

A Novel Signaling Pathway of Nitric Oxide on Transcriptional Regulation of Mouse κ Opioid Receptor Gene

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Nitric oxide (NO) suppressed the transcription of the mouse κ opioid receptor (KOR) gene, mediated by a rapid downregulation of *c-myc* gene expression. KOR was constitutively expressed in postnatal day 19 (P19) embryonal carcinoma stem cells and is suppressed by NO donors [sodium nitroprusside (SNP), 3-morpholinosydnonimine-1, and S-nitrosoglutathione] in P19 stem cells within 4 hr. The suppression was reversed by 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, an NO scavenger, but could not be blocked by dithiothreitol, ruling out S-nitrosylation as the underlying mechanism. The suppressive effect of NO on KOR occurred at the level of gene transcription, mediated by E boxes located in promoters I and II of this gene. Protein–DNA complexes that formed on these E boxes contained *c-myc*; *c-myc* expression was suppressed by NO in P19

stem cells within 2 hr of treatment. Furthermore, chromatin immunoprecipitation demonstrated reduced *c-myc* binding to the E boxes and hypoacetylation of histone H3 on the chromatin of endogenous KOR promoters in P19 stem cells treated with SNP. It is proposed that NO regulates KOR at the level of gene transcription, mediated by a rapid suppression of *c-myc* gene expression and its binding to KOR promoters, and followed by chromatin hypoacetylation of and reduced transcription from KOR promoters in P19 stem cells. A novel pathway mediating the potential interplay between NO and opioid systems is discussed.

Key words: nitric oxide; κ opioid receptor; *c-Myc*; gene regulation; transcription; P19

Opioids exert various pharmacological effects, including pain relief through their receptors, which are primarily detected in the brain and spinal cord (Slowe et al., 1999; Rosin et al., 2000). Three types of opioid receptors are present in animals, including the μ opioid receptor, δ opioid receptor, and κ opioid receptor (KOR); each receptor is encoded by a different gene (Goldstein and Naidu, 1989; Kieffer, 1995; Wei and Loh, 1996). The mouse KOR gene is constitutively expressed in postnatal day 19 (P19) embryonal carcinoma stem cells; the level of KOR expression changes as cells undergo differentiation (Chen et al., 1999; Bi et al., 2001). In addition to P19 carcinoma cells, various cancer cell lines also express KOR, including lung cancer (Maneckjee and Minna 1990; Kim et al., 1997), CNE2 human epithelial tumor (Diao et al., 2000), and human breast cancer (Hatzoglou et al., 1996).

The mouse KOR gene contains two promoters, promoter I (PI) and PII. PII is located in intron I of this gene. Its changing pattern of expression in differentiating P19 cultures provided a model to address the regulatory mechanisms controlling KOR gene expression. Recently, we demonstrated a negative pathway that controlled KOR gene transcription in both P19 cells and mouse embryos, which involved vitamin A hormones. In differentiating cultures treated for the long term, retinoic acid (RA), the active ingredient of vitamin A hormone, downregulated KOR transcription by inducing Ikaros that recruited histone deacetylases to an

Ikaros-binding site located in the second promoter (overlapping with intron I) of this gene (Hu et al., 2001). Interestingly, we observed a rapid and dramatically suppressive effect of nitric oxide (NO) on KOR expression in this cell-culture model.

It has been reported that long-term administration of either morphine (Bhargava et al., 1998) or cocaine (Bhargava and Kumar, 1997) increased neuronal NO synthase activity in certain brain regions. Furthermore, opioid alkaloid activation resulted in the release of NO in neutrophils, monocytes, and endothelial cells (Magazine et al., 1996; Kowalski, 1998; Stefano et al., 2000). Despite the demonstration of potential cross talks between the opioid and the NO systems as shown in these pharmacological studies, it remains unclear how the NO system interacts with the opioid system at the molecular level.

NO is a well defined signaling molecule that is involved in pathophysiological processes such as inflammation, apoptosis, regulation of enzyme activity, and gene expression. The actions of NO in gene expression were reported to involve, primarily, S-nitrosylation of proteins with subsequent alteration of transcription factors binding to the target promoter site (Lee et al., 2001; Marshall and Stamler, 2001), as well as the activation of soluble guanylate cyclase that stimulated the cGMP–protein kinase G pathway (Morris, 1995). Cadet et al. (2001) reported recently that NO downregulated the expression of the μ opioid receptor gene and suggested that the suppression possibly involved the apoptotic pathway mediated by peroxynitrite.

In this report, we demonstrate the identification of a novel signaling pathway of NO in the opioid receptor system, mediated by the immediate suppression of *c-myc* expression. As a result, occupancy of *c-myc* binding sites on both PI and PII of the endogenous KOR gene became reduced, chromatin histone on the two promoters became hypoacetylated, and KOR expression was rapidly suppressed in P19 stem cells.

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MATERIALS AND METHODS

Plasmid construction. A series of reporter constructs with luciferase inserted into the cloning site of pGL3B (Promega, Madison, WI) were as described by Hu et al. (2001). All of the deletion mutants were constructed in pGL3B. Deletions of PI include Kd36 (–1319 to –743), Kd37 (–1319 to –994), Kd38 (–1044 to –743), Kd39 (–1019 to –883), and Kd40 (–903 to –743). Deletions of PII include Kd50 (–404 to –210), Kd53 (–209 to –15), Kd54 (–163 to –15), Kd56 (–73 to –15), and Kd57 (–43 to –15).

Cell culture and transient transfection. P19 cells were maintained in α -MEM (Invitrogen Corporation, Grand Island, NY) supplemented with 2.5% FBS and 7.5% calf serum in 5% CO₂ at 37°C (Lu et al., 1997). COS-1 cells were grown in α -DMEM supplemented with 10% FBS. Transient transfection and reporter assays were as described previously (Lee et al., 1998). For cotransfection assays, the human *c-myc* expression vector (a gift from Dr. J. M. Bishop, University of California, San Francisco, CA) (Felsher et al., 2000) was used to express *c-myc* ectopically in P19 and COS-1 cells.

Reverse transcription-PCR of endogenous KOR and *c-Myc* genes. RNA was isolated and analyzed by reverse transcription-PCR (RT-PCR) as described previously (Wei et al., 2000). Primers specific to total KOR mRNA are 5'-ATCAGGGCTGAACAGCTA-3' and 5'-GCAAGGACATTCAATGAC-3'. Primers for *c-myc* mRNA are 5'-CCATATGCCCTCAACGTGAAC-3' and 5'-GGGATCCTTATGCACCAGAGTTT-3', which span a 1350 bp coding region.

Electrophoretic mobility shift assay. Two putative E boxes were found in the promoter fragments of both Kd40 and Kd57 constructs by computer alignment. P1-E and P2-E oligos correspond to PI (Kd40) and PII (Kd57), respectively. The electrophoretic mobility shift assay (EMSA) was conducted as described previously (von Knethen et al., 1999), with 10 μ g of nuclear extract incubated in a 10 μ l final reaction volume containing 2 ng of labeled DNA at 4°C for 30 min.

Chromatin immunoprecipitation assay. Control P19 cells or those treated with sodium nitroprusside (SNP) for 6 hr were cross-linked with formaldehyde as described previously (Hu et al., 2001). Cell lysates were subjected to immunoprecipitation overnight at 4°C using 2 μ g of anti-*c-myc* (Santa Cruz Biotechnology, Santa Cruz, CA), anti-acetylated-histone H3 (Upstate Biotechnology, Lake Placid, NY), or preimmune rabbit sera (Pierce, Rockford, IL). After reversed cross-linking, DNA was precipitated and detected by PCR with pairs of primers specific to the KOR promoter regions containing an E box (i.e., 5'-GATGCACAGTAGCTTTCC-3' and 5'-GCAAGGAAGCAAGTGGTA-3' for PI and 5'-TCCTTCCTTGGGATG-3' and 5'-CTGGAAAGCGAGAAAGTG-3' for PII).

Southwestern blot analysis. Nuclear extracts and whole-cell lysates (Sommer et al., 1998) were isolated, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was immediately neutralized and hybridized with an α -³²P-labeled probe for 5 hr at room temperature. The blot was extensively washed and exposed to a PhosphorImager screen (Amersham Biosciences, Piscataway, NJ). The same membrane was re probed with anti-*c-Myc* (Park et al., 2001).

RESULTS

Suppression of endogenous KOR gene activity by NO in P19 cells

To explore factors that might have an immediate effect on KOR gene expression in the P19 cell differentiation model, various agents were used to treat P19 stem cells, followed by the examination of the endogenous KOR mRNA expression. It was interesting to observe a rapid and dramatically suppressive effect of NO on KOR expression in P19 stem cells. As shown in Figure 1A, the steady-state KOR mRNA expression in P19 cells was dramatically suppressed after the addition of SNP, an NO donor, within 4 hr of treatment. The suppression continued for up to 24 hr (data not shown), suggesting a prolonged, suppressive effect of NO. Furthermore, the suppressive effect of SNP was dose dependent (Fig. 1B), supporting a specific effect of NO on the expression of KOR.

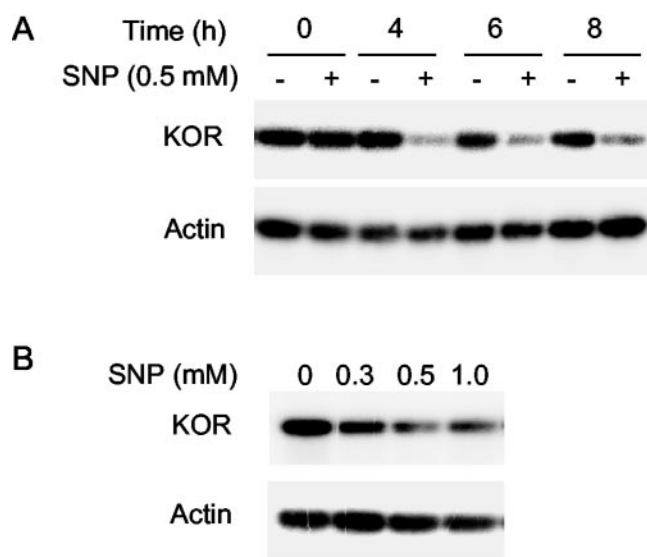


Figure 1. Suppression of endogenous KOR by NO. KOR mRNA isolated from SNP-treated P19 cells was analyzed by RT-PCR using primer pairs specific to KOR and actin. *A*, Different time points. *B*, Concentrations of SNP.

Suppression of KOR promoters by NO in P19 cells

Because the effect of SNP on KOR gene expression persisted over an extended period, we then determined whether this suppressive effect was mediated by the regulatory region of the KOR gene. We took advantage of a previously engineered KOR-luc reporter, which contained the regulatory region of both PI and PII of the mouse KOR gene, K45. As shown in Figure 2A, all of the NO donors tested, including SNP, 3-morpholininosydnonimine-1 (SIN-1), and *S*-nitrosoglutathione (GSNO), were equally effective in suppressing this reporter, suggesting that NO indeed was able to suppress KOR gene expression in P19 cells, and that the suppression was mediated by the regulatory region of the KOR gene. To substantiate the specificity of NO on KOR transcriptional regulation further, we used an NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO), to block the effect of SNP. As shown in Figure 2B, PTIO effectively blocked the suppressive effect of SNP; PTIO alone had no significant effect. To gain an insight into the cell-type specificity of SNP in relation to suppression of the KOR gene, the responses of the same reporter K45 to SNP in P19 cells and COS-1 cells were compared (Fig. 2C). It appeared that SNP had no effect on this reporter in the COS-1 background, suggesting the involvement of specific cellular factors of P19 in mediating the suppressive effect of NO on KOR transcription.

Suppression of KOR expression by NO through the E-box elements in PI and PII

The suppressive effect of SNP on both the endogenous KOR mRNA (Fig. 1) and the KOR reporter (Fig. 2) suggested a physiological relevance of NO on transcriptional regulation of the KOR gene. To determine the genetic elements that mediated this suppressive effect, transient transfection assays were conducted using serial deletion mutants of the K45 reporter. Because the KOR gene could use two promoters, PI and PII, we first determined which promoter(s) mediated the effect of SNP. It was interesting that both promoters were suppressed by SNP; therefore, we used two sets of deletion mutants to determine the

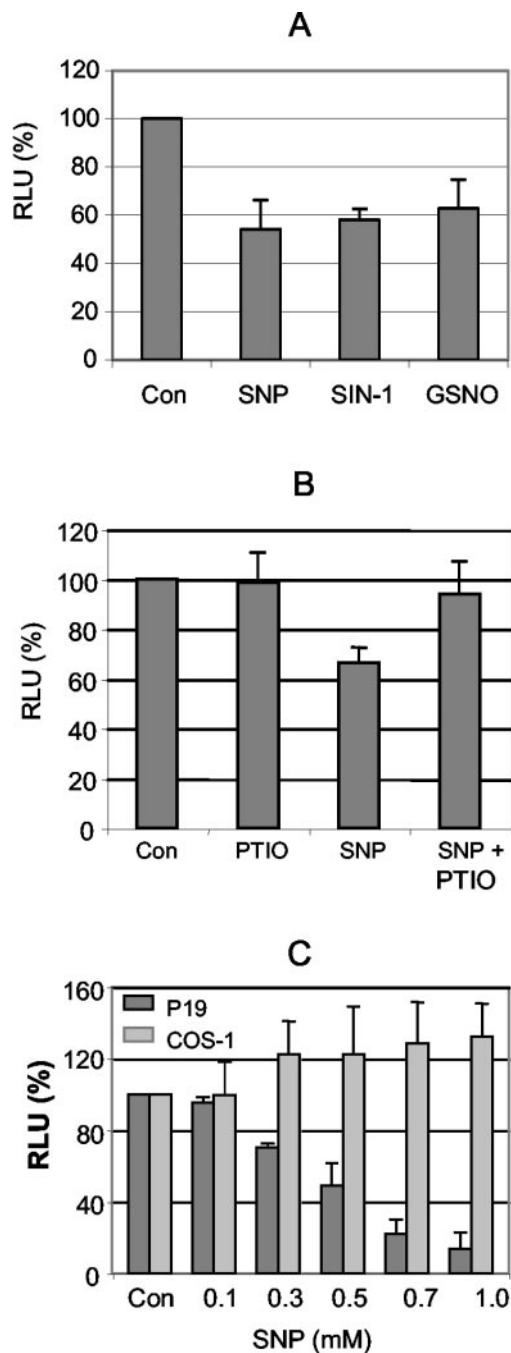


Figure 2. Effect of NO on the KOR promoter. P19 cells were transiently transfected with K45 and lacZ internal control. After 24 hr of transfection, cells were treated with SNP, SIN-1, and GSNO (*A*, each 0.5 mM) and with 50 μ M PTIO (*B*) in the presence or absence of SNP for 6 hr at 37°C. The relative luciferase units (RLU) were shown as the averages \pm SD of three independent experiments performed in triplicate. *C*, P19 and COS-1 cells transfected with K45 were exposed to various concentrations of SNP for 6 hr. The relative luciferase units are the averages \pm SD of three experiments. Con, Controls.

responsible DNA elements. Figure 3*A* shows the results using various deletions of PI (K19). Reporters K19, Kd36, Kd38, and Kd40 were all suppressed by SNP, whereas Kd37 and Kd39 were not affected, suggesting that the DNA fragment (–903 to –743) used in the smallest SNP-responsive construct, Kd40, contained the responding sequence. Figure 3*B* shows the results of deletion

constructs from PII (K18). It appeared that K18, Kd53, Kd54, Kd56, and Kd57 were also suppressed by SNP, but not Kd50, which was deleted in a *Pst*I fragment adjacent to the initiation site. The analysis of possible common sequences present in all of the SNP-responsive constructs revealed a putative *c-myc* binding site, an E box (CACGTG), in both promoters. The putative *c-myc* binding sites were located at –882 to –877 (CACTTG) and at –39 to –34 (CAAGTG) of PI and PII, respectively. These results suggest that NO might suppress KOR gene transcription through a putative *c-myc* binding site.

Suppression of protein–DNA complex formation on the E-box elements by NO

To first determine whether the E boxes were indeed the functional binding sites for nuclear factors of P19, presumably the *c-myc* transcription factor, we used EMSA with α -³²P-labeled DNA fragments spanning the putative *c-myc* sites as the probe. One major band was detected in EMSA using nuclear extracts prepared from untreated P19 cells, for both PI (P1-E) and PII (P2-E). Furthermore, this band could be efficiently competed out by cold DNA fragments for both P1-E and P2-E (Fig. 4*A*). Interestingly, as the SNP concentration increased, the intensity of the retarded band decreased (*lanes 4–6, 9–11*), suggesting decreased protein–DNA interaction on these E boxes as a result of SNP treatment. A stronger reduction in the binding intensity of P2-E than that of P1-E by SNP suggests that P2-E responds to SNP more effectively. Furthermore, the specifically shifted band, which could be competed out by cold DNA fragments (Fig. 4*B*, *lane 2*), disappeared only in the presence of the *c-myc* antibody (*lane 3*), suggesting that protein complexes formed on E boxes indeed contained *c-myc*, which was specifically blocked by this antibody. The formation of this complex was not affected by the preimmune serum (*lane 4*). In addition to EMSA, Southwestern blotting showed binding of multiple proteins to the E box of PII for both P19 and COS-1 nuclear extracts; one major band was competed specifically with the cold probe (Fig. 4*C*), which was confirmed as the *c-myc* protein by probing the same membrane with a *c-myc* antibody (Fig. 4*D*). The identity of *c-myc* binding to these E boxes was also confirmed on the endogenous KOR gene promoter regions using the chromatin immunoprecipitation (ChIP) assay (see Fig. 6).

The reduced binding of the *c-myc* complex in SNP-treated cultures suggested two possibilities. NO might induce post-translational modification of *c-myc*, such as *S*-nitrosylation, widely known as the major modification affecting protein–DNA interactions (Marshall and Stamler, 2001). Alternatively, NO might downregulate *c-myc* expression in P19 cells. To test these two possibilities, DTT, a compound that inhibits *S*-nitrosylation, was used to treat the nuclear extract of P19 in EMSA, as shown in *lanes 7 and 8* in Figure 4*A*. Interestingly, DTT could not recover protein binding to the *c-myc* binding site, ruling out *S*-nitrosylation as a cause for this reduced *c-myc* binding to DNA.

To examine the alternative possibility that *c-myc* expression was suppressed by SNP, *c-myc* mRNA and protein levels were examined with RT-PCR and Western blotting, respectively. It appeared that SNP treatment for 2 hr began to suppress the steady-state level of *c-myc* mRNA in P19 cells; the suppressive effect was even more dramatic at 4–8 hr (Fig. 5*A*). Furthermore, suppression of *c-myc* expression by SNP was also concentration dependent (Fig. 5*B*). The level of *c-myc* protein was examined by Western blot analyses as shown in Figure 5*C*. SNP also suppressed *c-myc* protein levels in a dose-dependent manner (*lanes*

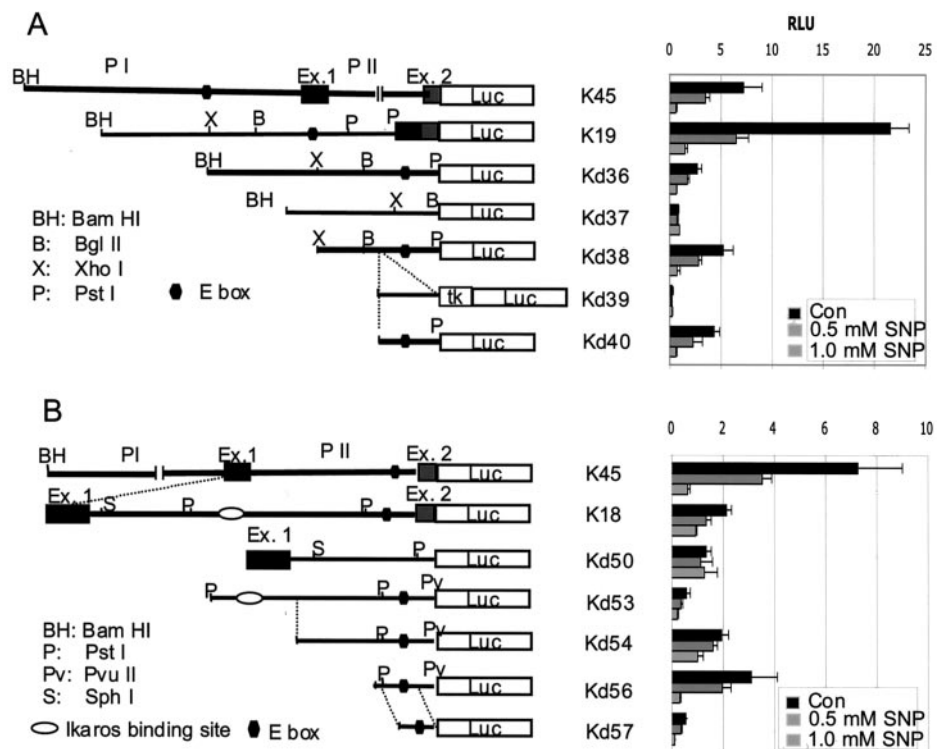


Figure 3. NO suppresses KOR PI and PII through E boxes. P19 cells were transiently transfected with various deletion constructs of PI (*A*) or PII (*B*) as indicated. The cells were untreated (*Con*) or treated with 0.5 or 1 mM SNP for 6 hr. The results presented at the right were shown as the averages \pm SD of two independent experiments performed in triplicate. The E boxes present in the smallest genomic fragments Kd40 and Kd57 are indicated. *Luc*, Luciferase; *RLU*, relative luciferase units.

1–4). The suppression of *c-myc* by SNP was recovered by treatment with an NO scavenger, PTIO (Fig. 5*D*), consistent with the results shown in Figure 2*B*. To confirm the effects of *c-myc* on KOR regulation, a *c-myc* expression vector was used to overexpress *c-myc*. As shown in Figure 5*E*, *c-myc* expression indeed induced KOR reporter activity approximately ninefold in both P19 and COS-1 cells, and SNP blocked the induction in P19 cells as predicted (Fig. 5*E*). Consistent with the result showing that NO failed to suppress KOR reporter expression in COS-1 cells (Fig. 2*C*), SNP has no effect either on the expression of *c-myc* protein in COS-1 cells (Fig. 5*C*, lanes 5–7) or on the elevation of KOR reporter activity by *c-myc* expression (Fig. 5*E*), supporting the cell-type specificity of NO-mediated suppression of the KOR gene in P19 cells, which was attributable to the suppression of *c-myc* mRNA and protein expression.

Suppression of *c-myc* binding and histone acetylation on endogenous KOR promoters by NO

It was shown that *c-myc* was able to recruit histone acetyltransferases (HATs), enzymes involved in acetylating histone proteins and inducing an open chromatin conformation for gene activation (McMahon et al., 2000). To examine whether the histone acetylation status of KOR promoters and the binding of endogenous *c-myc* to these KOR regulatory sequences in P19 cells were altered by NO treatment, ChIP assays were performed to detect the acetylation status of PI and PII and the binding of *c-myc* to the endogenous KOR promoters. As shown in Figure 6, SNP decreased *c-myc* binding to the endogenous E-box sequences of the KOR gene for both PI and PII (lanes 5 and 6). Acetylation of these DNA regions of the endogenous KOR gene was also consistently reduced (lanes 7 and 8). Furthermore, PII appears to be more sensitive to the SNP-triggered reduction of *c-myc* binding, consistent with the results of the gel-shift data shown in Figure 4. This provided the evidence that NO indeed reduced *c-myc* occupancy on the regulatory regions of the endogenous KOR gene,

which resulted in diminished histone acetylation on KOR promoter regions. Therefore, it was concluded that NO rapidly suppressed KOR gene transcription in P19 cells by a novel signaling pathway that involved immediate repression of *c-myc* expression, formation of *c-myc*–DNA complexes, and ultimately hypoacetylation of chromatin on the endogenous KOR promoter regions.

DISCUSSION

We have shown previously that the mouse KOR gene was constitutively expressed in P19 stem cells and exhibited a unique pattern of expression in developing embryos, primarily in the CNS (Chen et al., 1999; Hu et al., 1999). Using the P19 culture model, we tried to explore potential regulatory pathways underlying the specific expression of the KOR gene. Vitamin A was found to be a negative factor for KOR expression in differentiating P19 cells, which was mediated by the induction of Ikaros that recruited HATs to silence the KOR gene in differentiating cells (Bi et al., 2001; Hu et al., 2001). This study showed that NO could rapidly and dramatically suppress KOR expression in stem cells by reducing *c-myc* expression, revealing a novel pathway of NO signaling and an interesting interplay between the NO and KOR systems at the gene level in stem-cell populations.

All of the NO donors tested herein showed a similar suppressive effect on the KOR gene, which was reversed by an NO scavenger. NO was shown to affect the expression of a number of genes, such as intercellular adhesion molecule-1, gonadotropin-releasing hormone, MAP kinase phosphatase-3, and inducible NO synthase (Toyoshima et al., 1999; Belsham and Mellon, 2000; Rossig et al., 2000; Lee et al., 2001). However, in all of these studies, the action of NO was shown primarily to involve S-nitrosylation of Oct-1 (Lee et al., 2001), nuclear factor κ B (Marshall and Stamler, 2001), and c-Myb (Brendeford et al., 1998) transcription factors. Our finding that NO was able to repress the expression of *c-myc* transcription would be the first

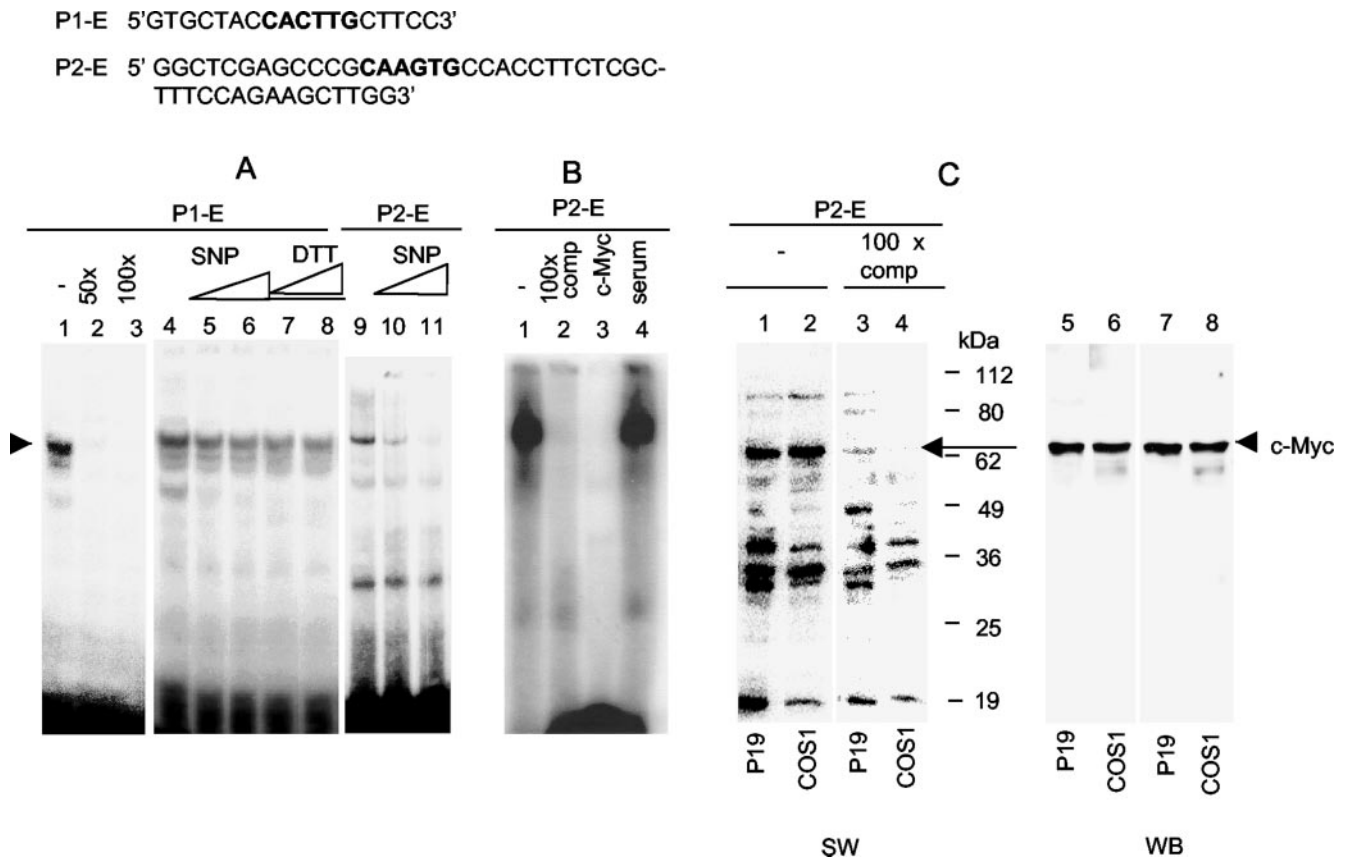


Figure 4. *c-myc* binds to the E boxes of KOR promoters. **A**, EMSA. P19 cells were incubated alone (lanes 1, 4, and 9) or with 0.5 SNP for 6 hr (lanes 5 and 10) or 1 mM SNP for 6 hr (lanes 6 and 11). DTT (1 or 5 mM) was added to P19 cells for 30 min, followed by treatment with 1 mM SNP (lanes 7 and 8) for 6 hr at 37°C. Nuclear extracts (10 μ g) were incubated with α -³²P-labeled probes of P1-E (lanes 1–8) in the presence of excess cold probes (lanes 2 and 3) or P2-E (lanes 9–11) for 30 min at 4°C. **B**, Antibody interference assay. Nuclear extracts were incubated with 100 \times excess of cold probe (lane 2), *c-myc* antibody (lane 3), or preimmune serum (lane 4) for 30 min at room temperature and then with α -³²P-labeled P2-E for 30 min at 4°C. **C**, Southwestern (SW) and Western (WB) blots. Nuclear extracts (75 μ g) of P19 cells and COS-1 cells were separated by 12% SDS-PAGE, transferred to PVDF membranes, and hybridized with α -³²P-labeled P2-E in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 \times excess of cold probe. The same membranes were reprobed with *c-myc* antibody as shown on the Western blot. *comp*, Competition. Arrowheads indicate *c-myc* complexes.

example of a novel signaling pathway of NO that regulated the expression level of a transcription factor. Because this effect is cell-type-specific, it would be interesting to identify the specific cellular factors or transcription machinery that are responsible for this rapid suppression of the *c-myc* gene by NO in stem cells. In addition to NO, we have observed that *c-myc* gene expression was suppressed by RA in P19 cells (data not shown). This finding is in agreement with the suppressive effect of RA on KOR expression (Bi et al., 2001).

A transient transfection assay with various deletions of PI and PII demonstrated that two E boxes [–882 to –877 (PI) and –39 to –34 (PII)] functioned as the NO-responsive element. As reviewed previously (Dang, 1999; Amati et al., 2001), the E box is a binding site of *c-myc* transcription factor. The action of *c-myc* involved its interaction with other transcription factors as well as enzymes for modifying histone proteins, the HATs (Xu et al., 2001). In other words, *c-myc* formed complexes with many other transcription factors, such as Max, to act on gene promoters (Blackwood and Eisenman, 1991), and recruited the transformation–transactivation domain-associated protein coactivator complex (McMahon et al., 1998; Bouchard et al., 2001), which contained the HAT component hGCN5 (McMahon et al., 2000). The recruitment of the HAT complex ultimately leads to histone acetylation of histone H3 and H4 of the chromatin, consequently

activating gene transcription (Amati et al., 2001; Xu et al., 2001). Our Southwestern blot results indeed suggested that protein complex binding to the E box of the KOR promoter contained proteins other than the confirmed *c-myc*. It would be interesting to identify specific protein factors that are associated with *c-myc* in protein complexes formed on the E box of KOR promoters. The results of the ChIP assay (Fig. 6) revealed hypoacetylation of histone proteins on the chromatin of KOR promoter regions, confirming that both the binding of *c-myc* complexes to E boxes and the acetylation of histone on KOR promoters were reduced in P19 stem cells after the cells were exposed to NO. This would provide a mechanistic explanation for the action of NO that resulted in a more prolonged effect on the KOR gene attributable to changes in its chromatin modification in specific cell types.

It has been demonstrated that many transcription factors are modulated by NO-induced S-nitrosylation of the proteins (Marshall et al., 2000; Marshall and Stamler, 2001). However, in the regulation of the KOR gene, DTT, a compound inhibiting S-nitrosylation (Brendeford et al., 1998), had no effect on either the DNA-binding ability of nuclear extracts from the cells treated with SNP or the reporter activity in the transient transfection assay. This result allowed us to rule out S-nitrosylation as the underlying mechanism. Alternatively, the transcription factors Oct-1 and CCAAT/enhancer-binding protein- β have been known

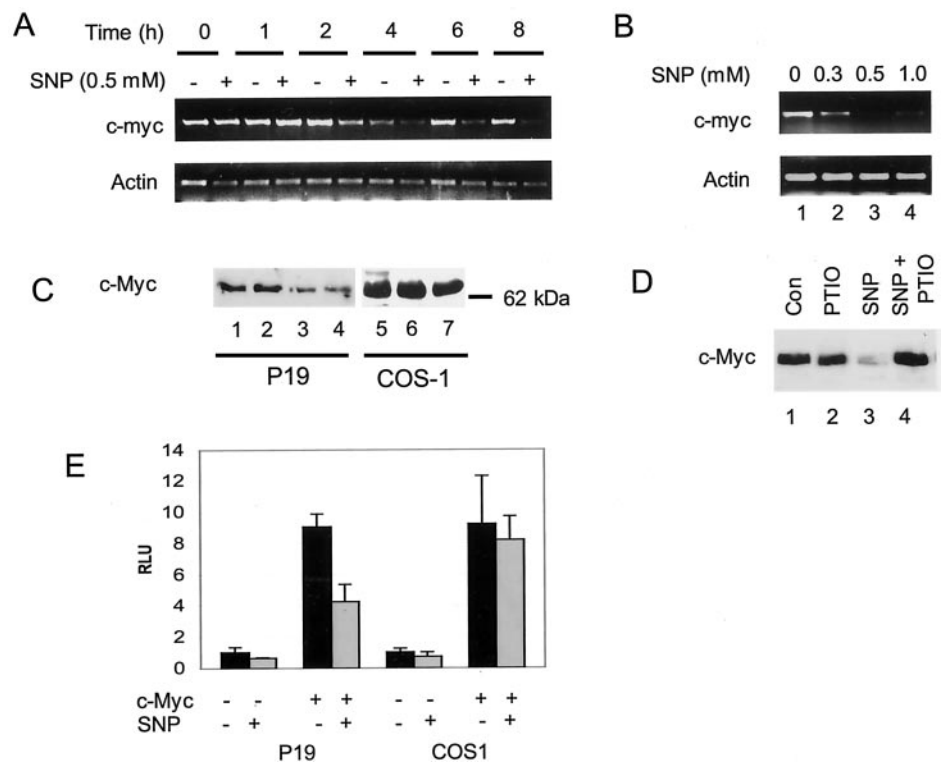


Figure 5. NO suppresses *c-myc* expression. *A*, *B*, *c-myc* mRNA was reverse-transcribed and amplified by PCR. The amounts of *c-myc* mRNA were analyzed against the time (*A*) or the dose (*B*) of SNP added. *C*, Western blot. Whole-cell lysate of P19 cells (50 μ g; lanes 1–4) or COS-1 cells (100 μ g; lanes 5–7) was immunoblotted with *c-myc* antibody. *D*, Western blot analysis, with anti-*c-myc*, of the whole-cell lysate (50 μ g) of P19 controls (lane 1), cells treated with PTIO (lane 2), or cells treated with 1 mM SNP in the absence (lane 3) or presence (lane 4) of 50 μ M PTIO for 6 hr. *E*, Induction of KOR reporter by *c-myc* expression in P19 and COS-1 cells. Cells were co-transfected with K45, a *c-myc* expression vector, and an internal lacZ control. Cells were then treated with vehicle or 0.5 mM SNP for 6 hr. The relative luciferase units (RLU) of two independent experiments are presented.

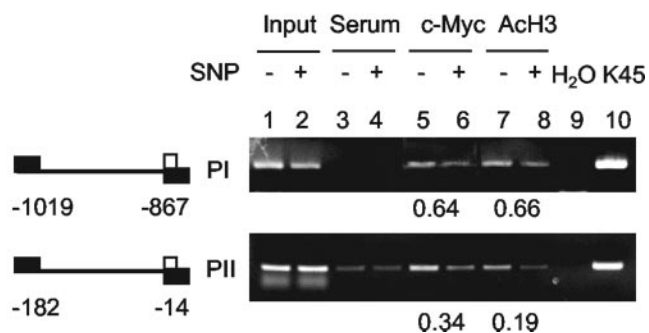


Figure 6. NO downregulates *in vivo* binding of *c-myc* and renders histone hypoacetylation on KOR promoters. +, SNP treatment for 6 hr; –, untreated cultures. ChIP assays were performed as described in Materials and Methods. *Left*, Schematic presentation of E boxes in PI and PII and the location of respective primer sequences for PCR analysis. The numbers below the promoters indicate the position of the PCR primers relative to the ATG codon. *Right*, Comparison of *in vivo* binding of *c-myc* and acetylated histone H3 (*ACh3*) between control and SNP-treated cultures. The relative intensities of the signals of SNP-treated samples to controls are presented below as the averages of two experiments. The input was the PCR product of total chromatin. A rabbit preimmune serum was used as a nonspecific control. *Con*, Control.

to be involved in the NO/cGMP-mediated repression of gonadotropin-releasing hormone gene expression (Belsham and Mellon, 2000). However, the potent cGMP analog 8-bromoadenosine 3',5'-cGMP had no effects either in the transient transfection assay using KOR promoter constructs or on *c-myc* protein expression in cells treated with SNP (data not shown). This result precluded the involvement of the cGMP–protein kinase G pathway in the regulation of the KOR gene by NO. Instead, NO reduced the expression of *c-myc* in P19 (Fig. 5). Furthermore, a detailed comparison of the kinetics of the suppression of *c-myc* and KOR in P19 cells treated with SNP also supported the sequential actions of NO (0

hr) followed by *c-myc* (2 hr) on the expression of the KOR gene (4 hr). More convincingly, SNP did not affect *c-myc* expression in COS-1 cells, in which SNP had no effect on KOR expression. Therefore, this novel signaling pathway of NO is cell-type-specific and involves certain cellular factors that affect the expression of *c-myc*; these factors remain to be identified.

The action of NO can occur at the processes of cellular apoptosis and/or differentiation (Brune et al., 1998; Chung et al., 2001). Peunova et al. (2001) suggested that NO might be an essential negative regulator of neuron precursor proliferation during brain development in vertebrates. Our finding that NO suppressed KOR expression in P19 stem cells is consistent with this idea. However, it remains to be determined whether the effect of NO on KOR expression is associated with P19 apoptosis or differentiation, because this effect occurred within a very short period in stem cells. The physiological implications of a rapid suppression of KOR by NO in stem cells remain to be determined experimentally.

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