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The tobacco smoke component acrolein induces glucocorticoid resistant gene expression via inhibition of histone deacetylase

Matthew J. Randall[*,‡], **Guido R.M.M. Haenen**[*], **Freek G. Bouwman**[†], **Albert van der Vliet**[‡], and **Aalt Bast**[*]

[*]Department of Toxicology, Maastricht University, P.O. Box 616, 6200MD Maastricht, The Netherlands [†]Department of Human Biology, Maastricht University, P.O. Box 616, 6200MD Maastricht, The Netherlands [‡]Department of Pathology, College of Medicine, University of Vermont, 89 Beaumont Avenue, Burlington, Vermont 05405 USA

Abstract

Chronic obstructive pulmonary disease (COPD) is the leading cause of cigarette smoke-related death worldwide. Acrolein, a crucial reactive electrophile found in cigarette smoke mimics many of the toxic effects of cigarette smoke-exposure in the lung. In macrophages, cigarette smoke is known to hinder histone deacetylases (HDACs), glucocorticoid-regulated enzymes that play an important role in the pathogenesis of glucocorticoid resistant inflammation, a common feature of COPD. Thus, we hypothesize that acrolein plays a role in COPD-associated glucocorticoid resistance. To examine the role of acrolein on glucocorticoid resistance, U937 monocytes, differentiated with PMA to macrophage-like cells were treated with acrolein for 0.5 h followed by stimulation with hydrocortisone for 8 h, or treated simultaneously with LPS and hydrocortisone for 8 h without acrolein. GSH and nuclear HDAC activity were measured, or gene expression was analyzed by qPCR. Acrolein-mediated TNFα gene expression was not suppressed by hydrocortisone whereas LPS-induced TNFα expression was suppressed. Acrolein also significantly inhibited nuclear HDAC activity in macrophage-like cells. Incubation of recombinant HDAC2 with acrolein led to the formation of an HDAC2-acrolein adduct identified by mass spectrometry. Therefore, these results suggest that acrolein-induced inflammatory gene expression is resistant to suppression by the endogenous glucocorticoid, hydrocortisone.

Keywords

COPD; HDAC; acrolein; glucocorticoid; inflammation

1.0 Introduction

Chronic obstructive pulmonary disease (COPD), characterized by increased mucus production, chronic inflammation, and destruction of the lung, continues to be one of the leading causes of cigarette smoke-related death worldwide [1]. In a study by Corradi *et al*.,

Correspondence: M.J. Randall, Department of Medicine, University of Vermont, 89 Beaumont Avenue, Given Building E405, Burlington, Vermont 05405 USA, Tel: +01 802-656-8046, Fax: +01 802-847-5368, mjrandal@uvm.edu.

exhaled breath condensates and sputum from both asthmatic and COPD patients were shown to contain higher levels reactive aldehydes [2], and lung tissue from COPD patients contain elevated levels of the reactive aldehyde 4-hydroxy-2-nonenal (4-HNE) as compared to patients without COPD [3]. Whether these reactive aldehydes have a causal role in COPD development is rather poorly understood.

Cigarette smoke contains many volatile compounds which include the reactive aldehydes, crotonaldehyde and acrolein [4]. Cigarette smoke contains approximately 45 μg of acrolein per cigarette [5] and can be found as high as 90 ppm in mainstream cigarette smoke [6]. Acrolein is an α,β-unsaturated aldehyde. Acrolein is categorized as a soft electrophile due to its electrochemical polarity. The oxygen of the aldehyde moiety is electron dense thus making the α- β-unsaturated bond electron poor at the β-carbon [7] (Figure 1). As a highly reactive electrophile acrolein has high affinity for adduction with cysteine (Cys) amino acid residues [7, 8] and upon adduction the biological activity of proteins is often drastically altered [9]. Similarly to cigarette smoke, acrolein has been shown to produce several characteristics of COPD including mucus hypersecretion [10], and secretion of pro-inflammatory cytokines such as interleukin 8 (IL-8) and tumor necrosis factor α (TNFα) [11].

Glucocorticoids, a class of steroid hormones, are frequently utilized for the treatment of chronic inflammation associated with COPD. Although glucocorticoids are the main therapeutic used for COPD, many patients are resistant to the effects of or completely unaffected by inhaled synthetic glucocorticoids [12]. Furthermore, innate suppression of inflammation requires the endogenously produced glucocorticoid hydrocortisone [13]. Both the endogenous glucocorticoid hydrocortisone, and the synthetic glucocorticoids that are administered to patients with chronic inflammatory diseases including COPD, function via histone deacetylase 2 (HDAC2)-regulated epigenetic modifications to suppress pro-inflammatory gene transcription [14]. HDACs along with histone acetyltransferases are the major regulators of histone deacetylation and acetylation respectively and thereby directly regulate gene transcription [15]. Moreover it is known that HDAC2 has a significant role in COPD pathophysiology [16]. HDAC enzymes have been demonstrated as being sensitive to oxidative modification [17]. Likewise, *S*-nitrosylation of HDAC on Cys^{274} facilitated the release of HDAC from chromatin [18] and alkylation of HDACs by 4-HNE and prostaglandin 15d-PGJ_2 inhibited deacetylase activity [19].

Asthmatics who smoke cigarettes were observed to have an impaired glucocorticoid response [20]. Although cigarette smoke is known to decrease protein levels and activity of HDACs within macrophages [21, 22], the possibility that acrolein can alkylate HDAC and thereby suppress glucocorticoid responses, has not been addressed to date and may be crucial for the cigarette-smoke mediated pathogenesis of COPD. Therefore, the current study was designed to further evaluate the causal role of acrolein in glucocorticoid resistance and COPD development. Our long-term goal is to develop a more efficacious therapeutic for COPD patients.

2.0 Methods and Materials

2.1 Cell culture and treatments

U-937 monocytes (CRL-1593.2™; ATCC®) were cultured at 37°C in 95% humidified air containing 5% CO_2 using RPMI medium supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin. For experimentation, cells were differentiated to macrophage-like cells by stimulation with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 h at a density of 5×10^5 cells/ml according to previous literature [23]. Cells were seeded in 24-well plates at 1×10^6 cells/well and allowed to differentiate for 48 h. Prior to treatment, cells were serum-starved overnight in RPMI media. Acrolein was administered in 1 ml Hank's Balanced Salt Solution (HBSS) (to avoid unwanted reactions of acrolein with other constituents present in the culture media), and cells were collected after 0.5 h for GSH and deacetylase activity measurements. When indicated, cells were treated with 1 ng/ml lipopolysaccharide (LPS) or 50 nM hydrocortisone for 8 h (Figure S1).

2.2 mRNA gene expression

Eight h after LPS/hydrocortisone treatment, cells were lysed using 200 µl *QIAzol lysis Reagent* (Qiagen, Venlo, NL). RNA was isolated with chloroform by phase separation and precipitated with isopropanol. RNA was re-suspended in RNase/DNase free water and incubated at 60 °C for 10 min. Synthesis of cDNA was performed with 100-500 ng RNA using *iScript cDNA Synthesis Kit* (Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Reactions for qPCR were made with primers for IL-8 and TNFα (Eurofins MWG Operon, Ebersberg, Germany) with SYBR green mix (Bio-Rad). PCR was performed using a (iCycler system, BioRad) with 25 µl total volume. Data are expressed as relative quantitative value as normalized to β-actin gene expression.

2.3 Analysis of cellular GSH levels

Cells were lysed 0.5 h post acrolein treatment with 200 µL 100 mM potassium phosphate buffer, pH 7.5, containing 10 mM EDTA and 0.1% Triton X-100. Protein was quantified via the bicinchoninic acid assay (BCA protein assay kit; Pierce, Rockford, IL, USA). Sulfosalicylic acid (SSA) was added to a concentration of 0.6% and samples were diluted 5x. GSH analysis was determined by 5,5'-Dithiobis-(2-Nitrobenzoic acid) (DTNB) reduction as described previously [24]. Briefly, cell lysates, with and without a 1 h pre-incubation with vinyl pyridine, were incubated with glutathione reductase and DTNB for 30 s after which NADPH was added and immediately, absorbance was measured kinetically over 3 minutes at 412 nm. The absorbance values measured from the lysates without vinyl pyridine were subtracted from their counterparts with vinyl pyridine to obtain the total amount of free GSH. Glutathione concentrations were calculated based on a standard curve and results are expressed as a percent of control values.

2.4 Nuclear deacetylase activity

Nuclear extracts were made 0.5 h after acrolein treatment. Cells were washed with PBS and incubated on ice for 15 min in hypotonic buffer. Cells were scraped into Eppendorf tubes, and nuclei were isolated and lysed according to Life Technologies™ Protocols. HDAC

activity in nuclear extracts was measured by Fluor de Lys® Fluorescent Assay System according to manufacturer's protocol (HDAC Fluorimetric Cellular Activity Assay; ENZO Life Sciences, Farmingdale, NY, USA).

2.5 Mass Spectrometry

Recombinant human HDAC2 (Cat #10009377; Cayman Chemical, Ann Arbor, MI, USA), was incubated with 100 μM acrolein in 25 mM Tris-HCl (pH 7.4) for 0.5 h followed by quenching of excess acrolein with the addition of 50 mM $NaBH_4$ for 1 h on ice. Solvents were evaporated, the residue reconstituted in 50 mM NH_4HCO_3 and digested for 4 h with 1 μg Sequencing Grade Modified Trypsin and 1 μl ProteaseMax™ (Cat #V5111 and #V2071; Promega, Fitchburg, WI, USA). Samples were spotted in 2.5 mg/ml α-matrix and spectra acquired by MALDI-TOF (4800 MALDI-TOF/TOF analyzer; Applied Biosystems, Foster City, CA, USA).

2.6 Statistical analyses

Data for each group were statistically analyzed in GraphPad Prism (GraphPad Software, San Diego, CA, USA) via t-test, 1-way ANOVA, or 2-way ANOVA with Tukey or Bonferroni posteriori analysis depending on the experimental design. Significance was assigned at a maximum cut off of $p < 0.05$.

3.0 Results

3.1 Acrolein-induced inflammatory cytokine expression is unaffected by hydrocortisone

Based on previous studies which identify the ability for acrolein to cause secretion of TNFα and IL-8 [11, 25], we investigated the acrolein-mediated upregulation of TNFα and IL-8 in PMA differentiated U937 cells. As shown in Fig. 2, the control inflammatory stimulant LPS amplified mRNA expression of both TNFα and IL-8. Acrolein (30 μM) significantly increased mRNA expression of TNFα although we observed no effect on IL-8 (Figure 2). Furthermore, we assessed the ability for the endogenous glucocorticoid, hydrocortisone, to suppress inflammatory gene expression. Hydrocortisone, which per se caused insignificant decreases of baseline mRNA expression, significantly suppressed the LPS-induced expression of both TNFα and IL-8 (Figure 3). However, acrolein-induced TNFα expression was unaffected by hydrocortisone and additionally, the baseline expression of IL-8 in acrolein-treated cells was not suppressed by hydrocortisone (Figure 3). These findings indicate that acrolein can induce expression of pro-inflammatory stimuli and furthermore acrolein can prevent glucocorticoid- dependent suppression of pro-inflammatory gene expression.

3.2 Thiol reactivity of acrolein. Ability for acrolein and LPS to deplete cellular GSH

It is known that the biological effects of acrolein are largely caused by its thiol reactivity [7]. The impact of the thiol reactivity of acrolein within the macrophage-like cells was evaluated by measuring cellular GSH. Acrolein (30 μM) was shown to significantly deplete GSH to 21 ± 3.2% of control validating the relevance of its thiol reactivity in our experimental setup. Conversely, LPS had no effect on GSH levels (Figure 4). These data would suggest that unlike acrolein, LPS does not have direct thiol reactivity. Therefore, the difference in

acrolein- and LPS-mediated pro- inflammatory gene expression and effects of hydrocortisone on this gene expression is most likely due to this difference in thiol reactivity.

3.3 Acrolein decreases nuclear deacetylase activity

Having identified that acrolein prevents hydrocortisone from suppressing TNFα and IL-8 gene expression as well as significantly depletes cellular GSH levels, we further examined the potency for acrolein to inhibit the nuclear deacetylase activity within these macrophage-like cells. Following 0.5 h treatment of acrolein, significantly less deacetylase activity was measured within the nucleus of acrolein-treated cells as compared to cells receiving HBSS (Figure 5). Together this would suggest that acrolein inhibits nuclear deacetylase activity ablating the anti- inflammatory effect of hydrocortisone.

3.4 Mass Spectrometric analysis of acrolein adduction to a cysteine thiol on HDAC2

The observed thiol reactivity of acrolein combined with the presence of critical thiol groups on HDAC2 [19], a critical enzyme for the functionality of glucocorticoids [26], prompted us to identify whether acrolein directly adducts cysteine residues on HDAC2. Recombinant human HDAC2 was reacted with acrolein for 0.5 h and analyzed by MALDI-TOF mass spectrometry. The addition of acrolein (56 Da), onto a peptide was identified by an additional mass of +58 Da since the acrolein-adduct was first stabilized by reduction to the corresponding alcohol with the addition of $NaBH_4$. In comparison to unreacted HDAC2 (Figure 6A), we observed a decrease in the relative intensity of the peak with mass of 994 *m/z* after incubation with acrolein (Figure 6B). In acrolein incubated samples, the decrease in the peak with 994 *m/z* corresponded with an increase in the peak mass of 1052 *m/z* (Figure 6D), as compared to the low level of 1052 *m/z* fragment detected in the unreacted HDAC2 (Figure 6C). Identified peptides were matched to the protein sequence (51% sequence coverage) (Figure 6E). The native 994 *m/z* peptide contains one cysteine residue (indicated as bolded and colored red), Cys^{274}, which is sensitive to adduction by α,β-unsaturated aldehydes [19] and therefore, this cysteine is considered to be adducted by acrolein.

4.0 Discussion

Acrolein, an abundant and reactive aldehyde found in cigarette smoke, was found to promote secretion of pro-inflammatory cytokines [11, 25]. In line with this finding, we confirm that acrolein induces the upregulation of pro-inflammatory cytokine TNFα in PMA-differentiated U937 cells as a model for macrophages. Acrolein and crotonaldehyde, are the main thiol reacting constituents of cigarette smoke [27, 28]. It is also known that acrolein directly modifies redox proteins [9, 29, 30] modulating activity of mitogen activated protein kinases (MAPKs) [29]. Due to the role of oxidative stress in NF-κB signaling it has also been suggested that acrolein can cause NF-κB activation [31]. Conversely, LPS causes increased expression of pro-inflammatory cytokines by activation of downstream signaling via Toll-like receptor 4 [32].

The induction of TNFα gene expression by 30 μM acrolein was greater than that of the TNFα gene expression induced by 1ng/ml LPS. Although acrolein has been shown to cause secretion of IL-8 [11], our results show no effect of acrolein on IL-8 gene expression. Furthermore, acrolein has the ability to suppress pro-inflammatory signaling of MAPKs or NF-κB [33, 34] and has been shown to specifically inhibit IL-8 production via NF-κB inhibition [35]. This dichotomy regarding the ability for acrolein to activate or inhibit inflammatory pathways may lend credence to the differences we observe in acrolein mediated pro-inflammatory gene expression [36]. Overall, acrolein appears to have a direct pro-inflammatory effect whether via MAPKs or NF-κB.

LPS-induced IL-8 and TNFα expression or secretion is effectively suppressed by hydrocortisone [23, 37]. However, our results demonstrate that acrolein-induced TNFα gene expression is resistant to the suppressive effects of hydrocortisone. Moreover, the hydrocortisone-mediated suppression of baseline IL-8 mRNA expression was hindered by acrolein. Thus, the anti- inflammatory effect of hydrocortisone is impaired by acrolein.

Cigarette smoking, a direct activator of inflammation [38], also indirectly causes inflammation via both inhibition of HDAC activity and decrease of HDAC protein levels within macrophages [21, 22], known characteristics of smokers and COPD patients [16, 39]. HDAC2 has a crucial role not only in glucocorticoid function but also in glucocorticoid-resistant COPD pathogenesis [40]. HDACs are known targets for nitrosative stress [18], oxidative stress [23, 41], and also alkylation by the α,β-unsaturated aldehyde, 4-HNE [19]. In the present studies, we have identified that recombinant HDAC2 can be adducted by acrolein. The reactivity of 4-HNE and acrolein, as electrophiles, resides in the α,β unsaturated aldehyde moiety present in both molecules [7, 8]. Due to the electrophilic characteristic of acrolein, nucleophilic thiol groups predominantly found in GSH and cysteine residues of proteins, readily react via 1,4-Michael addition [42]. The modified fragment which we identified contains one cysteine (Cys) residue, Cys^{274}, which is also the expected target of 4-HNE and 15-deoxy-Δ12,14-PGJ_2 [19]. Therefore, acrolein is most likely modifying HDAC2 via adduction to Cys^{274}. We verified that acrolein, but not LPS, significantly reduced the concentration of the cellular GSH implicating the thiol reactivity of acrolein in the initiation of glucocorticoid resistance. We infer from this that acrolein not only directly activates pro-inflammatory signaling, but also displays an indirect pro-inflammatory effect via suppression of anti-inflammatory mediators such as hydrocortisone-induced HDAC activity. This has potential significance in the establishment of a chronic inflammatory state which is a key feature of COPD.

Likewise, we observe a significant decrease in deacetylase activity in these macrophage-like cells following acrolein treatment, which may correspond to the ability for acrolein to form adducts on cysteine residues within HDAC2. Although the measured deacetylase activity is non-specific for HDAC2, meaning inhibition of other HDACs or sirtuins (Class III HDACs) might also be involved. Sirtuins, like HDAC2, contain critical cysteine residues [43] which are targets of 4- HNE interaction [44] and moreover, as deacetylase regulators of inflammation are suggested to be involved in COPD [45]. Therefore, acrolein adduction and inhibition of sirtuins would strengthen the idea that acrolein inhibits glucocorticoid signaling in COPD and may potentiate chronic inflammation.

In summary, our present findings strongly imply that acrolein-mediated pro-inflammatory cytokine gene expression is resistant to the endogenous glucocorticoid, hydrocortisone. Our data, as summarized in Table S1, establish that not only does acrolein promote inflammatory gene expression, but it also decreases HDAC activity, thus sustaining the expression of pro-inflammatory cytokines despite the presence of hydrocortisone. We attribute these effects to the thiol reactivity of acrolein as demonstrated by the observed acrolein-dependent depletion of GSH, whereas LPS which elicited no effect on GSH, did not hinder the function of deacetylase activity nor exhibit an increased expression of either IL-8 or TNFα mRNA in the presence of hydrocortisone. Therefore, we suggest that acrolein plays a key role in the development of COPD by directly effecting nuclear deacetylase activity by its adduction to, and inhibition of HDAC2 thus promoting a glucocorticoid resistant, sustained inflammatory response (Figure 7). These findings could lend credence to not only the glucocorticoid resistance observed in COPD patients but also the development of the chronic inflammatory feature of COPD. Our previous study has attempted to recognize protein modifications by acrolein as a reversible electrophile signaling mechanism [46]. Further studies should be performed to identify a therapeutic compound which can repair or modulate acrolein protein adduct levels and restore glucocorticoid sensitivity in COPD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

COPD	Chronic Obstructive Pulmonary Disease
HDAC	Histone Deacetylase
PMA	Phorbol 12-myristate 13-acetate
LPS	Lipopolysaccharide
GSH	Glutathione
qPCR	quantitative polymerase chain reaction
TNFα	Tumor necrosis factor α
4-HNE	4-hydroxynonenal
IL-8	Interleukin 8
Cys	Cysteine
FBS	Fetal bovine serum
RPMI	Roswell Park Memorial Institute media
HBSS	Hanks balanced salt solution
SSA	sulfosalicylic acid
DTNB	Ellman's reagent
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight

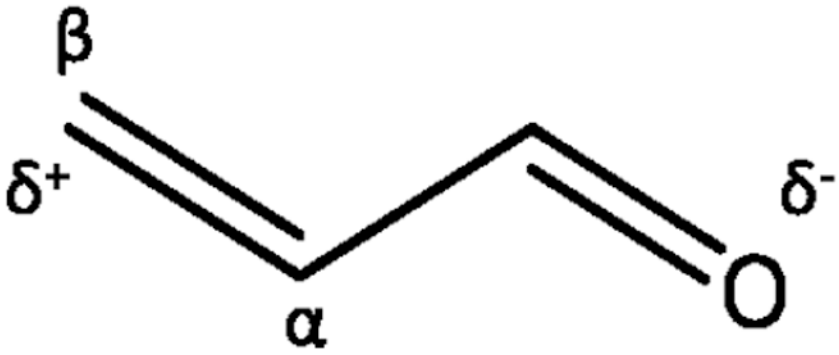


Figure 1. Acrolein skeletal structure, and electrochemical polarity

Acrolein is an α,β-unsaturated aldehyde which has a partial negative charge on the oxygen of the aldehyde moiety and a partial positive charge on the β-carbon.

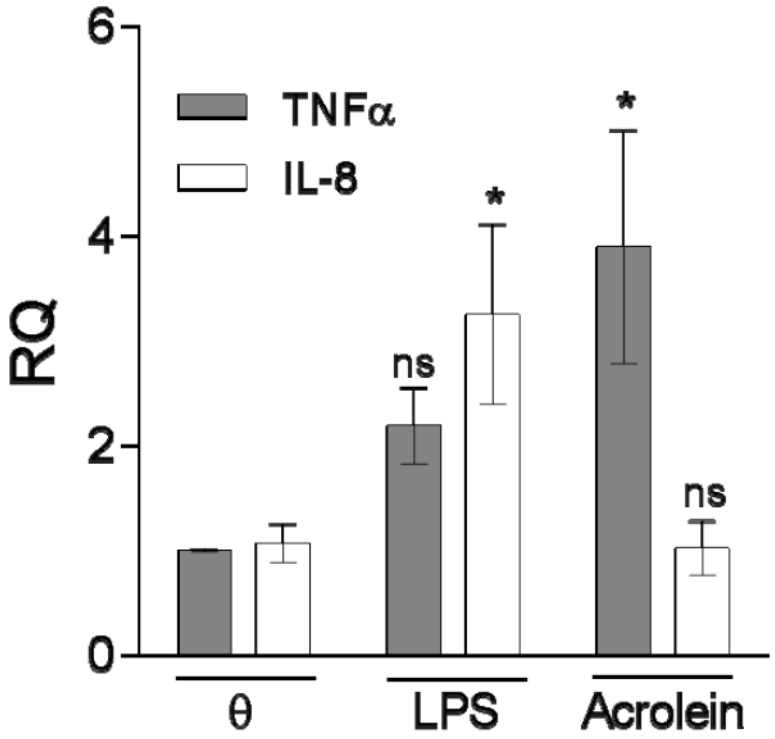


Figure 2. Acrolein promotes pro-inflammatory cytokine expression

PMA differentiated U937 monocytes were treated with either 1 ng/ml LPS (8 h) or 30 μM acrolein (8 h). PCR was performed to analyze gene induction of both IL-8 and TNFα. Data are expressed as a relative quantitative value (RQ) compared to β-actin. Data points represent mean ± SEM (n=5). *: $p < 0.05$; (ns = not significant). Significance was determined via 1-way ANOVA with Bonferroni correction. The TNFα and IL-8 data sets were analyzed separately.

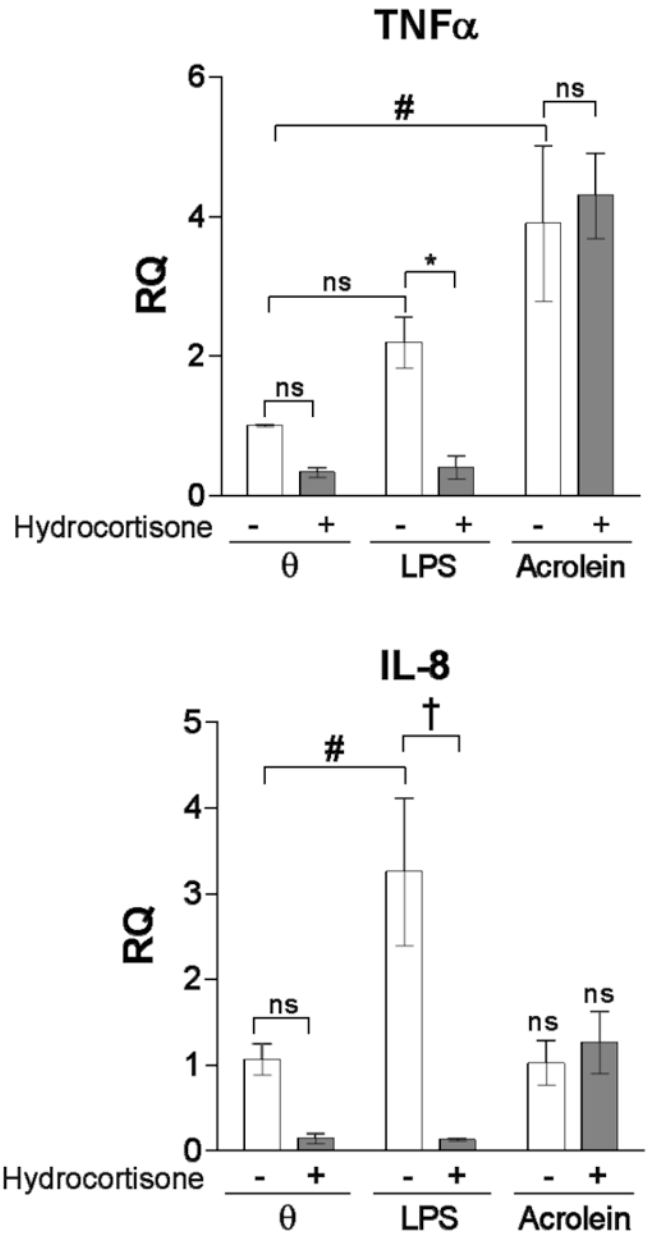


Figure 3. Effect of hydrocortisone on Acrolein-induced pro-inflammatory cytokine expression

PMA differentiated U937 monocytes were pretreated with 30 μM acrolein (0.5 h) or HBSS followed by treatment with 50 ng of hydrocortisone (8 h) or treatment of 50 nM hydrocortisone and 1 ng/ml LPS (8 h). PCR was performed to analyze the effect of hydrocortisone on LPS and acrolein induced TNFα and IL-8 expression. Data are expressed as a relative quantitative value (RQ) compared to β-actin. Data points represent mean ± SEM (n=5). *: $p < 0.05$, **: $p < 0.01$, #: p<0.001, †: p<0.0001, (ns = not significant). Significance was designated after analysis by 2-way ANOVA with Tukey correction.

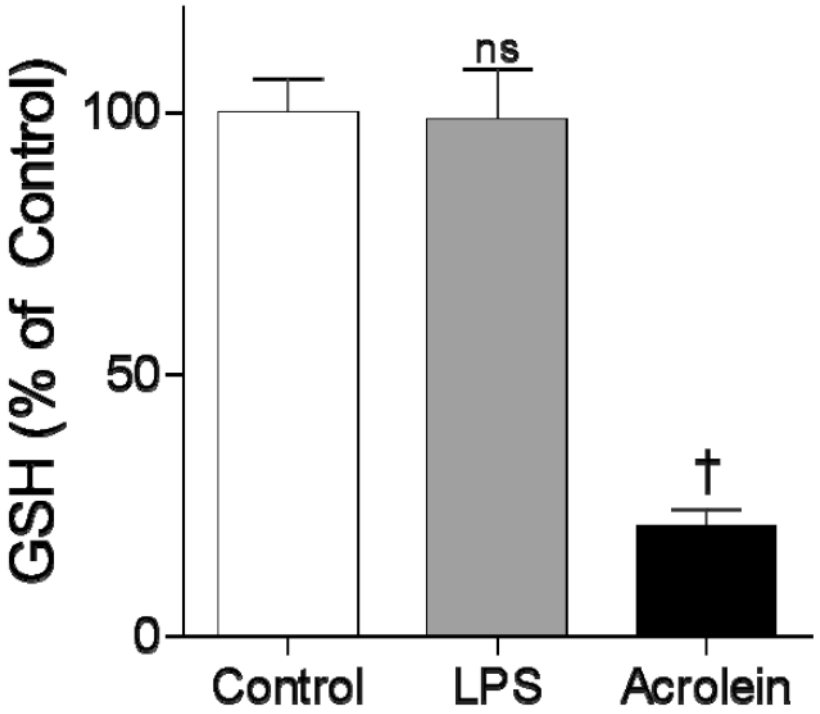


Figure 4. Effect of acrolein and LPS on cellular GSH level

GSH levels were measured in cell lysates from PMA differentiated U937 monocytes after exposure to 30 μM acrolein for 0.5 h or LPS for 1 h. Data points represent mean ± SEM (n=5). †: $p < 0.0001$, (ns = not significant). Significance was designated after analysis by 1-way ANOVA with Bonferroni correction in comparison to control.

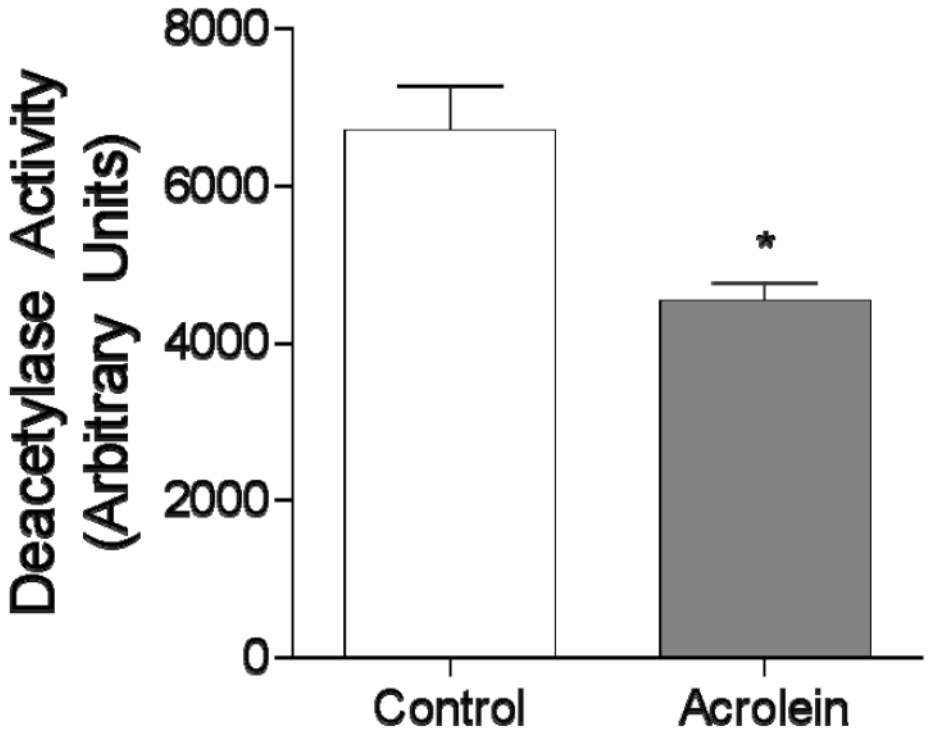
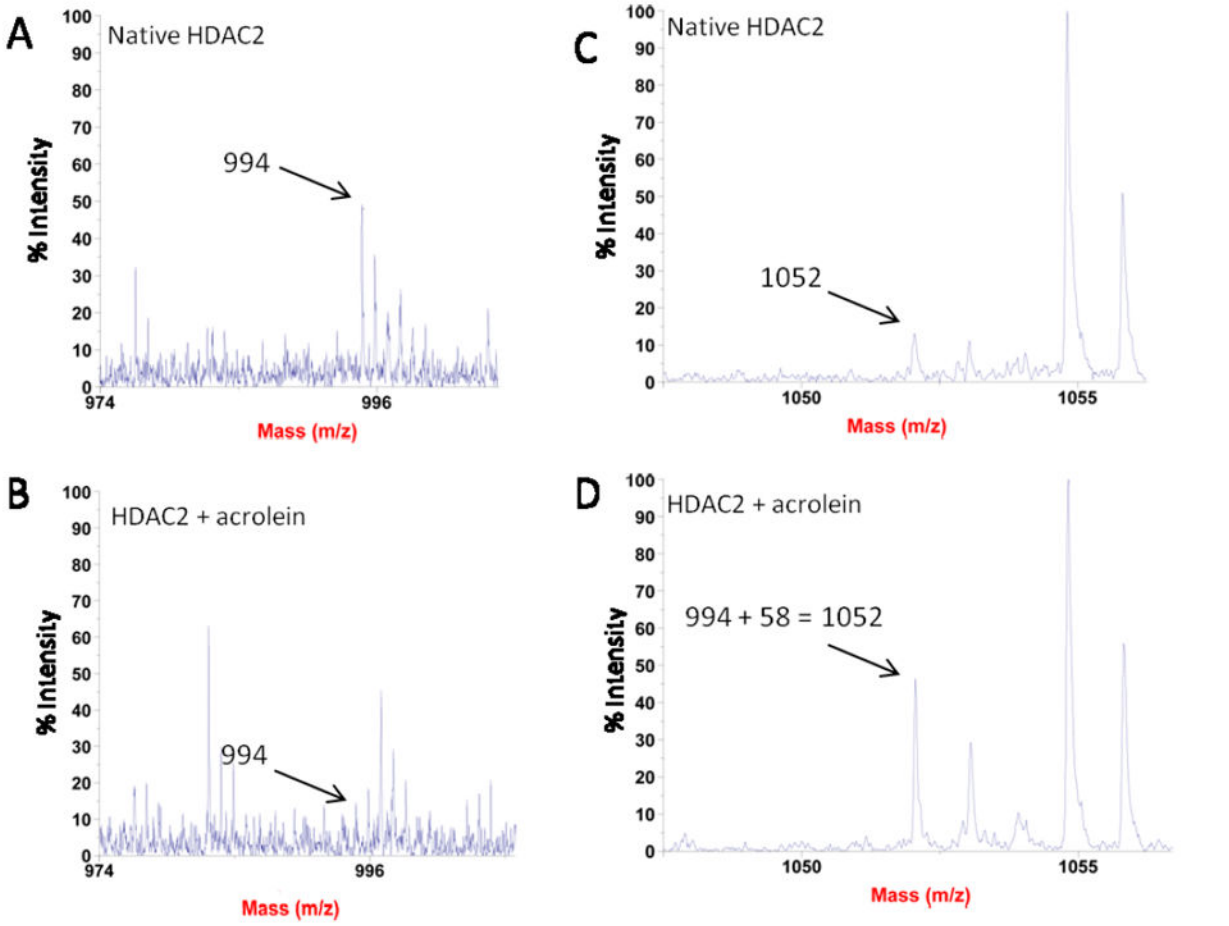


Figure 5. Effect of acrolein on nuclear deacetylase activity

PMA differentiated U937 monocytes were treated with 30 μM acrolein for 0.5 h. Nuclear extracts were used for analysis of deacetylase activity. Data points represent mean ± SEM (n=6). *: p =0.011. Significance was designated after analysis by two-tailed t-test.



E

1 M|AYSQGGGK|KK|VCYYYDGDIGNYYYGQGHPMKPHR|IR|MTHNLLLNYGLYR|KMEIYRPHK|ATAEEMTK
68 |YHSDEYIK|FLR|SIRPDNMSEYSK|QMQRFNVGEDCPVFDGLFEFCQLSTGGSVAGAVKLNR|QQTDMAVNW
137 AGGLHHAK|KSEASGFCYVNDIVLAILELLK|YHQR|VLYIDIDIHHGDGVEEAFYTTDR|VMTVSFHK|YGEYFPGT
210 GDLR|DIGAGK|GK|YYAVNFPMR|DGIDDESYGQIFKPIISK|VMEMYQPSAVVLQCGADSLSGDR|LGCFNLT
279 VK|GHAKCVEVVK|TFNLPLLMLGGGGYTIR|NVARCWTYETAVALDCEIPNELPYNDYFEYFGPDFK|LHISPSNM
352 TNQNTPEYMEK|IK|QR|LFENLR|MLPHAPGVQMQAIPEDAVHEDSGDEDGEDPDKRISIRASDKRIACDEEFSDS
425 EDEGEGGRRNVADHKKGAKKARIEEDKK|ETEDK|KTDVKEEDKSKDNSGEKTDTKGTKSEQLSNP

Figure 6. Identification of acrolein adduct on HDAC2

Recombinant HDAC2 was incubated with 30 μM acrolein for 0.5 h, and subsequently digested with trypsin for 4 h. Visualization of the 994 *m/z* peak (A, B) and 1052 *m/z* peak (C, D) in native or acrolein incubated samples. (E) Amino acid sequence of HDAC2 with matched peptides detected by MALDI-TOF/MS indicated in bold, representing 51% sequence coverage. Vertical lines indicate trypsin cleavages. Indicated in red and with increased font size, Cys^{274} indicates expected acrolein binding site.

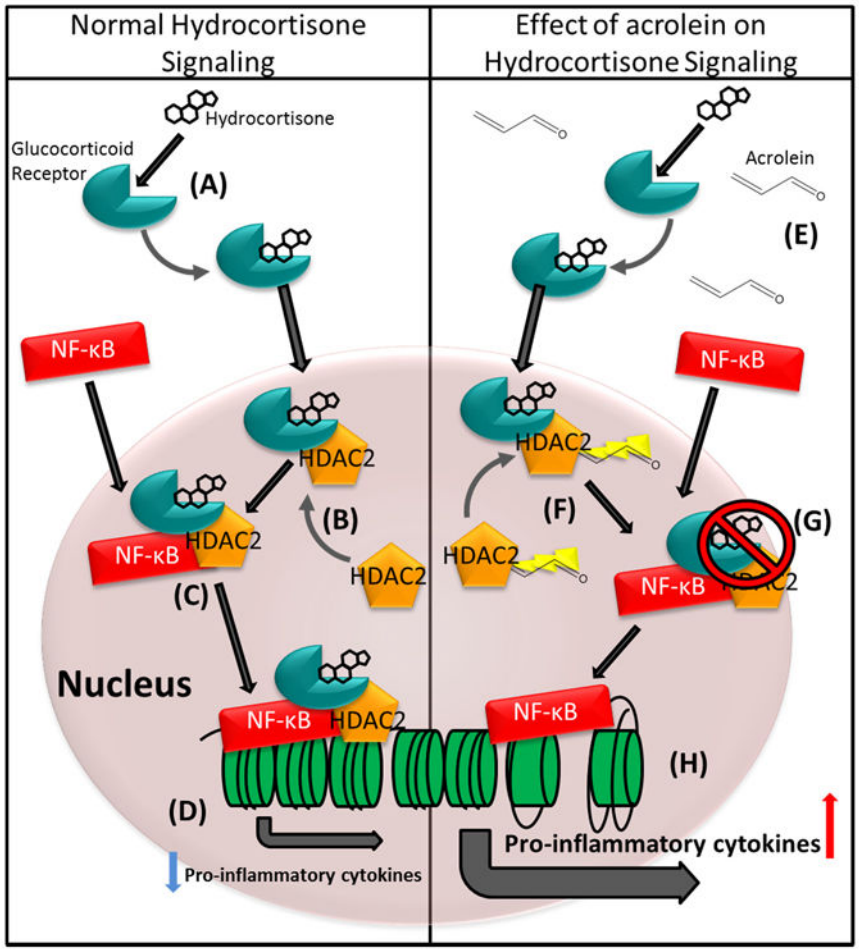


Figure 7. Mechanism of normal hydrocortisone signaling compared to our proposed mechanism by which acrolein elicits glucocorticoid resistance in macrophages

(A) Hydrocortisone ( ) functions via binding to the glucocorticoid receptor ( ) which in turn (B) recruits HDAC2 ( ). (C) This complex interacts with transcription factors such as NF- κB ( ). HDAC2 deacetylates the histones ( ) (D) thus suppressing the transcription of pro-inflammatory cytokines. We suggest that (E) upon acrolein ( ) exposure, (F) HDAC2 is targeted ( ) and consequently inhibited by acrolein which (G) disrupts ( ) glucocorticoid receptor function and (H) allows for the transcription of pro-inflammatory cytokine genes by transcription factors such as NF-κB.