Curcumin Restores Corticosteroid Function in Monocytes Exposed to Oxidants by Maintaining HDAC2

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Oxidative stress as a result of cigarette smoking is an important etiologic factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), a chronic steroid-insensitive inflammatory disease of the airways. Histone deacetylase-2 (HDAC2), a critical component of the corticosteroid anti-inflammatory action, is impaired in lungs of patients with COPD and correlates with disease severity. We demonstrate here that curcumin (diferuloylmethane), a dietary polyphenol, at nanomolar concentrations specifically restores cigarette smoke extract (CSE)- or oxidative stress–impaired HDAC2 activity and corticosteroid efficacy *in vitro* with an EC_{50} of approximately 30 nM and 200 nM, respectively. CSE caused a reduction in HDAC2 protein expression that was restored by curcumin. This decrease in HDAC2 protein expression was reversed by curcumin even in the presence of cycloheximide, a protein synthesis inhibitor. The proteasomal inhibitor, MG132, also blocked CSE-induced HDAC2 degradation, increasing the levels of ubiquitinated HDAC2. Biochemical and gene chip analysis indicated that curcumin at concentrations up to 1 μM propagates its effect via antioxidant-independent mechanisms associated with the phosphorylation-ubiquitin-proteasome pathway. Thus curcumin acts at a post-translational level by maintaining both HDAC2 activity and expression, thereby reversing steroid insensitivity induced by either CSE or oxidative stress in monocytes. Curcumin may therefore have potential to reverse steroid resistance, which is common in patients with COPD and asthma.

Keywords: cigarette smoke; corticosteroid; macrophages; chronic obstructive pulmonary disease; polyphenols

CLINICAL RELEVANCE

Curcumin, a dietary polyphenol, restores oxidative stress–impaired histone deacetylase-2 activity and corticosteroid efficacy in monocytes. Hence, curcumin has potential to reverse corticosteroid resistance, which is common in patients with chronic obstructive pulmonary disease and severe asthma.

Oxidative stress is a central feature of many inflammatory diseases and can be both an initiator and driving force of the disease (1). The resulting tissue damage that occurs as a result of oxidative stress can help drive an inflammatory response (2). In chronic obstructive pulmonary disease (COPD), oxidative stress due to cigarette smoke is considered to be the main etiologic factor in disease pathogenesis (3, 4). The disease is characterized by a chronic inflammatory response, leading to a progressive and poorly reversible airflow limitation (5) that is resistant to corticosteroid therapy (6, 7). The inflammatory response is characterized by an influx of leukocytes into the lung, in particular macrophages (8–10), as well as increases in inflammatory mediators such as TNF-α and IL-8 (11). Bronchoalveolar lavage (BAL) macrophages isolated from patients with COPD also display resistance to corticosteroid-mediated suppression of inflammation (12, 13). This apparent steroid insensitivity can also be induced in U937 monocytes and A549 epithelial cells exposed to oxidative stress (14).

Corticosteroids are considered to be among the most effective anti-inflammatories in clinical use at present. The suppression of pro-inflammatory gene expression by the glucocorticosteroid receptor (GR) has been shown to require the recruitment of the transcriptional co-repressor HDAC2 into an activated GR complex, referred to as transrepression (15–18). In contrast, the anti-inflammatory activity of corticosteroids was not dependent on GR-mediated gene expression through GR-DNA binding via the glucocorticoid response element, otherwise known as transactivation (19). Subsequent *in vivo* studies by Reichardt and coworkers (20) using the transgenic GR^{dim} mouse supported this hypothesis. HDAC2 is one of 18 isoforms within the HDAC family (21, 22). A common feature of HDACs is the ability to remove acetyl moieties from the ε-acetoamido group on lysine residues of acetylated proteins, such as histones (21). In general, this results in condensation of the chromatin structure through tighter winding of the DNA around the core histones. This displaces the transcriptional machinery and occludes further transcription factor binding, thereby resulting in gene silencing. In contrast, histone acetylation by histone acetyltransferases (HATs) disrupts the attractive electrostatic interaction between the DNA and histones. This leads to the unwrapping of the DNA from the core histones, allowing access for the transcriptional machinery, resulting in gene transcription (23, 24). HDAC2 is proposed to play a central role in gene repression by steroids where the steroid receptor recruits HDAC2, which in turn becomes associated into the NF-κB transcriptome complex, thereby specifically shutting off pro-inflammatory gene expression (15, 25, 26). Oxidative stress inhibits HDAC2 activity (27, 28), and chronic oxidative stress, as seen in the lungs of patients with COPD, caused both reduced HDAC2 activity and expression (29), thereby blocking steroid efficacy (14). Furthermore,

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inhibition of HDAC2 enhances pro-inflammatory gene expression (27, 30) by tilting the HDAC/HAT balance in favor of greater histone acetylation, opening up the chromatin structure to allow more pro-inflammatory transcription factor DNA binding. Thus, agents that restore HDAC2 may prove to be useful in restoring steroid efficacy and hence inflammatory response.

Curcumin, a dietary polyphenol, is the active constituent from the *Curcuma longa* plant, commonly known as turmeric. It has been reported to have both anti-cancer and anti-inflammatory properties (31–33) and inhibits a wide range of inflammatory and signaling molecules (34–38). Curcumin can also inhibit the formation of lipid-derived inflammatory mediators, such as leukotrienes, through the inhibition of PLA2, COX-2, and 5-LOX activity *in vitro* (39). Due to its polyphenolic structure, curcumin also exhibits antioxidant activity and is an effective scavenger of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) (40, 41). More recently, curcumin has been demonstrated to induce antioxidant defenses through increases in glutathione production (38), most likely as a result of induction of Nrf2-mediated glutamate-cysteine ligase transcription (42). Similarly, expression of phase II enzymes such as glutathione-S-transferase is also induced by curcumin (43). At the level of chromatin, two studies have shown that curcumin inhibits HAT activity with no apparent impact on HDAC activity (44, 45). However, neither study determined the impact of curcumin on chromatin modifications in inflammatory cells subjected to oxidative stress. Here we investigated whether curcumin had any effect on HDAC2 in oxidatively stressed monocytes, and thus have the potential to restore corticosteroid efficacy. Therefore, we studied the mechanism of action of curcumin on HDAC2 due to its antioxidant or free radical scavenging properties and/or post-translational impact on prevention of phosphorylation-ubiquitination-proteosomal degradation of HDAC2.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Aldrich, Inc. (St. Louis, MO). Hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS), ionomycin, CGS 2180, cycloheximide, dimethylsulfoxide (DMSO), protein A agarose, 3-(4,5-dimethylthiazol-2-yr)-2-5-diphenyltetrazolium bromide (MTT), pyrogallol, xanthine, xanthine oxidase, menadione and Hanks' Balanced Salt Solution (HBSS) were purchased from Sigma Aldrich (Poole, Dorset, UK). Research-grade cigarettes (Reference code 2R1/1R3F) were obtained from the University of Kentucky (Lexington, KY). Phorbol-12-myristate-13-acetate (PMA), CHAPS, and trichostatin A (TSA), were purchased from Merck Biosciences (Boulevard Industrial Park, Beeston, Nottingham, UK). Anti-HDAC1, anti–phospho-serine, and anti-ubiquitin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-acrolein and anti-4-hydroxy-2-nonenal (4-HNE) (carbonyl) was prepared as described previously (28, 49). Curcumin was purchased from Biomol (Affiniti Research Products, Exeter, UK). The PDE4 inhibitor (roflumilast) was obtained from Qventas (Branford, CT) and the anti-inflammatory corticosteroid (budesonide) from Sigma Aldrich. The fluorometric HDAC activity assay kit was purchased from Biovision (Mountain View, CA). MG-132, the proteasome inhibitor, was purchased from Calbiochem (La Jolla, CA). TNF-α was obtained from R&D Systems Europe (Abingdon, Oxfordshire, UK). Tempone-H-HCl was sourced from Axxora Biochemicals (San Diego, CA). Microarray gene chips were purchased from Affymetrix (Santa Clara, CA).

Electron Paramagnetic Resonance Spectroscopic Assessment of Antioxidant Properties

For these experiments, curcumin was made up at 100 mM in ethanol, diluted to 1 mM in ethanol and further dilution to the desired working concentration was made in HBSS. Xanthine was made up at 10 mM in 0.01 M NaOH, and menadione was made at 100 mM in DMSO. Both compounds were subsequently diluted to a final concentration of 100 μM in HBSS. Pyrogallol, xanthine oxidase, iron III (Fe^{3+}) chloride and hydrogen peroxide were all dissolved and diluted in HBSS. Electron paramagnetic resonance (EPR) measurements were made for a number of different radical generating systems (pyrogallol, 100 μM; xanthine/xanthine oxidase, 100 μM and 100 mU/ml; menadione, 50 μM; or iron [III] chloride + hydrogen peroxide, 50 μM + 10 μM, respectively) in the presence of a well-recognized spin trap (tempone-H; 1 mM) and the presence or absence of curcumin (1 nM–100 μM) after incubations at 1, 4, and 24 hours (37°C) using a benchtop EPR spectrometer (MS200 X-Band Spectrometer; 9.30–9.55 GHz microwave frequency; Magnettech GmbH, Berlin, Germany) set with the following parameters: B_0 Field, 3,365 Gauss; sweep, 50 Gauss; sweep time, 30 s; modulation, 1,500 mG; microwave power, 20 mW. Formation of the spin-adduct (4-oxo-tempo) by oxidizing radical species generates a characteristic three-line spectrum centered at approximately 3,365 Gauss, and the amplitude of the signal (arbitrary units) is proportional to the concentration of the adduct formed.

Preparation of Aqueous Cigarette Smoke Extract

Research-grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky. The composition of 1R3F research-grade cigarettes was: total particulate matter, 17.1 mg/cigarette; tar, 15 mg/cigarette; and nicotine, 1.16 mg/cigarette. Cigarette smoke extract (CSE, 10%) was prepared by bubbling smoke from one cigarette into 10 ml of culture medium at a rate of one cigarette every 2 minutes as described previously (46), using a modification of the method described earlier by Carp and Janoff (47). CSE preparation was standardized by measuring the absorbance (OD 0.76 ± 0.05) at a wavelength of 320 nm. The absorption spectrum observed at λ_{320} showed very little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture medium.

Cell Culture and Treatments

The human monocytic cell line (U937) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in complete growth medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. U937 were differentiated into an adherent "macrophage-like" morphology by exposure to PMA (40 ng/ml) for 4 hours in complete growth medium at 37°C. Cells were harvested by centrifugation (1,200 rpm, 4 min at 18°C), resuspended in fresh complete growth medium, and then subcultured into either in 96-, 12-, or 6-well culture plates (Corning, NY) at 0.2×10^6, 1×10^6, or 2×10^6/well, respectively, and kept at 37°C for a further 48 hours. The status of differentiation or adherence was assessed under light microscopy. In most cases, over 75% of the total population adhered to the surface with a distinctive altered morphology (macrophage-like). Cell toxicity was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell viability was assessed by measuring LDH release using cytotoxicity detection kit (Roche, Indianapolis, IN). After differentiation, cells were starved overnight in phenol red–free RPMI 1640 medium with 0.5% FCS. The cells were then subjected to oxidative stress for 4 hours using either H_2O_2 (100 μM) or CSE (1%) in phenol red–free RPMI-1640 medium only. The medium was then replaced and incubated with/without test compounds (curcumin 1 nM–10 μM; trichostatin A 100 nM, cycloheximide 10 μg/ml). MG132 was treated for 30 minutes and 4 hours before and after the oxidative insult, respectively. For cell-based functional assays, the cells were subsequently treated with corticosteroid in the presence or absence of LPS (10 ng/ml) for a further 18 hours. TNF-α and IL-8 release was measured by sandwich enzyme-linked immunosorbent assay (R&D Systems) in the culture supernatants. After compound treatment for up to 18 hours, the cells were harvested and cellular protein or RNA extracted as described below.

Cell Lysis, Immunoprecipitation, HDAC Activity Assay, and Western Blotting

All details are as previously described (28). Briefly, cell protein extracts were prepared using modified RIPA buffer (50 mM Tris HCL pH7.4,

150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1% CHAPS, 1 mM EDTA with freshly added complete protease and phosphatase inhibitor cocktail II (Calbiochem). Protein concentration was determined using the Pierce BCA protein assay kit (Rockford, IL). Immunoprecipitation was conducted with anti-HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HDAC2 (Abcam, Cambridge, UK) antibodies. HDAC activity on total cell lysates or immunoprecipitates was assessed using a commercial fluorometric assay kit (Biovision, Mountain View, CA). RIPA cell lysates or immunoprecipitates were subjected to western blot after SDS-PAGE using mouse monoclonal anti-HDAC2 (Abcam). Alternatively, to determine post-translational modifications of HDAC2, blots were probed with either anti–phospho-serine, anti-acrolein, anti−4-HNE, or anti-ubiquitin antibodies. Blots were reprobed by stripping with Chemicon Re-Blot Plus western recycling kit (Chemicon International, Temecula, CA), blocked, and then reprobed with the appropriate antibody.

Microarray Gene Chip Analysis

U937 differentiated cells, either untreated or ROS exposed as described above, were treated with curcumin (1 μM) for either 4 or 18 hours. The cells were harvested and total RNA extracted using RNeasy (Promega, Madison, WI). RNA integrity and yield were analyzed and quantified using the Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). Preparation of cDNA, hybridization, and scanning of the HG-U133 Plus2.0 GeneChip oligonucleotide arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). GeneChip images were quantified and gene expression values were calculated by Affymetrix Microarray suite version 5.0 (MAS 5.0; Affymetrix). Normalization and downstream analysis was performed using Genespring 7.2 (Agilent Technologies). Probesets that were absent and/or had a raw expression signal less than 100 in all samples were removed. Statistically significant genes were identified

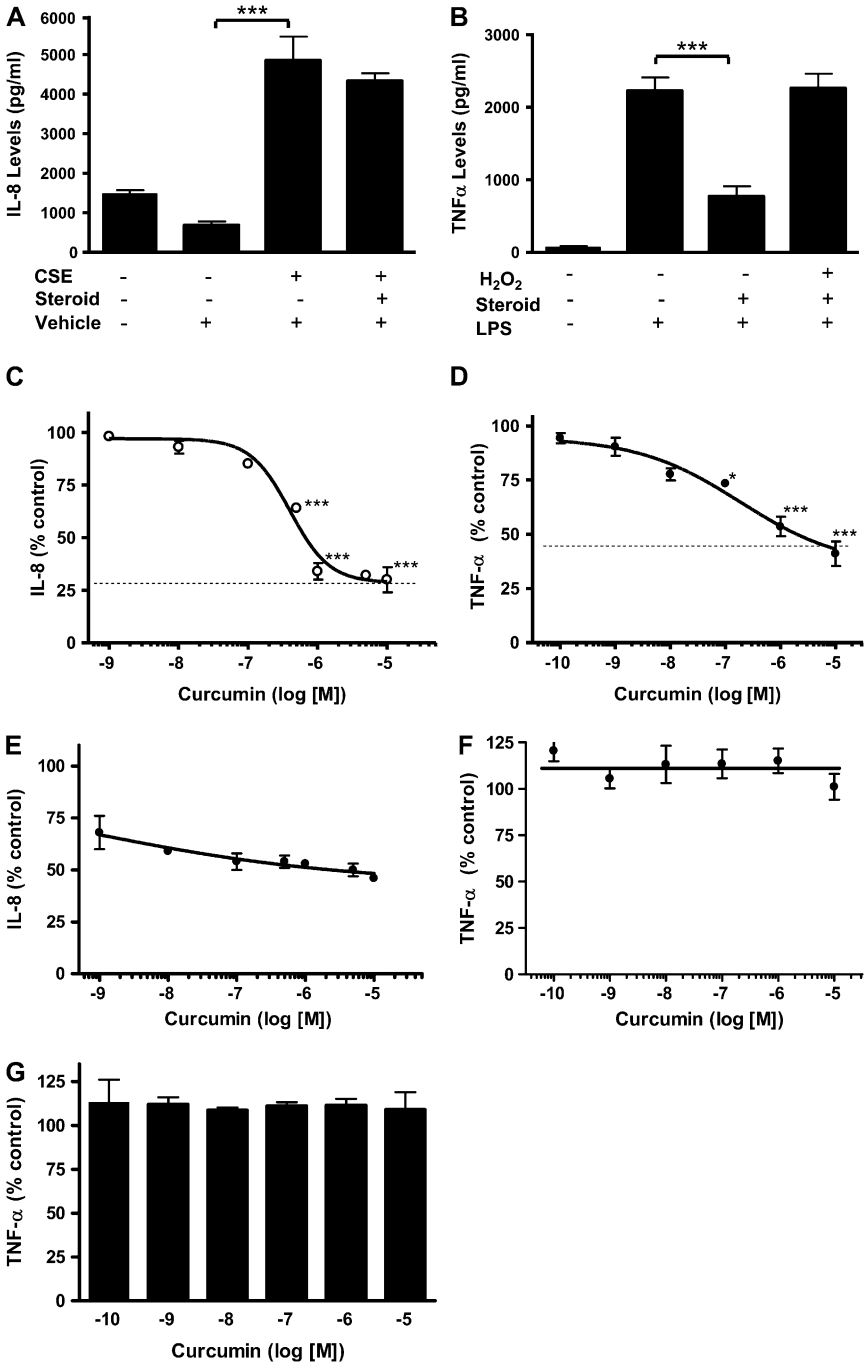

Figure 1. Curcumin restores reactive oxygen species (ROS)- and cigarette smoke extract (CSE)-impaired corticosteroid efficacy by inhibiting the pro-inflammatory cytokines in human monocytes. Phorbol-12-myristate-13-acetate (PMA)-differentiated U937 cells were stressed for 4 hours with CSE (1%), then left for a further 18 hours (*A*, *C*, and *E*), or with H_2O_2 (100 μM) followed by LPS (10 ng/ml) for 18 hours (*B*, *D*, *F*, and *G*). Immediately after ROS and CSE exposures, the cells were treated with increasing concentrations of curcumin alone (*E* and *F*) or in combination with corticosteroid; dexamethasone, 100 nM (*C*) or budesonide, 1 nM (*D* and *G*). As internal controls for *C* and *D*, *dotted horizontal lines* represent cytokine levels in naïve cells or LPS-treated naïve cells in the presence of budesonide (1 nM), respectively. ROS-stressed cells were also treated with a curcumin-budesonide combination in the presence of trichostatin A (TSA), 100 nM (*G*). TNF-α and IL-8 release was evaluated by enzyme-linked immunosorbent assay as described in Materials and Methods. The data are displayed as the mean ± SEM of at least three independent experiments, except for *G*, where the data represent the mean ± SEM for duplicate experiments. Where indicated the data is normalized against control; cells stimulated with CSE only (*C* and *E*) or cells treated with LPS only (*D*, *F*, and *G*). $*P < 0.05$, $***P < 0.001$ versus control using ANOVA with Bonferroni *post hoc* analysis. Vehicle, DMSO.

using a 1.8-fold change cutoff and a P value of < 0.05 (Welch's t test, parametric with variances assumed not equal). Differentially expressed genes from naïve versus ROS (H_2O_2, 100 μM)-exposed cells with/without subsequent curcumin treatment were used to generate hierarchical clustering plots using Pearson's correlation and displayed as heatmaps. Gene chip data have been deposited with the GEO database at the NCBI with accession number GSE10896.

Data and Statistical Analysis

Data points were plotted as the mean ± SEM of "n" independent experiments. Concentration–response curves were analyzed by least square non linear regression using "Prism" curve fitting software (Graph Pad, San Diego, CA). Statistical analysis was conducted using one- or two-way ANOVA with Dunnett's or Bonferroni *post hoc* analysis, as appropriate. For the gene chip arrays, Welsh t test was employed to determine significance. $P < 0.05$ was considered significant.

RESULTS

Curcumin Restores CSE- and Oxidant-Induced Steroid Insensitivity

We determined the anti-inflammatory efficacy of corticosteroids on the pro-inflammatory effects of exposure to CSE and a potent oxidant hydrogen peroxide (H_2O_2) in monocytic cells (human monocytic cell line, U937). Exposure of U937 cells to either CSE or H_2O_2 resulted in an inability of corticosteroid to suppress the ensuing pro-inflammatory response (Figures 1A and 1B). CSE (1%) exposure caused a significant ($P < 0.01$) increase in IL-8 release from U937 cells (Figure 1A). Subsequent treatment of the CSE exposed cells for 18 hours with dexamethasone failed to suppress the IL-8 release. In contrast, when U937 cells were exposed to LPS alone, the corticosteroid budesonide was able to significantly inhibit pro-inflammatory mediator release as measured by TNF-α (Figure 1B). However, pre-exposure of the differentiated U937 cells to oxidative stress in the form of H_2O_2 before LPS treatment led to an inability of budesonide to suppress LPS induced TNF-α release to levels observed with budesonide on LPS treatment alone (Figure 1B). Interestingly, H_2O_2 treatment alone did not have much effect on TNF-α release and moreover, when H_2O_2 was used in conjunction with LPS there was a small enhancement in TNF-α release over that for LPS stimulation alone (data not shown). All these treatments did not show any significant cytotoxic effect as measured by LDH release and MTT assay.

We next investigated whether curcumin could potentiate or restore the impaired anti-inflammatory efficacy of corticosteroid in cells exposed to oxidative stress. U937 cells were pre-exposed to CSE, and then treated with increasing concentrations of curcumin, either in the presence (Figure 1C) or absence (Figure 1E) of corticosteroid (dexamethasone). Alternatively, U937 cells were pre-exposed to H_2O_2, then treated with various concentration of curcumin in the presence (Figure 1D) or absence (Figure 1F) of corticosteroid (budesonide) followed by LPS (10 ng/ml) stimulation for 18 h (Figures 1D and 1F). In both the CSE and H_2O_2 exposure systems, curcumin showed a concentration-dependent restoration of corticosteroid-mediated suppression of pro-inflammatory cytokine release with an EC_{50} of between 200 and 300 nM (Figures 1C and 1D). In the case of the CSE system (Figure 1C), the ability of corticosteroid in the presence of curcumin to suppress the inflammatory IL-8 response was virtually complete, with the resulting IL-8 levels similar to that for unstimulated cells. Similarly, in the H_2O_2–LPS system, curcumin again restored the ability of corticosteroid to suppress the LPS-induced TNF-α release to levels observed in naïve unstressed cells (*dotted horizontal line* in Figure 1D). In contrast, in both ROS exposure systems (CSE and H_2O_2), curcumin in the absence of corticosteroid did not show a significant concentration-dependent inhibition in pro-inflammatory mediator release (Figures 1E and 1F). However, in the case of CSE (Figure 1E) there is clearly an overall anti-inflammatory shift throughout the concentration range studied relative to CSE alone (100% control). In Figure 1G, addition of trichostatin A, a specific and potent HDAC inhibitor, completely removed the concentration-dependent restoration of corticosteroid efficacy by curcumin observed in Figure 1D. Coupled with the impact curcumin has on restoring HDAC2 activity (Figure 2), this was suggestive that restoration of corticosteroid efficacy by curcumin was indeed HDAC2 dependent. We also used another human non–PMA-stimulated macrophage cell line, MonoMac6 (49), to confirm the results, which also shown similar response as described for U937 cells (data not shown).

Effect of Curcumin on Total and Isoform-Specific HDAC Activity

Treatment of CSE- or H_2O_2-stressed U937 cells with curcumin resulted in a restoration of total HDAC activity compared with un-stressed cells alone (Figure 2A). Initially, exposure to either CSE (1%) or H_2O_2 (100 μM) for 4 hours, significantly ($P < 0.01$) reduced total HDAC activity by 40% relative to untreated cells (Figure 2A), whereas CSE or H_2O_2 exposure did not show

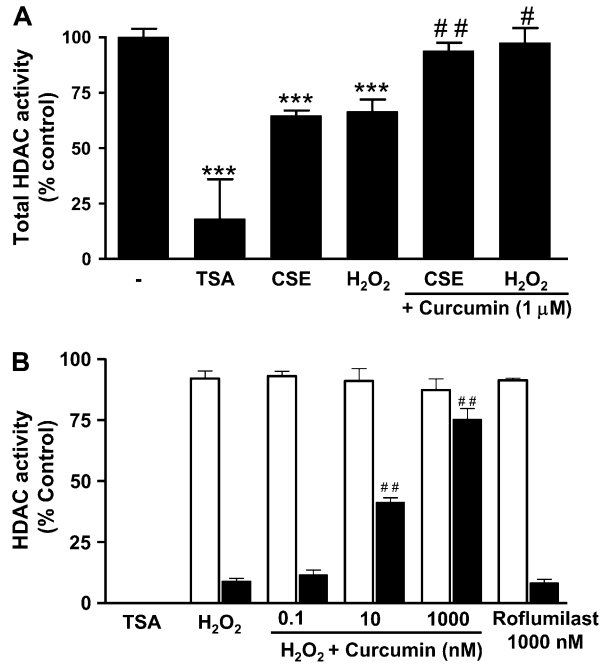

Figure 2. Curcumin restores ROS-impaired histone deacetylase (HDAC)2 in human monocytes. (*A*) ROS- and CSE-stressed (100 μM H_2O_2 or 1% CSE for 4 h) U937 were treated with and without curcumin (1 μM) for 18 hours at 37°C before measuring total cellular HDAC activity. (*B*) Isoform-specific HDAC activity was assessed in immunoprecipitates of HDAC1 (*open bars*) and HDAC2 (*solid bars*) from lysates of U937 cells that had been pre-exposed to H_2O_2 (100 μM) for 4 hours, followed by treatment with curcumin as indicated. HDAC activity is displayed as the mean ± SEM of at least three independent experiments and normalized against control (naïve untreated cells). The PDE4 inhibitor (roflumilast) was used in parallel as a negative control to validate the action of curcumin and was unable to restore HDAC2 activity. ***$P < 0.001$ versus control. #$P < 0.05$, ##$P < 0.01$ versus H_2O_2-/CSE-stressed cells only.

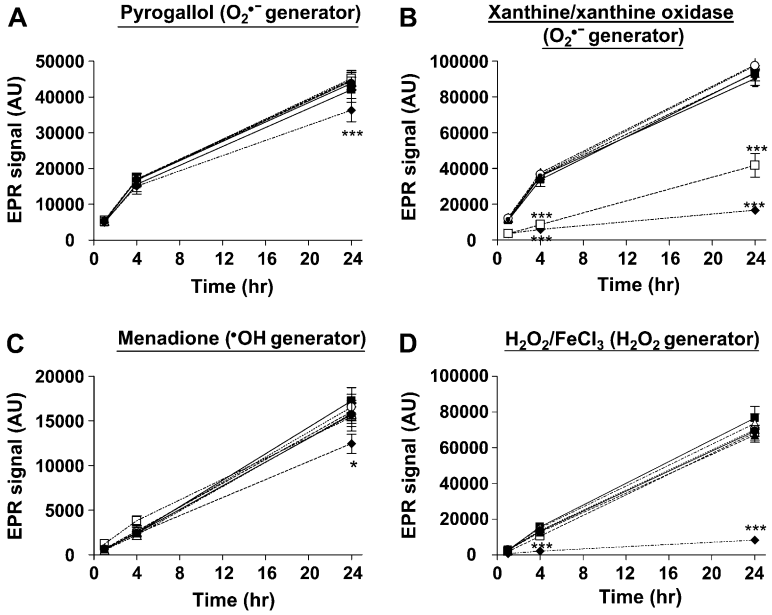

Figure 3. Evaluation of the antioxidant/radical quenching properties of curcumin by electron spin resonance spectroscopy. Curcumin at various concentrations (1 nM to 100 μM) was mixed with four different free radical electron–generating systems: (*A*) pyrogallol, (*B*) xanthine/xanthine oxidase, (*C*) menadione, and (*D*) $H_2O_2/FeCl_3$. The antioxidant/radical quenching capacity was evaluated by measuring the electron paramagnetic resonance (EPR) signals (arbitrary units, AU) due to oxidation of Tempone-H (1 mM). Decreased EPR signals show increased antioxidant capacity and vice versa. The data shown is the mean ± SEM for six experiments. *$P <$ 0.05, ***$P < 0.001$ versus control. Curcumin concentrations are shown with the following symbols: *solid diamonds*, 100 μM; *open squares*, 10 μM; *solid squares*, 1 μM; *open triangles*, 100 nM; *solid triangles*, 10 nM; *open circles*; 1 nM; *solid circles*, no curcumin.

any significant change in cytotoxicity as measured by LDH release (data not shown). As a positive control, the HDAC inhibitor TSA inhibited total HDAC activity by as much as 85%. However, when either the CSE or H_2O_2 exposed cells were then treated with curcumin (1 μM), there was a significant increase in total HDAC activity returning back to pre-oxidant exposure levels (Figure 2A). The impact of curcumin on HDAC activity was unique to CSE- or H_2O_2-exposed cells, as naïve cells treated with curcumin had no impact on HDAC activity (data not shown).

As HDAC2 has been shown to be an essential co-factor for the anti-inflammatory efficacy of corticosteroids, we investigated the impact of curcumin on HDAC2 activity and a close isoform of HDAC2, namely HDAC1, which contains 83% identity (21). Total cellular lysates derived from curcumin-treated U937 cells with/without pre-oxidant stress were subjected to immunoprecipitation with anti-HDAC1 or anti-HDAC2 polyclonal antibodies. The resulting immunoprecipitates were then used for the measurement of isoform-specific HDAC activity as described earlier in MATERIALS AND METHODS. The results displayed in

TABLE 1. EARLY PHASE (4 h) CURCUMIN-REGULATED GENES WITH KNOWN FUNCTION

Accession Number	Gene Name	Biological Function	Cell Status	Expression Status
AI217992	Pleckstrin homology domain containing, family H member 2	Cell adhesion	Naïve	↑
AI770005	Polycystic kidney and hepatic disease 1 (autosomal recessive)	Cell adhesion	Naïve	↑
W72626	Boc homolog (mouse)	Cell adhesion	ROS	↑
S78505	Prolactin receptor	Cell signaling	Naïve	↑
AV700865	SET-binding factor 2	Cell signaling	Naïve	↑
AW974499	Rho GTPase–activating protein 30	Cell signaling	Naïve	↓
BE673800	Family with sequence similarity 80, member A	Cell signaling	ROS	↑
AL512701	Protein kinase C, eta	Cell signaling	ROS	↑
AK021928	RAB3 GTPase activating protein subunit 2 (noncatalytic)	Cell signaling	ROS	↓
BC040296	Exocyst complex component 4	Intacellular transport	Naïve	↑
BC042091	Sec1 family domain containing 2	Intacellular transport	Naïve	↓
NM_003759	Solute carrier family 4, sodium bicarbonate cotransporter, member 4	Intacellular transport	ROS	↑
BE818251	ATPase, Class II, type 9B	Intacellular transport	ROS	↓
BG222394	Mitogen-activated protein kinase 8 interacting protein 1	Intacellular transport	ROS	↓
BC015196	Geranylgeranyl diphosphate synthase 1	Metabolic pathways	Naïve	↑
AW390231	Hypothetical protein KIAA1434	Metabolic pathways	Naïve	↑
BC011938	TGFB1-induced anti-apoptotic factor 1	Metabolic pathways	Naïve	↑
NM_004795	Klotho	Metabolic pathways	ROS	↑
AW022496	Zinc finger, A20 domain containing 1	Protein degradation	Naïve	↓
AI742722	Ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	Protein degradation	ROS	↓
AK093340	STAM-binding protein-like 1	Protein degradation	ROS	↓
BC017989	Similar to ribosomal protein L31	Gene regulation	Naïve	↑
NM_002147	Homeobox B5	Gene regulation	Naïve	↑
AK024514	Suppressor of zeste 12 homolog (*Drosophila*)	Gene regulation	Naïve	↓
AF007135	Jumonji, AT rich interactive domain 1A (RBBP2-like)	Gene regulation	ROS	↓
NM_014258	Synaptonemal complex protein 2	Cell cycle/Apoptosis	Naïve	↑
AK024940	Tumor necrosis factor, alpha-induced protein 8	Cell cycle/Apoptosis	Naïve	↑

TABLE 2. LATE PHASE (18 h) CURCUMIN-REGULATED GENES WITH KNOWN FUNCTION

Accession Number	Gene Name	Biological Function	Cell Status	Expression Status
BC035328	Microsomal glutathione S-transferase 2	Antioxidant/Detoxification	ROS	↑
AF130082	Collagen, type III, alpha 1	Structural proteins	Naïve	↓
AK057448	Syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	Muscle contraction	Naïve	↓
BF057731	Major histocompatibility complex, class II, DP beta 2 (pseudogene)	Inflammation/immunity	Naïve	↓
AF315688	Interferon, kappa	Inflammation/immunity	Naïve	↓
AA779991	Calcium binding atopy-related autoantigen 1	Inflammation/immunity	ROS	↑
AW301806	ADP-ribosylation factor-like 6 interacting protein 2	Inflammation/immunity	ROS	↓
BF508564	Ectonucleoside triphosphate diphosphohydrolase 1	Cell adhesion	Naïve	↓
AA053711	EGF-like repeats and discoidin I–like domains 3	Cell adhesion	Naïve	↓
BE219446	Muscle RAS oncogene homolog	Cell signaling	Naïve	↑
AI862674	Membrane-spanning 4-domains, subfamily A, member 1	Cell signaling	Naïve	↑
NM_004274	A kinase (PRKA) anchor protein 6	Cell signaling	Naïve	↑
AU147360	Protein tyrosine phosphatase, receptor type, A	Cell signaling	ROS	↑
AK091846	Hypothetical protein MGC35295	Cell signaling	ROS	↓
AI590659	AlkB, alkylation repair homolog 8 (*Escherichia coli*)	Cell signaling	ROS	↓
NM_002314	LIM domain kinase 1	Cell signaling	ROS	↓
NM_021094	Solute carrier organic anion transporter family, member 1A2	Intacellular transport	Naïve	↑
AL050263	Solute carrier family 1 (glial high-affinity glutamate transporter), member 3	Intacellular transport	Naïve	↑
AA960991	Sideroflexin 1	Intacellular transport	Naïve	↓
AF085911	Solute carrier family 5 (sodium/glucose cotransporter), member 11	Intacellular transport	Naïve	↓
AK024543	Neuron navigator 1	Intacellular transport	Naïve	↓
BF446577	Mitochondrial carrier triple repeat 6	Intacellular transport	ROS	↑
AI718937	Potassium channel tetramerisation domain containing 12	Intacellular transport	ROS	↓
AI963713	Enabled homolog (*Drosophila*)	Intacellular transport	ROS	↓
BC000879	Kynureninase (L-kynurenine hydrolase)	Metabolic pathways	Naïve	↑
AW297143	HBS1-like (*Saccharomyces cerevisiae*)	Metabolic pathways	Naïve	↓
W72516	Dihydropyrimidinase-like 3	Metabolic pathways	ROS	↑
BF692729	Exosome component 6	Metabolic pathways	ROS	↓
AI190575	Farnesyl pyrophosphate synthetase like	Metabolic pathways	ROS	↓
AW510697	Ubiquitin C	Protein degradation	Naïve	↓
NM_005133	RCE1 homolog, prenyl protein peptidase (*S. cerevisiae*)	Protein degradation	ROS	↑
AI309207	Membrane-associated ring finger (C3HC4) 7	Protein degradation	ROS	↓
AL359578	Zinc finger protein 287	Gene regulation	Naïve	↑
BC028160	Zinc finger protein 589	Gene regulation	Naïve	↓
BF665176	KRR1, small subunit (SSU) processome component, homolog (yeast)	Gene regulation	Naïve	↓
R44780	Ets variant gene 1	Gene regulation	Naïve	↓
AF265440	Mitochondrial translational initiation factor 3	Gene regulation	ROS	↑
BC001800	Orthopedia homolog (*Drosophila*)	Gene regulation	ROS	↑
AW149379	Ribosomal protein L41	Gene regulation	ROS	↑
BF241405	Exosome component 3	Gene regulation	ROS	↓
AI248610	Forkhead box P1	Gene regulation	ROS	↓
BC029395	M-phase phosphoprotein 6	Cell cycle/Apoptosis	Naïve	↓
AI057404	Cyclin I	Cell cycle/Apoptosis	ROS	↓
NM_013347	Replication protein A4, 34kDa	Cell cycle/Apoptosis	ROS	↓

Figure 2B show that ROS (hydrogen peroxide) exposure had no significant impact on HDAC1 activity. In contrast, ROS abolished HDAC2 activity by almost 90% compared with that in normal naïve cells. Treatment with increasing concentrations of curcumin restored HDAC2 activity back to normal levels found in naïve cells in a concentration-dependent manner, with an approximate EC_{50} of 30 nM. The PDE4 inhibitor (roflumilast) was used in parallel as a negative control to validate the action of curcumin and was unable to restore HDAC2 activity. As a positive control, U937 cells that had been treated with trichostatin A displayed very little or no deacetylase activity in both the HDAC1 and HDAC2 isoforms tested. Similar responses were also observed in MonoMac6 cells exposed to CSE (data not shown).

Antioxidant Properties of Curcumin in Free Radical–Generating Systems

Curcumin is a polyphenol with known antioxidant properties at high concentrations (32). In view of the observation that ROS exposure reduced HDAC2 activity and nanomolar concentrations of curcumin were able to restore HDAC2 activity and corticosteroid efficacy with approximate EC_{50} of 30 nM and 200 nM, respectively, we investigated whether or not curcumin could still act as an antioxidant at nanomolar concentrations. To assess the pharmacologic nature of curcumin's antioxidant properties, a dose–response effect for curcumin in several different free radical– or oxidant-generating systems was determined (Figure 3). EPR spectroscopy was used to evaluate the antioxidant free radical–scavenging potential of curcumin. Below 1 μM, curcumin did not possess any significant free radical–scavenging activity in the four systems studied here. However, at 10 μM, curcumin did show some weak antioxidant capacity in the xanthine/xanthine oxidase free radical–generating system. By 100 μM the antioxidant capacity of curcumin is clearly evident, as observed by a significant reduction in the EPR signal at the 24-hour time point in all four free radical–generating systems studied (Figure 3). Therefore, at concentrations less than 10 μM,

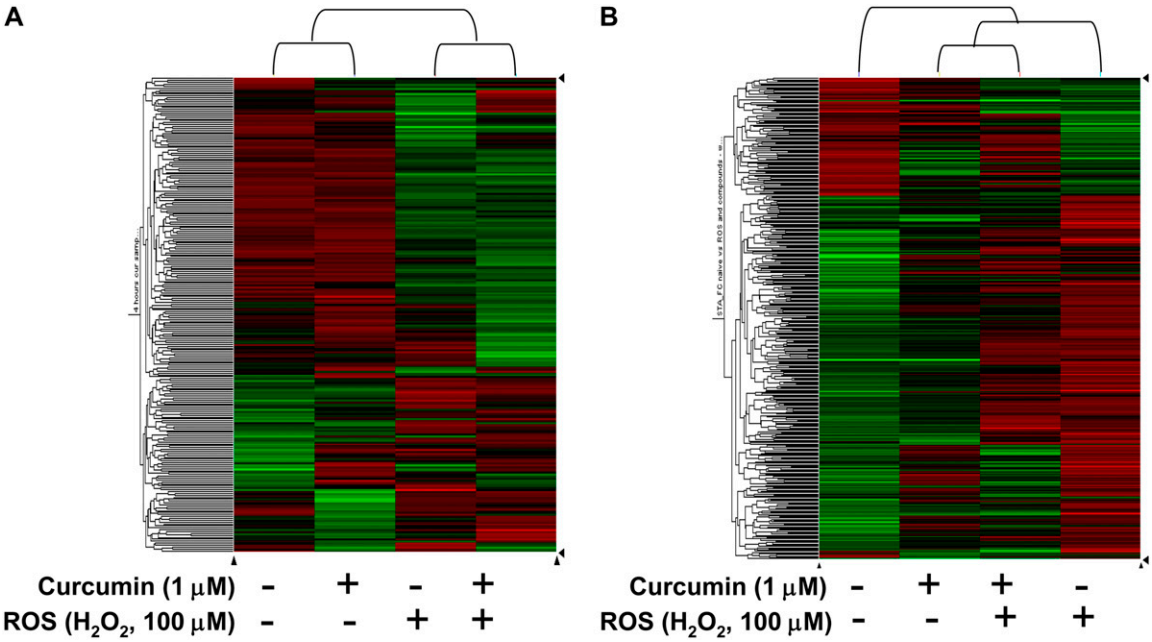

Figure 4. Effect of curcumin of gene expression in response to ROS stress. Heatmap showing hierarchical clustering of differentially expressed genes between naïve versus ROS-stressed human monocytes after curcumin (1 μM) treatment for (*A*) 4 and (*B*) 18 hours. Condition clustering (*vertical*) shows the average of three independent samples. Only genes with a statistically significant change greater than 1.8-fold, as defined by Welsh's *t* test ($P < 0.05$), are shown. *Red* indicates increased gene expression, *green* decreased expression, and *black* no change.

curcumin was unable to act as an antioxidant in the four systems studied, which would imply that the impact of curcumin at nanomolar concentrations on HDAC2 activity observed here was unlikely to be due to any direct antioxidant effects, as our data show.

Impact of Curcumin on Gene Expression

Given that curcumin (1 μM) was able to induce maximal effects on restoration of both HDAC activity and corticosteroid responses, we investigated what impact a similar concentration of curcumin would have on differentiated U937 cell gene expression. Of particular interest were those genes involved in inflammation, antioxidant protection, and HDAC, especially when differentiated U937 cells had been pre-exposed to ROS (H_2O_2). Differentiated U937 cells, with or without ROS stress, were cultured with 1 μM curcumin for up to 18 hours. Total RNA extracted from two distinct time points, 4 and 18 hours after curcumin addition, was probed on Affymetrix HG-U133 plus2.0 gene chips. Microarray gene expression data was collected from three independent experiments and analyzed using Genespring software (Agilent technologies). A cutoff threshold of greater than 1.8-fold change in expression was chosen. Only those genes that significantly met this threshold as defined by Welsh's *t* test ($P < 0.05$) are listed in Tables 1 and 2 and graphically displayed as a heatmap in Figure 4. A comparison of naïve versus ROS-stressed cells indicated that 4 hours after ROS stress a total of 298 genes are significantly affected, and that this drops to 71 genes 18 hours after ROS stress. Curcumin can be seen to affect both normal and ROS-stressed U937 cells at both 4- and 18-hour time points. After 4 hours, there were 77 curcumin-responsive genes that significantly changed in curcumin-treated U937 cells. Only 40 of these genes code for proteins with known function (Table 1). By 18 hours, a different set of curcumin-responsive genes had undergone significant changes in expression, and the number of genes had increased to 98, of which 44 were genes with known function (Table 2). Analysis of both these gene lists (Tables 1 and 2) revealed that curcumin (1 μM) did not induce any known antioxidant response genes in our experiments. Moreover, as curcumin is reported to activate the antioxidant transcription factor NF-E2–related factor (Nrf-2), no Nrf-2 responsive genes as identified by Thimmulappa and colleagues (48) were evident among those inducible genes seen here using this low concentration and earlier time points of curcumin treatments (Tables 1 and 2). Similarly, 1 μM curcumin had no widespread impact in down-regulating any pro-inflammatory genes induced by ROS. With respect to HDAC gene expression, no changes

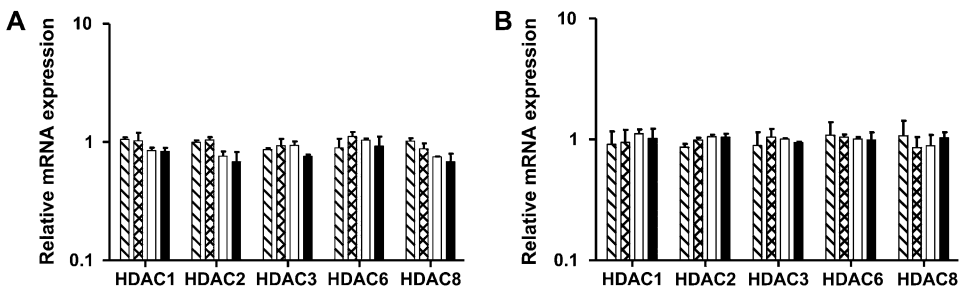

Figure 5. Gene expression profile showing the impact of curcumin (1 μM) on different HDAC isoforms in naïve and ROS-stressed human monocytes. Only those HDAC isoforms shown to be significantly expressed as detected by microarray analysis from three independent samples are shown. (*A*) Four hours after ROS, (*B*) 18 hours after ROS exposure. *Hatched bars*, control; *cross-hatched bars*, curcumin (1 μM); *open bars*, H_2O_2 (100 μM); *solid bars*, curcumin + H_2O_2.

in gene expression were evident among any of the HDAC genes detected. Indeed, 4 hours of ROS-mediated stress had very little impact on HDAC gene expression. The added impact of curcumin exposure again did not significantly change HDAC gene expression (Figure 5). These data suggest that curcumin mediated restoration of HDAC2 activity and that corticosteroid efficacy was not due to HDAC2 gene induction or pro-inflammatory gene suppression via NF-κB–dependent mechanisms *per se*. Interestingly, genes associated with protein degradation through the ubiquitin cycle at both the 4- and 18-hour time points (such as the zinc finger–A20 domain containing protein, ubiquitin-conjugating enzyme E2E, STAM-binding protein, and ubiquitin C protein) were significantly down-regulated by curcumin (Tables 1 and 2).

Curcumin Retains Post-Translational Protein Expression of HDAC2 after ROS Stress

Previously, we have shown that HDAC2 is modified at the post-translational level in response to oxidative stress (28, 49). To determine any post-translational impact of curcumin on HDAC expression, Western blots against HDAC2 were conducted. Figure 6A demonstrates that CSE exposure caused a reduction in HDAC2 protein expression, compared with H_2O_2 exposure (Figure 6B). As such, subsequent experiments (Figure 7) investigated whether curcumin in both the presence (Figure 7B) and absence (Figure 7A) of cycloheximide had any impact on HDAC2 protein expression. When U937 cells were also exposed to the protein synthesis/translation inhibitor cylcoheximide for 4 hours, there was a noticeable loss in HDAC2 protein expression, suggestive that there is a rapid turnover of HDAC2 (Figure 7B). Again, CSE is seen to reduce HDAC2 protein levels which when followed with cycloheximide for 4 hours after CSE stress, resulted in further loss of HDAC2 protein levels. However, when curcumin was incubated in the presence of cycloheximide for 4 hours after CSE stress, there was a concentration-dependent restoration in HDAC2 levels from those seen with CSE stress and cycloheximide to levels seen with cycloheximide alone. As cycloheximide is known to block the *de novo* synthesis of new protein, in this case HDAC2; this leads one to summarize that curcumin may block the degradation of existing HDAC2 protein induced by CSE. Moreover, the concentration range over which curcumin apparently blocks this HDAC2 degradation is similar to the range in which it restores HDAC2 activity and corticosteroid function. This prevention of CSE-induced reduction in HDAC2 protein expression by curcumin, could also be mimicked by the proteasomal inhibitor MG132 (Figure 8A). Indeed, the CSE-induced increase in ubiquitinated HDAC2 was increased even further by MG132 (Figure 8B). Interestingly, MG132 alone on naïve cells also induced an increase in ubiquitinated HDAC2 (Figure 8B), which suggests that HDAC2 itself may have a high turnover. In Figure 8C, we also show that CSE induced serine phosphorylation of HDAC2 and curcumin reversed this phosphorylation in U937 cells. This would indicate that HDAC2 undergoes a classical pathway of phosphorylation-ubiquitination-degradation upon exposure to CSE.

In view of reactive oxidants and reactive aldehydes present in cigarette smoke, we investigated the effect of curcumin on covalently modified HDAC2 by acrolein and 4-HNE, the reactive aldehydes that are present in cigarette smoke and formed as a result of lipid peroxidation. Covalent modification of HDAC2 protein was assessed by immunoprecipitation, followed by Western blot analysis using anti-acrolein or anti–4-HNE antibody. There was a significant increase in carbonylation of HDAC2 (HDAC2-acrolein and HDAC2–4-HNE interaction) in oxidant-treated differentiated U937 cells, which was significantly reduced by curcumin treatment (Figure 8D). A similar response was also observed in CSE-treated MonoMac6 cells (data not shown). This would support the concept that curcumin protects the cells from reactive oxidant/aldehyde-mediated post-translational modifications.

DISCUSSION

Glucocorticoid resistance is known to occur in COPD and severe asthma due to increased oxidative stress. It has been shown that corticosteroids recruit HDAC2 to the promoter of pro-inflammatory genes, thereby suppressing pro-inflammatory gene transcription (15, 18, 19, 50). Oxidative stress induced by CSE and H_2O_2 reduced both HDAC2 activity and corticosteroid efficacy in monocytes. No effect of oxidative stress was observed on HDAC1. Curcumin restored both HDAC2 activity and corticosteroid efficacy, in a concentration-dependent manner, with an EC_{50} of around 30 nM and 200 nM, respectively.

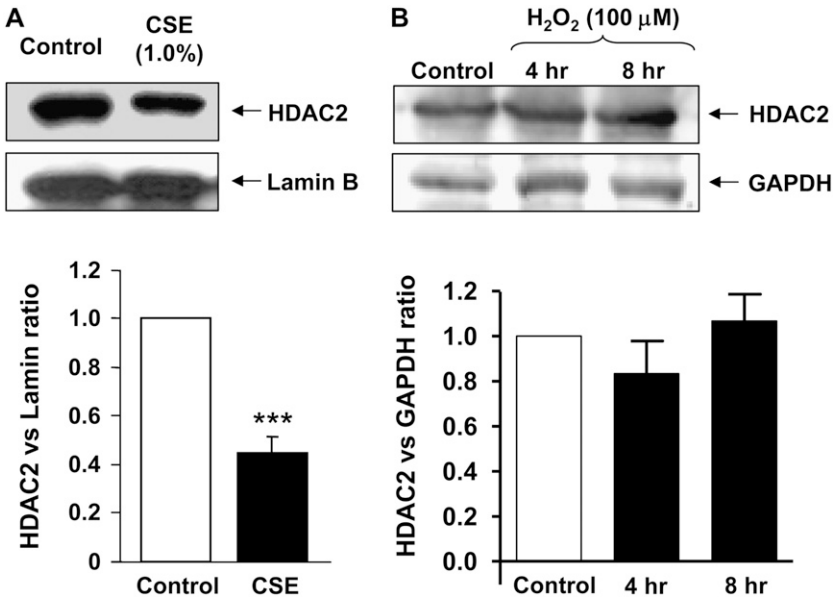

Figure 6. CSE, but not H_2O_2, decreases HDAC2 levels in human monocytes. (*A*) U937 cells were exposed to CSE (1%) for 4 hours and the HDAC2 levels were assessed by Western blot. (*B*) U937 cells were exposed to H_2O_2 (100 μM) and then analyzed for HDAC2 by Western blot immediately after ROS exposure (4 h) and 4 hours after ROS exposure (8 h). As internal loading controls, blots were stripped and reprobed for either GAPDH or lamin B protein levels. The *blots* shown are representative of the experiment being repeated at least three times ($n = 3$). ***$P < 0.001$, significant compared with control.

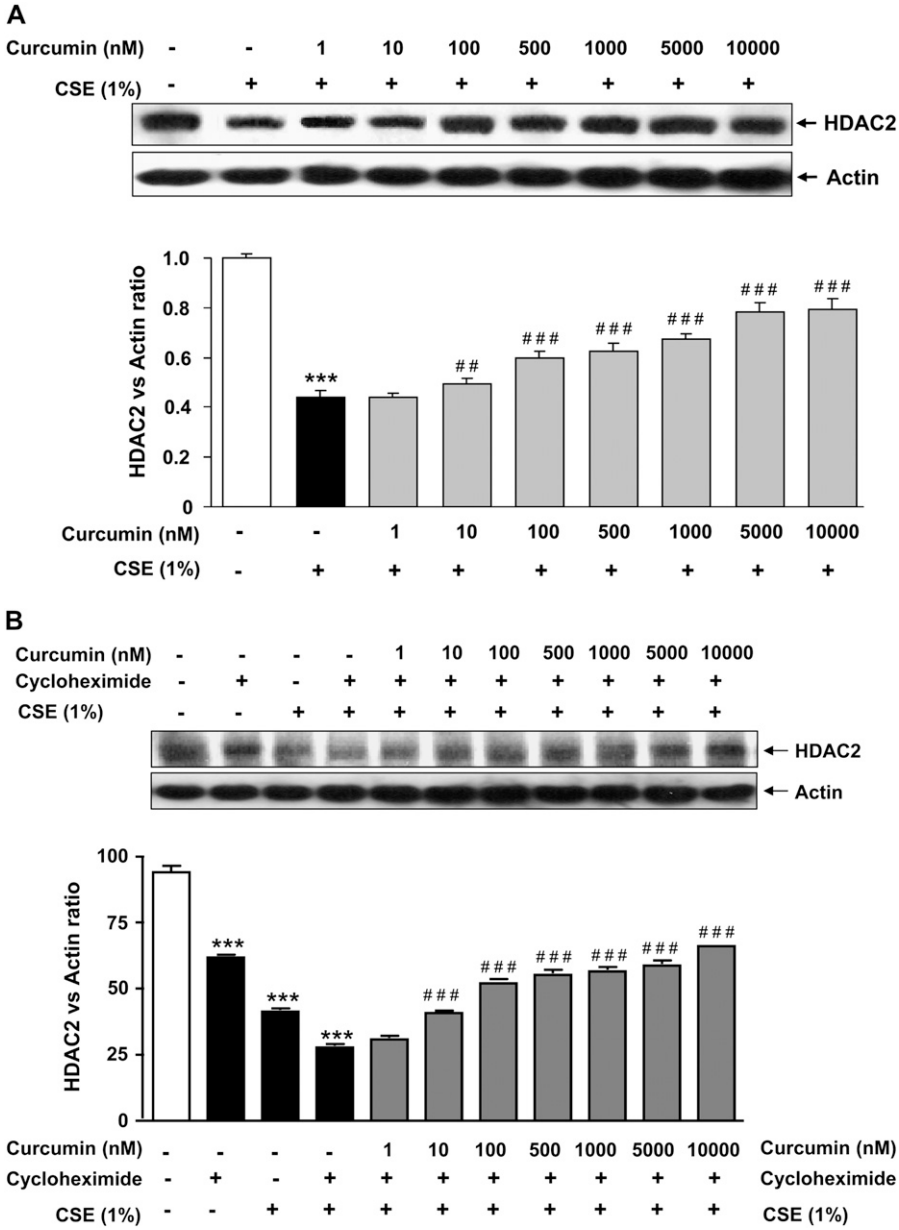

Figure 7. Curcumin restored HDAC2 protein level in response to CSE exposure, which was not masked by cycloheximide treatment. (*A*) U937 cells exposed to CSE (1%) for 4 hours were then treated with various concentrations of curcumin for a further 4 hours in the absence (*A*) or presence (*B*) of cycloheximide (10 μg/ml). HDAC2 levels were assessed by Western blot in the nuclear fraction. Actin was assessed as a loading control. The results shown are representative of the experiment being repeated at least three times ($n = 3$). $^{***}P < 0.001$, significant compared with untreated cells; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, significant compared with CSE- or CSE+cycloheximide-treated group.

Restoration of ROS-impaired corticosteroid function by curcumin was also shown to be HDAC dependent, as a global HDAC inhibitor, TSA, abolished the effect of curcumin. The impact of curcumin, at concentrations less than 1 μM, on HDAC activity and corticosteroid function in pre–oxidant-stressed cells could not be attributed to either the inherent antioxidant properties of curcumin, or the indirect antioxidant effects through gene induction. Indeed, both the direct and indirect antioxidant effects are reported to occur at curcumin concentrations greater than 10 μM (38, 42). These concentrations are at least 100- to 1,000-fold higher than the observed effects on HDAC activity reported here. However, we did observe that CSE caused a reduction in HDAC2 protein levels. Moreover, curcumin was able to reverse this decline in HDAC2 protein expression, even in the presence of the *de novo* protein synthesis inhibitor cycloheximide, suggesting that curcumin inhibited the CSE-induced degradation of HDAC2.

Theophylline, a compound structurally unrelated to curcumin, can also induce HDAC activity and restore corticosteroid efficacy in BAL macrophages from patients with COPD (13). However, theophylline has a narrow window of efficacy on HDAC2 and thus is not the drug of choice for use in the treatment of steroid-resistant COPD. It has been postulated that a major anti-inflammatory role of corticosteroids is to recruit HDAC2 activity to the promoter sites of pro-inflammatory gene expression (15, 18, 19, 50). This results in localized chromatin deacetylation and condensation, thereby silencing pro-inflammatory gene expression at these sites (15). The identification of curcumin's ability to restore HDAC activity and corticosteroid efficacy, similar to that of theophylline, raises interesting questions as to the mechanism by which both compounds are able to achieve this. Ito and coworkers (15) demonstrated that incubating theophylline with immunoprecipitates of HDAC2 did not have a direct impact in elevating HDAC activity (15). Similarly, we have also observed that curcumin did not have any direct impact on deacetylase activity in immunoprecipitates of HDAC2 from ROS-stressed monocytes (P. A. Kirkham and colleagues, unpublished observations). However, as curcumin did restore HDAC2 activity in intact ROS-stressed cells, this would imply an indirect effect of

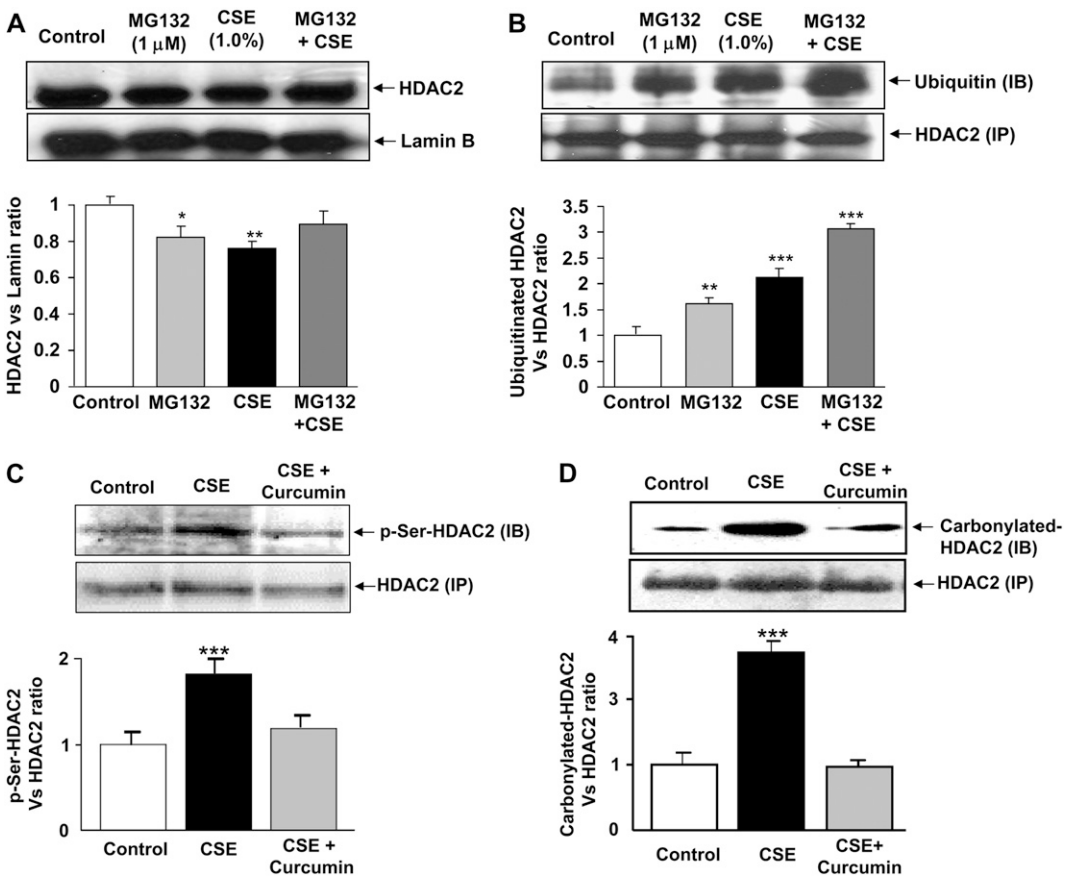

Figure 8. CSE-induced reduction in HDAC2 protein expression is mediated via proteasomal degradation, and curcumin reverses CS-induced post-translational modifications. Differentiated U937 cells were treated with either MG132 (1 μM) alone, exposed to CSE (1%) alone, or both together for 4 hours, then treated with or without MG132 (1 μM) alone for a further 4 hours as indicated. Cell lysates were probed for HDAC2 by Western blot using lamin B as an internal nuclear loading control (*A*). Alternatively, immunoprecipitates of HDAC2 were probed by Western blot for ubiquitin content using an HDAC2 Western blot as an internal loading control (*B*) or serine phosphorylation (*C*). The level of HDAC2-carbonyl adduct (acrolein or 4-HNE) was increased in response to CSE treatment, which was attenuated by curcumin (1 μM) treatment (*D*). The blots shown are representative of the experiment being repeated at least three times ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant compared with control value.

curcumin in regulating HDAC2 activity. Interestingly, curcumin did not have any impact on HDAC2 activity in naïve "non–ROS-stressed" monocytes. This latter finding is in agreement with that of Kang and coworkers, who found that curcumin at concentrations up to 100 μM had no impact on HDAC activity in hepatic Hep3B cells (45). This would suggest that under basal conditions HDAC2 remains constitutively active and that it is only under certain conditions, such as ROS stress, in which HDAC activity is reduced, that curcumin is able to restore HDAC activity back to basal levels. By maintaining HDAC activity in such a high state, it would not only keep a check on unnecessary pro-inflammatory gene expression (30), but would also allow the cells to respond rapidly to external stimuli by recruiting HDAC activity as appropriate to where it is needed. Nevertheless, regulation of HDAC activity can be accomplished in several ways, none of which are mutually exclusive; through post-translational modification, protein–protein interaction, subcellular localization, and protein expression status (51).

We and others have recently shown that oxidative stress, both *in vitro* and *in vivo*, can cause changes in post-translational modification of HDAC2, such as tyrosine nitration, carbonylation, and phosphorylation (27, 28, 49). Moreover, while these modifications have been demonstrated by us to affect activity (28, 49), they have also been shown to tag proteins for ubiquitination and eventual degradation (52). Our results here would suggest that curcumin does not impact on HDAC2 gene expression, but restores HDAC2 activity through regulation of its protein expression status, by preventing CSE-induced degradation of HDAC2. Moreover, our gene expression data lend additional support to this conclusion, as curcumin was seen to down-regulate gene expression for proteins associated with protein degradation, such as the zinc finger-A20 domain–containing protein, ubiquitin-conjugating enzyme E2E, STAM-binding protein, and the ubiquitin C protein (Tables 1 and 2). Equally plausible, however, is the possibility that curcumin acts earlier by preventing or even reversing any post-translational modifications that tag HDAC2 for eventual degradation through the ubiquitination pathway. The impact of CSE on reducing HDAC2 protein expression, as shown here, was clearly greater than that achieved with H_2O_2, even though both CSE and H_2O_2 caused a reduction in HDAC activity and corticosteroid efficacy. This may simply reflect the possibility that CSE, unlike H_2O_2, is a heterogenous mixture of chemicals containing both ROS and reactive aldehydes/carbonyls and therefore more likely to have a greater impact on the type of post-translational modifications (carbonyl-adducts formation) that can arise on any exposed proteins. This in turn could make any extensively modified proteins more susceptible to ubiquitination and eventual degradation by 26S proteasomes. Indeed, we observed that exposure of monocytes to CSE resulted in increased HDAC2 ubiquitination. Alternatively, post-translational modifications can also affect protein–protein interactions, in particular the co-repressors SDS3, Mi2, Sin3A, NCoR, and CoREST, which are essential for HDAC activity (53, 54), the disruption of which would have a detrimental effect on HDAC activity (55). What is clear, however, is that curcumin clearly acts at a post-translational level by reducing the level of protein carbonylation and serine phosphorylation on HDAC2, as well as restoring its activity.

Curcumin at high concentrations (~ 100 μM) has been shown to inhibit IκB kinase (56, 57), blocking NF-κB activation (34, 58) and subsequent IL-8 expression in A549 cells by pro-inflammatory stimuli such as TNF-α and ROS (38). Recently, Sandur and colleagues (59) reported that curcumin mediates its apoptotic and

anti-inflammatory activities through modulation of the redox status of the cell at μM concentrations. However, in ROS-stressed monocytes, curcumin alone (up to 10 μM) did not show any anti-inflammatory effect toward LPS-induced TNF-α release. Moreover, curcumin did not display any significant dose-dependent anti-inflammatory effects against ROS-induced pro-inflammatory cytokine release in the face of CSE alone. This suggests that restoring HDAC alone is not anti-inflammatory unless it is recruited to the site of pro-inflammatory gene expression. Similar observations have also been described for theophylline in U937 cells (13). The discrepancy between the impact of curcumin at low and high concentration on ROS-induced inflammation may be due to two factors: the intrinsic antioxidant properties of curcumin at high concentrations, and the ability to induce antioxidant as well as suppress pro-inflammatory gene expression at lower concentrations. Our data show that curcumin does not possess any antioxidant potential at concentrations below 10 μM in any of the free radical generating systems tested. Moreover, the impact of 1 μM curcumin on gene expression was equally restricted. Unlike previously reported gene array data using higher concentrations of curcumin (60), no evidence of induction of Nrf2-responsive genes such as the antioxidant genes, or suppression of pro-inflammatory gene sets, was evident. This would help explain the limited impact that low concentrations of curcumin (< 1 μM) alone would have on ROS-induced inflammation, whether or not LPS was also present. More surprisingly, Kang and colleagues (45) have demonstrated that curcumin can act as a HAT inhibitor at concentrations of 50 μM or greater, resulting in chromatin hypoacetylation. In view of the fact that histone H4 hypoacetylation is associated with pro-inflammatory gene silencing (15), some of the anti-inflammatory properties of curcumin at such high micromolar concentrations may also be attributed to inhibition of HAT activity. However, in the light of such facts, it is highly unlikely that HAT inhibition by curcumin at the concentrations used in the experiments described here will have played any significant role. Moreover, given that curcumin is considerably more efficacious in restoring HDAC2 in ROS-stressed cells at low nanomolar levels, this in turn would help to restore the HAT/HDAC imbalance that exists under oxidative stress (30), curtailing the magnitude of any inflammatory response. Consequently, this might help to explain, in part, why curcumin is considered to be more efficacious as an anti-inflammatory under conditions of oxidative stress. Furthermore, our data have implications for the treatment of conditions in which corticosteroid resistance occurs, particularly in response to oxidative stress by cigarette smoke.

In summary, we have shown that curcumin is able to restore HDAC2 activity and corticosteroid efficacy in ROS-stressed monocytes *in vitro*. Our data also provide further support for the critical role HDAC2 plays in the anti-inflammatory efficacy of corticosteroids. The molecular signaling mechanism by which this occurs is unclear at present, but it appears likely to involve the post-translational impact on HDAC2 by reversing protein phosphorylation and carbonylation that ultimately prevents its proteolytic degradation. Indeed, gene array analysis indicates that curcumin down-regulates gene expression of proteins involved in proteasomal degradation. Most significantly, the concentration range at which we observed the effect of curcumin on restoring HDAC2 activity ($EC_{50} \sim 30$ nM) and corticosteroid efficacy ($EC_{50} \sim 200$ nM) was at least 100-fold lower than the effective concentration required to have any impact on previously published *in vitro* targets (36, 39, 44, 45). The identification of the molecular target propagating these nanomolar effects of curcumin on HDAC2 would allow better therapeutic agents, with improved bioavailability, for example, to be developed for use in corticosteroid-resistant chronic inflammatory diseases, such as COPD. These agents could then be used in combination with conventional corticosteroid therapies to restore HDAC2 activity and thereby improve/enhance the anti-inflammatory efficacy of corticosteroids.

Conflict of Interest Statement: K.K.M. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK). S.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.K.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.S. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK) and also holds shares in Novartis. J.A.M. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK) and also holds shares in Novartis. P.C. was an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham UK) up until July 2007. D.F. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK). P.W. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK) and also holds shares in Novartis. I.L.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.A.K. is currently an employee of Novartis Pharmaceuticals PLC within the Novartis Institutes for Biomedical Research (Horsham, UK) and also hold shares in Novartis. I.R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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