mice and could be blocked in vivo by inhibitors of GR and PI3K.

GR mediates the non-nuclear activation of PI3K/Akt by corticosteroids. To determine on a molecular level whether GR mediates PI3K activation, we performed studies in COS7 cells that lack endogenous GR (38). In mock-transfected cells, treatment with dexamethasone did not

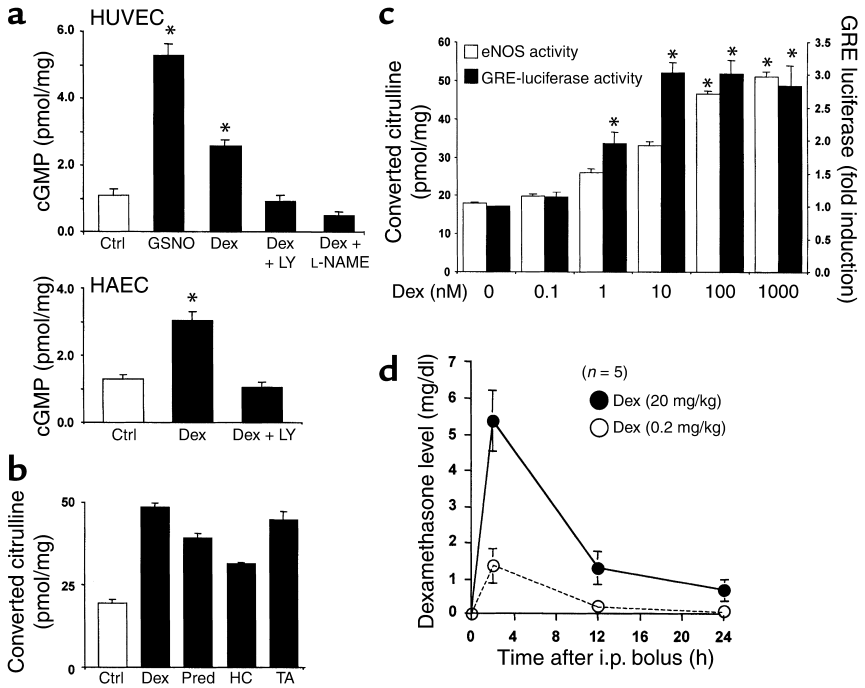

Figure 5
Nontranscriptional activation of eNOS by high-dose corticosteroids. (**a**) Shown are cGMP levels in human ECs after dexamethasone treatment. The upper panel shows cGMP levels in HUVECs stimulated with S-nitrosoglutathione (GSNO, 300 μM) or dexamethasone (100 nM) with or without LY294002 (LY, 30 μM) or L-NAME (1 mM, $n = 4$). The lower panel shows cGMP levels in HAECs stimulated with dexamethasone with or without LY294002 ($n = 4$). Ctrl, control. *$P < 0.05$ vs. control. GSNO, S-nitrosoglutathione; LY, LY294002. (**b**) Effects of 100 nM of dexamethasone, prednisolone, hydrocortisone, or triamcinolone acetonide on eNOS activity in HUVECs ($n = 2$). Pred, prednisolone; HC, hydrocortisone; TA, triamcinolone acetonide. (**c**) Dose–response effects of dexamethasone on eNOS activity (HUVECs, $n = 5$) and GRE-luciferase activity (bovine aortic endothelial cells transfected with pGRE-luc, $n = 6$). *$P < 0.01$ vs. control. (**d**) Dexamethasone serum levels in mice after intraperitoneal bolus administration of low- and high-dose dexamethasone.

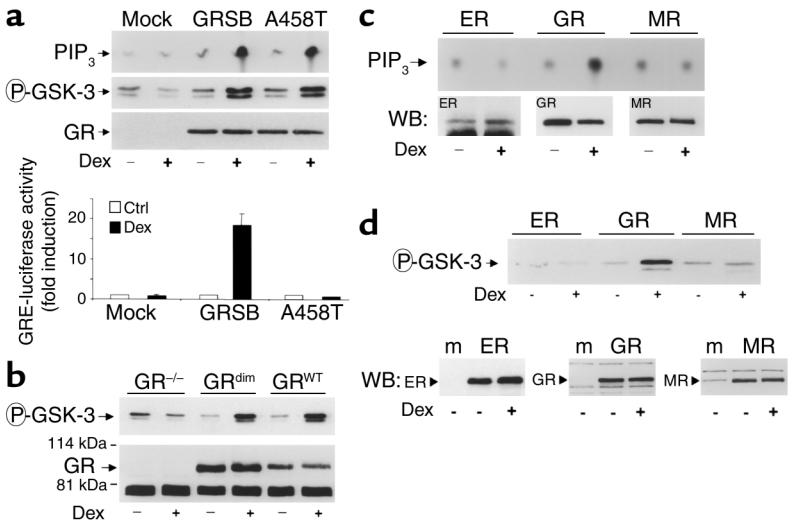

Figure 6

Nontranscriptional activation of PI3K by GR. (a) Nontranscriptional activation of PI3K and Akt kinase by GR: The upper panels show PI3K and Akt kinase assays in COS7 cells transfected with expression plasmids encoding GRα (GRSB) or dimerization-defective GR (A458T). Lysates were immunoblotted against GR. The lower panel shows GRE-reporter activity of COS7 cells cotransfected with either GRSB or A458T expression plasmid with GRE-luc and stimulated with dexamethasone (100 nM). (b) MEFs obtained from wild-type, *GR*$^{-/-}$, and dimerization-defective *GR*^{dim} mice were assayed for Akt activity and expression of GR by Western blotting. (c) Dexamethasone activates the PI3K/Akt pathway through GR. The upper panel shows immunoprecipitation of ERα, GR, or MR followed by PI3K assay or immunoblotting against ERα, GR, and MR. (d) Akt kinase assay in COS7 cells transfected with expression plasmids encoding ER, GR or MR. Lysates of mock-transfected or receptor-transfected cells were immunoblotted against ER, GR, or MR. Representative experiments are shown; m, mock transfected. WB, Western blot; P-GSK-3, phospho-GSK-3.

activate PI3K or Akt, whereas transfection of human GR enabled activation of the PI3K/Akt pathway by dexamethasone. Furthermore, transfection of a dimerization-defective GR mutant (A458T), which is unable to bind DNA and transactivate target genes (38), still activated the PI3K/Akt pathway in a ligand-dependent manner (upper panels in Figure 6a). These results suggest a nontranscriptional mechanism of PI3K/Akt activation, since GR mutant A458T was unable to induce significant transcriptional activity (lower panel in Figure 6a).

Nontranscriptional activation of PI3K was further evaluated in mouse embryonic fibroblasts (MEFs) from genetically modified mice (Figure 6b). While dexamethasone did not activate Akt in *GR*$^{-/-}$ MEFs, activation was readily observed in wild-type MEFs, expressing endogenous levels of GR, and in cells with a dimerization-defective GR (*GR*^{dim}) that renders GR unable to bind to DNA and induce gene expression (5). These results indicate that GR is required for nontranscriptional activation of PI3K and downstream effectors. These findings also suggest specific recruitment of PI3K to GR. Indeed, we have recently described the association of GR with the p85 α regulatory subunit of PI3K in a ligand-dependent manner (23). Corticosteroids can bind to and activate the mineralocorticoid receptor (MR). To determine whether the nontranscriptional effects of dexamethasone were specific for GR activation, PI3K activity associated with different steroid receptors was examined in HUVECs (Figure 6c). Treatment with dexamethasone readily stimulated GR-associated PI3K activity but did not stimulate association of PI3K activity with either MR or ERα, suggesting that PI3K specifically associates with ligand-bound GR.

To corroborate the specificity of high-dose dexamethasone action toward GR, we evaluated Akt activation in COS7 cells transfected with expression plasmids for ERα, GRα, or MR (Figure 6d). Although all receptors were sufficiently expressed after transfection, only GR-mediated activation of Akt by dexamethasone. These findings suggest that although high doses of steroids are required for non-nuclear actions, the effects are nevertheless specifically mediated by GR.

Discussion

Clinical studies of corticosteroids in stroke have yielded conflicting results, which may reflect differences in dosage regimens, the time to onset of treatment, or the specific clinical condition. Although several trials in patients with presumed ischemic stroke have failed to show a reduction in mortality (39–42), at least one study has demonstrated a significant improvement in functional outcome (43). In experimental stroke, physiological levels of corticosteroids increase the severity of ischemic injury after global ischemia (44). However, our finding that corticosteroids are neuroprotective is consistent with previous studies showing reduction in stroke size after focal cerebral ischemia (20–22). Similar to these earlier studies, we found that pharmacological concentrations of corticosteroids were required for acute neuroprotection, whereas lower doses had no effect. It is interesting to note that this dose–response relationship has also been observed in spinal cord injury, where low-dose corticosteroids are not protective, whereas high doses improve clinical outcome when given within the first 8 hours after injury (45, 46).

Several lines of evidence support the conclusion that the neuroprotective effect of high-dose steroids is mediated by nontranscriptional activation of eNOS through the PI3K/Akt pathway. Rapid augmentation of ischemic CBF and stroke protection by steroids is mediated by eNOS, since these effects are reversed in the absence of eNOS. Although neuroprotection is mediated by eNOS, eNOS expression levels were unchanged by steroid treatment in our studies (i.e., <24 hours), suggesting nontranscriptional enhancement of eNOS activity. This is in

contrast to a previous report showing that longer-term steroid therapy (i.e., >72 hours) leads to the downregulation of eNOS and an increase in systemic blood pressure (24). Thus, the beneficial and detrimental effects of steroid therapy with regard to eNOS expression are, in part, dependent on the duration and dose of treatment. In addition, two previous studies suggest that injury from MCAo could lead to increased eNOS expression (47, 48). This is in contrast to the findings of our study, which did not show significant change in eNOS expression in the brain after MCAo in any treatment group. Differences between our study and previous studies include the animal species (i.e., mouse vs. rat), the model of cerebral ischemia (transient vs. permanent occlusion), and the method of measuring eNOS expression (real time PCR vs. immunostaining or semiquantitative RT-PCR).

We found that inhibition of PI3K reversed the beneficial effects of dexamethasone on stroke. Steroids activate eNOS in vivo and in vitro through the PI3K/Akt pathway. Although the activation of PI3K by corticosteroids requires GR, no GRE-mediated gene transcription is necessary for PI3K activation. Instead, activation of PI3K involves the association of the regulatory p85 subunit of PI3K with GR (23). This non-nuclear effect of GR is similar to that of the estrogen receptor, which interacts with and activates PI3K in a ligand-dependent manner (11). Furthermore, the peak serum dexamethasone level in mice treated with neuroprotective doses corresponded to the in vitro concentration that maximally stimulated eNOS and PI3K. Together, these data strongly suggest that nontranscriptional actions of GR mediate neuroprotection through activation of PI3K and eNOS, leading to increases in CBF.

In our studies, the non-nuclear and protective actions of GR require steroid doses at least 10 times as high as those required for the genomic response by the receptor. Yet, these nongenomic actions are specifically mediated by GR. Clearly, further studies are needed to address this discrepancy between transcriptional and nontranscriptional actions. One might speculate that activation of PI3K requires so-far-undiscovered adaptor molecules bound to a subset of GR that alter the binding affinity of the receptor for steroids.

Postischemic inflammation and microvascular occlusion contribute to ischemic brain injury, which can be counteracted by endothelial-derived NO (13, 49). The inhibitory effects of corticosteroids on endothelial-leukocyte adhesion are mediated by GR (50). However, the mechanism by which GR inhibits endothelial-leukocyte interaction is not well understood. By enhancing endothelial NO production, corticosteroids might inhibit platelet aggregation and leukocyte adhesion, thereby enhancing blood flow. The features of neuroprotection by GR resemble the role of NO in stroke protection, since endothelial-derived NO decreases stroke size by improving regional CBF (14). On the other hand, NO donors lose their protective efficacy when administered more than 2 hours after ischemia, an effect that is similar to the effects of dexamethasone (32, 51). Indeed, the ability of dexamethasone to induce NO-dependent relaxation in the mouse aorta probably also contributes to its ability to increase eNOS activity and CBF in the nonischemic regions of the brain. This is a novel feature of corticosteroids that is distinct from its anti-inflammatory effects.

Protection from ischemia in our model is primarily dependent on CBF. However, in a different setting, nonnuclear activation of PI3K and Akt by GR could exert perfusion-independent protective effects, since Akt directly inhibits apoptosis in neural tissue and the myocardium (52, 53). We have recently shown that high-dose corticosteroids decrease myocardial infarct size through the PI3K/Akt pathway (23). Although steroid therapy reduced myocardial infarct size, there was no change in the area at risk between treatment groups, suggesting additional blood flow–independent effects of corticosteroids on ischemic myocardium. Indeed, corticosteroids can promote survival through an Akt-related protein kinase, serum- and glucocorticoid-regulated kinase (SGK) (54, 55). Because SGK is a downstream target of PI3K (56), the ability of ligand-bound GR to activate PI3K may, therefore, provide an additional mechanism by which corticosteroids can regulate survival.

The therapeutic use of corticosteroids is limited by side effects, which occur at relatively low concentrations and are typically associated with long-term treatment. Thus, it appears that these detrimental effects might be related to the genomic actions of corticosteroids. By linking GR to PI3K, the biological and pharmacological actions of corticosteroids could be considerably broadened. This would require the development of a novel class of drugs that selectively activate the nontranscriptional actions of GR. Indeed, synthetic compounds that separate specific GR functions, such as transactivation from transrepression, have recently been identified (57).

Acknowledgments

We thank R. Mandle and F. Rehman (Center for Blood Research, Boston, Massachusetts, USA) for assistance in performing real-time PCR. Plasmids GRSB and A458T were kindly provided by A.C.B. Cato (Forschungszentrum Karlsruhe, Karlsruhe, Germany), expression plasmids for human GR and MR by R. Evans (Salk Institute, La Jolla, California, USA), and human ERα by W.W. Chin (Eli Lilly and Co., Indianapolis, Indiana, USA). This work was supported by grants from the National Institutes of Health (HL-70274, HL-52233, HL-48743, and NS-10828), the American Heart Association Bugher Foundation Award, the Ministero per l'Università e la Ricerca Scientifica e Tecnologica (MURST), the Deutsche Forschungsgemeinschaft (Li-948 1/1 to F.P. Limbourg), and the American Heart Association, New England Affiliate Inc. (to J.-C. Plumier).

1. Hollenberg, S.M., et al. 1985. Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature.* **318**:635–641.
2. Beato, M., Herrlich, P., and Schutz, G. 1995. Steroid hormone receptors: many actors in search of a plot. *Cell.* **83**:851–857.

3. Cole, T.J., et al. 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.* **9**:1608–1621.
4. Reichardt, H.M., Umland, T., Bauer, A., Kretz, O., and Schutz, G. 2000. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. *Mol. Cell. Biol.* **20**:9009–9017.
5. Reichardt, H.M., et al. 1998. DNA binding of the glucocorticoid receptor is not essential for survival. *Cell.* **93**:531–541.
6. Orchinik, M., Murray, T.F., and Moore, F.L. 1991. A corticosteroid receptor in neuronal membranes. *Science.* **252**:1848–1851.
7. Steiner, A., Vogt, E., Locher, R., and Vetter, W. 1988. Stimulation of the phosphoinositide signalling system as a possible mechanism for glucocorticoid action in blood pressure control. *J. Hypertens.* **6**(Suppl.):S366–S368.
8. Inagaki, N., et al. 1992. Studies on the anti-allergic mechanism of glucocorticoids in mice. *J. Pharmacobiodyn.* **15**:581–587.
9. Kousteni, S., et al. 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell.* **104**:719–730.
10. Chen, Z., et al. 1999. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J. Clin. Invest.* **103**:401–406.
11. Simoncini, T., et al. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature.* **407**:538–541.
12. Haynes, M.P., et al. 2000. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ. Res.* **87**:677–682.
13. Iadecola, C. 1997. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* **20**:132–139.
14. Huang, Z., et al. 1996. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J. Cereb. Blood Flow Metab.* **16**:981–987.
15. Huang, Z., et al. 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science.* **265**:1883–1885.
16. Morikawa, E., et al. 1994. L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat. *Stroke.* **25**:429–435.
17. Endres, M., et al. 1998. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA.* **95**:8880–8885.
18. Anderson, D.C., and Cranford, R.E. 1979. Corticosteroids in ischemic stroke. *Stroke.* **10**:68–71.
19. Libby, P., Maroko, P.R., Bloor, C.M., Sobel, B.E., and Braunwald, E. 1973. Reduction of experimental myocardial infarct size by corticosteroid administration. *J. Clin. Invest.* **52**:599–607.
20. Bertorelli, R., Adami, M., Di Santo, E., and Ghezzi, P. 1998. MK 801 and dexamethasone reduce both tumor necrosis factor levels and infarct volume after focal cerebral ischemia in the rat brain. *Neurosci. Lett.* **246**:41–44.
21. Slivka, A.P., and Murphy, E.J. 2001. High-dose methylprednisolone treatment in experimental focal cerebral ischemia. *Exp. Neurol.* **167**:166–172.
22. de Courten-Myers, G.M., Kleinholz, M., Wagner, K.R., Xi, G., and Myers, R.E. 1994. Efficacious experimental stroke treatment with high-dose methylprednisolone. *Stroke.* **25**:487–492.
23. Hafezi-Moghadam, A., et al. 2002. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat. Med.* **8**:473–479.
24. Wallerath, T., et al. 1999. Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. *Proc. Natl. Acad. Sci. USA.* **96**:13357–13362.
25. Walsh, J.P., Caldwell, K.K., and Majerus, P.W. 1991. Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. *Proc. Natl. Acad. Sci. USA.* **88**:9184–9187.
26. Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A., and Sessa, W.C. 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100**:3131–3139.
27. Laufs, U., et al. 2000. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J. Clin. Invest.* **106**:15–24.
28. Mulvany, M.J., and Halpern, W. 1977. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* **41**:19–26.
29. Swanson, et al. 1990. A semiautomated method for measuring brain infarct volume. *J. Cereb. Blood Flow Metab.* **10**:290–293.
30. Yamada, M., et al. 2000. Endothelial nitric oxide synthase-dependent cerebral blood flow augmentation by L-arginine after chronic statin treatment. *J. Cereb. Blood Flow Metab.* **20**:709–717.
31. Huang, P.L., et al. 1995. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature.* **377**:239–242.
32. Dalkara, T., Morikawa, E., Panahian, N., and Moskowitz, M.A. 1994. Blood flow-dependent functional recovery in a rat model of focal cerebral ischemia. *Am. J. Physiol.* **267**:H678–H683.
33. Dimmeler, S., et al. 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature.* **399**:601–605.
34. Fulton, D., et al. 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* **399**:597–601.
35. Rameh, L.E., and Cantley, L.C. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. *J. Biol. Chem.* **274**:8347–8350.
36. Burgering, B.M., and Coffer, P.J. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature.* **376**:599–602.
37. Moreno, M., et al. 2002. Are the effects of T3 on resting metabolic rate in euthyroid rats entirely caused by T3 itself? *Endocrinology.* **143**:504–510.
38. Heck, S., et al. 1994. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J.* **13**:4087–4095.
39. Bauer, R.B., and Tellez, H. 1973. Dexamethasone as treatment in cerebrovascular disease. 2. A controlled study in acute cerebral infarction. *Stroke.* **4**:547–555.
40. Norris, J.W., and Hachinski, V.C. 1986. High dose steroid treatment in cerebral infarction. *Br. Med. J. (Clin. Res. Ed.)* **292**:21–23.
41. Norris, J.W. 1976. Steroid therapy in acute cerebral infarction. *Arch. Neurol.* **33**:69–71.
42. Mulley, G., Wilcox, R.G., and Mitchell, J.R. 1978. Dexamethasone in acute stroke. *Br. Med. J.* **2**:994–996.
43. Patten, B.M., Mendell, J., Bruun, B., Curtin, W., and Carter, S. 1972. Double-blind study of the effects of dexamethasone on acute stroke. *Neurology.* **22**:377–383.
44. Sapolsky, R.M., and Pulsinelli, W.A. 1985. Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. *Science.* **229**:1397–1400.
45. Bracken, M.B., et al. 1984. Efficacy of methylprednisolone in acute spinal cord injury. *JAMA.* **251**:45–52.
46. Bracken, M.B., et al. 1990. A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N. Engl. J. Med.* **322**:1405–1411.
47. Leker, R.R., et al. 2001. Expression of endothelial nitric oxide synthase in the ischemic penumbra: relationship to expression of neuronal nitric oxide synthase and vascular endothelial growth factor. *Brain Res.* **909**:1–7.
48. Danton, G.H., Prado, R., Truettner, J., Watson, B.D., and Dietrich, W.D. 2002. Endothelial nitric oxide synthase pathophysiology after nonocclusive common carotid artery thrombosis in rats. *J. Cereb. Blood Flow Metab.* **22**:612–619.
49. Del Zoppo, G.J., and Hallenbeck, J.M. 2000. Advances in the vascular pathophysiology of ischemic stroke. *Thromb. Res.* **98**:73–81.
50. Cronstein, B.N., Kimmel, S.C., Levin, R.I., Martiniuk, F., and Weissmann, G. 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* **89**:9991–9995.
51. Zhang, F., and Iadecola, C. 1994. Reduction of focal cerebral ischemic damage by delayed treatment with nitric oxide donors. *J. Cereb. Blood Flow Metab.* **14**:574–580.
52. Datta, S.R., et al. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* **91**:231–241.
53. Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R.N., and Walsh, K. 2000. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation.* **101**:660–667.
54. Brunet, A., et al. 2001. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol. Cell. Biol.* **21**:952–965.
55. Moran, T.J., Gray, S., Mikosz, C.A., and Conzen, S.D. 2000. The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. *Cancer Res.* **60**:867–872.
56. Park, J., et al. 1999. Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *EMBO J.* **18**:3024–3033.
57. Vayssiere, B.M., et al. 1997. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol. Endocrinol.* **11**:1245–1255.