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Epigenetic regulation of inflammatory gene expression in macrophages by selenium☆

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Abstract

Acetylation of histone and non-histone proteins by histone acetyltransferases plays a pivotal role in the expression of proinflammatory genes. Given the importance of dietary selenium in mitigating inflammation, we hypothesized that selenium supplementation may regulate inflammatory gene expression at the epigenetic level. The effect of selenium towards histone acetylation was examined in both *in vitro* and *in vivo* models of inflammation by chromatin immunoprecipitation assays and immunoblotting. Our results indicated that selenium supplementation, as selenite, decreased acetylation of histone H4 at K12 and K16 in COX-2 and TNFα promoters, and of the p65 subunit of the redox sensitive transcription factor NFκB in primary and immortalized macrophages. On the other hand, selenomethionine had a much weaker effect. Selenite treatment of HIV-1-infected human monocytes also significantly decreased the acetylation of H4 at K12 and K16 on the HIV-1 promoter, supporting the down-regulation of proviral expression by selenium. A similar decrease in histone acetylation was also seen in the colonic extracts of mice treated with dextran sodium sulfate that correlated well with the levels of selenium in the diet. Bone-marrow-derived macrophages from $Trsp^{fl/fl}Cre^{LysM}$ mice that lack expression of selenoproteins in macrophages confirmed the important role of selenoproteins in the inhibition of histone H4 acetylation. Our studies suggest that the ability of selenoproteins to skew the metabolism of arachidonic acid contributes, in part, to their ability to inhibit histone acetylation. In summary, our studies suggest a new role for selenoproteins in the epigenetic modulation of proinflammatory genes.

Keywords: Selenium; p300; Epigenetic regulation; Inflammatory gene expression; Cyclopentenone prostaglandins; Selenoproteins

1. Introduction

Transcription, DNA repair and replication are associated with changes in chromatin [1,2]. The nucleosome, a basic unit of chromatin, is composed of dimers of histones H2A, H2B, H3 and H4, and 147 base pairs of DNA are wrapped around the histone core. The highly basic N-terminal tails of the histones are exposed on the surface of the nucleosome and serve as the main sites for posttranslational modifications (PTMs). Among these, reversible acetylation of histones at lysine residues plays a role in controlling the structure of the chromatin [3,4], where highly acetylated histones make the chromatin more accessible to host factors, signifying active transcription. Histone acetyltransferases (HATs) are the class of enzymes that catalyze the acetylation of histones (and certain nonhistone proteins), wherein they transfer the acetyl group from acetyl-CoA to the lysine-amino tails of histones and other proteins at specific lysine residues. Thus, HATs are also referred to as lysine acetyltransferases (KATs). Acetylation of histone H4 at K5, K8, K12 and K16 by HATs such as p300/CBP, Gcn5, PCAF, ATF2, Esa1 and Tip60 is known to be involved in transcriptional activation [5–9].

Dysfunction of HATs and the associated acetylation events have been implicated in a wide variety of diseases like neurodegeneration, cancer, HIV–AIDS, and inflammation [10–13]. The association of HAT activity with initiation and progression of cancer has made p300, Gcn5 and PCAF attractive targets for anticancer and anti-inflammatory therapies. Many of the inhibitors of these enzymes are peptide conjugates of CoA or natural products and their derivatives (reviewed in [14]). HAT activity is also essential for the initiation and elongation events during HIV transcription. Acetylation of HIV transactivator of transcription (Tat) by p300 is critical for the elongation of HIV transcripts [15–17]. Inhibition of p300 has been shown in literature to inhibit HIV replication [18].

Dietary selenium modulates epigenetic events like DNA methylation by inhibiting DNA methyltransferases (DNMTs) in a prostate cancer cell line [19]. It has also been suggested that alpha keto-derivatives of organic selenocompounds inhibit histone deacetylase (HDAC) activity in prostate and colon cancer cell lines [20,21]. Together, HATs and HDACs maintain the balance between gene expression and repression in cells, with acetylation of histones leading to relaxation of the

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chromatin and active transcription, while deacetylation leads to gene repression and reestablishment of chromatin structure. Little is known about the effects of selenium on HAT activity in immune (nontransformed) cells.

Recently, Gandhi et al. have reported a positive correlation between selenium (in the form of selenite) supplementation and the expression of a critical enzyme in the prostaglandin (PG) biosynthesis pathway, hematopoietic prostaglandin D synthase (H-PGDS), *in vitro* and *ex vivo* in murine macrophages, culminating in an increased production of cyclopentenone PGs (CyPGs) [22]. This results in a shift in cyclooxygenase (COX)-mediated prostaglandin production from proinflammatory PGE_2 to anti-inflammatory CyPGs, Δ^{12}-PGJ_2 and 15d-PGJ_2 [22]. As a consequence of such a shunting of eicosanoids, supplementation with selenium polarizes macrophages towards alternatively activated (anti-inflammatory) phenotypes [23]. Previous studies from our laboratory have also shown that Cys^{1438} in the critical substrate-binding site of p300 HAT domain is a target for covalent modification by CyPGs, which results in the inhibition of the enzymatic activity of p300 [24]. Our laboratory has also shown that selenoprotein biosynthesis via the cotranslational insertion of Sec (from tRNA[Ser]^{Sec}; *Trsp*) is essential for the anti-inflammatory effects of selenium, including CyPG production [22]. In the current study, we describe the ability of selenium, via the production of CyPGs and selenoproteins, to inhibit histone acetylation and regulate inflammatory gene expression *in vitro* in inflamed macrophages and a model of HIV infection, and *in vivo* in a murine model of dextran sulfate sodium (DSS)-induced inflammatory bowel disease.

2. Materials and methods

2.1. Analysis of histone acetylation in macrophages

Murine macrophage-like RAW264.7 cells [cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 5% fetal bovine serum (ATCC, 7 nM selenium), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin] were treated with 100 ng/ml lipopolysaccharide (LPS) for 2 h, followed by incubation with increasing doses of selenium in the form of sodium selenite, selenomethionine (SeMet; Sigma-Aldrich) or 1,4-phenylenebis(methylene)selenocyanate (p-XSC; provided by Dr. Shantu Amin, Penn State College of Medicine, Hershey, PA, USA) for 72 h (as indicated), with or without indomethacin (indo; 10 μM, COX inhibitor; Cayman Chemicals) or HQL-79 (25 μM, H-PGDS inhibitor; Cayman Chemicals). Histones were isolated from these cells [24] and analyzed for their acetylation status using anti-H4 acetyl (K5/K8/K12/K16) antibodies (Active Motif). Histone H3 (anti-H3 C-terminal, Active Motif) was used as a control to normalize loading. Murine primary macrophages (bone-marrow-derived macrophages; BMDMs) isolated from mice ($Trsp^{fl/fl}Cre^{LysM}$ or wild-type littermates) maintained on selenium-deficient diets [22] were cultured in DMEM (Invitrogen) in above-mentioned media with 10% (v/v) L929 fibroblasts conditioned medium. Following treatment with the inhibitors (or vehicle as control) for 12 h, the BMDMs were stimulated with 10 ng/ml LPS for 2 h, after which they were cultured with sodium selenite at different concentrations for 72 h, with or without inhibitors. BMDMs were then treated with 100 ng/ml LPS for 12 h and harvested. Histones were isolated and analyzed as described above.

2.2. Analysis of histone acetylation in the colon of a DSS-induced murine colitis model

Selenium-deficient (<1 ppb selenium; Def), selenium-adequate (80 ppb as sodium selenite in diet; Ade) and selenium-supplemented (400 ppb; Sup) mice were treated with water containing 4% (w/v) DSS for 5 days *ad libitum*. The mice were given regular water for the next 5 days. On the 10th day, the mice were sacrificed and their colons were isolated. Histones were isolated from these colons and analyzed for acetylation of H4K12 by immunoblotting.

2.3. Analysis of p65 acetylation

RAW264.7 macrophages were treated with 100 ng/ml LPS for 2 h, followed by incubation with different concentrations of selenium. These cells were harvested, and the nuclear and cytoplasmic fractions were separated. The nuclear fractions were analyzed by immunoblotting for the acetylation status of p65 at K^{310}. The blot was also probed for total p65 as control.

2.4. Chromatin immunoprecipitation (ChIP) assay

BMDMs and RAW264.7 cells were cultured with or without sodium selenite at indicated concentrations for 72 h, followed by stimulation with 100 ng/ml LPS for 2 h. These cells were harvested poststimulation and used for cross-linking ChIP. Briefly, the cells were treated with 1% formaldehyde for 10 min, followed by 125 mM glycine to terminate the cross-linking reaction. The cells were washed with ice-cold phosphate-buffered saline and harvested. Approximately 10 million cells were lysed with ChIP lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) and subjected to sonication (Diagenode Bioruptor; 30 s ON, 60 s OFF for 20 cycles). Five micrograms of the sonicated chromatin, diluted to 1 ml with the ChIP dilution buffer (0.5% Triton-X 100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl), was pretreated with 5 μl of protein A/G agarose (Origene) for 30 m. The agarose beads were spun down by centrifugation at 1500×*g* for 5 m at 4 °C. The chromatin was now incubated with antibodies to H4 acetyl (ac)-K12 or H4 ac-K16 (2 μl) and 5 μl of protein A/G agarose overnight at 4 °C. Five micrograms of chromatin from the samples was also subjected to IP with an unrelated rabbit polyclonal antibody (immunoglobulin G) as control. The beads were then washed with 300 μl of ChIP wash buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl) thrice, followed by 500 μl of Tris-EDTA buffer (1 mM Tris, pH 8.0, 0.1 mM EDTA) twice. The immunoprecipitated chromatin was eluted off the beads using the ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$). The samples were subjected to reverse cross-linking by incubating them with 300 mM NaCl, 0.5 μg of RNase A (Sigma) and 3 U of Proteinase K (New England Biosciences) per ChIP reaction at 65 °C in a water bath overnight. DNA was isolated and concentrated using the ChIP DNA clean and concentrator kit (Zymo Research) according to the manufacturer's instructions. DNA was eluted in 50 μl of elution buffer. The immunoprecipitated DNA was then analyzed by quantitative polymerase chain reaction (qPCR) using primers detecting the promoter regions of COX-2 (forward GAGCAGCGAGCACGTCA, reverse TCCAGTGGGGGCCTAAA) and TNFα (forward CACACACACCCTCCTGATTG, reverse TCGGTTTCTTCTCCATCGC) that encompass a proximal NF-κB response element. U1/HIV-1 cells were incubated without or with selenium (500 nM) for 3 days. These cells were stimulated with 20 ng/ml PMA for 6 h, harvested and processed for ChIP assay as described above. The immunoprecipitated DNA was analyzed by qPCR with primers targeting the HIV LTR (forward CGAGAGCTGCATCCGGAGTA, reverse GAGGCTTAAGCAGTGGGTTCC). All ChIP data have been presented as fold change in the signal-to-input ratio compared to control.

2.5. HDAC assay

RAW264.7 macrophages were cultured in different concentrations of selenium (as sodium selenite) for 72 h. These cells were then stimulated with 100 ng/ml LPS for 2 h. The cells were then washed with complete medium thrice and incubated with or without selenium for an additional 6 h. The cells were harvested, and nuclear extracts were prepared. Twenty micrograms of nuclear protein was used in the HDAC assay (performed as per manufacturer's instructions; Millipore, catalog # 17-320). Briefly, biotin-conjugated histone H4 peptide was labeled with [^{3}H]acetyl CoA using PCAF enzyme (supplied with kit). The radiolabeled peptide was immobilized on streptavidin-agarose beads. Ten thousand counts per minute of immobilized peptide was incubated with the nuclear extract in HDAC assay buffer for 24 h at room temperature on an orbital shaker. The beads were pelleted, and the supernatant was subjected to scintillation counting to assay the released [^{3}H]acetate.

2.6. Statistics

All data have been represented as mean±S.E.M. of at least three independent experiments. Data have been analyzed by unpaired *t* test, one-way analysis of variance (ANOVA) or two-way ANOVA where appropriate using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). *, @, # and $ represent $P<.05$, $P<.005$, $P<.0005$ and $P<.0001$, respectively.

3. Results

3.1. Selenium supplementation inhibits histone acetylation in macrophages in vitro

We tested the inhibition of HAT activity upon selenium supplementation in a murine macrophage model of LPS-induced inflammation. RAW264.7 macrophages were treated with LPS, followed by incubation with selenium for 72 h at the indicated concentrations. Histones were isolated from these cells, and the acetylation status of histone H4 at positions K5, K8, K12 and K16 was analyzed by immunoblotting (Fig. 1A). Changes in histone H4 K12 acetylation in intact RAW264.7 macrophages treated with LPS were also examined by immunofluorescence (Fig. S1). As shown in Fig. 1A, acetylation of histone H4 in inflamed macrophages was reduced by ~70% upon selenium supplementation (at 250 nM; as selenite). Primary murine BMDMs isolated from selenium-deficient mice were treated with 10 ng/ml LPS for 2 h and subsequently incubated with various concentrations of selenium