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Redox-sensitive gene regulatory events controlling aberrant matrix metalloproteinase-1 expression

Toni Bartling^{1,*}, Sita Subbaram^{2,*}, Ryan Clark¹, Akshaya Chandrasekaran¹, Supriya Kar³, and J. Andres Melendez^{1,§}

¹College of Nanoscale Science and Engineering, State University of New York, Albany, NY 12203, USA

²Albany Medical College, Center for Cell Biology & Cancer Research, Albany, NY 12208, USA

³Albany Medical College, Pediatrics, Albany, NY 12208, USA

Abstract

Aberrant matrix metalloproteinase-1 (MMP-1) expression contributes to the pathogenesis of many degenerative disease processes that are associated with increased oxidative damage or stress. We and others have established that shifts in steady-state H₂O₂ production resulting from enforced antioxidant gene expression, senescence or UV irradiation control MMP-1 expression. Here we establish that histone deacetylase-2 (HDAC2) protein levels and its occupancy of the MMP-1 promoter are decreased in response to enforced manganese superoxide dismutase expression (Sod2). Inhibition of histone deacetylase (HDAC) activity further accentuates the redox-dependent expression of MMP-1. Sod2-dependent decreases in HDAC2 are associated with increases in a proteasome-sensitive pool of ubiquitinated HDAC2 and MMP-1 specific histone H3 acetylation. Sod2 overexpression also enhanced recruitment of Ets-1, c-Jun, c-Fos, and the histone acetyltransferase (HAT) PCAF to the distal and proximal region of the MMP-1 promoter. Furthermore, the Sod2-dependent expression of MMP-1 can be reversed by silencing the transcriptional activator c-Jun. All of the above Sod2-dependent alterations are largely reversed by catalase coexpression, indicating that the redox-control of MMP-1 is H₂O₂-dependent. These findings identify a novel redox-regulation of MMP-1 transcription that involves site specific promoter recruitment of both activating factors and chromatin modifying enzymes, which converge to maximally drive MMP-1 gene expression.

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[§] Address correspondence to: J. Andrés Melendez, College of Nanoscale Science and Engineering, State University of New York, 257 Fuller Rd., NFE-4313, Albany, NY 12203, USA, Ph: (518) 956-7360, Fax: (518) 437-8687, jmelendez@albany.edu.

^{*} Authors contributed equally to this work

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Keywords

matrix metalloproteinase-1 (MMP-1); manganese superoxide dismutase (Sod2); oxidative stress; hydrogen peroxide; redox-dependent; epigenetic modification; acetylation; histone deacetylase-2; steady state H_2O_2 concentration (SS- $[\text{H}_2\text{O}_2]$)

Introduction

Matrix metalloproteinases (MMPs) are endopeptidases involved in the breakdown of extracellular matrix (ECM) in normal physiological processes, such as embryogenesis, wound healing, tissue repair and normal matrix turnover [1]. MMP-1 or interstitial collagenase cleaves collagens, types I, II, and III, and under normal physiological conditions its expression levels are extremely low and tightly regulated to maintain ECM homeostasis. In contrast, aberrant high level MMP-1 expression is associated with numerous degenerative disease processes, such as arthritis [2], atherosclerosis [3], periodontal disease, pulmonary fibrosis, stroke injury and metastatic disease [4–6].

High level MMP-1 expression is commonly linked to elevation in steady-state $[\text{H}_2\text{O}_2]$ (SS- $[\text{H}_2\text{O}_2]$) production. Redox-dependent MMP-1 expression has been shown to be regulated through juxtaposed Ets-1 and AP-1 DNA consensus binding site at –1607 of its promoter in response to oxidant dependent activity of the MAPK family members ERK and JNK [7, 8]. ROS have been shown to be obligatory for fibronectin-stimulated MMP expression in articular chondrocytes [9] as well as UV-mediated expression in dermal fibroblasts [10]. A number of studies have established that overexpression of Sod2 is accompanied by high level MMP-1 expression that is attributed to shifts in SS- $[\text{H}_2\text{O}_2]$ [8, 11, 12].

Redox-sensitivity of the MMP-1 promoter is in large part attributed to the distal promoter region that includes the Ets-1/AP-1 (c-Jun/c-Fos) consensus binding sites at position –1607 [7, 13]. As illustrated in Fig 1A, the distal promoter region spans –1663 and –1464 and encompass the –1607 Ets-1/AP-1 (c-Jun/c-Fos) redox responsive element and other putative ETS family member binding domains. The proximal promoter region spans bases –212 to –34 and contains two phorbol responsive regions at –213 to –177 which binds Jun D/c-Fos and c-Fos/c-Jun heterodimers and the –73 AP-1 site that binds Jun D and Fra-2 [14].

In addition to transcription factor binding, post translational modifications of histones serves to maximally activate gene transcription. Lysine acetylation on histones H3 and H4 loosens the DNA/histone interaction and increases access of transcriptional activators to the DNA. Thus, histone acetylation is a key determinant in regulating chromatin access following transcription factor recruitment. Epigenetic modifications around the MMP-1 loci affect MMP-1 expression. In RA synovial fibroblasts, elevated MMP-1 levels are correlated to increases in H4 acetylation in the distal MMP-1 promoter [15]. Martens *et al.* have shown that acetylation, histone H3 lysine 4 di- and trimethylation and histone H3 serine 10 phosphorylation are important steps in the activation of MMP-1 [16]. We have previously reported that both histone acetyl transferase, P300 and histone H3 acetylation on the MMP-1 promoter is sensitive to shifts in SS- $[\text{H}_2\text{O}_2]$ [7]. It has been suggested that histone acetylation can be affected by the presence of ROS through the activation of MAPK pathways [17–19].

Hydrogen peroxide has been shown to increase histone acetylation in alveolar epithelial cells leading to aberrant IL-8 expression [20–24]. Alveolar macrophages derived from cigarette smokers [22] and cystic fibrosis airway epithelial cell models [25] show changes in acetylation, likely due to elevated exposure to ROS. These observations indicate that oxidants play an important role in regulating the accessibility of chromatin to remodeling factors that allow for efficient gene transcription.

The fact that ROS is a known inducer of histone acetylation, and that MMP-1 is redox-regulated led us to explore the participants that drive MMP-1 epigenetic regulation and transcriptional activation. Using a series of redox-engineered cell lines to modulate SS-[H₂O₂] we have identified a number of redox responsive signaling components that regulate MMP-1 transcription. The current work identifies the various chromatin remodeling factors and transcriptional activators that control the redox-responsive MMP-1 promoter. Using chromatin immunoprecipitation (ChIP) we have determined that c-Jun, c-Fos, Ets-1 as well as P300/CBP-associated factor (PCAF) recruitment to the MMP1 promoter is H₂O₂-dependent. MMP-1 transcriptional initiation is also enhanced by redox-dependent, but H₂O₂-independent, modifications of HDAC2. Overall, these studies demonstrate that alterations in SS-[H₂O₂] enhance kinase signaling which modify components of the transcription initiation complex leading to maximal MMP-1 production.

Materials and Methods

Cell Culture and Reagents

All cell lines were maintained in 75cm² flasks in MEM containing 10% fetal calf serum, 1000U/ml penicillin, 500µg/ml of streptomycin, 50µg/ml Zeocin and or 1mg/ml G418 sulfate, in a 37°C incubator containing 5 % CO₂. Cell lines, recombinant Mn-SOD and catalase plasmids, and transfections have been described elsewhere [26, 27]. In brief, the redox-engineered cell lines were generated in our laboratory from HT1080 human fibrosarcoma cells. The cell lines generated included cells that were transfected with control plasmid (CMV), *Sod2* targeted to the mitochondria (Sod2), and cells co-transfected with catalase directed to the cytosol (Sod2Cat). Sod2 enzyme activity and catalase levels were confirmed (Supplemental Figure 1). MG-132 and 3-amino 1, 2, 4 triazole is from Sigma (St Louis, MO); Trichostatin A was obtained from Upstate Biotechnology/Millipore (Billerica, MA).

Antibodies

Total histone H3 antibody (06–755) was from Upstate Biotechnology/Millipore (Billerica, MA). c-Fos (sc-52x) and Ets-1 (sc-350) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). c-Jun antibody (9162) was obtained from Cell Signaling Technologies (Danvers, MA); HDAC2 antibody was from Zymed Laboratories (South San Francisco, CA) (51–5100). MMP-1 antibody is from R and D systems (Minneapolis, MN) (MAB901); c-Jun western blot antibody is from cell signaling technologies (Danvers, MA) (9165L), GAPDH was from Ambion/Applied Biosystems (Foster City, CA) (4300). Mouse monoclonal Ab to polyubiquitinated proteins clone FK2 (PW8810) is from BIOMOL

International (Plymouth meeting, PA) and mouse monoclonal antibody clone 6C1 (U0508) is from Sigma (St Louis, MO).

Immunoprecipitation experiments

Cells were treated with or without MG-132 or Trichostatin A (500 ng/ml) for 18 hours, washed with ice cold PBS and resuspended with PBS-EDTA. Nuclear extracts were prepared from each sample, protein assays performed and equivalent fractions of nuclear protein lysates were resuspended in 500 µl of lysis buffer. Samples were incubated 18 hours on a rotating platform with 1.5 µg of the indicated antibody. 50 µl of protein G beads were added to each sample and gently shaken for 2 h. Protein G beads were centrifuged at 4000 rpm for 30 sec, washed thrice with lysis buffer. Loading buffer was added and samples boiled for 5 min, centrifuged at 14000 rpm for 5 min and supernatants were electrophoresed and immunoblotted with the indicated antibodies. All incubations were performed at 4°C.

Western blot analysis

Samples prepared from different treatments are analyzed for protein concentration using the BCA protein assay according to the manufacture's instruction (Pierce/Thermo Scientific, Rockford, IL). Electrophoresis was performed on SDS-polyacrylamide gels using equal amount of total protein. After electrophoresis, the proteins were transferred to nitrocellulose or PVDF membrane, blocked by incubation for 1 hr in 1X Tween-TBS (pH 7.6) containing 5% non-fat dry milk or BSA. The membrane was incubated with the desired primary antibody either in milk or BSA, followed by washing with 1X TTBS and incubation with desired dilution of secondary antibody. Detection of the proteins was performed by the addition of Pierce/Thermo Scientific (Rockford, IL) SuperSignal Chemiluminescent substrate for 5 min and exposure to Kodak MS radiographic film (Kodak, Rochester, NY). Data plotted as mean relative densitometric intensity (RDI) normalized to loading control for three independent experiments.

Construction and Transient Transfection of MMP-1 Promoter Constructs

The full-length human MMP-1 promoter/luciferase reporter plasmids (1G and 2G) contained the firefly luciferase gene under the transcriptional control of the human MMP-1 promoter in a pGL3 basic reporter vector (Promega, Madison, WI) and were kindly provided by Dr. Constance Brinckerhoff (Dartmouth University). Transfection and analysis of MMP-1 promoter luciferase activity have been described in detail by Nelson *et al* [28].

MMP-1 Immunoblotting

was performed as described by Nelson *et al* [28].

Chromatin Immunoprecipitation assays

Cells were fixed with 1% formaldehyde for 10 minutes in serum free media. Reaction was stopped with 1.25 M glycine. Cells were lysed and chromatin was sheared using Active Motif's Enzymatic Shearing Kit following the manufacturer's instructions (53006; Carlsbad, CA). Enzymatic shearing for 4 minutes resulted in chromatin fragments of 100–1,000 bp in size. Chromatin immunoprecipitation (ChIP) was performed using Millipore's EZ-Magna

ChIP A/G Chromatin Immunoprecipitation Kit following the manufacturer's instructions (17–10086; Billerica, MA). An immunoprecipitation with an isotype specific IgG antibody was performed as a control for non-specific binding. Real time PCR was performed using MMP-1 promoter specific primers or negative control regions using Applied Biosystems' SYBR Green PCR Master Mix following the manufacturer's instructions (4309155) (Foster City, CA). For data analysis, ChIP samples were all normalized to the amount of input DNA for each sample. This normalization was performed after the QPCR and before any statistical analysis. Data is presented as enrichment after ChIP, with the IgG control sample set to 1. Data is plotted as the mean of three independent IPs with standard error bars representing the standard error of the mean. P values obtained from one-way ANOVA test.

Primers

Distal MMP-1 promoter region:

Forward primer- 5' CCCTCTTGAACACATGTTATGCC 3'

Reverse primer- 5' CCTTTCCCACTGTATCAGGTTTGC 3'

Proximal MMP-1 promoter region:

Forward primer- 5' AGAGTGTGTCTCCTTCGCACACAT 3'

Reverse primer- 5' ATAGAGTCCTTGCCCTTCCAGAAAGC 3'

β Globin promoter:

Forward primer- 5' AGGACAGGTACGGCTGTCATC

Reverse primer- 3' TTTATGCCCAGCCCTGGCTC

GAPDH control primers (from Millipore (Billerica, MA) EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit 17–10086):

Forward primer- 5' TACTAGCGGTTTTACGGGCG 3'

Reverse primer- 5' TCGAACAGGAGGAGCAGAGAGCGA 3'

cDNA preparation and real time PCR

Cell lines were treated as indicated. Real time PCR was performed as previously described by Nelson *et al* [28].

Primers:

Forward MMP-1 primer- 5' AGTGAAGTGGGAAACCAGATGCTGA3',

Reverse MMP-1 primer-5'GCTCTTGGCAAATCTGGCGTGTA3',

Forward Actin primer-5'ACCAACTGGGACGACATGGAGAAA3',

Reverse Actin primer-5'TAGCACAGCCTGGATAGCAACGTA3,

Forward c-Jun primer-5'AGATGAAGTCTTTCTGGCCTGCCT3'and Reverse c-Jun primer-5'ACACTGGGCAGGATACCCAAACAA3'.

Forward NQO1 primer 5' AGGACCCTCCGGAGTAAGA 3'

Reverse NQO1 primer 5' TCAGTTGGGATGGACTTGCC 3'
 Forward HMOX1 primer 5' CTTCAAGCTGGTGTATGGCCT 3'
 Reverse HMOX1 primer 5' GCTTGAACCTGGTGGCACTG 3'
 Forward DNAJB2 primer 5' GCATCACCACACGCAGAATC 3'
 Reverse DNAJB2 primer 5' GTTTGGTTGCTTCACCCCTC 3'
 Forward HSPA1L primer 5' GGGAGACTAGGCCTCAGAGA 3'
 Reverse HSPA1L primer 5' CACAAGGACTTTGGGCTTGC 3'
 Forward FTL primer 5' CTTGCCAACCAACCATGAGC 3'
 Reverse FTL primer 5' CAGCTGGCTTCTTGATGTCC 3'

siRNA transfections

The indicated cells were grown in a 6 well plate to near confluency in 2.5ml antibiotic free media. The cells were transfected according to the manufacturer's protocol (http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf/protocols.Par.26001.File.dat/HT1080_RNAiMAX.pdf). After siRNA transfection the cells were incubated for 24 or 48hrs. siRNA c-Jun (NM_002228) from Invitrogen (Carlsbad, CA) with the following concentrations was used: 50nM, 100nM and 200nM.

SOD activity gel

The levels of Sod2 were determined using Sod activity gels. Briefly, cells were lysed by sonication in 50 mmol/L potassium phosphate buffer, pH 7.8 containing 0.1 mmol/L EDTA and 25 µg of whole-cell lysates were separated on a 10% nondenaturing PAGE. The gel was stained with a solution containing 0.125 mmol/L nitroblue tetrazolium, 0.4 µmol/L riboflavin, 0.075 mmol/L TEMED, and 50 mmol/L potassium phosphate (pH 7.8)/0.1 mmol/L EDTA for 15 min in the dark, rinsed with distilled H₂O and exposed to light to visualize zones of Sod activity.

Catalase Assays

Cells were lifted with PBS-EDTA, washed, sonicated and resuspended in potassium phosphate buffer (pH 7.4). Catalase assay was carried out as described by Claiborne, A.; Fridovich, I [29].

Statistical Analysis

All data are presented as the sample average with error bars representing standard error of the mean. For ChIP data, all p-values were determined using a one-way ANOVA test. Comparisons were made between the Sod2 cell line compared to the control cell line and also the Sod2 cell line compared to the Sod2Cat cell line. For all other data, a two-tailed, two-sample equal variance t test was used to compare the means of groups with equal variance.

Results

Redox-dependent MMP-1 expression is associated with decreased HDAC and increased HAT recruitment that is accompanied by enhanced histone acetylation

The HDAC family of enzymes deacetylate lysines in histones tails, initiating rewinding of DNA and gene silencing. Alterations in HDAC2 levels and activity disturb the HAT/HDAC balance and lead to dysregulation of gene expression and signaling in several disease pathologies (HDAC2 in health and disease reviewed by Krämer [30]). Due to their importance in gene silencing we used Chromatin immunoprecipitation (ChIP) to determine promoter occupancy of HDAC2 at the distal and proximal regions of the MMP-1 promoter (Fig 1A) in redox-engineered cells. These redox-engineered cells have been well characterized and shown to display robust shifts in SS[H₂O₂] resulting from either Sod2 or catalase expression [7, 13, 31, 32]. For comparative purposes antioxidant enzyme levels of the cells used in this study are shown in Supplemental Figure 1. HDAC2 recruitment to the distal and proximal MMP-1 promoter was decreased in response to Sod2 overexpression. Coexpression of catalase partially rescued HDAC2 binding suggesting that its recruitment to the MMP-1 promoter is in part responsive to shifts in SS[H₂O₂]. A ChIP control IP was performed with an IgG matched antibody to control for non-specific binding (Fig 1B). β Globin and GAPDH promoter primers were also used as ChIP negative control regions, and no redox-dependent alterations in HDAC binding were observed (Supplemental Figure 2 & 3).

The histone acetyltransferase PCAF also plays a prominent role in acetylation and primarily acetylates lysine 14 of H3 [33]. ChIP analysis revealed that recruitment of PCAF to the distal and proximal MMP-1 promoter was dramatically enhanced in response to Sod2 overexpression and was attenuated by catalase coexpression (Fig. 1D). Again, β Globin and GAPDH promoter negative control regions showed no redox-dependent alterations PCAF binding (Supplemental Figure 2 & 3).

Histone acetylation is critical for transcriptional activation, and a reduction in HDAC with a corresponding increase in PCAF levels would likely result in elevated histone acetylation at responsive promoters. ChIP analysis revealed that histone H3 acetylation around both the distal and proximal MMP-1 promoter, is redox-dependent. Increased numbers of histones with acetylated lysine residues were found at the MMP-1 promoter in cells overexpressing Sod2. Coexpression of catalase significantly reduced the level of H3 acetylation, however it did not reduce it to control levels (Fig 1C). The β Globin promoter negative control regions showed no redox-dependent alterations in acetylated H3lysine residues (Supplemental Figure 2).

Redox-dependent MMP-1 expression is enhanced by HDAC inhibition

The impairment in HDAC2 recruitment to the MMP-1 promoter indicates that it plays a suppressive role in restricting redox-dependent MMP-1 gene activation. To evaluate the importance of HDACs in regulating MMP expression cells were treated with the broad spectrum inhibitor of Class I and II HDACs, Trichostatin-A. Sod2 overexpression significantly increases MMP-1 transcript levels, and this is further exacerbated with TSA

treatment. Coexpression of catalase caused a partial reversal of the TSA-dependent induction of MMP-1 when Sod2 was overexpressed (Fig 2A). These data suggest HDACs participate in restricting the oxidant-dependent induction of MMP-1, and their inhibition increases MMP-1 transcription.

HDAC2 is ubiquitinated and targeted for proteasomal degradation in cells overexpressing Sod2

—Since HDAC activity appears to be involved in the regulation of MMP-1 in response to Sod2 overexpression, levels of HDAC2 were assessed in the redox engineered cell lines. HDAC2 is predominantly a nuclear protein and is sensitive to oxidant-mediated inactivation and subsequent degradation [34–37]. HDAC2 is decreased in response to ROS in samples from individuals with inflammatory lung disease and smokers. Lung biopsies from patients with asthma [38] and primary nasal tissue from patients with cystic fibrosis [39] show reduced HDAC2 protein expression. Lung biopsies from smokers also reveal reduced protein expression of HDAC2 [22]. Analysis of nuclear HDAC2 revealed that Sod2 overexpression decreases nuclear HDAC2 levels which is rescued with the coexpression of catalase (Fig 2B). To ascertain if decreases in HDAC2 levels are a consequence of its redox-dependent degradation, cells were treated with the proteasome inhibitor, MG132. Proteasome inhibition slightly enhanced HDAC2 accumulation in Sod2 overexpressing which was accentuated in the Sod2/catalase coexpressing cells (Fig 2C).

HDAC2 turnover can be regulated by poly-ubiquitination and proteasomal degradation initiated by oxidation [35, 36, 40]. Analysis of ubiquitinated HDAC2 following MG132 treatment revealed it to be heavily ubiquitinated upon Sod2 overexpression, and this was not reversed by catalase overexpression (Fig 3A & B). These data suggest that HDAC2 ubiquitination is sensitive to the overexpression of Sod2 but is not reversed by catalase coexpression.

It has also been reported that Nuclear Factor (erythroid-derived 2)-like 2 depletion increases oxidative stress and decreases HDAC2 levels [41]. Sod2-mediated increases in steady state H₂O₂ may promote adaptive gene responses through Nrf2 and alter gene expression profiles. Analysis of a subset of Nrf2-dependent gene response indicated that there was no change in their expression in response to Sod2 expression (Supplemental Figure 4). HDAC2 itself has also been shown to negatively regulate Nrf2 function and HDAC inhibition activates Nrf2. Treatment with HDAC inhibitor increased Nrf2-dependent gene expression in both control and Sod2 expressing cells, with the exception of NQO1 whose expression was decreased by TSA treatment.

Enhanced recruitment of the transcriptional activators Ets-1, c-Jun and c-Fos to the MMP-1 promoter

—We have previously shown that the proximal Ets-1 and AP-1 binding sites in the MMP-1 promoter are required for maximal H₂O₂-dependent expression [7]. Using ChIP analysis we next investigated the redox-dependent binding of Ets-1, c-Jun, and c-Fos to the distal and proximal MMP-1 promoter. We find a significant increase in Ets-1 recruitment at both the distal and proximal MMP-1 promoter sites in response to increases in SS-[H₂O₂] mediated by Sod2 overexpression that was partially reversed by H₂O₂ removal by catalase (Fig 4A and B). No redox-dependent alterations in Ets-1, c-Jun,

or c-Fos binding was observed at the β Globin promoter negative control region (Supplemental Figure 2).

Both AP-1 family members, c-Jun and c-Fos display redox-dependent recruitment to the distal and proximal MMP-1 promoter. Again this increase in transcription factor binding was partially reversed with catalase coexpression (Fig 4A and B). Taken together these findings indicate that Ets-1, c-Jun and c-Fos are key transcriptional activators that are recruited to the distal and proximal MMP-1 promoter and participate in its maximal redox-dependent expression.

c-Jun is required for the transcriptional activation of MMP-1 in response to oxidants—We have previously reported the c-Jun-N-terminal kinase (JNK) is in large part responsible for redox-responsiveness of the MMP-1 promoter [7]. As c-Jun is a primary target of JNK and we have shown here that its recruitment to the MMP-1 promoter is redox-sensitive, we next assessed whether loss of c-Jun would render the MMP-1 promoter redox-insensitive. Analysis of varying siRNA concentrations demonstrated a maximal loss of c-Jun 48 hrs post transfection with 200nM of siRNA oligomers in the Sod2 expressing cells (Fig 5A). Densitometric analysis revealed an approximate 84% decrease in c-Jun immunoreactivity (Fig 5A, lower panel). Levels of secreted MMP-1 from Sod2 overexpressing cell lines treated with c-Jun siRNA displayed a significant decrease in MMP-1 immunoreactive protein (Fig 5B). In addition mRNA levels of both c-Jun and MMP-1 were analyzed by real time PCR. SiRNA mediated loss of c-Jun was associated with a significant decrease in redox-dependent MMP-1 mRNA expression (Fig 5C). Furthermore, c-Jun silencing severely impaired the redox-dependent expression of luciferase driven by the distal MMP-1 promoter element containing the highly active 2G containing single nucleotide polymorphism. (Fig 5D). These results indicate c-Jun to be essential for the full redox-dependent transcriptional activation of the MMP-1 promoter.

Discussion

Redox signaling has emerged as a primary mode for the propagation of signal transduction events in response to a diverse array of cellular stimulus. The primary targets for redox-control are the many signaling kinases and their inhibitory phosphatases. One example of this control is the redox-dependent activation of c-Jun-N-terminal kinase which engages downstream expression of AP-1 responsive genes. However, redox-dependent JNK activation only engages a small subset of genes of which MMP-1 is one of the most responsive [7]. Thus, control of redox-signaling must also occur downstream of kinase signaling to regulate gene specific transcription. Our analysis identifies key proteins and regions of the MMP-1 promoter that participate in its redox-responsiveness and converge to exacerbate its transcriptional activity. Our predicted model for redox-control of MMP-1 transcription includes recruitment of transcription factors (Ets-1 and AP-1) and histone modifying protein (PCAF) and increased HDAC2 turnover which culminates in maximal MMP-1 gene activation.

The distal MMP-1 promoter region is known to play an important role in augmenting MMP-1 expression both basally and in response to oxidants [7, 8, 28, 42]. We show an

oxidant-dependent recruitment of the transcriptional activator proteins Ets-1, c-Jun, c-Fos, and PCAF to the distal and proximal MMP-1 promoter (Figures. 1D, 4A, and B). This increase in promoter binding is, largely, H₂O₂-dependent. The proximal MMP-1 promoter region includes the AP-1 at position -80 and an additional consensus element at -182 that confers phorbol ester responsiveness [16]. The resolution of our ChIP analysis reveals that increased SS-[H₂O₂] results in residence of transcriptional activator complexes with HAT activity at both the distal and proximal MMP-1 promoter. While PCAF has intrinsic HAT activity itself, it is often found associated in a larger complex with CBP/P300 [43–46]. CBP and P300 are two closely related transcriptional coactivators with HAT activity themselves. CBP/P300 has been shown to bind Ets-1[47], c-Jun [48], and c-Fos [49]. Further analysis may reveal that the assembled redox-dependent complex contains additional chromatin modifying enzymes that prime the MMP-1 loci for maximal transcription.

This study further identified that the histone deacetylase HDAC2 is negatively regulated in response Sod2 overexpression. While TSA has a modest effect on basal MMP-1 expression it dramatically enhances Sod2-dependent MMP-1 expression. This suggests that acetylation state of the MMP-1 promoter is pivotal in regulating the amplitude of its transcription in response to Sod2-driven increases steady state H₂O₂ production. Similarly, Sod2 overexpression engages a multitude of signaling responses that converge to drive MMP-1 expression [50] and the levels of HDAC2 are key to controlling the maximal level of its expression.

HDAC2 depletion, through oxidation, has been linked to driving lung inflammation in chronic obstructive pulmonary disease and our findings support these observations [51]. It has been established that H₂O₂ can result in decreased HDAC2 protein in a concentration dependent manner [34, 52]. This is consistent with our observed decrease in HDAC2 protein after Sod2 overexpression, and resulting increase in SS-[H₂O₂]. The combination of loss of HDAC2 and increased occupancy of PCAF binding to the MMP-1 promoter would serve to enhance histone H3 acetylation in response to Sod2 overexpression. UV irradiation has also been shown to drive HDAC depletion with an accompanying increase in the p300 HAT recruitment to the MMP-1 promoter in human dermal fibroblasts[53]. The precise mechanism of HDAC depletion and p300 HAT activation likely involves the UV-dependent increases in ROS production and may parallel the current findings.

We establish that HDAC2 is decreased in the nucleus upon Sod2 overexpression and is modified by ubiquitination leading to its likely degradation. HDAC2 ubiquitination is Sod2-dependent and not reversed by catalase coexpression. This observation may explain why catalase does not completely bring MMP-1 levels back to their basal uninduced state (Figures. 2 and prior work)[7, 8]. HDAC2 nitration can also result in its proteasomal degradation, and can lead to aberrant gene transcription [34, 37, 52, 54–56]. It is possible that shifts in cellular nitrating capacity may result from the overexpression of Sod2 that would not respond to the overexpression of catalase. The nitration status of HDAC2 in response to Sod2 overexpression is currently being explored. It is also important to emphasize that while total cellular HDAC2 levels were modestly affected by Sod2 overexpression the pool of ubiquitinated HDAC2 was clearly increased. The ubiquitinated pool of HDAC2 may represent a small fraction of total HDAC2 at any particular moment

due to its rapid degradation in the absence of proteasome inhibitor. Nonetheless, loss of a small fraction of HDAC2 appears to have a profound effect on the oxidant-dependent expression of MMP-1.

This finding indicates that increases in SS-[H₂O₂] enhance the recruitment of coactivators (PCAF, Ets-1 and AP-1) to the MMP-1 promoter driving transcription. Inhibition of HDAC activity further propagates redox-dependent transcription of the MMP-1 promoter. The observation that HDAC2 recruitment and H3 acetylation display H₂O₂-sensitive profiles similar to that of PCAF recruitment suggests that Sod2-dependent increases in SS-[H₂O₂] serve to maximally enhance MMP-1 promoter acetylation. Interestingly, TSA treatment leads to an increase in the mRNA levels of a number of NRF2 responsive genes which would aid in ROS detoxification (Sup. Figure 4). In addition, Sod2 overexpression did not impact the expression of the NRF2 regulated genes that were analyzed. While this was not a comprehensive assessment of the NRF2 response the findings do suggest that the Sod2-mediated increase in SS-[H₂O₂] may not activate NRF2. Nonetheless, MMP-1 levels were dramatically elevated in response to TSA treatment suggesting that the acetylation state of the MMP-1 promoter is the driving force behind maximal MMP-1 expression and that NRF2 activation is not as effective as catalase in restricting Sod2-dependent MMP-1 expression.

In summary, by manipulating intracellular antioxidant status we have identified a set of redox-responsive coactivators that regulate transcriptional activity of MMP-1. Aberrant MMP-1 expression is implicated in a number of degenerative disease pathologies that involve shifts in steady state oxidant production. These findings also suggest that preservation of HDAC activity may serve to restrict MMP expression under oxidizing conditions. The antioxidant, N-acetyl cysteine prevents redox-dependent increases in MMP-1 expression [7] and is used clinically for the treatment of pulmonary fibrotic injury [57]. It is exciting to speculate that N-acetyl cysteine and other antioxidants likely exert their therapeutic effects by preserving HDAC activity and limiting H₂O₂-dependent signals that drive the pathologic expression of not only MMPs but other similar oxidant responsive genes. Moreover, we have unveiled the molecular triggers that drive aberrant MMP-1 expression in response to high level Sod2 activity. Growth factors and cytokines can induce Sod2 and modulate the intracellular redox environment. In this fashion Sod2 may contribute to the aberrant proteolysis that accompanies chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sod2-dependent increases in MMP-1 expression are associated with altered HAT/HDAC recruitment.
- Shifts in HAT/HDAC balance is accompanied by enhanced histone acetylation.
- Redox alterations augment HDAC2 ubiquitination.
- Increased steady-state H₂O₂ drives AP-1 and ETS-1 recruitment to the MMP-1 promoter
- c-Jun ablation prevents the Sod2-dependent increases in MMP-1 expression

Figure 1. Redox-dependent MMP-1 expression is associated with decreased HDAC and increased HAT recruitment that is accompanied by enhanced histone acetylation

Cells were subjected to ChIP as described in materials and methods. Protein-DNA complexes were pulled down using specific antibodies to HDAC2, PCAF, and acetylated H3. IgG antibody was used as a negative control for non-specific binding. **A**) Schematic diagram of transcription elements on the MMP-1 promoter indicating primer spanning regions used in ChIP. Distal and proximal primers span -1491 to -1696 and -245 to -37 upstream of the transcription start site respectively. **B**) HDAC2 promoter occupancy at the distal and proximal MMP-1 promoter. * $p=0.0149$ compared to control, ** $p<0.0001$ control vs. Sod2 or Sod2Cat, Sod2 vs. Sod2Cat **C**) Acetylation of Histone H3 at the distal and proximal regions of the MMP-1 promoter. * $p=0.0007$ or ** $p<0.0001$ compared to control **D**) Promoter occupancy of PCAF to the distal and proximal MMP-1 promoter. * $p<0.0001$ or ** $p<0.05$ for Sod2 compared to control, or Sod2 compared to Sod2Cat. $N=3$ independent immunoprecipitations. For data analysis, ChIP samples were all normalized to the amount of input DNA for each sample. Data is presented as enrichment after ChIP, with the IgG control sample set to 1. Mean values are plotted with error bars representing standard error of the mean.

Figure 2. HDACs contribute to the redox-responsiveness of MMP-1 transcription

A) The indicated cell lines were treated with or without TSA (500 ng/ml) for 18 hrs and resulting real-time RT-PCR analysis of MMP-1 mRNA levels normalized to β -actin is shown. Data is representative of two independent experiments, $*=p<0.005$ compared to untreated. Means are plotted with error bars indicating standard deviation. **B)** Representative western blots are shown for cytosolic and nuclear HDAC2 and Actin in the indicated cell lines treated with 10 μ M MG-132 for 4 hours. **C)** Relative densitometric intensity (RDI) of

HDAC2 normalized to Actin is presented. RDI of HDAC2 was obtained and expressed as % of untreated control group from three independent experiments, $*=p<0.05$ and $**=p<0.01$.

Figure 3. HDAC2 ubiquitination is increased in response to oxidant stress and regulates MMP-1 expression

A) Cells were either treated with 10 μ M MG-132 or left untreated for 18 hours followed by immunoprecipitation with anti HDAC2 antibody and blotted for anti-ubiquitin. Blot is representative of three separate experiments. **B)** Relative densitometric intensity of ubiquitinated HDAC2 normalized to total HDAC2 levels. The control was set to 1. Data is representative of three independent experiments. *, $p < 0.05$ as compared to control. Means are plotted with error bars indicating standard error of the mean.

Figure 4. Redox-dependent recruitment of Ets-1, c-Jun and c-Fos to the MMP-1 promoter

Cells were subjected to ChIP as described in materials and methods. Protein-DNA complexes were pulled down using specific antibodies to Ets-1, c-Jun, and c-Fos. IgG antibody was used as a negative control for non-specific binding. **A)** Relative promoter occupancy at the distal region of the MMP-1 promoter by Ets-1, c-Jun and c-Fos. **B)** Relative promoter occupancy of Ets-1, c-Jun and c-Fos at the proximal region of the MMP-1 promoter. * $p < 0.0001$ for Sod2 compared to control or Sod2 compared to Sod2Cat. $N = 3$ independent immunoprecipitations. For data analysis, ChIP samples were all normalized to the amount of input DNA for each sample. Data is presented as enrichment after ChIP, with the IgG control sample set to 1. Mean values are plotted with error bars representing standard error of the mean.

Figure 5. Redox-regulated MMP-1 transcription is c-Jun dependent in response to Sod2 overexpression

A) Western blot analysis of c-Jun and GAPDH in Sod2 overexpressing cells transfected with c-Jun specific or scrambled siRNA. *Lower panel*, Relative c-Jun levels in siRNA transfected cells normalized to that of scrambled control (n=3). Average percentage in reduction in c-Jun protein levels is indicated. **B)** Western blot of MMP-1 protein levels in media from Sod2 overexpressors treated as indicated. *Lower Panel*, **Relative** densitometric analysis of MMP-1 levels in siRNA transfected cells as compared to scrambled controls (n=3). **C)** Real-time RT-PCR analysis of MMP-1 and c-Jun in Sod2 overexpressors treated as above (n=3). mRNA levels normalized to β -actin in the indicated cells **D)** MMP-1 promoter luciferase reporter assay in Sod2 overexpressing cells treated as indicated (n=3). All immunoblots are representative of three independent experiments. *, $p < 0.007$ as compared to scrambled control. **, $p = 0.0002$ as compared to scrambled control. Means are plotted with error bars indicating standard error of the mean.