Nitric oxide (NO) is a pluripotent regulatory gas with diverse bio-messenger functions including regulation of gene expression. Transcriptional studies using sensitive luciferase reporter systems have suggested that NO inhibits the promoter activity of a variety of genes. Here we report that NO donors (sodium nitroprusside, 2′,2′-(hydroxyxynitrosyldrazono)bis-ethanimine, and (±)-4-ethyl-2-[(Z)-hydroxyiminol]-5-nitro-3-hexen-1-yl-nicotinamide) decrease luciferase activity in a promoter-independent fashion in both viral and eukaryotic promoters, with a reduction to nearly 50% in the presence of 100 μM NO donor. Addition of an SV40 enhancer downstream of the luciferase coding region shifted NO donor inhibition to the right, with inhibition at ~300 μM. In contrast, when studied in a chloramphenicol acetyltransferase reporter, two promoters indicating inhibition by NO were unaffected. The decrease in luciferase activity was not caused by NO suppression of the luciferase enzyme. Real-time PCR data showed that luciferase mRNA half-life decreased by nearly half in the presence of NO donor (from 75 to 45 min). The SV40 enhancer prolonged luciferase mRNA half-life and somewhat blunted the NO effect. Our data suggest that exogenous NO inhibits luciferase activity in a dose-dependent manner through decreasing luciferase mRNA stability. Thus, the use of luciferase reporter systems to study transcriptional regulation by NO should be attempted with caution.

Nitric oxide (NO) is an important molecule with diverse biological effects on numerous enzymes, receptors, structural proteins, and transcriptional factors (1). Studies aimed at understanding the mechanisms by which NO has these myriad actions have represented one of the most rapidly growing areas of research during the last decade. Many reports have shown that NO alters proteins by regulating gene transcription through alterations in promoter specific activity; i.e. NO has been reported to suppress both Egr-1 (2) and PKG-Iα (3) through control of their respective promoters. A partial list of similarly inhibited gene expression through regulation of promoter activity includes cytokines (4), cytochrome P450 enzymes (5), growth factors (6) as well as secondary inhibition of gene expression stimulatory by factors such as 1α,25(OH)2D3 (7) and estradiol (8). On the other hand, the ability of nitric oxide to up-regulate promoter activity has been less well reported, although many proteins are increased after exposure to nitric oxide (9–11). Thus, the ability of nitric oxide to inhibit promoters seems to be widespread.

We also found that nitric oxide inhibited the expression of a protein we were interested in; when we turned to transcriptional assays, we also measured effects of the permeable molecule on promoter-driven firefly luciferase. However, while studying a series of promoter deletions, we found that the effect of nitric oxide seemed to be nonspecific. This led us to consider whether our findings might be explained by a more general effect of NO on the luciferase reporter system. Since the first descriptions of firefly luciferase as a highly sensitive, rapid, and easy-to-perform assay (12–14), luciferase assay has become perhaps the most commonly used method for monitoring promoter activity. We chose three structurally different NO donors to test both viral and eukaryotic promoters linked to the luciferase reporter gene. Our experience, reported here, shows that NO donors significantly repressed luciferase activity in a promoter-independent fashion. Our data also show a direct effect of nitric oxide to shorten the half-life of the luciferase message, revealing at least one mechanism by which NO might have nonspecific effects on this reporter system.

MATERIALS AND METHODS

Materials—Sodium nitroprusside (SNP), 2′,2′-(hydroxyxynitrosyldrazono)bis-ethanimine (Deta/NO), and (±)-4-ethyl-2-[(Z)-hydroxyiminol]-5-nitro-3-hexen-1-yl-nicotinamide (NOR4) were obtained from Sigma/Cell Signaling & Neuroscience. pGL3-Control “pGL3-SV40-Luc” and the pGL3-Promoter “pGL3-SV40-Luc” were purchased from Promega (Madison, WI). pcDNA/CAT and LipofectAMINE were from Invitrogen. The 4xVDRE was inserted into pTK-Luc vector. The macrophage colony stimulating factor (MCSF) promoter and the receptor activator of NF-κB ligand (RANKL) promoter were inserted into both pGL3-Basic and pGL3-Enhancer. pCAT-MCSF was generously provided by Dr. M. Harrington (Indiana University School of Medicine, Indianapolis, IN) (15). DNase I was obtained from Ambion (Austin, TX). All other reagents were purchased from Sigma or are specifically noted otherwise.

Cell Cultures and Transfection—The ST-2 murine bone marrow stromal cell line was purchased from Riken Cell Bank (Tsukuba Science City, Japan) and maintained in a minimal essential medium with 10% FBS. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbeco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. ST-2 cells and HepG2 cells were obtained from Clontech Laboratories.
were seeded at a density of 150,000/well in six-well plates. After 18–20 h, cells were transfected with 1.6 μg/well of DNA and 8 μl of LipofectAMINE in the absence of serum. Five hours later, an equal volume of medium with serum was added to bring the final concentration of serum to 10%. The next day, media was changed, and NO donors were added to cultures as specified. 

Luciferase Assay—To assess luciferase activity, the Promega luciferase assay system was used. Briefly, report lysis buffer was added and incubated at room temperature for 15 min before centrifugation to remove cell debris. For the assay, 20 μl of cell lysate was mixed with 100 μl of luciferase substrate, and light emission was measured with the LumiCount Luminometer (PerkinElmer Life Sciences). In some experiments, cells were co-transfected with pcDNA3/CAT to allow for normalization to CAT activity. CAT normalized results were not different from those normalized by protein content (Bio-Rad detergent-compatibility protocol).

CAT Assay—ST-2 cells were transfected with CAT reporter vector and treated with Deta/NO for 24 h. Cell lysates were collected 48 h after transfection and mixed with 100 μl of luciferase substrate, and light emission was measured with the LumiCount Luminometer (PerkinElmer Life Sciences). In some experiments, cells were co-transfected with pcDNA3/CAT to allow for normalization to CAT activity. CAT normalized results were not different from those normalized by protein content (Bio-Rad detergent-compatibility protocol).

RNA Isolation—After ST-2 cells were treated with Deta/NO, total RNA was extracted by TRIzol as described in the Invitrogen protocol. DNase I treatment alone could not sufficiently eliminate plasmid DNA contamination in our samples, as shown by persistent presence of significant ampiclons as assessed by nonsignificant differences in cycle thresholds (Ct values) between RNA samples for negative control (i.e. not reverse-transcribed) and samples that had been reverse-transcribed. To decontaminate preparations of plasmid DNA, RNA samples (5–6 μg) were incubated with 30 U RsaI, which generated multiple cuts in the luciferase coding region (particularly inside the luciferase PCR amplicon; see below). To study possible NO effects on the CAT reporter message, the lysates containing plasmid were cut with SspI. After incubation for 1 h, the samples were heated at 65 °C for 10 min, and RNA was re-extracted with the RNaseasy mini kit (QIAGEN, Valencia, CA). Finally, 10 units of DNase I (DNA-free DNase treatment and removal reagents; Ambion) was added to each sample at 37 °C for 30 min followed by application of the DNase I removal reagent. The samples were stored at −70 °C.

Real-time PCR—Analysis of Luciferase, CAT, and 18 S mRNA was performed using the iCycler (Bio-Rad). Reverse transcription of 1 μg of total RNA was performed with random decamers (Ambion) and SuperScript II reverse transcriptase (Invitrogen) in total volume of 20 μl. For real-time PCR, amplification reactions were performed in 25 μl containing primers at 0.5 μM and dNTPs (0.2 mM each) in PCR buffer and 0.03 TAq units of TAq polymerase (Invitrogen) and SYBR-green (Molecular Probes, Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 10–10,000-fold for 18 S and 5–625-fold for luciferase as well as CAT to generate relative standard curves to which sample cDNA was compared (16, 17). For luciferase, forward and reverse primers were 5'-GCC TGA AGT CTC TGA TTA AGT-3' and 5'-ACA CCT GCG TCG TAC AAG T-3', respectively, creating an amplicon of 96 bp (18). Real-time PCR to identify CAT mRNA, represented by a 133-bp amplicon, used the forward primer sequence 5'-GCC TGT TAC GGT GAA AAC CT-3' and the reverse primer sequence 5'-GGG CGA AGA AGT TGT CCA TA-3'. For 18 S, an amplicon of 345 bp was generated with forward primer 5'-GAA CGT CTC CCC TAT CAA CAA CT-3' and reverse primer 5'-CCA AGA TCC AAC TAC GAG CT-3' (17). Standards and samples were run in triplicate. Dilution curves showed that PCR efficiency was more than 90% for
luciferase, CAT and 18 S amplicons. Negative controls, such as samples without RT and PCR mixtures lacking both cDNA and RNA were also set up for each real-time PCR to ensure elimination of plasmid DNA. PCR product arising from RNA samples that were not reverse-transcribed had CT values of 27.9/11006.56 for luciferase, 28.4/11006.3 for CAT, and 32.1/11006.95 for 18 S; this was not significantly different from data generated from sample buffer alone with CT values of 29.03/11006.38 for luciferase and 32.25/11006.99 for 18 S. Luciferase values were normalized for the amount of 18 S in the same RT sample, which was also standardized on a dilution curve from RT sample as described in the literature (16, 19).

Statistical Analysis—Results are expressed as the mean ± S.E. Statistical significance was evaluated by Dunnett one-way analysis of variance or t test (Prism; GraphPad Software, San Diego, CA).

RESULTS
Nitric Oxide Donors Inhibit the Luciferase Expression of Multiple Promoter Constructs—Nitric oxide has been shown to repress the activities of multiple promoters studied with luciferase reporter plasmids, yet no specific sequences have been associated with this down-regulation. To assess whether this effect might be nonspecific, we tested several constructs, including both those derived from pGL3-Basic and those where a SV40 enhancer was placed downstream of the poly(A) signal as in the “pGL3-Enhancer” vector.

ST-2 bone stromal cells were transfected with the luciferase reporter constructs and treated with the nitric oxide donor SNP for 20 h (overnight) at variable concentrations. Fig. 1, b–e, shows that the luciferase activity in multiple constructs significantly decreased with the addition of 100 μM SNP. These constructs represent both viral (CMV promoter, Fig. 1b; SV40 promoter, Fig. 1d), as well as a concatameric consensus sequence representing the vitamin D response element associated with a minimal thymidine kinase promoter (Fig. 1c) and 774-nucleotide sequence from the murine MCSF promoter (Fig. 1e) (20). Further reduction in luciferase activity was seen with the addition of 300 μM SNP to cells transfected with the constructs. When the SV40 enhancer was included as part of the promoter construct, added downstream of the poly(A) signal, the inhibitory effect of SNP was blunted with significant inhibition by 40% achieved only at 300 μM SNP; this was seen with both the viral and the eukaryotic promoters studies in Fig. 1, f and g.

To ascertain whether these general effects were confined to the specific NO donor SNP, we also studied two other NO donors: Deta/NO, which is a long lasting NO donor (half-life 20 h at 37 °C) (21, 22) and NOR4, a short lasting NO donor (half-life ~1 h). ST-2 cells were cultured in same condition as the SNP group above but treated with Deta/NO or NOR4. The NOR4 was added twice because of its short half-life, at 0 and 6 h. For data shown in Fig. 2, ST-2 cells were transfected with pGL3-CMV-Luc (without SV40 enhancer) or pGL3-SV40 (SV40Enhancer)-Luc before treatment with 0–300 μM of Deta/NO for 20–24 h. Deta/NO at 300 μM decreased luciferase activities in both reporter constructs. However, luciferase activity was inhibited at 100 μM of Deta/NO in pGL3-CMV-Luc, in which there was no SV40 enhancer downstream of the lu-
ciferase gene; activity was similar at 100 μM SNP (Fig. 2a) but required 300 μM to significantly repress a construct containing the SV40 enhancer (Fig. 2b). Similar results were found when the donor NOR4 was studied along with SNP in ST-2 cell cultures transfected with the pGL3-MCSF-Luc or pCAT-MCSF and treated with 300 μM Deta/NO for 24 h. Cell lysates were assayed for luciferase or CAT expression. There was a decrease in luciferase expression in presence of NO but CAT levels were not changed from the control group. This experiment was repeated three times with similar data. *, p < 0.01, significantly different from untreated group.

Nitric Oxide Donors Fail to Inhibit the Same Promoters Driving a CAT Reporter—The inhibitory effect of NO donors on luciferase activity in multiple unrelated promoter constructs could be explained either by a direct effect of nitric oxide to inhibit promoter activity or a direct effect on luciferase mRNA processing or luciferase activity. To examine the first possibility, the effect of NO donors was examined using two promoters studied previously in the luciferase reporters but here with a CAT reporter system: pcDNA3/CAT (CMV as promoter) and pCAT-MCSF, containing the same nucleotide sequence of the MCSF promoter studied in Fig. 1 (Fig. 3a).

ST-2 cells were transfected with these two constructs. When cells transfected with pcDNA3/CAT (CMV promoter) were treated with Deta/NO overnight (~48 h after transfection), CAT activities did not change even when 500 μM of Deta/NO was added (Fig. 3b) in contrast with the inhibitory effect seen in Fig. 2a. We also compared the promoter activities when treated with Deta/NO in either pCAT-MCSF or pGL3-MCSF-Luc. As shown in Fig. 3c, 300 μM of Deta/NO had no significant effect on CAT activity (unchanged from control levels), whereas the expected 50% reduction in luciferase activity was seen in the same promoter driving the luciferase reporter. These results suggested that the inhibitory effects of NO donors on luciferase activity were not caused by NO effects on promoter-initiated transcription.

NO Donors Decrease Steady-state Luciferase mRNA Levels by Decreasing Message Half-life—We next investigated whether the NO donor or its metabolites might directly inhibit luciferase activity in the sample lysates. Cell lysates were made from ST-2 cells transfected with pTK-4xVDRE-Luc plasmid. Cell lysates were treated with NOR4 (0–500 μM) for 30 min before luciferase assay. Data showed that NOR4 did not directly inhibit luciferase activity. b, neither the NO donor nor its metabolite inhibited luciferase activity. ST-2 cells were transfected with pGL3-MCSF-Luc. Transfected ST-2 cell lysates were mixed with reporter lysis buffer or untreated ST-2 cell lysate or lysates from ST-2 cells treated with 300 μM of Deta/NO. Data showed that there were no differences in luciferase activity among groups.
tion of ST-2 cell lysates containing NO or its metabolites did not change luciferase activity (Fig. 4b).

We further examined whether NO donor repression of luciferase activity was caused by changes in luciferase mRNA levels. For these experiments, we used 300 μM Deta/NO to treat ST-2 cells transfected with pGL3-MCSF-Luc. Real-time PCR analysis was used to measure amounts of luciferase mRNA that were normalized to 18 S mRNA levels from real-time PCR in each sample. Care was taken to rule out plasmid DNA contamination (from the transfection of plasmid) in the RNA samples. In our hands, neither the protocol outlined by Cok and Morrison (18), in which RNA samples were treated twice with DNase I, nor the Ambion protocol, in which samples were heated before DNase I digestion, was able to ensure that DNA contamination was removed: both protocols showed the same threshold cycles of either RNA samples only (without RT as negative control, equivalent amount of RNA for RT reaction) or from RT products for luciferase detection (data not shown). To overcome this obstacle, we added a new step in which samples were treated with the endonuclease Rsal, which generated multiple cuts in the luciferase gene, including one within the luciferase PCR amplicon specified by the primers used for amplification. Rsal was deactivated before continuing with DNase I treatment. With this additional step, the average CT value for luciferase from RT product was 20.2 ± 0.6 cycles compared with the average CT value of 27.9 ± 0.6 cycles from RNA sample (without RT). The luciferase CT value from contaminating plasmid DNA were sufficiently shifted out of the range relevant for RNA analysis (23).

As shown in Fig. 5a, 300 μM Deta/NO caused a 25% decrease in steady-state luciferase mRNA expression as measured by comparison with real-time PCR products in cells not exposed to the NO donor. Our next step was to examine whether the decrease in luciferase mRNA was caused by changes in message stability. Cells transfected with pGL3-MCSF-Luc construct were treated for 2 h with the NO donor SNP (300 μM). At this point, actinomycin D was added to inhibit further mRNA transcription. In ST-2 cells, the control luciferase mRNA had a very short half-life of about 75 min. In those cells treated with SNP, the half-life was decreased to 45 min, a significant decrease seen in two repeats of this experiment (Fig. 5b). Thus, the substantial decreases in luciferase activity caused by NO donors are reflected in the nearly 50% decrease in the stability of luciferase mRNA. In contrast, luciferase mRNA half-life was longer (~220 min) in ST-2 cells transfected with pGL3-Control, in which the SV40 enhancer was placed downstream of the luciferase coding region and poly(A) (Fig. 5c). Comparing luciferase mRNA half-life between control and NO treatments, there was a slight decrease to ~200 min in the presence of 300 μM SNP.

As another control, CAT mRNA levels were also measured to evaluate whether NO donors affected this message. ST-2 cells were transfected with pCAT-MCSF and total RNA collected after treatment with Deta/NO (300 μM) for 24 h. As shown in Fig. 5d, CAT mRNA expression, measured by real-time PCR,
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The NO Effect on Luciferase Activity Is Seen in HepG2 Cells—HepG2, a hepatocyte carcinoma cell line was used to evaluate whether the NO donor repression of luciferase was restricted to the ST-2 bone stromal cell line. The constructs pGL3-SV40 (SV40Enhancer)-Luc and pGL3-MCSF (SV40Enhancer)-Luc were transfected into HepG2 cells. The cells were then treated with NOR4 (0–300 μM) as was done previously in the ST-2 cell line. As shown in Fig. 6a, high dose of NOR4 (300 μM) decreased luciferase activity of the pGL3-SV40 (SV40Enhancer)-Luc as shown previously. The effect of the MCSF or SV40 promoter without the enhancer showed that SNP repressed activity at less than 300 μM (Fig. 6b).

DISCUSSION

Nitric oxide has been shown to modulate the gene expression of many proteins through cGMP and cGMP independent pathways as well as altering DNA binding capabilities of transcription factors through S-nitrosylation (1, 24). Besides genes that seem to be up-regulated, such as ecSOD (9), there are many genes whose expression is inhibited after treatment with nitric oxide (e.g. IGFBP-1 (6), cGMP-dependent protein kinase Iα (3), and the cytochrome P450 enzyme CYP3A4 (25)). Identification of the mechanism of gene repression often involves assessing the specific promoter activity in the presence of an NO donor. The luciferase gene is widely used in constructs employed to evaluate whether the NO donor repression of luciferase was dose-dependently inhibited promoters assayed with a luciferase reporter. For instance, when NO donors were shown to decrease vitamin D receptor-retinoid X receptor-VDR complex formation, the next study was to assay a VDRE fused to minimal thymidine kinase promoter with a luciferase reporter: Deta/NO was shown to decrease luciferase activity by 50% at 300 μM (7). A repressive effect of NO on vitamin D stimulation of the CYP2A4 gene was also suggested to rely on a transcriptional effect of NO; the authors showed that the NO donor NORM dose dependently decreased the CYP3A4 promoter linked to a luciferase reporter with an ED50 of around 300 μM (5, 25). Similarly, the expression of endogenous cGMP-dependent protein kinase Iα shown to be sensitive to NO, was studied with a PKG-Iα promoter/luciferase construct, revealing a dose-dependent inhibition of luciferase activity after treatment with three structurally unrelated NO donors (3). In this case, the activity of IGFBP-1 promoter/luciferase deletion constructs was shown to be down-regulated by Deta/NO; although NO stimulated cGMP, inhibitors of cGMP failed to block NO associated gene repression, leading the authors to conclude that NO had an inhibitory effect separate from cGMP (3). Reports such as these led to an examination of NO involvement in gene repression in our laboratory. Consideration of multiple promoter constructs in our laboratory showed many to be sensitive to NO donor inhibition, leading us to examine the generality of NO promoter repression.

To assess NO effect on the promoter-luciferase assay, we studied both weak and strong promoters and those with an SV40 enhancer used to increase sensitivity. As shown in Fig. 1, all the luciferase reporter constructs we studied had decreased luciferase activity in the presence of NO donors (including SNP, Deta/NO, and NORM). Those lacking the SV40 enhancer, perhaps the most common type of construct used to study promoter activity, were all inhibited by NO donors with apparent 50% inhibition at ~300 μM. Similar results are presented for other promoters studied with luciferase in the literature as cited above. One group did show increased repression of the CYP2D6 promoter compared with a control luciferase, in that case pGL3-control; however, it seems that the control reporter contained the SV40 enhancer, whereas their specific promoter did not (25). Our results show that constructs containing the SV40 enhancer are less sensitive to the repressive effect of the NO donor. Inclusion of the SV40 enhancer conveyed resistance to suppression of luciferase activity until 300 μM (Fig. 2). Many firefly luciferase vectors are marketed; for instance Promega’s pGL3-control and the pGL3-enhancer contain the SV40 enhancer, whereas the pGL3-basic and the pGL3-promoter do not. Consideration of the SV40 enhancer region as a stabilizer that is, perhaps, not sensitive to NO effects suggests that construct design is important for analysis of NO effect: Sugawara inserted the insulin-like growth factor binding protein 1 promoter into pGL3 promoter vector (6); Haru and Sellak used the pGL3-basic construct (3, 25). In those constructs derived from pGL3-Basic 100 μM of SNP, Deta/NO, or NORM was sufficient to decrease luciferase activity.

One example from the literature was careful to show a control luciferase construct that seems to be resistant to NO repression. In this article by Sogawa et al., NO donors inhibited the luciferase expression driven by a promoter containing four hypoxia-response element (HRE3) sequences upstream of an SV40 promoter, as well as one HRE sequence set before a VEGF promoter (26). The inhibition of these HRE-containing sequences was highly significant at low doses of SNP, with an ED50 under 10 μM. The control luciferase reporter vector, also preceded by a SV40 promoter, was not inhibited by the NO
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donors—however, the luciferase construct did differ from those containing HRE by the inclusion of an SV40 enhancer downstream of luciferase coding frame and the poly(A) signal (26). Despite this, it is likely that this extremely sensitive repression is markedly different that the promoter responses we have studied in this work.

We ruled out that NO donors caused a generalized decrease in gene transcription by using two of the previously NO “inhibited” promoters linked to non-luciferase-based reporters. We had previously tried to use the β-galactosidase reporter, but this was also significantly inhibited by Deta/NO in a dose-response manner in ST-2 cells in multiple promoter constructs (data not shown). As shown in Fig. 3, NO failed to inhibit CAT expression driven by a viral or eukaryotic promoter that was significantly “inhibited” by NO donors in luciferase assay. The CAT assay results revealed that neither the MCSF nor CMV involved in the regulation of NO are complex and involve multimers and had no direct effect on the luciferase assay, we inserted into the luciferase reporter vectors to improve the sensitivity of the luciferase message finding a half-life of significantly less than 80 min, it may be particularly susceptible to regulation through altering message stability. Our results indicate that luciferase reporter may not be a good choice for studying the activity of promoters that seem to be regulated by nitric oxide. At the very least, adequate controls, and appreciation of specifics of reporter elements such as inclusion of activity enhancing sequences, are necessary for any conclusion regarding significant effects of nitric oxide.

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Nitric Oxide Donors Inhibit Luciferase Expression in a Promoter-independent Fashion
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