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Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration

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Abstract

Oxidative stress is a characteristic of chronic inflammatory diseases. The reactive oxygen intermediate hydrogen peroxide (H_2O_2) is an important signaling molecule that modulates gene expression. We have demonstrated that H_2O_2 significantly enhanced cytokine production in BEAS-2B cells, with a maximal effect at 4 h. This did not result from enhanced NF-κB activation, but through decreased activity of histone deacetylase (HDAC)2. This results in increased inflammatory gene expression following acetylation of specific histone residues. Decreased HDAC2 activity was associated with tyrosine nitration status. Peroxynitrite and SIN-1, a peroxynitrite generator, were also able to reduce HDAC2 activity via tyrosine nitration. Our data suggest that oxidative stress contributes to worsening inflammation via reduction of HDAC2 activity through HDAC2 nitration. This novel mechanism of inflammation may be important in increasing the severity and chronicity of inflammatory diseases.

Keywords: Oxidative stress; Histone acetylation; Gene expression; Inflammation; Peroxynitrite

Oxidative stress is characteristic of chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, and inflammatory bowel disease [1,2]. Elevated intracellular reactive oxygen species (ROS) are generated under various physiological and pathological conditions, including inflammation, ischemia and reperfusion, and sepsis. They can be derived from sources as different as activated inflammatory cells, oxidized lipoproteins, cigarette smoke, and structural cells [3]. Major ROS are superoxide anions (O^{2-}), hydroxyl radicals (·OH), and H_2O_2. In addition, peroxynitrite is a potent radical formed from a rapid interaction between superoxide anions (O^{2-}) and nitric oxide (NO) [4]. ROS mediate some biological responses, such as cytokine (IL-6, IL-8, and TNFα) induction by IL-1β or TNFα [4,5]. This is reported to be due to enhanced NF-κB activation in response to H_2O_2 [4–6].

Chromatin structure and binding of proteins to DNA can be modulated by reversible acetylation of lysine residues within the N-terminal tails of core histones. In the resting cell, DNA is tightly compacted to prevent transcription factor accessibility. During activation of the cell, this compact inaccessible DNA is made available to transcription factors through histone acetylation. This chemical modification is carried out by histone acetyltransferases (HAT) and counteracted by histone deacetylases (HDAC) [7] and [8] many transcriptional co-activators, such as CREB-binding protein (CBP), have intrinsic HAT activity. IL-1β and TNFα can both stimulate the binding of NF-κB (p65 subunit) to CBP, increase HAT activity, and induce histone acetylation, thus leading to increased inflammatory gene (such as GM-CSF and IL-8) transcription [9,10]. Hydrogen peroxide (H_2O_2) and other ROS can also induce enhanced inflammatory mediator release from cells, a process that is associated with changes in histone acetylation [11].

Several reports have shown that HDACs 1–3 can also be associated with inactive p65 and play a role in the regulation of NF-κB-mediated gene transcription without altering the degree of DNA binding [9,12–14]. Thus, changes in HDAC activity associated with p65 can enhance or repress NF-κB-mediated gene expression [12].

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We have previously reported that HDAC2 expression and activity is decreased in smokers, COPD subjects, and mild asthma patients and that there is a good correlation between cytokine production and HDAC activity in alveolar macrophages from smokers and non-smokers [11]. Reduced HDAC2 may be a key factor in the development of inflammation in airway obstructive disease. There is abundant evidence for increased oxidative stress and peroxynitrite formation in COPD and severe asthma [2]. We show that nitration of HDAC2 following oxidative stress may account for the reduced HDAC activity seen in cells from patients with oxidant stress related diseases.

Materials and methods

Materials. Thirty percentage of H_2O_2 and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (Sigma, Poole, UK); IL-1β was from R&D (Abingdon, UK); DCFH-DA and SIN-1 were from Molecular Probes (Leiden, Netherlands); and peroxynitrite was from Cayman Chemicals (Ann Arbor, MI, USA). Anti-p65 (sc-109, sc-7151), anti-phosphotyrosine (sc-508), anti-HDAC2 (sc-7899), and anti-HDAC1 (sc-6298) antibodies were obtained from Santa Cruz Biotec (Santa Cruz, California, USA), and anti-nitrotyrosine (1A6) was obtained from Upstate (Charlottesville, VA, USA).

Cell culture. BEAS-2B cells were grown to 50% confluence in keratinocyte conditioned medium (Gibco, Paisley, UK). Before experimentation, cells were serum-starved for 24 h in medium without EGF and bovine pituitary extracts. Cells were stimulated by IL-1β (1 ng/ml) in the presence or absence of H_2O_2 (100 μM) or SIN-1 (500 μM).

Cytokine ELISA. Determination of GM-CSF and IL-8 expression was measured by sandwich ELISA (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

DCF assay for oxidative stress. Oxidative stress was detected by the modified method described by Wang and Joseph [15]. Viable cells were plated into 96-well culture plates 1 day before the experiments. On the day of the experiments, after removing the medium, the cells in the plates were washed with Krebs–Ringer–Hepes–glucose–glutamine buffer (KRH buffer) and then incubated with 100 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the loading medium in 5% CO_2/95% air at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed and incubated with KRH buffer with SIN-1 (500 μM) or H_2O_2 (100 μM) and the fluorescence of the cells from each well was measured and recorded. The excitation filter was set at 485 nm and the emission filter was set at 530 nm.

Nuclear extraction. Cells were collected and resuspended in mild lysis buffer [10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, and complete protease inhibitor cocktail (Boehringer–Mannheim)] for 10 min. Nuclei were collected by microcentrifugation (10,000 rpm, 5 min, 4 °C) and resuspended in Tris-based high salt buffer (10 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol, and complete protease inhibitor cocktail). After 30 min, supernatant was collected and diluted with Tris-based, no salt buffer (10 mM Tris–HCl, pH 8.0, 0.25 mM EDTA, 10 mM of 2-mercaptoethanol, and complete protease inhibitor cocktail) to 40 mM NaCl final concentration.

Immunoprecipitation. Extracts were prepared using 100 μl of modified RIPA buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% Triton X-100, 0.5% NP-40, 0.1% SDS, 0.5% deoxycholate, and complete protease inhibitor cocktail (Boehringer–Mannheim)] as previously described [9]. For the HDAC assay, immunoprecipitates were washed twice with HDAC buffer (10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 0.25 mM EDTA, and 10 mM of 2-mercaptoethanol) and for Western blotting the buffer was aspirated completely and resuspended in Laemmli buffer.

Western blotting. Immunoprecipitates or nuclear extractions were analyzed by SDS–PAGE and Western blot analysis using ECL as previously described [9].

NF-κB activation. NF-κB activation was measured with TransAM NF-κB kit (Active Motif, California, USA).

Histone deacetylase assay. HDAC assays were performed as previously described [9].

Immunocytochemistry. BEAS-2B cells (0.5×10^6) were cultured in 8-well slide chambers with IL-1β (1 ng/ml) in the presence or absence of H_2O_2 and immunocytochemistry for p65 performed as previously described [9]. Stained cells were observed by confocal microscopy. Confocal scanning laser microscopy images were collected with a Leica confocal microscope, equipped with a 488/514 nm dual band argon ion laser. An oil-immersion objective was used and images were collected using TCSNT software.

Chromatin immunoprecipitation (ChIP) assay. BEAS-2B cells pretreated with H_2O_2 (4 h) or trichostatin A (TSA, 10 min) were treated with IL-1β (1 ng/ml) as described above. After a 0.5-h incubation, protein–DNA complexes were fixed by formaldehyde (1% final concentration) and treated as previously described [9]. Acetylated H4 or NF-κB–p65 binding IL-8 promoter (−121 to +61) was quantified by real-time PCR using a QuantiTect SYBR Green PCR kit (Qiagen) on a Rotor-Gene 3000 (Corbett Research, NSW, Australia).

Statistics. Results are expressed as means ± standard error of the mean (SEM). A multiple comparison was made between the mean of the control and the means from each individual treatment group by Dunnett's test using SAS/STAT software (SAS Institute, Cary, NC, USA). All statistical testing was performed using a two-sided 5% level of significance.

Results

H_2O_2 enhances IL-1β-stimulated cytokine expression

Pretreatment of human airway epithelial cells (BEAS2B) with H_2O_2 (100 μM) for 4 h slightly enhanced basal IL-8 production but markedly potentiated IL-1β-stimulated IL-8 production (2497 ± 226 ng/ml versus 1066 ± 64) (Fig. 1A) without affecting cell survival (data not shown). This enhancement by pretreatment of H_2O_2 was maximal at 4 h (Fig. 1B). Four hour pretreatment of H_2O_2 also enhanced GM-CSF production as well as IL-8 (211 ± 22 vs 138 ± 36). This effect was blocked by the anti-oxidant *N*-acetyl-L-cysteine (NAC, 10 mM, Fig. 1C) (IL-1β only 1066 ± 64, IL-1β + H_2O_2, 2497 ± 226, and IL-1β + H_2O_2 + NAC 1334 ± 09 ng/ml).

H_2O_2 does not enhance NF-κB nuclear translocation

IL-1β-induced p65 nuclear translocation was rapid (detectable at 10 min), peaked at 30 min, and remained elevated for at least 2 h (data not shown). H_2O_2 (100 μM) induced some p65 nuclear translocation but this was minimal compared to that seen with IL-1β and peaked at 2 h (Fig. 2A). H_2O_2 did not significantly enhance IL-1β-induced p65 nuclear translocation (Fig. 2B). These results were confirmed by immunocytochemistry (Fig. 2C). Furthermore, H_2O_2 did not

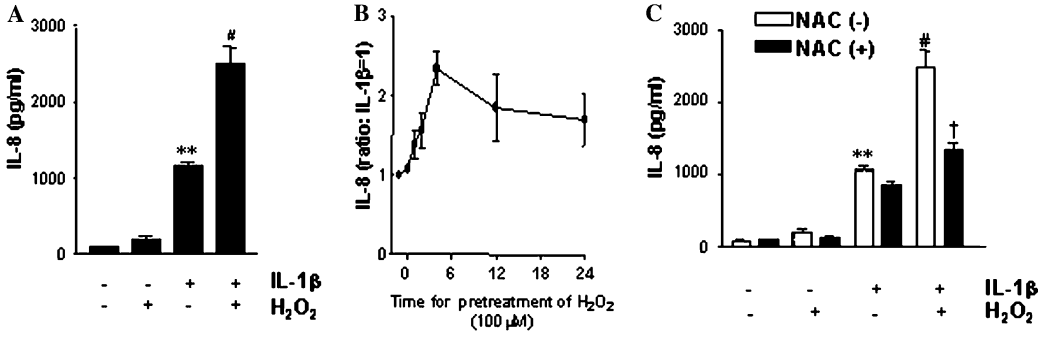

Fig. 1. Effect of H_2O_2 on IL-1β-induced inflammatory cytokine production. (A) BEAS-2B cells were stimulated by IL-1β (1 ng/ml) for 24 h in the presence or absence of H_2O_2 (100 μM) and IL-8 was measured by ELISA. (B) Effect of different pretreatment periods with H_2O_2 on IL-1β-induced IL-8 production. (C) The effect of *N*-acetyl-L-cysteine (10 mM) added 10 min before H_2O_2 pretreatment. Cells were stimulated with IL-1β 4 h after treatment and IL-8 was measured after overnight incubation. Results are expressed as means ± SEM ($n = 3$–5), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control; $^{\#}p < 0.05$ compared with IL-1β-stimulated; and $^{\dagger}p < 0.05$ compared with IL-1β plus H_2O_2.

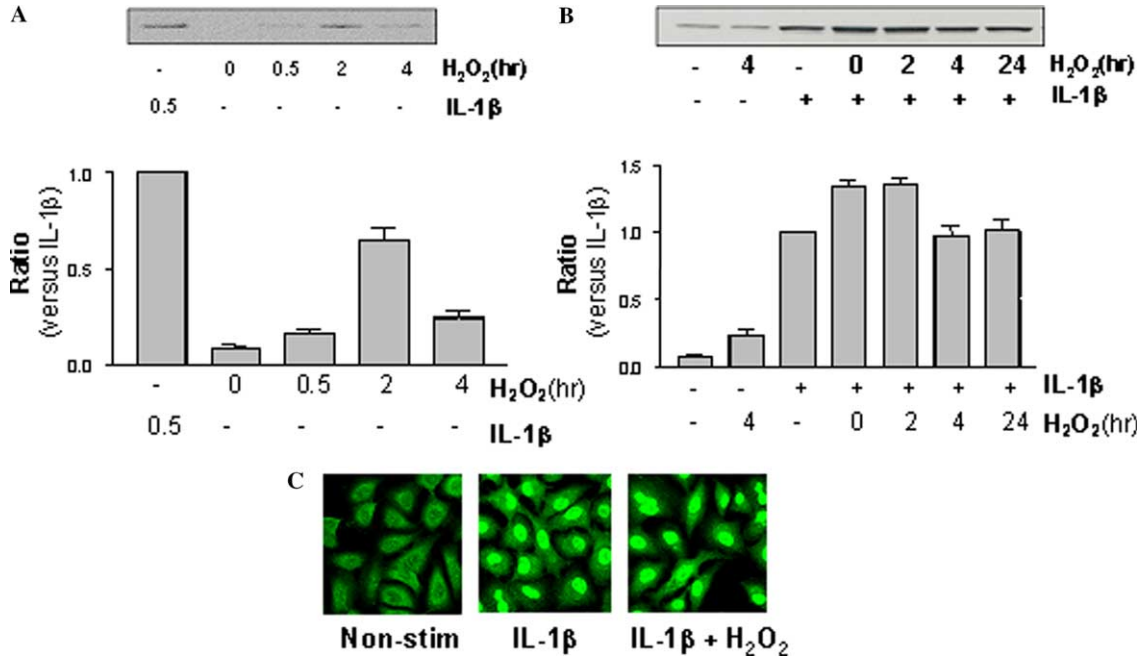

Fig. 2. H_2O_2 does not markedly enhance IL-1β-induced NF-κB nuclear translocation. (A) Representative Western blot analysis of NF-κB p65 subunit nuclear translocation in BEAS-2B cells. The cells were treated with IL-1 (1 ng/ml) or H_2O_2 (100 μM) and collected at the indicated time (h). Densitometric analysis of each band is plotted below. Results are expressed as means ± SEM as the ratio versus IL-1β alone, $n = 3$ independent experiments. (B) Representative Western blot analysis of NF-κB p65 subunit nuclear translocation in BEAS-2B cells treated with IL-1β (1 ng/ml) in the presence of H_2O_2 (100 μM). H_2O_2 was pretreated for indicated time. Cells were collected 30 min after IL-1β stimulation. Densitometric analysis of each band is plotted below. Results are expressed as means ± SEM as the ratio versus IL-1β alone. (C) Immunocytochemistry of p65 in BEAS-2B cells. Cells were pre-treated with H_2O_2 for 4 h and fixed 30 min after IL-1β stimulation.

significantly enhance IL-1β induced p65 activation measured with TransAM kit (absorbance: basal, 0.21 ± 0.045; IL-1β, 1.43 ± 0.16; and IL-1β + H_2O_2, 1.58 ± 0.16). Trichostatin A (10 ng/ml), a histone deacetylase inhibitor, also enhanced IL-1β-induced IL-8 production (2821 ± 171 vs. 1066 ± 64 ng/ml) without activation of NF-κB (data not shown). Pathways, other than NF-κB activation, must therefore be responsible for the marked elevation of IL-1β-stimulated cytokine release.

H_2O_2 enhances GM-CSF promoter-associated histone acetylation

We analyzed the effect of H_2O_2 (100 μM) on IL-1β-induced increase in histone 4 acetylation associated with the IL-8 promoter in BEAS-2B cells by quantitative chromatin immunoprecipitation. Following IL-1β treatment p65 immunoprecipitates showed a marked enrichment of IL-8 promoter (−121 to +61) DNA (Fig. 3A). Immunoprecipitation with an antibody

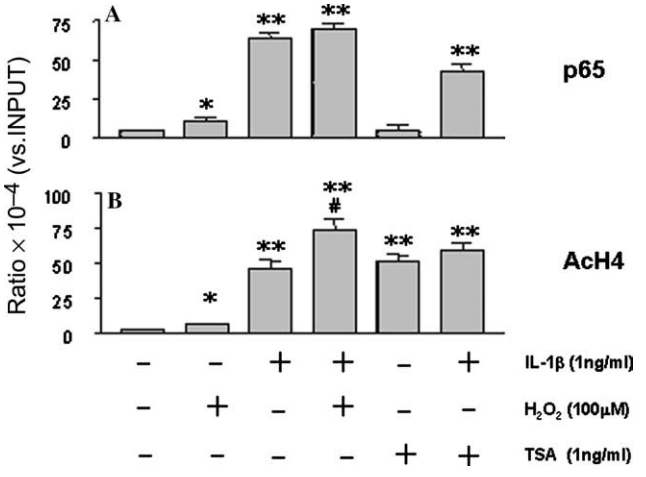

Fig. 3. Chromatin immunoprecipitation assay shows that H_2O_2 does not enhance p65 DNA binding but increases histone H4 acetylation at the IL-8 promoter. BEAS-2B cells were pretreated with H_2O_2 (100 μM) for 4 h before incubation with IL-1β (1 ng/ml). After 30 min, proteins and DNA were cross-linked by formaldehyde treatment and chromatin pellets were extracted. Following sonication, NF-κB p65 subunit (A) and acetylated histone H4 (B) were immunoprecipitated and the associated DNA was amplified by PCR. Results are representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control; $^{\#}p < 0.05$ compared with IL-1-stimulated.

against acetylated histone 4 resulted in the enrichment for the DNA segments encompassing the GM-CSF promoter following IL-1β treatment (Fig. 3B). H_2O_2 did not effect IL-1β-induced enrichment of p65-associated IL-8 promoter fragments but caused an enhancement in the enrichment of acetylated H4-associated IL-8 promoter fragments (Fig. 3). This suggests that enhanced histone acetylation at the NF-κB site is related to either enhanced HAT activity or reduced HDAC activity.

H_2O_2 represses HDAC activity and expression

Pretreatment with H_2O_2 (100 μM, 4 h) reduced basal HDAC activity (330 ± 15 versus 499 ± 91 dpm/μg protein) and inhibited IL-1β-stimulated HDAC activity (487 ± 20 versus 898 ± 64 dpm/μg protein) (Fig. 4A). This effect occurred prior to changes in HDAC2 protein expression since H_2O_2 decreased HDAC2 expression only after 24 h (Fig. 4b). This effect of H_2O_2 was concentration-dependent and reached plateau at 100 μM (Fig. 4C).

Oxidative stress inhibits HDAC activity

IL-1β induced tyrosine phosphorylation of HDAC2, which was associated with a concomitant increase of HDAC activity (data not shown). This IL-1β-induced increase in HDAC2 activity was inhibited by alkaline phosphatase pre-treatment (basal, 381 ± 84; IL-1β, 667 ± 140; IL-1β + alkaline phosphatase, 410 ± 94 dpm/μg protein).

H_2O_2 induced nitration of HDAC2 from 0.5 h after stimulation, which peaked at 4 h, and was still elevated at 24 h (Fig. 5A). Peroxynitrite (500 nM) also enhanced tyrosine nitration of HDAC2 but this did not affect basal HDAC2 activity, but inhibited IL-1β-stimulated HDAC activity (Fig. 5B). Furthermore, SIN-1 (500 μM), a peroxynitrite generator, did not affect basal HDAC activity but significantly ameliorated the IL-1β-induced HDAC2 activity (basal, 107 ± 12; SIN-1, 76 ± 8; IL-1β, 165 ± 7; and IL-1β + SIN-1, 76 ± 6 dpm/μg protein) (Fig. 5C). This correlated with the ability of SIN-1 to enhance IL-1β-induced IL-8 production (2196 ± 22 versus 1091 ± 89 ng/ml) (Fig. 5C). SIN-1 also

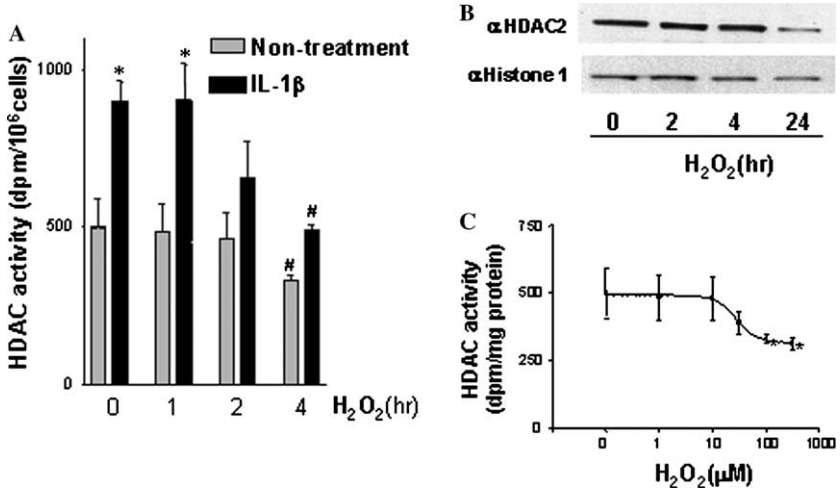

Fig. 4. Time- and concentration-dependent effect of H_2O_2 on HDAC activity and expression. (A) H_2O_2 (100 μM) directly inhibits HDAC activity in BEAS-2B cells at 4 h. Cells were collected at 1 h after IL-1β (1 ng/ml) stimulation, nuclear protein was extracted, and total HDAC activity was measured using [^{3}H]acetate incorporated histone. Results are expressed as means ± SEM for three experiments. $^{*}p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to $t = 0$. (B) Western blot analysis of HDAC2 in nuclear extracts of BEAS-2B cells shows decreased expression following H_2O_2 (100 μM) stimulation for 24 h. Histone 1 protein expression was detected as control. (C) Concentration-dependent inhibition of HDAC activity by H_2O_2 after 4 h incubation. $^{*}p < 0.05$ compared to control

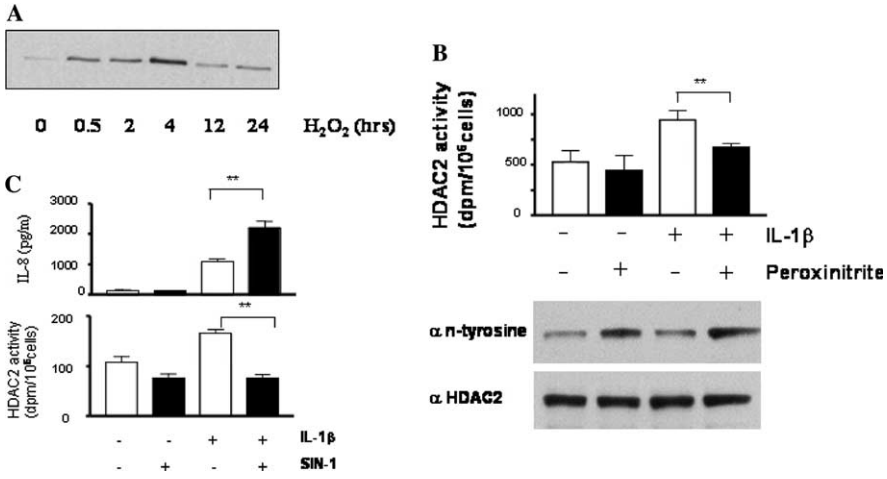

Fig. 5. H_2O_2 and peroxynitrite induce HDAC2 tyrosine nitration and suppression of HDAC activity. (A) Western blotting analysis of H_2O_2-induced tyrosine nitration of HDAC2 in BEAS-2B cells. Cells were collected after the indicated time, HDAC2 immunoprecipitated, and nitrotyrosine levels were determined. (B) Peroxynitrite directly regulates HDAC2 activity and nitration. Cells were stimulated with IL-1β (1 ng/ml) or control medium and after 1 h were collected, lysed with immunoprecipitation buffer, and immunoprecipitated with anti-HDAC2 antibody. Peroxynitrite (500 nM) was incubated with the immunoprecipitates from IL-1β-treated or non-treated cells for 10 min at 30 °C. HDAC activity assay and Western blotting for nitro-tyrosine and HDAC2 protein were performed. Results are expressed as means ± SEM ($n = 3$). $^{**}p < 0.01$ compared to IL-1β-stimulation. (C) SIN-1 (500 μM) attenuates HDAC2 immunoprecipitated HDAC2 activity and enhanced IL-8 production. Results are expressed as means ± SEM ($n = 3$). $^{**}p < 0.01$ compared to IL-1β-stimulation.

decreased HDAC1 and HDAC3 activity by 71% and 65% of IL-1β-induced HDAC activity, respectively. H_2O_2 (100 μM) and SIN-1 (500 μM) produced peroxynitrite at 3.6 ± -0.65 and 17.4 ± 0.61 μM, respectively.

Discussion

In this study we have demonstrated that H_2O_2 and peroxynitrite enhanced IL-1β-induced expression of inflammatory cytokines, such as GM-CSF and IL-8. This effect could be blocked by the anti-oxidant NAC. Using chromatin immunoprecipitation assays in cells treated with IL-1β plus H_2O_2, the IL-8 promoter region was associated with much greater levels of acetylated histone H4 than those after IL-1β stimulation alone. Under the same conditions, there was no change in p65-associated IL-8 promoter suggesting no increased NF-κB DNA binding at this time. A lack of enhancement of NF-κB activation was confirmed by Western blotting, immunocytochemistry, and DNA binding activity assay (TransAM).

H_2O_2 alone reduced HDAC2 activity after 4 h but its expression reduced only at 24 h or longer. H_2O_2 also reduced IL-1β-stimulated HDAC activity at 4 h-pretreatment and the reduction in total HDAC activity correlated with the enhancement of IL-1β-induced cytokine production. At this time point there was no reduction in HDAC expression, suggesting that a post-translational modification of HDAC is involved in attenuating HDAC activity. These data suggested that enhancement of IL-1β-induced cytokine production by H_2O_2 is not associated with NF-κB activation, but with enhancement of histone acetylation by inhibition of HDAC2 activity.

Peroxynitrite, the product of the reaction between nitric oxide (NO) and superoxide anions, is a much stronger oxidant than NO or H_2O_2. As well as having a strong cytotoxic effect [2], it is also reported to change protein function via the nitration of the *ortho*-position of tyrosine residues. For example, peroxynitrite-mediated nitration of a single tyrosine residue in the cell cycle kinase cdc2 prevents tyrosine phosphorylation in endothelial cells [16]. In addition, peroxynitrite-induced nitration of tyrosine residue of EGF receptor prevents their dimerization [17]. Peroxynitrite-mediated nitration of tyrosine residues has also been shown to inactivate the mitochondrial Mn superoxide dismutase [18], the lipid aggregatory activity of surfactant protein A [19], and glutamine synthetase activity [20]. In our system, nitration of HDAC inhibited the phosphorylation-associated increase in HDAC activity induced by IL-1β (data not shown) and confirm previous data showing that enhanced HDAC activity is associated with phosphorylation [21]. This initial increase in HDAC activity may be associated with a feedback resolution of gene transcription [22]. H_2O_2 nitrated HDAC2, which is peaked at 4 h. This correlated with the HDAC activity and enhancement of IL-1β-induced cytokine production. SIN-1, a peroxynitrite generator, gave the same effect as peroxynitrite. In these cells we obtained similar levels of peroxynitrite formation with SIN-1 and H_2O_2 possibly due to the low endogenous levels of cNOS present in the cells since iNOS is not induced until much later time points [23]. Further experiments using mass

spectrometry and peptide fingerprinting are required to determine whether nitration of distinct tyrosine residues antagonizes the enhanced HDAC activity associated with phosphorylated tyrosine residues.

Neutrophils, eosinophils, and alveolar macrophage produce ROS and nitric oxide, which interact to produce peroxynitrite. Nitro-tyrosine, which is a footprint of peroxynitrite formation, is increased in COPD and bronchial asthma [2]. Reduction in HDAC activity by oxidative stress might be one of the factors that worsen inflammatory disease.

In summary, we have shown in vitro ROS enhanced IL-1β-induced inflammatory cytokine release by reduction of HDAC activity acting via tyrosine nitration. This is a novel mechanism for the enhancement of inflammation. These studies suggest that there is a potential to develop novel therapeutic agents with improved anti-inflammatory properties that have improved HDAC activation properties and highlight the potential for anti-oxidant therapy in the treatment of chronic inflammatory diseases.

Acknowledgments

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