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

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Summary

Interleukin (IL)-1β 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-1β 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-1β 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-1β 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 1β • 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 also have the potential to influence the development of T cell cytokine production profiles (17). Here, we report that exposure to IL-1β (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-1β 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-1β 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 (TSA), and 5-aza-2′-deoxycytidine (5-Aza-C); AzadC, 5-aza-2′-(1-iminoethyl)-lysine; NF-κB, nuclear factor κB; N F-κB, nuclear factor κB; NMA, N-methyl arginine; NO, nitric oxide; NOS, NO synthase; PK, pyruvate kinase; RIN, RINm5F; R T, reverse transcription; SIN, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SSPE, saline-sodium phosphate-EDTA; TCA, trichostatin A.
other reagents were of the best quality commercially available. Restriction enzymes were from Promega or New England Biolabs. All deoxycytosine (poly dI-dC) were from Boehringer Mannheim. Re- comed Amersham plc. Glutathione (GSH) and poly deoxyinosine-deoxytosine (poly dl-dC) were from Boehringer Mannheim. Other reagents were of the best quality commercially available.

Cell Culture. Insulin-producing rat RIN m5F (R IN) 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 μg/ml streptomycin, and 2.5 μg/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 guanidinium 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 out as described previously (18, 19). Amplification of hypoxanthine phosphoribosyltransferase gene (HPT) in RIN cells was assessed using murine primers. Human specific primers were used for HPT mRNA analysis in Jurkat T cells and fresh peripheral lymphocytes (7). Reverse transcription (R-T)-PCR of ATPa se 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 μg 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% formalide, 6× saline-sodium phosphate-EDTA (SSPE), 5× Denhardt’s solution, 0.5% SDS, 100 μg/ml herring sperm DNA. Wash conditions were: 2× SSPE, 0.1% SDS at room temperature and 0.1× 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 μg of nuclear protein extract resolved on 5% SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and subjected to immunodetection using a 1,000,000 dilution of primary antibody and an enhanced chemiluminescence detection (13).

Southern Blot. DNA samples were prepared from cultured cell lines by standard procedures. 10 μg of genomic DNA was digested overnight with the restriction enzymes EcoRI-EagI or HindIII-SacI, EagI and SacII being sensitive to methylation. R estriction 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 PM SF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml RNase A after centrifugation. Nuclear extracts were prepared by resuspension of the crude nuclei in high salt buffer. 15–25 μg of proteins was incubated for 2 h at 37°C with 4 μg of poly (dl-dC) as template and 5.25 μM 32P-labeled S-adenosylmethionine (1 μCi; 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%) following 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 (dl-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-1β 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-1β, and complete suppression of the gene resulted with longer exposures (Fig. 1 a). Since IL-1β is known to be a powerful stimulus for induction of NO 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-1β, and that the IL effect is fully prevented by the simultaneous addition of L-NMA, an inhibitor of NO. Preventive effect was not observed when we used D-NMA (not shown). To further demonstrate that IL-1β exerts gene silencing via NO production, we used specific iNOS inhibitors such as AMT, E1T, and L-NIL and found that all of them also prevented the action of IL-1β (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-1β or addition of NO pre-
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-1β, the application of NO precursors (500 μM SNP and 100 μM SIN) resulted in complete suppression of FMR1 expression (Fig. 2a; see also Fig. 3b), 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, particularly 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. 2b 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-1β) plus AzadC, we incubated the cells for 24 h as has been rec-

Figure 1. FMR1 expression in RIN cells treated with IL-1β 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-ATPase (250 bp) and GADPH (600 bp) were used as control. (a) Time course of IL-1β (25 U/ml) induced FMR1 repression in RIN cells. (b) 16 h treatment with IL-1β (25 U/ml), SNP (100 μM), or IL-1β (25 U/ml) plus NMA (100 μM). (c) 16 h treatment with IL-1β (25 U/ml) or IL-1β plus a specific iNOS inhibitor (20 μM AMT, 100 μM EtT, or 10 μM L-NIL). (d) Different exposure times of RIN cells with ActD alone (5 μg/ml) or added simultaneously with SNP (100 μM). Data shown in this and the other figures are representative of two to six experiments.

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 μM) or SIN (100 μM). GADPH 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 μM) or SIN (100 μM) (lanes 2 and 4); cells treated for 24 h simultaneously with SNP (500 μM) plus AzadC (2 μg/ml) or SIN (100 μM) plus AzadC (2 μg/ml) (lanes 3 and 5); cells incubated for 16 h with SIN (100 μM) (lane 6); and cells exposed for 16 h to SIN (100 μM), washed, and incubated for 24 h with AzadC (2 μg/ml) (lane 7). (c) Methylation pattern induced by 3, 4, 5, or 6 h incubation with SNAP (100 μM). (d) DNA Methylase activity in RIN and Jurkat T cells treated for 16 h with IL-1β (25 U/ml) and for 6 h with SIN (100 μM), respectively, expressed as cpm/μg protein (100 cpm/μg protein corresponds to a specific activity of 0.015 pmol/h/μg protein).
formed rodent cells overexpressing c-fos within the range of those recently published in transfection assays. It is expected that addition of this product would prevent demethylation induced methylation of the CpG island is abolished by the demethylating agent AzadC (see Fig. 2b), except for the condition shown in lane 6. 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. 2b), it is expected that addition of this product would prevent FMR1 silencing induced by either IL-1β or NO. Fig. 3a illustrates that FMR1 suppression produced by IL-1β 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-1β. Similarly, demethylation also prevented FMR1 suppression resulting from exposure of the cells to NO donors (Fig. 3b). These data indicate that NO-dependent FMR1 gene silencing results from methylation of the CpG island, an effect mediated by activation of DNA methylase.

The major mammalian DNA methylase 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 methylase 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 assessed whether the effect of NO on DNA methylase activity was due to activation of guanylyl cyclase, by incubation of Jurkat T cells with 2 mM dibutyryl cGMP for 24 h. cGMP had no effect on either DNA methylase activity or the expression of FMR1 gene (not shown). The Ras signaling pathway has been shown to increase DNA methylase 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 methylase 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 methylase. We have not studied the expression of the recently described DNA methylase A and DNA methylase B (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 methylase 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. 4b also shows that NO did not modify the magnitude or size of any of the bands obtained with the polyclonal antibody against the DNA methylase, 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. 5a shows that application of an NO donor (SNAP) to a nuclear protein extract induced a dose-dependent increase of DNA methylase 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. 5b illustrates the time course of DNA methylase activation induced by 50 μM 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 methylase activity was observed (P < 0.001, n = 3 for SNAP; P <
Effect of NO on DNA MεTase activity in a nuclear protein extract of Jurkat T cells. (a) Dose-dependent effect of 3 h incubation with SNAP (●), sodium nitrite (■), and peroxynitrite (▲) on the activity of DNA MεTase. (b) Time course of DNA MεTase 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 MεTase 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 MεTase 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 MεTase activity in basal conditions was 0.017 ± 0.005 (n = 15) pmol/h/μg protein. In each experiment, the basal DNA MεTase activity was set to 100%.

Table I. Effect of NO on DNA MεTase Activity in a Nuclear Protein Extract of Jurkat T Cells

| Activity induced by SNAP (% of control) | DNA MεTase | LDH | HK | PK |
|----------------------------------------|------------|-----|----|----|
| 366 ± 90* (n = 6)                     | 366 ± 90*  | 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.
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 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 μM 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-1β are mediated by the induction of iNOS and the resulting NO production (42, 43). We show that by activating DNA methylase, NO can induce methylation of 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-1β 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 κB (NF-κB) in B cells can induce specific demethylation of the Igκ locus (46), and in some cells, including RIN cells, IL-1β receptor stimulation induces a cascade that activates NF-κB (47, 48). In this work, we have shown that IL-1β clearly represses gene expression by a mechanism involving methylation. Our data indicate that the possible demethylating activity of NO–κB 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-κB (49), does not prevent the inhibitory action of NO on gene expression (results not shown). However, it could be speculated that IL-1β transiently represses housekeeping genes having Cpg islands via NO production and methylation while inducing tissue/stage-specific gene expression by activating demethylation via NF-κB. For instance, it has been reported that R as 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-1β 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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