Biological Chemistry

The Core Promoter and Redox-sensitive *Cis*-elements as Key Targets for Inactivation of the Lysyl Oxidase Gene by Cadmium

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Exposure of humans to cadmium (Cd) either from environmental contamination or from cigarette smoke, often induces lung emphysema and cancers. Lysyl oxidase (LOX), a copper-dependent enzyme essential for crosslinking of the extracellular matrix, displays antagonistic effects on emphysema and cancer pathogenesis. Our previous studies showed down-regulation of LOX in Cd-resistant (CdR) rat fetal lung fibroblasts (RFL6) derived from parental cells *via* long-term Cd exposure. The cloned rat LOX gene promoter -804/-1 (relative to ATG) with the maximal promoter activity contains the Inr-DPE core promoter, putative NFI binding sites, metal response elements (MRE) and antioxidant response elements (ARE). ChIP assays reported here further characterize the rat LOX gene promoter in response to Cd. CdR cells exhibited enhanced methylation of CpG at the LOX core promoter region and reduced activities of the NFI binding sites and MRE, but increased activity of the ARE in a dose-dependent manner. The collective effect of Cd on the LOX promoter is trans-inhibition of the LOX gene as shown by suppression of histone H3 acetylation in the LOX core promoter region. Thus, the LOX core promoter and redox-sensitive *cis*-elements are key Cd targets for down-regulation of LOX relevant to mechanisms for Cd-induced emphysema and lung cancers. *Journal of Nature and Science, 1(2):e38, 2015.*

Cadmium | lysyl oxidase | LOX | Inr-DPE core promoter | redox-sensitive *cis*-elements | DNA methylation | histone H3 acetylation

Lysyl oxidase (LOX), a copper-dependent enzyme, oxidizes peptidyl lysine residues in substrates, e.g., collagen, elastin and histone H1, essential for organization and stabilization of the extracellular matrix (ECM) and the cell nucleus [1, 2]. This enzyme also exhibits a dual role in carcinogenesis, i.e., as a tumor suppressor, as well as a metastasis promoter. As reported, transfected LOX cDNA suppressed Ha-*ras*-induced cell transformation indicating a *ras*-suppressor effect of LOX [3]. On the other hand, overexpression of LOX enhanced tumor metastasis under hypoxia conditions [4, 5]. Thus, LOX plays multiple functions in biology implicating in various human pathologies, *e.g.*, organ fibrosis, atherosclerosis, emphysema, carcinogenesis, *etc.* [1, 2].

Cadmium (Cd) is a toxic metal without any biological availability. In addition to occupational exposure, cigarette smoke constitutes a major source of Cd exposure for humans [2, 6]. Cd targets the lung with a biological half-life of 9.4 years inducing emphysema and cancers [6]. Previous studies have illustrated down-regulation of LOX by Cd at mRNA, protein and catalytic levels in lung cell and animal models [7, 8]. The gene cloning studies have shown the rat LOX gene promoter containing the Inr-DPE core promoter with multiple transcription start sites and several redox-sensitive *cis*-acting elements such as the NFI binding site, metal response element (MRE), antioxidant response element (ARE), etc. [9]. To further assess molecular basis for Cd damage to the lung LOX, we have studied Cd effects on the LOX promoter regulation using Cd resistant (CdR) lung cells as a chronic Cd exposure model.

Here, we report that CdR cells with different degrees of Cd-resistance exhibited enhanced methylation of CpG at the LOX promoter region, and inhibited nuclear factor I (NFI) binding to the LOX NFI binding sites and MRE-binding transcription factor 1 (MTF1) binding to the LOX MRE, but increased NF-E2-related factor 2 (Nrf2) binding to the LOX ARE in a dose-dependent manner. The collective effect of such core promoter and *cis*-elements in response to Cd is trans-inactivation of the LOX gene as indicated by suppression of histone H3 acetylation in the LOX core promoter region. These results show the molecular mechanisms for Cd modulation of LOX gene promoter activation consistent with its suppression of the LOX gene expression at mRNA, protein and catalytic levels in cell and animal models [7, 8].

Materials and Methods

Materials

Cadmium chloride with 99.9% pure, was from Aldrich Chemicals (Milwaukee, WI, USA). Mouse anti-RNA polymerase II (RNA-PolyII), NFI, MTF1, Nrf2, acetylated histone H3**,** and glyceradehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotech. (Santa Cruz, CA, USA). [α-^{32}P]UTP was from PerkinElmer (Boston, MA, USA). Synthetic oligonucleotide primers used for the PCR were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). All tissue culture products were from Invitrogen Co. (Carlsbad, CA, USA).

Cell culture and metal exposure

The rat fetal lung fibroblasts (RFL6) obtained from ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 and 95% air incubator as previously described [7]. Stock cultures were derived from the frozen cell line and passaged every 4 days. A total of 6 passages were used for experiments. To identify effects of chronic Cd exposure on cell phenotype changes, Cd resistant (CdR) RFL6 cells were isolated by incubation of cells with graded concentrations of Cd as described [7]. Different degrees of CdR cells used in this study such as those resistant to 10, 20, and 40 μM Cd were referred to as CdR10, CdR20, and CdR40 cells [7, 10]. To obtain growth-arrested cultures, cells were incubated in 0.3% FBS/DMEM for 3 days, changed to fresh medium and used for experiments [7].

The nuclear run-on assay

To assess LOX transcription initiation, relative rates of LOX transcription in control and CdR cells were evaluated by the nuclear run-on assay as described [11]. Cell pellets were gently resuspended in a nuclear isolation buffer and incubated on ice with intermittent microscopic examination for nuclear integrity. The

Conflict of interest: No conflicts declared.

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nuclei were centrifuged at 500 × g and resuspended in a nuclear freezing buffer either for direct use or for storage in liquid nitrogen [12]. For the nuclear run-on reaction, 100 µl of thawed nuclei were mixed with 30 µl of a 5 × run-on buffer with NTP containing 100 µCi [α-^{32}P]UTP and 5 µl of the Sarkosyl stock to give a final concentration of 0.06%. The mixture was incubated for 30 min at 30°C, then 15 µl of DNase I (1U/µl) were added and the incubation continued for another 15 min. RNA was isolated by a single step Trizol extraction and the incorporation of ^{32}P determined by γ–counting. Plasmids containing LOX cDNA and GAPDH cDNA were slot-blotted onto the nitrocellulose membrane using a BioRad BioDot SF apparatus. The blots were prehybridized in 1% SDS/10% dextran sulfate, 1.4 M NaCl and 325 µg/ml each of herring sperm DNA and yeast tRNA for 2 h at 60°C followed by treatment with RNasin plus DTT. Radiolabeled RNAs were hybridized onto filters for 2 days. The filters were then washed, dried and autoradiographed on preflashed film. The densities of labeled RNA bands on the film were analyzed by the 1D Scan software as described [13]. Experiments presented here and below were repeated at least three times with the reproducible data

Assay for methylation of the LOX core promoter region

Genomic DNA from cell lines was isolated using the DNA Mini Preparation kit (Qiagen, Inc.). PCR assays were performed by using the promoter methylation PCR kit (Panomics, Redwood City, CA, USA) as described [14]. Briefly, 2 µg of genomic DNA were digested with 10 units Mse I (New England Biolabs, Boston, MA, USA) which recognizes the TTAA site to produce small fragments of DNA retaining the CpG islands. Following incubation with methylation binding protein (MBP) to form a protein/DNA complex, methylated DNA was isolated by centrifugation using a separation column and amplified at the following PCR program: 94°C for 5 min, 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min for 35 cycles. PCR products were analyzed on 2.2% agarose gel. Based the TTAA site distribution in the LOX promoter, the primer pair including the forward (F) oligomer, 5'-TTCAGACACTGTGCGCTCTC-3' and the reverse (R) oligomer, 5'-AGGAGGGAGACCTCTTCGAG-3' was designed and used for amplification of the methylated LOX fragment on the promoter region (205 bp) containing 15 CpG islands.

Chromatin immunoprecipitation (ChIP) assay

To determine transcription factor binding to the LOX promoter region, the ChIP assay was performed as described [9] with the EpiQuik Chromatin Immunoprecipitation Kit based on the protocol provided by the supplier (Epigentek Group Inc., Brooklyn, NY, USA). Cellular components were cross-linked by incubation of control and CdR cells at the same number (2 x 10^6) with 1% formaldehyde at room temperature for 10 min. The cross-linking reaction was stopped by addition of glycine to a final concentration of 125 mM. Nuclei were extracted with a nuclear isolation buffer, resuspended in a nuclear lysis buffer with protease inhibitor cocktail and then sonicated to shear DNA to lengths between 200-1000 bp. After centrifugation, cell debris was discarded and DNA containing supernatants were diluted with the ChIP dilution buffer and aliquots of samples were removed out. Diluted DNA samples were transferred into the strip wells that were precoated with the antibodies against rat RNA-PolyII, NFI, MTF1, Nrf2 and acetylated histone H3 (Santa Cruz Biotech., Santa Cruz, CA), and incubated at room temperature for 90 min with shaking. Note that in each experiment, nonspecific rat IgG from Santa Cruz Biotech was included using as a negative control (data not shown). After successively washing, wells with the washing buffer and finally with the TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), precipitated DNA-protein complex samples were treated with proteinase K (250 µg/ml) in the DNA release buffer for 15 min and then incubated in the reverse buffer for 90 min at 65°C. The DNA samples were collected by the P-spin columns, washed with 70% and 90% ethanol successively, and then eluted with the elution buffer. Using purified DNA as a template, PCR was conducted under the following conditions: initial denaturation at 94° for 2 min, 30 cycles each with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 5 min. Primers were used in ChIP assays as follows:

1) in LOX pro-mRNA synthesis and processing assay:
F, 5'-GAAGAGGTCTCCCTCCTTCG-3' and
R, 5'-ACTGCAGCTGTCCCAGAAAG-3'
for amplifying the LOX 5'-UTR fragment (136 bp);

F,5'-CAAAGCAAGCTTCTGTCTGGA-3' and
R, 5'-GTCTGATTCAGGCACCAGGTA-3'
for amplifying the LOX coding region fragment (214 bp);

F, 5'-GGCAAACGGAAAAAC AACAA-3' and
R, 5'-TGCACCCACAGAATTGAAAC -3'
for amplifying the LOX 3'-UTR fragment (158 bp);

F, 5'-TTGCTTGGCTTCTTCTTTGG-3' and
R, 5'-GAGACGAGGCTG GTACTCCA-3'
for amplifying the GAPDH gene fragment (160 bp), an internal control.

2) in the MTF1 binding to the MRE assay:
F, 5'-CTTCAGACACTG TGCGCTCT-3' and
R, 5'-GCAGGGACTGGTGCCAAG-3'
for amplifying the MTF1-bound LOX MRE fragment (150 bp);

F, 5'-TTGCTTGGCTTCTTCTTTGG-3' and
R,5'-GAGACGAGGCTGGTACTCCA-3'
for amplifying the RNA-Poly II-bound GAPDH gene fragment (160 bp), an internal control.

3) in the NFI binding to the NFI binding site assay:
F, 5'-GGAAAGGGGAGAGGAGGAC-3' and
R, 5'-AGGAGGGAGACCTCTTCGAG-3'
for amplifying the NFI bound LOX NFI binding site fragment (142 bp);

F, 5'-GATGTTAGCGGGATCTCGCTCCTG-3' and
R, 5'-GTTCAACG GCACAGTCAAGGCTGAG-3'
for amplifying the RNA-PolyII binding region in the GAPDH promoter (90 bp), an internal control.

4) in the Nrf2 binding to the LOX ARE assay:
F, 5'-TTTGGCCCTCATCGCTCT-3' and
R, 5'-GACTTAATCTGGGCCGAACA-3'
for amplifying the Nrf2-bound LOX ARE fragment (162 bp);

F, 5'-TTGCTTGGCTTCTTCTTTGG-3' and
R,5'-GAGACGAGGCTGGTACTCCA-3'
for amplifying the RNA PolyII-bound GAPDH gene fragment (160 bp), an internal control.

5) in acetylated histone H3 binding to the core promoter assay:
F, 5'-GAAGAGGTCTCCCTCCTTCG-3' and
R, 5'- ACTGCAGCTGTCC CAGAAAG-3'
for amplifying the acetylated histone H3-bound LOX core promoter region (136 bp)

F, 5'-TTGCTTGGCTTCTTTTGG-3' and
R,5'-GAGACGA GGCTGGTACTCCA-3'
for amplifying the RNA-PolyII-bound GAPDH gene fragment (160 bp), an internal control.

PCR products were analyzed on a 2.2% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator. Densities of PCR-amplified gene fragments on the gel were measured with the 1D Scan software as described [13].

Results

Reduction of the relative transcription rate of LOX in CdR cells

The nuclear run-on assay was carried out to evaluate changes in the LOX transcription initiation [11]. As shown in Fig.1, using the internal control GAPDH as reference, levels of [^{32}P]-labeled transcripts hybridized to the LOX cDNA were markedly diminished in nuclei of Cd-exposed cells, amounting to 22%, 8% and 5% of the RFL6 control, respectively, in CdR10, CdR20 and CdR40 cells (Fig.1). Thus, reduction of new LOX transcript synthesis by Cd is a critical mechanism for down-regulation of LOX mRNA in chronic Cd exposed cells.

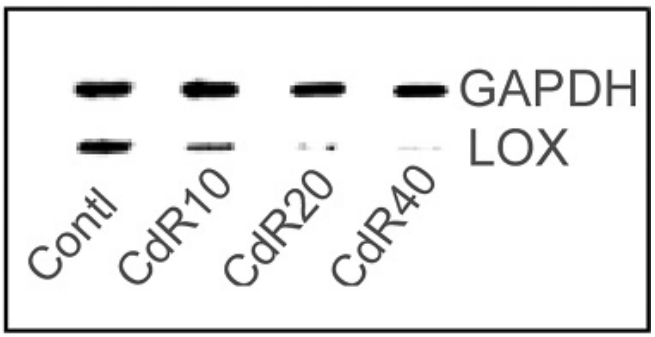

Fig.1. Reduction of the relative transcription rate of LOX in CdR cells as revealed by the nuclear run-on assay. Nuclei were freshly isolated from control and CdR cells under the same conditions as described in Methods. Nascent transcripts were labeled with 32P-UTP and hybridized to a previously prepared filter containing cDNAs for LOX and GAPDH (an internal control). Hybridized radiolabeled RNAs onto filters were washed, dried and autoradiographed on preflashed film. Densities of labeled RNA bands on the film were measured with the 1D Scan software. Experiments here and below were repeated at least 3 times and a typical one presented.

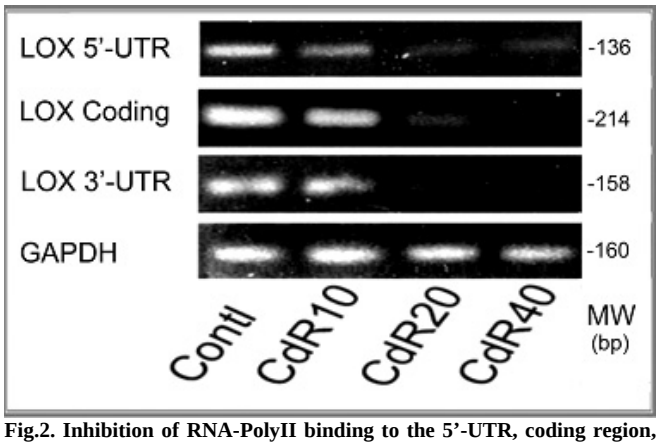

Fig.2. Inhibition of RNA-PolyII binding to the 5'-UTR, coding region, and 3'-UTR of the LOX gene in CdR cells as revealed by ChIP and PCR assays. DNAs were isolated from control and CdR cells each with 2 x10^6, sonicated and immunoprecipitated with an antibody against RNA-PolyII. Using immunoprecipitated DNA as a template, the PCR with four primer pairs as shown under Methods amplified the 5'-UTR fragment with 136 bp, the coding region fragment with 214 bp and the 3'-UTR fragment with 158 bp of the LOX gene and the promoter fragment of the GAPDH gene (an internal control) with 160 bp, respectively. PCR products were analyzed on 2.2% agarose gels. Densities of PCR-amplified gene fragments on the gel as described here and below were measured with the 1D Scan software.

Inhibition of LOX pre-mRNA synthesis and processing in CdR cells

RNA-PolyII plays a central role in eukaryotic mRNA synthesis and processing [15, 16]. This multimeric enzyme is tightly associated with a transcribed gene from the 5'-start site to the 3'-termination end. To further define Cd modulation of LOX transcription, we used ChIP assays to directly compare measurements of RNA-PolyII binding to the different LOX gene fragments including the 5'-UTR, coding, and 3'-UTR regions in control and CdR cells. The immunoprecipitated chromatins from cells with the same number were used as template for PCR amplification with specific designed primer pairs. The PCR products encompass parts of the Inr-DPE core promoter/5'-UTR, the coding region and the 3'-UTR. PCR products were analyzed on 2% agarose gels, As shown (Fig.2), CdR10, CdR20 and CdR40 cells displayed marked decreases in levels of RNA-PolyII binding to the LOX gene amounting to 50, 18 and 13% of the control in the 5'-UTR region, 60, 10 and 0% of the control in the coding region, and 60, 0 and 0% in the 3'-UTR region. In contrast, there were no significant changes in RNA-PolyII binding to the GAPDH gene, an internal control, in various degrees of Cd-resistant cells. Thus, the distribution along the LOX gene of RNA-PolyII in CdR cells exhibited a gradual reduction from the 5'-UTR to the 3,-UTR in a Cd-dose dependent manner suggesting a strong down-stream inhibition of LOX pre-mRNA synthesis and processing existing in CdR cells.

Aberrant methylation of the LOX gene promoter in CdR cells

Modification of DNA, particularly the promoter region, interferes with the interaction of DNA with proteins. Cytosine methylation in the CpG islands is involved in stable transcriptional repression [17, 18]. The rat LOX promoter -804/-1 with the maximal activity contains approximately 38 CpG dinucleotides, of which some overlap with *cis*-elements, e.g., the core promoter, MREs, etc. [9]. To answer the question whether downregulation of LOX transcript initiation, synthesis and processing as shown in Figures 1 and 2 in CdR cells is due to methylation of CpGs in the promoter region, we examined the methylation status of the LOX gene in these cells. Methyl-CpG-binding domain protein (MBP)-bound DNA was isolated and amplified by the PCR. The PCR product is a 205 bp DNA fragment encompassing the LOX gene promoter region from -279 to -75 relative to ATG [9]. It contains 15 CpG. As shown in Fig. 3, CdR 10, 20 and 40 cells displayed an increased methylation of CpG in the examined LOX promoter fragment reaching 132, 146 and 171% of the control, respectively. Thus, methylation of LOX promoter is a critical mechanism for silencing the LOX gene by Cd.

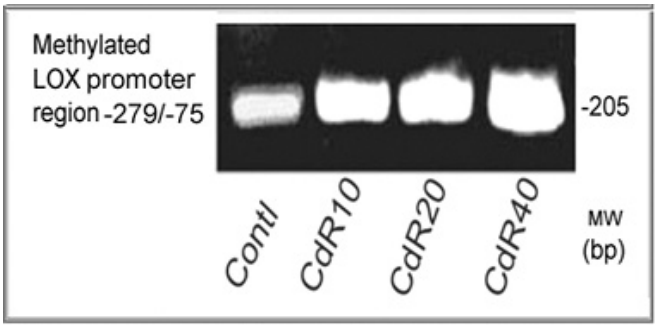

Fig.3. Enhancement of methylation at the LOX promoter region in CdR cells as determined by using the methylation promoter PCR kit. The same amount of genomic DNA isolated from control and CdR cells were digested with restriction enzyme Mse I then incubated with MBP to form a protein/DNA complex, methylated DNA was isolated using a separation column and amplified by PCR. PCR products as a 205 bp DNA fragment encompassing one RNA synthesis star site at -78 and MREs were analyzed on 2.2% agarose gel.

Inhibition of MTF1 binding to the MRE of the LOX gene in CdR cells

Upregulation of metallothionein (MT) is a major phenotypic change in association with downregulation of LOX in Cd treated cells [7, 8]. Both MT and LOX promoters contain MREs which are activated by MTF1 for the gene transactivation [9, 19, 20]. There are at least two MREs mapped at –269/–263 and –248/–241 in the cloned rat LOX promoter (–804/–1 relative to ATG) [9]. To assess Cd effects on the MRE activation, we examined MTF1 binding to MREs in the LOX promoter by ChIP assays as described [9]. As shown in Fig.4, the PCR products contain 150 bp DNA encompassing the promoter fragment from -279 to -130. In reference with the internal control, the GAPDH DNA part bound with the RNA-PolyII, unexpectedly, Cd resistant cells showed

reduced, rather than enhanced MTF1 binding to the MREs in the LOX promoter amounting to 82, 51 and 32% of the control, respectively, in CdR10, 20 and 40 cells. Thus, in contrast to MT [19, 20], chronic exposure to Cd inhibited the MRE activation in the LOX gene.

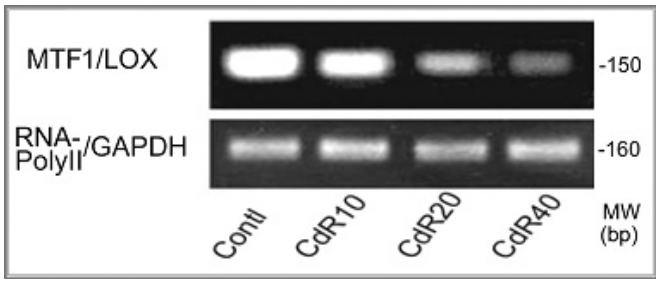

Fig.4. Inhibition of MTF1 binding to the MRE of the LOX gene in CdR cells as revealed by ChIP and PCR assays. The ChIP assay was performed as described in Fig.2 using antibodies against the MTF1 and the RNA-PolyII, respectively. Molecular weights of PCR-amplified the MTF1-bound LOX MRE fragment and RNA-PolyII-bound GAPDH fragment are 150 bp and 160 bp, respectively.

Inhibition of NFI binding to the NFI binding site of the LOX gene in CdR cells

NFI is a critical one of redox-sensitive transcription factors [21]. At least two functional NFI binding sites (–594/–580 and –147/–133) are active in the 804 base pair promoter region of the LOX gene [9]. To assess the active status of the NFI binding sites in the LOX gene in response to Cd, we examined the NFI binding to the LOX gene in CdR cells by ChIP assays. As shown in Fig.5, using the designed primer pair, the PCR product was a DNA oligonucleotides with a 142 bp MW from -218 to -77 encompassing the NFI binding site –147/–133 (relative to ATG). In reference to the internal control, the GAPDH fragment bound with the RNA-PolyII, CdR cells exhibited an inhibition of the NFI binding to the LOX gene declining to 52, 12 and 20% of the control for CdR10, 20 and 40 cells, respectively. These results indicated that Cd inactivated the NFI binding sites of the LOX gene.

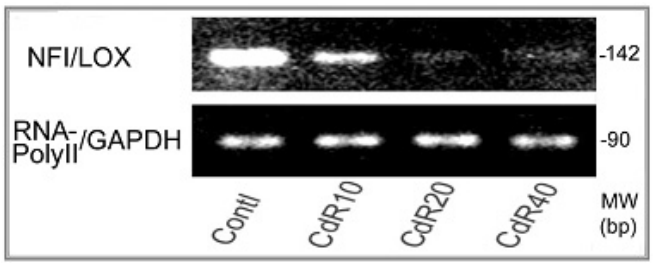

Fig.5. Inhibition of the NFI binding to the NFI binding site of the LOX gene in CdR cells as revealed by ChIP and PCR assays. The ChIP assay was performed as described in Fig.2 using antibodies against the NFI and the RNA-PolyII, respectively. Molecular weights for the PCR-amplified the NFI-bound LOX MRE fragment and the RNA-PolyII-bound GAPDH fragment are 142 bp and 90 bp, respectively.

Enhancement of Nrf2 binding to the ARE of the LOX gene in CdR cells

LOX gene promoter -804/-1 contains one ARE with the core sequence 5'-RTGACNNNGC-3' (R = purine, N = any nucleotides) [22] at the region –581/–572 [9]. The transcriptional factor interacted with the ARE is the Nrf2 which drives expressions of a variety of xenobiotic metabolizing enzymes for antioxidant-detoxification [22]. Cd is known to induce oxidative stress in the biological system [23]. To identify Cd effects on the ARE activity of the LOX gene, we tested the Nrf2 binding to the LOX ARE in CdR cells by the ChIP/PCR assays. Using the Nrf2 specific antibody to immunoprecipitate the Nrf2 bound ARE fragment of the LOX gene followed by the PCR, the amplified ARE containing DNA fragment with 162 bp encompasses the part of the LOX promoter from -675 to -514. As shown in Fig.6, CdR cells displayed an increased Nrf2 binding to the LOX gene reaching 1.75, 1.96 and 2.01-fold of the control, respectively, for CdR10, 20 and 40 cells. In contrast, there were no significant changes found in the RNA-PolyII binding to GAPDH, an internal control. Thus, Cd enhanced the LOX ARE activity in chronically treated cells.

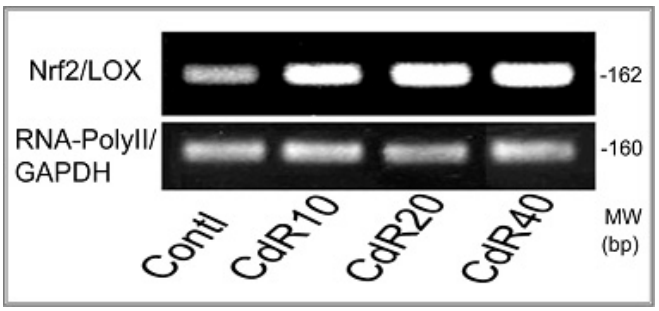

Fig.6. Enhancement of Nrf2 binding to the ARE of the LOX gene in CdR cells as determined by ChIP and PCR assays. The ChIP assay was performed as described in Fig.2 using antibodies against the Nrf2 and the RNA-PolyII. Molecular weights for the PCR-amplified the Nrf2-bound LOX ARE fragment and the RNA-PolyII-bound GAPDH fragment are 162 bp and 160 bp, respectively.

Inhibition of binding of acetylated histone H3, a marker for gene transactivation, to the core promoter of the LOX gene in CdR cells

Acetylation of histone N-terminal lysines is intimately linked to chromatin remodeling for transcription regulation. Reversible acetylation of histones such as H3 facilitates access of transcriptional machinery to DNA. Thus, acetylated histones are critical markers for special gene activation [24]. To elucidate the active status of the LOX core promoter in response to Cd, quantitation of acetylated histone H3 at the LOX Inr-DPE region was performed by the ChIP assay. The anti-diacetylated histone H3 antibody was used to precipitate DNA fragments isolated from control and CdR cells. Using primers as described, the PCR amplified a 136 bp fragment (-95/+41, relative to ATG) containing the LOX core promoter and an intact transcription start site cluster from -78 to -51 [9]. As shown in Fig.7, in comparison to the internal control, the GAPDH fragment bound with the RNA-PolyII, the histone H3 acetylated at the tested region of the LOX promoter in CdR cells was reduced to 63, 15, and 6% of the control for CdR 10, 20 and 40 cells, respectively. These results further illustrated Cd inhibition of LOX transcription as a key mechanism for downregulation of this enzyme at the molecular level.

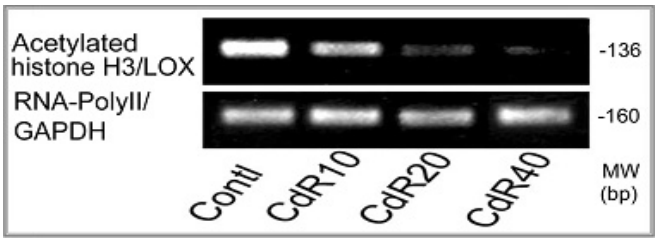

Fig.7. Inhibition of binding of acetylated histone H3, a marker for gene transactivation, to the core promoter of the LOX gene in CdR cells as identified by ChIP and PCR assays. The ChIP assay was performed as described in Fig.2 using antibodies against the acetylated histone H3 and the RNA-PolyII. Molecular weights of the PCR-amplified the acetylated histone H3-bound LOX core promoter fragment and the RNA-PolyII-bound GAPDH fragment are 136 bp and 160 bp, respectively.

Discussion

This study focuses on investigating Cd modulation of the LOX gene expression at pre-mRNA, core promoter and *cis*-element levels using CdR cells as a chronic Cd exposure model. Our data showed reduced LOX mRNA initiation rates (Fig.1) in Cd treated cells. Furthermore, assays for Cd effects on LOX pre-mRNA

synthesis and processing were performed by examining RNA-PolyII binding to 5'-UTR, coding, and 3'-UTR regions of the LOX gene. RNA-PolyII is a large complex solely responsible for synthesis of mRNAs [15, 16]. The ChIP assay was successfully used to monitor accumulation of RNA-PolyII along different regions of several genes [25]. The advantage of this method is that amplified specific DNA levels represent the amount of the specific protein bound to that portion of the gene. Thus, the RNA-PolyII binding to the different regions of a gene directly indicates the pre-mRNA synthesis and processing [25]. In our case, Cd induced a dose-dependent inhibition of RNA-PolyII binding to the same DNA region in CdR 10, 20 and 40 cells, and a stronger inhibition of RNA-PolyII binding to the down-stream regions in the same CdR cells (Fig.2) suggesting Cd targeting the LOX gene transcription not only for the initiation factors but also for the processing or termination factors.

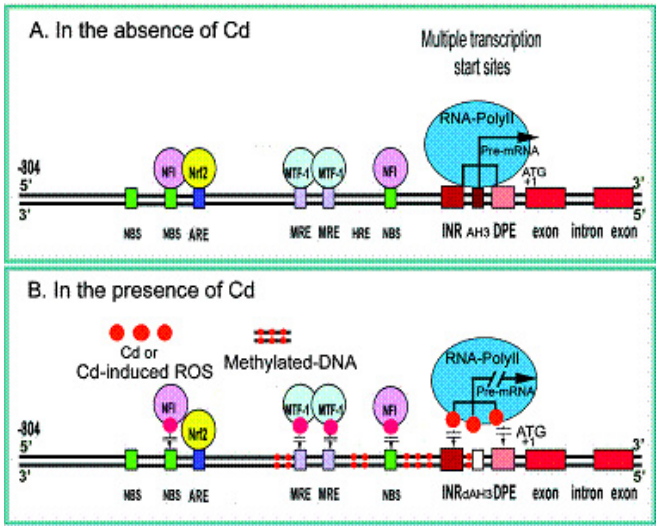

Fig.8. The schematic linear map of the cloned rat LOX promoter and its response to Cd. A. In the absence of Cd, the RNA-PolyII and transcription factors binding to the core promoter and *cis*-elements initiating LOX pre-mRNA synthesis. **B.** In the presence of Cd, it blocks the RNA-PolyII and transcription factors binding to the core promoter and *cis*-elements inhibiting LOX pre-mRNA synthesis. ATG, the translational start site; Inr, the initiator element; DPE the down-stream core promoter element; NBS, the NFI binding site; MRE, the metal response element; ARE, the antioxidant response element; RNA-PolyII , RNA-PolymeraseII; NFI, nuclear factor I; MTF1, MRE-binding transcription factor 1; Nrf2, NF-E2-related factor 2; AH3, acetylated histone H3; dAH3, de-acetylated histone H3; Cd, cadmium; ROS, reactive oxygen species.

The cloned rat LOX gene promoter from-804/-1 with the maximal promoter activity contains the Inr (5'-TCATTTTT-3') and the DPE (5'-GGACG-3') elements mapped from -53 to -14 without the typical TATA box (Fig 8A) [9]. The Inr and DPE coordinately function as a single core promoter for the RNA-PolyII-directed gene transcription. Three assays regarding the LOX core promoter activity were carried out in CdR cells: 1) RNA-PolyII binding (Fig.2), 2) acetylated histone H3 incorporation (Fig.7), and 3) CpG methylation (Fig. 3). RNA- PolyII is a central component of the transcription machinery [15, 16]. Cd reduction of RNA-PolyII binding to the the 5'-UTR where the core promoter is located (Fig.2) may result from its direct or indirect modulation of the core promoter DNA, or the up-stream *cis*-elements. Acetylation of histone H3, a major nuclear structural protein, promotes access of transcription factors to DNA by increasing transient nucleosome unwrapping or enhancing histone octamer mobility [26]. Thus, histone acetylation at promoters is a hallmark of actively transcribed genes [27]. Cd was reported to inhibit histone acetyltransferase activity in cells [28]. In this study, Cd declined acetylated histone H3 binding to the LOX core promoter reflecting a low LOX transcription rate in Cd-exposed cells.

Between 60% and 90% of all CpGs are methylated in mammalian genes [29]. Abnormal CpG methylation in the promoter results in transcriptional silencing that can be inherited by daughter cells following division [30]. Aberrant methylation of genomic DNAs and increased DNA methyltransferase activities were detected in Cd transformed cells [30]. Methylation-inactivated LOX gene was reported in human gastric cancers [31]. Importantly, methylated DNA can bind to methyl-CpG-binding proteins (MBDs) which then recruit additional proteins to the locus such as histone deacetylases, thereby forming compact, inactive heterochromatins [29]. The rat LOX promoter -804/-1 contains approximately 38 CpGs [9]. Cd enhanced the CpG methylation (Fig. 3) in the LOX promoter from -279 to -75 encompassing 15 CpGs in overlay with least one transcription start site at -78, one NFI and two MREs, expectedly to interfere with the interaction of DNAs with proteins. This may be a critical mechanism for Cd permanently silencing the LOX gene involving Cd carcinogenesis.

Other transcription factor binding sites are generally located upstream of the core promoter. Since Cd induces oxidative stress in the biological system [23], we further identified the redox-sensitive *cis*-elements of the LOX gene in response to Cd. The cloned rat LOX promoter contains the NFI binding sites, MREs and the ARE, etc. (Fig.8A) [9]. Corresponding transcription factors NFI, MTF1 and Nrf2 are very susceptible to oxidative damages. Thus, their interactions were examined in this study.

The cloned rat LOX promoter –804/–1 contains three putative NFI-binding sites [9]. NFI binds to the consensus sequence TTGGC(N5)GCCAA (N = any nucleotides) on duplex DNA as a dimer. Notably, it can also bind to the individual TTGGC or GCCAA with a somewhat reduced affinity [21]. At least two NFI binding sites at -594/-580 and -147/-133 were functionally active in the rat LOX gene promoter. The NFI-binding sites act as an enhancer transactivating the LOX gene [9]. The highly conserved N-terminal residues of NFI contain the DNA binding domain. Four cysteine residues are conserved in all DNA binding domains of rat NFI isoforms. One cysteine residue in the DNA binding domain is susceptible for oxidative damage [21]. The ChIP assays in this study showed reduction of NFI binding to the LOX promoter in CdR cells suggesting inactivation of the NFI binding site at the promoter region as a potential mechanism for Cd inhibition of LOX transcription.

MRE was initially found in multiple copies in metallothionein (MT) genes [19, 20]. A protein that binds specifically to MREs is termed as MTF1. In response to metal stimuli, MTF1 binds to MREs activating MRE containing genes [20]. MTF1 is a Zn finger protein sensitive to oxidative stress mediating expressions of MT genes (MTI-IV) which contain more copies of MREs. Two putative MREs (TGCRCNC, R = purine, N = any nucleotide) are located at –269/–263 and –248/–241 in the rat LOX promoter. Our study has shown inhibition of MTF1 binding to the LOX gene promoter consistent with downregulation of LOX in CdR cells (Fig. 4). Note that down-regulation of LOX by Cd was coupled with up-regulation of MT [7, 8]. Cd enhances the MT gene expression *via* transactivation of MRE by MTF1. MTI and MTII gene promoters each contain at least 6 MREs [20] which may compete with MTF1 binding to the LOX promoter in cell response to Cd. Thus, LOX MREs may have a lower affinity for MTF1 binding. Different affinities of MREs for MTF1 have been reported in MT genes although they share the highly conserved core sequence [32].

LOX gene promoter contains one ARE (RTGACNNNGC, R = purine, N = any nucleotides) at the region –581/–572 [9, 22]. The transcriptional factor interacted with the ARE is the Nrf2 which drives expressions of a variety of xenobiotic metabolizing enzymes for antioxidant-detoxification [22]. Nrf2 is inactive in the cytoplasm by binding to the cysteine-rich Keap1 protein in the actin filaments. Keap1 bound Nrf2 can be degraded by the ubiquitin proteasome pathway. Upon oxidative stress, modification of cysteine residues in the Keap1 protein induces the Nrf2 release and nuclear translocation. After forming the heterodimer with Maf,

Nrf2 binds to the ARE enhancing expressions of Nrf2 target genes [22]. As shown in this study (Fig. 6), Cd enhanced Nrf2 binding to the LOX promoter reflecting the active status of the ARE of the LOX promoter and oxidative stress occurred in CdR cells under our culture conditions.

Overall, as shown (Fig.8B), results in this study provide strong evidence that the LOX core promoter and redox-sensitive-*cis* elements are critical targets for Cd epigenetic toxicity. Cd induced enhancement of the LOX promoter methylation, reduction of histione H3 acetylation at the core promoter region, inactivation of the upstream LOX *cis*-elements such as the NFI binding sites and MREs but not the ARE, declining of pre-mRNA initiation, synthesis and processing coordinately contributing to inhibition of LOX gene transcription, a critical basic mechanism for down-regulation of LOX in Cd emphysema and carcinogenesis.

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