

Methylation-dependent Gene Silencing Induced by Interleukin 1 β Via Nitric Oxide Production

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Summary

Interleukin (IL)-1 β is a pleiotropic cytokine implicated in a variety of activities, including damage of insulin-producing cells, brain injury, or neuromodulatory responses. Many of these effects are mediated by nitric oxide (NO) produced by the induction of NO synthase (*iNOS*) expression. We report here that IL-1 β provokes a marked repression of genes, such as fragile X mental retardation 1 (*FMR1*) and hypoxanthine phosphoribosyltransferase (*HPRT*), having a CpG island in their promoter region. This effect can be fully prevented by *iNOS* inhibitors and is dependent on DNA methylation. NO donors also cause *FMR1* and *HPRT* gene silencing. NO-induced methylation of *FMR1* CpG island can be reverted by demethylating agents which, in turn, produce the recovery of gene expression. The effects of IL-1 β and NO appear to be exerted through activation of DNA methyltransferase (DNA MeTase). Although exposure of the cells to NO does not increase DNA MeTase gene expression, the activity of the enzyme selectively increases when NO is applied directly on a nuclear protein extract. These findings reveal a previously unknown effect of IL-1 β and NO on gene expression, and demonstrate a novel pathway for gene silencing based on activation of DNA MeTase by NO and acute modification of CpG island methylation.

Key words: interleukin 1 β • nitric oxide • *FMR1* • CpG island methylation • gene repression

Methylation status of control regions in the genome plays a critical role in the regulation of gene expression (1–3). In susceptible genes containing 5' CpG islands, cytosine methylation favors a repressive chromatin structure that prevents the binding of transcriptional activators to the promoter (4, 5). The molecular link between methyl groups on the DNA and the positioning of nucleosomes to form an inactive chromatin configuration has been recently elucidated (6). Well-known examples of methylation-dependent gene silencing are X-linked gene inactivation (7) and genomic imprinting (8), and changes in the pattern of DNA methylation occur during cell differentiation (9, 10) and tumorigenesis (11–14). Hypermethylation of CpG islands can be associated with the silencing of some genes, such as fragile X mental retardation 1 (*FMR1*),¹ causing inherited mental diseases (15, 16). Differential genomic DNA meth-

ylation also has the potential to influence the development of T cell cytokine production profiles (17). Here, we report that exposure to IL-1 β (acting through inducible nitric oxide synthase [*iNOS*] induction) or direct application of NO donors induces in several cell types the suppression of the expression of *FMR1* and other housekeeping genes containing a CpG island in their promoter. This effect is shown to be produced by DNA methylation resulting from activation of DNA methyltransferase (DNA MeTase). These observations demonstrate that IL-1 β and NO, which are messenger molecules involved in a wide variety of pathophysiological processes, can have a direct effect on gene expression.

Materials and Methods

Materials. IL-1 β and IFN- γ were purchased from Genzyme. *S*-nitroso-*N*-acetylpenicillamine (SNAP), *N*-methyl arginine (L-NMA and D-NMA), actinomycin D (ActD), trichostatin A

¹Abbreviations used in this paper: ActD, actinomycin D; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; AzadC, 5-aza-2'-deoxycytidine; β -ME, β -mercaptoethanol; DNA MeTase, DNA methyltransferase; DTT, dithiothreitol; EIT, *S*-ethylisothiouria; *FMR1*, fragile X mental retardation 1 gene; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HK, hexokinase; HPRT, hypoxanthine phosphoribosyltransferase; *iNOS*, inducible NOS; LDH, lactate dehydrogenase; L-NIL, 1-*N*₆-(1-iminoethyl)-lysine; NF- κ B, nuclear factor κ B; NMA, *N*-methyl argi-

nine; NO, nitric oxide; NOS, NO synthase; PK, pyruvate kinase; RIN, RINm5F; RT, reverse transcription; SIN, 3-morpholinopyridone hydrochloride; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; SSPE, saline-sodium phosphate-EDTA; TSA, trichostatin A.

(TSA), 5-aza-2'-deoxycytidine (AzadC), dithiothreitol (DTT), β -mercaptoethanol (β -ME), and protease inhibitors were obtained from Sigma Chemical Co. Sodium nitroprusside (SNP) was from Fluka. 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), S-ethylisothiourea hydrobromide (EIT), and 1-N₆-(1-iminoethyl)-lysine hydrochloride (L-NIL) were from Tocris Cookson, Ltd. 3-morpholinopyridone hydrochloride (SIN) was from ICN Iberica. Rediprime[®] DNA labeling system, [³²P]dCTP, and cold and ³H-labeled S-adenosylmethionine were from Nycomed Amersham plc. Glutathione (GSH) and poly deoxyinosine-deoxycytosine (poly dI-dC) were from Boehringer Mannheim. Restriction enzymes were from Promega or New England Biolabs. All other reagents were of the best quality commercially available.

Cell Culture. Insulin-producing rat RINm5F (RIN) cell, Jurkat T cell, and mouse leukemic monocyte-macrophage cell (RAW 264 cell) lines were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B under 5% CO₂ at 37°C. Human lymphocytes were obtained from peripheral blood of healthy donors as reported previously (18).

Reverse Transcription PCR. Total RNA was extracted from cell lines or fresh peripheral lymphocytes by the guanidine phenol method. RNA was reverse transcribed using random hexamers, and the cDNA was amplified using specific primers. PCR amplification of the CGG repeats at the FRAXA site and KH domains was carried as reported previously (18, 19). Amplification of hypoxanthine phosphoribosyltransferase gene (*HPRT*) in RIN cells was assessed using murine primers. Human specific primers were used for *HPRT* mRNA analysis in Jurkat T cells and fresh peripheral lymphocytes (7). Reverse transcription (RT)-PCR of *ATP*-ase or glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was used as control. PCR products were visualized on agarose gel stained with ethidium bromide.

Northern and Western Blots. Northern blot of *FMR1* gene was performed using 10 μ g total RNA and 10 ng/ml of human *FMR1* cDNA probe labeled with [α -³²P]dCTP. Hybridization conditions were: 16 h at 42°C in 50% formamide, 6 \times saline-sodium phosphate-EDTA (SSPE), 5 \times Denhardt's solution, 0.5% SDS, 100 μ g/ml herring sperm DNA. Wash conditions were: 2 \times SSPE, 0.1% SDS at room temperature and 0.1 \times SSPE, 0.1% SDS at 55°C. DNA *MeTase* expression was assayed with the same protocol using a specific 5-kb cDNA probe. Northern blot of *iNOS* and *GAPDH* was assayed by standard procedures. Western blot analysis of DNA *MeTase* was performed using 20–40 μ g of nuclear protein extract resolved on 5% SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and subjected to immunodetection using a 1:2,000 dilution of primary antibody and an enhanced chemiluminescence detection (13).

Southern Blot. DNA samples were prepared from cultured cell lines by standard procedures. 10 μ g of genomic DNA was digested overnight with the restriction enzymes EcoRI-EagI or HindIII-SacII, EagI and SacII being sensible to methylation. Restriction fragments were separated by electrophoresis on 0.8% agarose gel, Southern blotted, and hybridized with radiolabeled StB12.3 probe as described previously (20).

DNA *MeTase* Assay. DNA *MeTase* activity was determined in nuclear protein extracts by the assay developed by Adams et al. (21) with minor modifications. Cells were lysed in buffer containing 20 mM Tris-HCl, pH 8, 137 mM NaCl, 5 mM MgCl₂, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 100 μ g/ml RNase. After centrifugation, nuclear extracts were prepared by resuspension of the crude nuclei in high salt buffer. 15–25 μ g of proteins was incubated for

2 h at 37°C with 4 μ g of poly (dI-dC) as template and 5.25 μ M ³H-labeled S-adenosylmethionine (1 μ Ci; Amersham Pharmacia Biotech) as methyl donor. Reactions were stopped, proteins extracted, and DNA template was recovered by ethanol precipitation. RNA was removed by resuspension of the precipitates in NaOH; DNA was spotted on Whatman filters, dried, and then washed with trichloroacetic acid (5%) followed by ethanol, then ether. Filters were placed in the scintillation mixture, and DNA *MeTase* activity was determined by scintillation counting. Results were expressed as cpm per microgram of protein; all experiments were performed in duplicate. Background levels were determined in assays where poly (dI-dC) was omitted. Statistical analyses were performed using Student's *t* test.

Other Enzymatic Assays. Lactate dehydrogenase (LDH) and pyruvate kinase (PK) were measured by standard procedures in the 12,000 *g* supernatant of Jurkat T cell homogenate as described previously (22). Hexokinase (HK) was measured in the homogenate of Jurkat T cells as reported elsewhere (23). Statistical analyses were performed using Student's *t* test.

Cell Proliferation Assay. Cellular proliferation was determined by a colorimetric assay system using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) following the manufacturer's instructions (Cell Proliferation Kit I; Boehringer Mannheim).

Results and Discussion

Fragile X syndrome, the most common form of hereditary mental retardation (24), results from repression of the *FMR1* gene due to the expansion of the CGG repeats in its first exon and methylation of the 5' CpG island. The latter alteration appears to be the primary cause of the disease, since hypermethylation of the CpG island in the active X chromosome is only observed in affected individuals, whereas there are cases with full expansion of the CGG repeats but with an unmethylated island that do not manifest the syndrome (25, 26). Furthermore, in vitro reactivation of the *FMR1* gene by demethylating agents has been reported recently (27). We have observed a marked inhibitory effect of IL-1 β on *FMR1* gene expression in RIN cells assessed by RT-PCR of both KH domains and CGG repeats (Fig. 1, a–c). Inhibition of *FMR1* expression was appreciable after 12 h of incubation with IL-1 β , and complete suppression of the gene resulted with longer exposures (Fig. 1 a). Since IL-1 β is known to be a powerful stimulus for induction of *NOS* in RIN and other cell types (28, 29), we investigated whether NO acted as a mediator of *FMR1* repression. Fig. 1 b shows that SNP, an NO donor, mimics the action of IL-1 β , and that the IL effect is fully prevented by the simultaneous addition of L-NMA, an inhibitor of *NOS* activity. This preventive effect was not observed when we used D-NMA (not shown). To further demonstrate that IL-1 β exerts gene silencing via NO production, we used specific *iNOS* inhibitors such as AMT, EIT, and L-NIL and found that all of them also prevented the action of IL-1 β (Fig. 1 c). To determine if *FMR1* mRNA stability was altered by NO, ActD was used to inhibit RNA synthesis. As shown in Fig. 1 d, the time course of *FMR1* mRNA degradation was not modified by the presence of SNP. Thus, production of NO by IL-1 β or addition of NO pre-

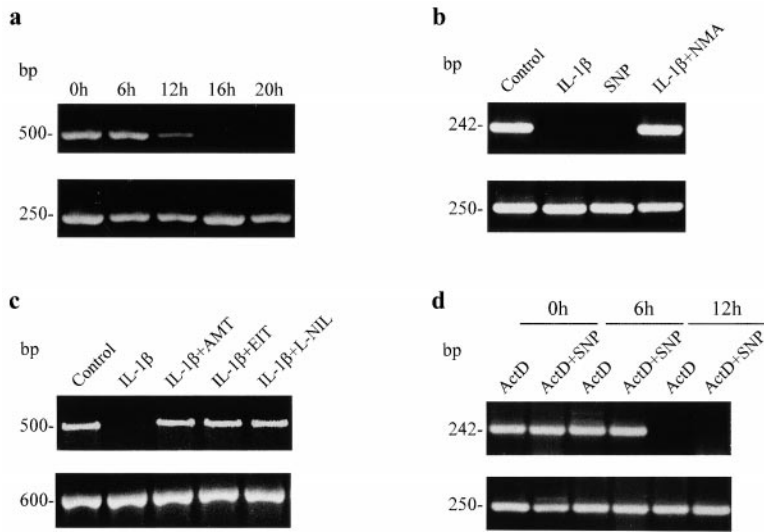


Figure 1. *FMR1* expression in RIN cells treated with IL-1 β and NO donors. In this and other figures, RT-PCR of KH domains and CGG repeats of *FMR1* give bands of 500 and 242 bp, respectively; *Na/K ATP-ase* (250 bp) and *GAPDH* (600 bp) were used as control. (a) Time course of IL-1 β (25 U/ml) induced *FMR1* repression in RIN cells. (b) 16 h treatment with IL-1 β (25 U/ml), SNP (100 μ M), or IL-1 β (25 U/ml) plus NMA (100 μ M). (c) 16 h treatment with IL-1 β (25 U/ml) or IL-1 β plus a specific iNOS inhibitor (20 nM AMT, 100 nM EIT, or 10 μ M L-NIL). (d) Different exposure times of RIN cells with ActD alone (5 μ g/ml) or added simultaneously with SNP (100 μ M). Data shown in this and the other figures are representative of two to six experiments.

cursors can produce *FMR1* gene silencing. In preliminary experiments, we have observed that IFN- γ , which induces NO synthesis, as well as NO donors can also inhibit *FMR1* expression in a monocyte-macrophage cell line (RAW 264 cells).

To elucidate the mechanisms underlying the effect of NO on gene regulation, we studied the expression of *FMR1* in human cells (Jurkat T cells and fresh lymphocytes) where the complete map of the *FMR1* promoter is known (30). Although these cells are not stimulated by IL-1 β , the application of NO precursors (500 μ M SNP and 100 μ M SIN) resulted in complete suppression of *FMR1* expression (Fig. 2 a; see also Fig. 3 b), thus allowing a more detailed analysis of the repression of *FMR1* gene by NO. Since it has been reported that NO can produce DNA damage by disruption of nucleotide bonds (31), we tested if the effect of NO could be explained by direct interaction with CG sites, par-

ticularly abundant in (CGG) n repeats and CpGs of *FMR1*. The fact that we were unable to induce CG cleavage in the *FMR1* gene by NO donors in cellular and cell-disrupted preparations (not shown) led us to hypothesize that NO could be part of a signaling pathway regulating methylation of the CpG island in the *FMR1* promoter region. This was tested by Southern blot using methyl-sensitive restriction enzymes and the StB12.3 probe currently applied to study the length and the methylation status of *FMR1* in fragile X patients (20, 24). Fig. 2 b shows that treatment of Jurkat T cells with SNP or SIN produced full CpG island methylation (lanes 2, 4, and 6), which was totally prevented when the cells were treated with the demethylating agent AzadC in the presence of any of the NO donors (lanes 3 and 5). In the experiments where we applied an NO donor (or IL-1 β) plus AzadC, we incubated the cells for 24 h as has been rec-

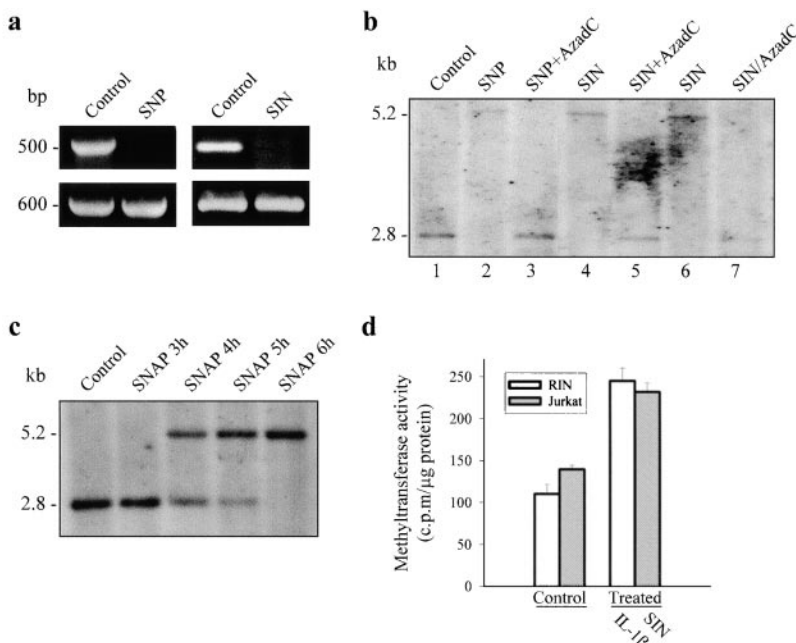


Figure 2. NO-induced *FMR1* silencing and methylation of *FMR1* CpG island in Jurkat T cells. (a) RT-PCR of *FMR1* KH domains after 16 h incubation with SNP (500 μ M) or SIN (100 μ M). *GAPDH* was used as control. (b) Southern blot of genomic DNA digested to completion with EcoRI-EagI. The 2.8-kb fragment results from cleavage by both enzymes, whereas the 5.2-kb fragment corresponds to digestion with EcoRI, indicating protection from restriction by methylation. Untreated cells (lane 1); cells incubated for 24 h with SNP (500 μ M) or SIN (100 μ M) (lanes 2 and 4); cells treated for 24 h simultaneously with SNP (500 μ M) plus AzadC (2 μ g/ml) or SIN (100 μ M) plus AzadC (2 μ g/ml) (lanes 3 and 5); cells incubated for 16 h with SIN (100 μ M) (lane 6); and cells exposed for 16 h to SIN (100 μ M), washed, and incubated for 24 h with AzadC (2 μ g/ml) (lane 7). (c) Methylation pattern induced by 3, 4, 5, or 6 h incubation with SNAP (100 μ M). (d) DNA MeTase activity in RIN and Jurkat T cells treated for 16 h with IL-1 β (25 U/ml) and for 6 h with SIN (100 μ M), respectively, expressed as cpm/ μ g protein (100 cpm/ μ g protein corresponds to a specific activity of 0.015 pmol/h/ μ g protein).

ommended when using the demethylating agent (7). After incubation with and washout of SIN, addition of AzadC also reverted the methylating action of NO (lane 7), indicating that the effect of AzadC was not due to direct chemical interaction with SIN. CpG methylation induced by NO donors (SIN or SNAP) progressed with time after the initial 3 h of treatment, and full methylation was observed at 6 h (Fig. 2 c).

To further investigate the NO-dependent methylation process, we measured the activity of DNA MeTase, the major DNA methylating enzyme that produces 5' methylcytosine (32). Incubation of RIN cells with IL-1 β increased DNA MeTase activity to about twice the level found in control cells ($P < 0.001$, $n = 3$). A qualitatively similar effect was observed in Jurkat T cells exposed to an NO donor ($P < 0.001$, $n = 3$; Fig. 2 d). These increases in activity are within the range of those recently published in transformed rodent cells overexpressing *c-fos* (33). Since NO-induced methylation of the CpG island is abolished by the presence of the demethylating agent AzadC (see Fig. 2 b), it is expected that addition of this product would prevent *FMR1* silencing induced by either IL-1 β or NO. Fig. 3 a illustrates that *FMR1* suppression produced by IL-1 β was almost completely reverted by incubation with the demethylating agent. It is also shown that AzadC did not diminish the level of expression of *iNOS* induced by IL-1 β . Similarly, demethylation also prevented *FMR1* suppression resulting from exposure of the cells to NO donors (Fig. 3 b). These data indicate that NO-dependent *FMR1* gene silencing results from methylation of the CpG island, an effect mediated by activation of DNA MeTase.

The major mammalian DNA MeTase is a large protein with an NH₂-terminal putative regulatory domain comprising two thirds of the protein with eight cysteine residues, and a COOH-terminal catalytic domain with the adenosylmethionine binding region and a proline-cysteine catalytic center (34). The signals and mechanisms involved in regulation of DNA MeTase activity are poorly understood. The NH₂ terminus is unnecessary for catalysis, but its cleavage from the COOH terminus causes a large stimulation of the initial velocity of methylation of unmethylated DNA (34). The NH₂ terminus contains a major phosphorylation site (serine 514) although its relevance in catalysis is

uncertain, since treatment of the enzyme with phosphatases and kinases results in no significant effect on the catalytic rate (35). We assayed whether the effect of NO on DNA MeTase activity was due to activation of guanyl cyclase, by incubation of Jurkat T cells with 2 mM dibutyryl cGMP for 24 h. cGMP had no effect on either DNA MeTase activity or the expression of *FMR1* gene (not shown). The Ras signaling pathway has been shown to increase DNA MeTase transcription (36), and recently it has been suggested that *fos* may transform cells through alterations in DNA methylation (33). Therefore, we tested if the expression of DNA MeTase could be altered by exposure of Jurkat T cells to an NO donor. Northern and Western blot analyses, shown in Fig. 4, a and b, respectively, indicate that NO does not affect the expression of the major human DNA MeTase. We have not studied the expression of the recently described DNA MeTase3A and DNA MeTase3B (37); however, it is very unlikely that they mediate the effects of NO described here, since these two enzymes are present mainly in embryonic tissues (37). Moreover, NO was able to activate DNA MeTase in a nuclear protein extract (see below), thus strongly suggesting that the regulation of the enzyme by NO is not dependent on transcription.

The Western blot in Fig. 4 b also shows that NO did not modify the magnitude or size of any of the bands obtained with the polyclonal antibody against the DNA MeTase, thus indicating that cleavage of the NH₂-terminal regulatory domain is probably not involved in the mechanism of action of NO. However, the presence of several cysteine residues in the protein suggested the possibility of a direct reaction of NO with thiols. Fig. 5 a shows that application of an NO donor (SNAP) to a nuclear protein extract induced a dose-dependent increase of DNA MeTase activity. In addition, sodium nitrite and peroxynitrite at different concentrations did not increase the enzymatic activity. On the contrary, high concentration of peroxynitrite drastically inhibited the reaction. Fig. 5 b illustrates the time course of DNA MeTase activation induced by 50 μ M of different NO donors (SNAP, SIN, and SNP) in the nuclear protein extract. In all cases, after 3 h incubation with the NO donor a statistically significant increase in the DNA MeTase activity was observed ($P < 0.001$, $n = 3$ for SNAP; $P <$

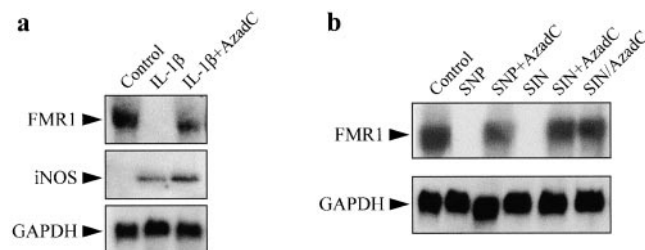


Figure 3. *FMR1* expression in RIN and Jurkat T cells incubated with IL-1 β or NO donors and the demethylating agent AzadC. (a) Northern blot of *FMR1* and *iNOS* in RIN cells incubated for 24 h with IL-1 β (25 U/ml) in the absence or presence of AzadC (2 μ g/ml). (b) Northern blot of *FMR1* in Jurkat T cells following the same protocol as in the legend to Fig. 2 b, except for the condition shown in lane 6. *GAPDH* expression was used as control.

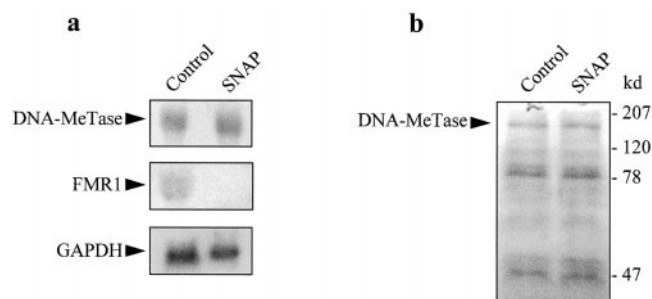


Figure 4. Effect of NO on the expression of DNA MeTase in Jurkat T cells. (a) Northern blot of DNA MeTase in cells incubated for 16 h with 100 μ M SNAP. *FMR1* and *GAPDH* were used as control. (b) Western blot of DNA MeTase using a polyclonal antibody in cells incubated for 16 h with 100 μ M SNAP.

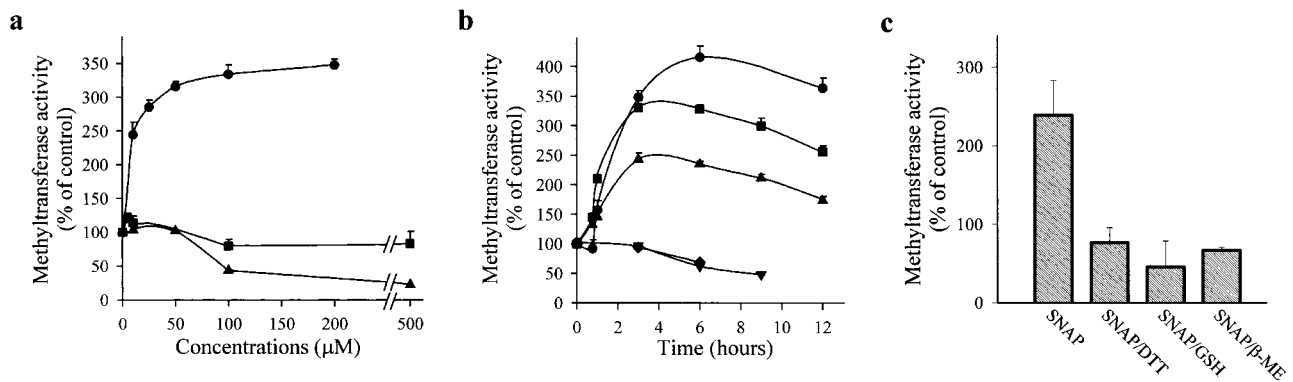


Figure 5. Effect of NO on DNA MeTase activity in a nuclear protein extract of Jurkat T cells. (a) Dose-dependent effect of 3 h incubation with SNAP (●), sodium nitrite (■), and peroxyntirite (▲) on the activity of DNA MeTase. (b) Time course of DNA MeTase activity in the presence of 50 μM SNAP (●), SNP (■), SIN (▲), expired SIN (▼), or expired SNAP (◆). Values in plots a and b are expressed in percentage of control values by mean ± SEM of three or two independent experiments in duplicate. (c) Effect of reducing agents on DNA MeTase activity stimulated by 50 μM SNAP. The nuclear protein extract was incubated for 3 h without (control) or with 50 μM SNAP. Nuclear protein extracts were recovered, after washing, by centrifugation or by filtration in a Sephadex G-25 spin column. Samples were further incubated for 2 h with no addition (control and SNAP) or in the presence of 5 mM DTT (SNAP/DTT), 100 μM GSH (SNAP/GSH), or 100 μM β-ME (SNAP/β-ME). Values are expressed in percentage of control values by mean ± SEM of at least three independent experiments in duplicate. All the values of DNA MeTase activity in the presence of the reducing agents were significantly different ($P < 0.001$) from the value of enzymatic activity in the absence of the agents. The average DNA MeTase activity in basal conditions was 0.017 ± 0.005 ($n = 15$) pmol/h/μg protein. In each experiment, the basal DNA MeTase activity was set to 100%.

0.001, $n = 2$ for SIN and SNP); however, no change was obtained when expired SNAP or SIN was used (more than eight half-lives). Thiol oxidation independent of NO did not seem to play a role in the action of NO donors on DNA MeTase activity, since similar effects were seen when superoxide dismutase and catalase were added to the reaction mixture (not shown). Enhancement of DNA MeTase activity induced by SNAP was completely reversed by further incubation, after washout of the NO donor, with reducing agents such as DTT, GSH, or β-ME (Fig. 5 c). These results strongly suggest that NO, either directly or through mediators present in the nuclear extract, regulates DNA MeTase activity possibly by nitrosation of some cysteines present in the protein.

To evaluate the selectivity of the stimulatory effect of NO on DNA MeTase, we measured in cell extracts the activity of other enzymes, such as lactate dehydrogenase (LDH), hexokinase (HK), and pyruvate kinase (PK), encoded by genes that are constitutively expressed in all cells and are involved in housekeeping functions. Cell extracts were incubated for 3 h in the absence or presence of the stimulus. Basal activities (given by mean ± SD) were 0.02 ± 0.006 ($n = 6$) pmol/h/μg protein for DNA MeTase, 110 ± 22 ($n = 5$) pmol/

min/μg protein for LDH, 12 ± 5 ($n = 3$) pmol/min/μg protein for HK, and 27 ± 11 ($n = 3$) pmol/min/μg protein for PK. Table I shows that whereas 50 μM SNAP induced a marked increase in DNA MeTase activity, it had no effect on the activities of any of the other enzymes studied. Thus, in cell extracts, NO exerts a selective effect on DNA MeTase.

Given that the inhibitory effect of NO on *FMR1* expression can be explained by activation of DNA MeTase and methylation of the CpG island, we explored if a similar action is exerted on other genes, such as *HPRT*, known to contain a CpG island in the promoter region. Fig. 6 shows that exposure of RIN and Jurkat T cells to IL-1β (a) and NO donors (b), respectively, resulted in abolishment of *HPRT* expression. In both cases, demethylation with AzadC produced recovery of gene expression. NO-dependent methylation of CpG islands observed in transformed cells (such as RIN and Jurkat T cells) was also clearly apparent in fresh human lymphocytes. Fig. 6 c illustrates the silencing of *FMR1* and *HPRT* genes by NO donors and the reversion of this effect by AzadC. The expression of genes such as *GAPDH* or *Na/K ATP-ase*, which do not contain CpG-rich promoters, is unaffected by NO (Fig. 6, a–c; see also Figs. 1–3).

Table I. Effect of NO on the Activity of Several Cellular Enzymes

	DNA MeTase	LDH	HK	PK
Activity induced by SNAP (% of control)	$366 \pm 90^*$ ($n = 6$)	105 ± 17 ($n = 5$)	100 ± 1 ($n = 3$)	107 ± 12 ($n = 3$)

Cell extracts were incubated with 50 μM SNAP for 3 h. Results are expressed by mean ± SEM of independent experiments in duplicate (number of experiments in parenthesis).

*Value significantly different from the corresponding control.

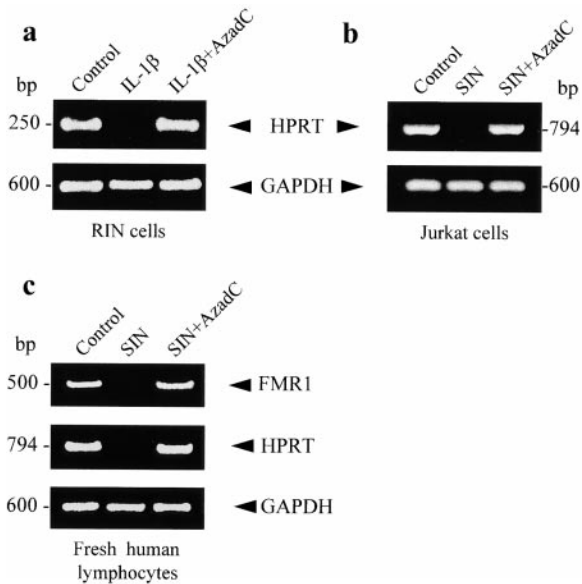


Figure 6. *HPRT* expression in RIN and Jurkat T cells assessed by RT-PCR. (a) RIN cells unstimulated and treated for 24 h with IL-1 β (25 U/ml) in the absence or presence of AzadC (2 μ g/ml). (b) Similar protocol used with Jurkat T cells stimulated with 100 μ M SIN. (c) RT-PCR of *FMR1* and *HPRT* from fresh peripheral lymphocytes incubated for 24 h with SIN (100 μ M) in the absence or presence of AzadC (2 μ g/ml). RT-PCR of *GAPDH* was used as control.

NO-induced DNA methylation was maintained for several hours after washout of the signal; however, it was a reversible phenomenon. In mitotically active cells (such as Jurkat T cells) previously exposed to NO donors, a unique and methylated band was observed by Southern blot 48 h after removal of the stimulus, whereas after 72 h two bands, methylated and unmethylated, were present (Fig. 7 a). After 48 or 72 h incubation, we observed a clear increase in the number of cells (Fig. 7 b). These results indicate that

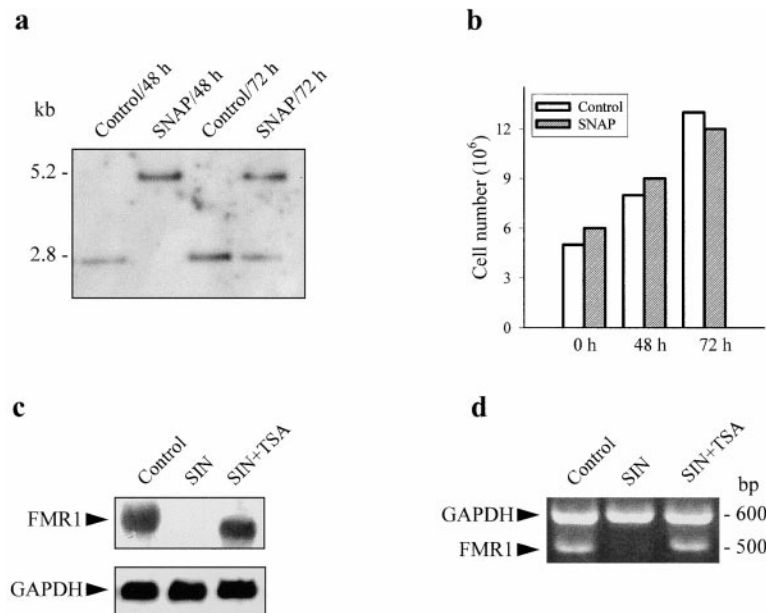


Figure 7. Reversibility of CpG island methylation in dividing cells and reversion of *FMR1* expression by a deacetylase inhibitor. (a) Jurkat T cells were incubated for 16 h with 100 μ M SNAP. Methylation of the CpG island of *FMR1* was measured by Southern blot at 48 and 72 h after washout of the stimulus. Control cells were treated in the same way but without exposure to SNAP. (b) Cell number was measured in the same experimental conditions as in a, with the MTT cell proliferation kit. (c) Northern blot of *FMR1* in Jurkat T cells incubated for 24 h in the absence or presence of 2 μ M TSA, an inhibitor of deacetylases. (d) RT-PCR of KH domain of *FMR1* in the same experimental conditions as in c. In both cases, GAPDH was used as control.

DNA methylation appears to be transient, due to either loss of methylation in the new generation of dividing cells or the existence of active DNA demethylation. This latter mechanism is suggested by the fact that reversibility of NO-induced methylation was also observed in nondividing cells such as quiescent fresh lymphocytes (not shown), and is in accordance with a recent report describing the existence of DNA demethylases in human cells (38).

DNA methylation causes repression of gene expression by promoting the condensation of chromatin. Methylated sites on DNA bind the 5-methylcytosine binding protein (MeCP2) that exists in a complex with Sin3A and histone deacetylase, resulting in a compact chromatin structure (6). To investigate whether NO-mediated gene silencing requires histone deacetylation, we tested the effect of TSA, an inhibitor of deacetylases (33). Fig. 7, c and d, show that suppression of *FMR1* expression (determined by both Northern blot and RT-PCR, respectively) was prevented by incubation of the cells with 2 μ M TSA, hence supporting the view that NO-induced gene repression is due to increased recruitment of histone deacetylases by methylated DNA.

NO is a broadly distributed signaling molecule involved in numerous physiological and pathophysiological processes (39–41), but its action on the genome is poorly understood. Moreover, many actions of IL-1 β are mediated by the induction of *iNOS* and the resulting NO production (42, 43). We show that by activating DNA MeTase, NO can induce methylation of 5' CpG islands and, hence, repress gene expression. Methylation/demethylation of DNA is known to be associated with X-linked gene inactivation, imprinting, and fragile X syndrome (7, 8, 15). Changes in DNA methylation are also observed during development, the acquisition of T cell cytokine production profiles, and tumorigenesis (9–14, 17), and several transcription factors actively promoting DNA demethylation have been reported (9, 44). Our findings provide the first case of *FMR1* gene

silencing in situations other than fragile X syndrome, although the methylation status in the two conditions shows some differences. Methylation induced by NO was lost with time, and TSA reversed the inhibitory effect on gene expression induced by NO (see above). In contrast, it has been recently reported that TSA has no effect on transcription in cells from fragile X patients due to additional modifications in histone-DNA association (45). The marked repressive effect of IL-1 β and NO on the expression of housekeeping genes, such as *FMR1* and *HPRT*, with a CpG island in the promoter might be part of a general adaptive mechanism triggered in cells challenged by stressing situations.

It has been reported that nuclear factor κ B (NF- κ B) in B cells can induce specific demethylation of the *Ig κ* locus (46), and in some cells, including RIN cells, IL-1 β receptor stimulation induces a cascade that activates NF- κ B (47, 48). In this work, we have shown that IL-1 β clearly represses gene expression by a mechanism involving methylation. Our data indicate that the possible demethylating activity of NF- κ B in RIN cells does not counterbalance the increase in activity of the DNA *MeTase*. Similarly, in Jurkat T cells the incubation with PMA plus a calcium ionophore (A23187), which induce NF- κ B (49), does not prevent the inhibitory action of NO on gene expression (results not shown). However, it could be speculated that IL-1 β transiently represses housekeeping genes having CpG islands via

NO production and methylation while inducing tissue/stage-specific gene expression by activating demethylation via NF- κ B. For instance, it has been reported that Ras induces an increase in demethylase activity in parallel to its induction of transcription of DNA *MeTase* (36).

Methylation of cytosine also gives an explanation for the high occurrence of genomic C-T transitions observed under exposure to NO (50). The abundance of 5-methylcytosine in methylated DNA favors the transition to thymine by simple deamination, which can occur spontaneously and is potentiated by NO (51). Finally, given the resemblance between certain viruses and housekeeping promoters (1), the reported antiviral action of NO (52) could be explained by methylation-dependent silencing of the viral genome. Interestingly, DNA *MeTase* is a housekeeping gene without 5' CpG island and, thus, its expression is insensitive to NO (see above). In contrast, it has a specific promoter containing activating protein (AP)-1, AP-2, and glucocorticoid response elements (53), suggesting a potentially high level of regulation by cellular signal transduction pathways.

In conclusion, we report here a novel action of IL-1 β mediated by NO production. NO induces a posttranscriptional increase in the activity of DNA *MeTase*, resulting in CpG island methylation and suppression of gene expression. These results give new insights into the pathophysiological regulation of genes with CpG-rich promoters.

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