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Cigarette Smoke Condensate and Dioxin Suppress Culture Shock Induced Senescence in Normal Human Oral Keratinocytes

Li Zhang^a, Ran Wu^a, R.W. Cameron Dingle^a, C. Gary Gairola^b, Joseph Valentino^c, and Hollie I. Swanson^a

a Department of Molecular and Biomedical Pharmacology, University of Kentucky College of Medicine, Lexington, KY

b Graduate Center for Toxicology, University of Kentucky College of Medicine, Lexington, KY

c Department of Surgery, University of Kentucky College of Medicine, Lexington, KY

Abstract

The aryl hydrocarbon receptor is a ligand activated transcription factor which regulates biological responses to a variety of environmental pollutants, such as dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) and cigarette smoke. The purpose of this study was to determine whether cigarette smoke condensate (CSC) is capable of activating the AHR in normal human oral keratinocytes (NHOK) and inhibiting their ability to senesce. Towards this end, NHOK were isolated from human subjects and were cultured in the presence or absence of either TCDD or CSC. While neither TCDD nor CSC treatments altered the lifespan of NHOK in culture, both were capable of suppressing a culture induced premature senescence as indicated by their ability to decrease the mRNA and protein levels of the senescence markers p16^{INK4a}, p53, p21 and p15^{INK4b}. A role of the AHR in mediating these events is indicated by the observations that the TCDD and CSC-induced decreases in p15^{INK4b}, p16^{INK4a} and p53 expression was accompanied by a corresponding increase in the expression levels of the AHR target gene, CYP1A1. In addition, cotreatment with the AHR antagonist, 3'-methoxy-4'-nitroflavone (MNF) blocked the effects of TCDD and CSC on p53 and CYP1A1 expression. The findings of this study indicate that in NHOK, CSC is capable of altering a key cell fate decision, i.e., commitment to premature senescence, that is in part, dependent on the AHR. These results support the idea that progression of CSC-induced tumorigenesis may include an AHR-mediated inhibition of senescence that contributes to immortalization and agents that block the actions of the AHR may be effective components of novel cancer therapeutics.

Keywords

oral keratinocytes; cigarette smoke condensate; senescence; aryl hydrocarbon receptor

Introduction

The AHR is a cytosolic ligand-activated transcription factor that initiates a genome dependent xenobiotic clearance program upon exposure to a variety of ubiquitous environmental contaminant agonists.^{1, 2} The chronic presence of many of these agonists results in toxic and/

Correspondence: Dr. Hollie Swanson, Department of Molecular and Biomedical Pharmacology, MS 305, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536, T: 859-323-1463, F: 859-323-1981, Email: hswan@uky.edu

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or carcinogenic endpoints that have been shown, in the main part, to be dependent on activation of the AHR signaling pathway. CSC (in which several have been identified as complete carcinogens, tumor initiators and/or tumor promoters) has been shown to be capable of activating the AHR.^{3–5} Previous studies performed in laboratory animals have suggested that the AHR may mediate some of the pro-carcinogenic effects of tobacco smoke,⁶ a major etiological factor associated with the development of oral cavity squamous cell carcinomas.^{6, 7} This idea is supported by studies using human patients in which a number of AHR target genes, such as CYP1A1 and CYP1B1 have been found to be upregulated in the epithelial cells of the respiratory tract of smokers as compared to that of nonsmokers.^{8, 9}

Senescence is an irreversible growth arrest state maintained even in the presence of mitogenic stimuli.^{10, 11} A senescent phenotype can be induced in normal cells by many distinct signals. While replicative senescence results from prolonged proliferation in culture, premature senescence can be induced by various damage or cellular stress signals and is independent of replicative age. In all cases, cell-cycle arrest that accompanies senescence is associated with the induction of tumor suppressors, such as p15^{INK4b}, p16^{INK4a} and p53. Classically identified as a barrier to the unlimited growth of primary cells in culture, senescence has now become recognized as a more general cell fate decision in response to varied stresses (e.g., oxidative stress, constitutive oncogene activity) within certain cellular contexts. Still, it has only been very recent studies that have conclusively shown that senescence is a bona fide *in vivo* cellular tumor suppressive mechanism akin to apoptosis.^{12, 13} From these studies, it is apparent that in certain contexts, the senescence response must be breached for the carcinogenic process to proceed. Thus, the abnormal cell proliferation that accompanies the progression of head and neck cancers may be a consequence of an inhibition of not only apoptosis and/or differentiation,¹⁴ but also an inhibition of senescence.

Our previous studies have demonstrated that activation of the AHR signaling pathway (via use of its prototypical ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) can inhibit a senescent response in neonatal human epidermal keratinocytes.^{15, 16} With these results in mind, we propose herein that cigarette smoke induced activation of the AHR signaling pathway allows normal human oral keratinocytes (NHOK), a tissue chronically exposed to AHR ligands in the smoking population, to overcome the senescent tumor suppressant barrier and thereby contribute to tobacco smoke induced cancers. Our results indicate that, like that observed in the neonatal human epidermal keratinocytes, TCDD inhibits senescence of NHOK. In addition, CSC suppresses the expression of p15^{INK4a}, p16^{INK4a} and p53 indicating that the ability of CSC to inhibit senescence in the NHOK may occur via its inhibition of these key regulators of cell fate. Finally, a role of the AHR in mediating at least some of the senescence-inducing actions of CSC is indicated.

Materials and Methods

Cell Culture

NHOK were obtained from the oral cavity tissue of patients, 5–12 years of age, who were undergoing routine tonsillectomies (Protocol 05-0091-P3G was approved by Human Subjects Institutional Review Board of University of Kentucky). Once the discarded surgical specimens were obtained, the epithelium was trimmed from the tonsil and the keratinocytes were isolated using the Keratinocyte Primary Isolation Kit (Cascade Biologics) and were cultured in EpiLife medium containing 0.06 mM Ca²⁺ and EpiLife Defined Growth Supplement (Cascade Biologics) in 12-well plates. The cells were used within 3–4 passages. For evaluation of replicative senescence, the NHOK cells obtained from four different donors were cultured separately in EpiLife medium (0.5×10^5 cells per 60 mm dish) in the presence of the indicated chemicals. The cells were passaged when approximately 70% confluent. At each passage, the cells were counted and the population doublings (PD) were determined. To initiate premature

(culture-induced) senescence, cells prepared from three different donors were cultured until nearly confluent (approximately 90% confluent). The media was then switched to DMEM (Mediatech, Inc) that contained 10% fetal bovine serum (FBS, HyClone) and 1.5 mM Ca^{2+} as previously described.^{15, 16} At the same time, the indicated chemicals were added and the cells were harvested at various time points after continuous exposure.

Chemicals

The cigarette smoke condensate (CSC) was prepared from the University of Kentucky Reference Cigarettes, 1R4F (9 mg tar and 0.8 mg of nicotine/cig). Briefly, the smoke particulates were collected on a Cambridge filter from cigarettes smoked under standard Federal Trade Commission protocol (35 ml puff volume of a 2 s duration)¹⁷ and dissolved in DMSO (Me_2SO) at 40 mg/ml. The solution was aliquoted into small vials and stored frozen at -80°C . The AHR agonist (TCDD) and antagonist (MNF, 3'-methoxy-4'-nitroflavone) which were dissolved in DMSO were kind gifts from Dr. Stephen H. Safe (Texas A & M University, College Station, TX, USA) and Dr. Thomas A. Gaseiwicz (University of Rochester, Rochester, NY, USA). Unless otherwise mentioned, all other chemicals were purchased from Sigma or Fisher Scientific.

RT Real Time PCR

At the indicated time points, the cells were harvested and total RNA was extracted using TRIzol reagent (Invitrogen). For RT real time PCR, the cDNA was prepared with Omniscript RT Kit (Qiagen) and random primers (Invitrogen) and analyzed with Brilliant SYBR Green QPCR Master Mix (Stratagene). Human QPCR Reference RNA (Stratagene) was used as controls. The following oligonucleotide primers (synthesized by Integrated DNA Technologies, Inc.) were specifically designed using Vector NTI 9.0.0 (InforMax) to amplify regions spanning exon junctions: HIS372, 5'-ATTGCCCTCAACGACCACTTTGTC-3' and HIS373, 5'-AGGTCCACCACCCTGTTGCTGTA-3' for GAPDH (80 bp); HIS374, 5'-CAAAACCTTTGAGAAGGGCCACATC-3' and HIS375, 5'-GACAGCTGGACATTGGCGTTCTC-3' for CYP1A1 (99 bp); HIS378, 5'-ACTAAGCGAGCACTGCCCAACAAC-3' and HIS379, 5'-CGGAACATCTCGAAGCGCTCC for p53 (116 bp); HIS402, 5'-GCTGCCCAACGCACCGAATA-3' and HIS403, 5'-CCGTGGAGCAGCAGCAGCTC for p16^{INK4a} (93 bp); HIS462, 5'-GAAGGTGCGACAGCTCCTGGAA-3' and HIS463, CTGCCCATCATCATGACCTGGAT-3' for p15^{INK4b} (90 bp).

Western Blot Analysis

At the indicated time points, the cells were harvested and whole lysates were prepared. Aliquots (100 μg of protein) were subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membrane and non-specific binding was blocked with 5% milk in PBST (5 g non-fat milk and 1 ml Tween 20 in 100 ml PBS). The membrane was incubated with anti-p15^{INK4b}, anti-p16^{INK4a}, anti-p53, anti-CYP1A1 (Santa Cruz Biotechnology, Inc) and anti- β -actin (Sigma) immunoglobulins in 5% milk in PBST followed by an incubation containing anti mouse or rabbit conjugated HRP.

Statistical Analysis

The data were analyzed and figures were plotted using GraphPad 3.0 (GraphPad Software Inc., San Diego, CA USA). Statistical differences among treatments at each time point were determined by two-way ANOVA and the Bonferroni posttest while significant differences among time points in DMSO treatments were analyzed by a one-way ANOVA Tukey's Multiple Comparison Test. The error bars are expressed as mean $\pm$ SEM.

Results

TCDD and CSC fail to alter the lifespan of cultured NHOK

Given our previous observations that activation of the AHR by its prototypical agonist, TCDD increases the lifespan of neonatal human epidermal keratinocytes, we first questioned whether similar results would be obtained using NHOK cells, a representative cell type that would be frequently exposed to AHR ligands via inhalation of tobacco smoke. Towards this end, we isolated NHOK cells from human subjects and cultured them in the absence or presence of TCDD. As shown in Figure 1, maximum population doublings in the cells treated with the vehicle control was reached after 20 days of culture (~ 8 population doublings) and was not significantly altered by the presence of TCDD. Similar results were obtained using CSC (data not shown). While treatment with the AHR antagonist, MNF failed to alter the population doublings of the NHOK cells, treatment with both MNF and TCDD resulted in a significant decrease in population doublings (i.e., at day 25, DMSO = $8.5 \pm .18$ and TCDD + MNF = $6.6 \pm .66$) at the latter time points.

TCDD alters the mRNA levels of CYP1A1 and regulators of premature senescence, p15^{INK4b}, p16^{INK4a} and p53 in NHOK cells

While replicative senescence is triggered by a replication-induced loss of telomeric DNA, premature senescence is regulated primarily by the p16^{INK4a}/Rb and p53 signaling pathways.^{10, 11} We first verified that treatment of NHOK cells with the prototypical AHR agonist, TCDD resulted in appropriate upregulation of its classic target gene (i.e., CYP1A1) during our culture shock-induced premature senescence protocol in a manner similar to that previously observed using the neonatal human epidermal keratinocytes.^{15, 16} As shown in Figure 2A, TCDD treatment resulted in an induction of CYP1A1 mRNA levels that was at least 700 fold that of the vehicle (DMSO) control at all time points examined. The induction of senescence using these conditions is indicated by the time dependent increase in mRNA levels of p15^{INK4b} (Figure 2B), p16^{INK4a} (Figure 2C) and p53 (Figure 2D) observed in the DMSO control in Days 3, 5 and 7 as compared to that at observed at Day 1. Similar to that observed in the neonatal human epidermal keratinocytes,^{15, 16} TCDD decreased the mRNA expression levels of these gene products at most time points examined. These results imply that these conditions are sufficient for inducing senescence of NHOK cells in culture and that this induction is blocked by TCDD.

We then determined whether AHR agonists present in CSC are similar to TCDD in their ability to upregulate CYP1A1 mRNA levels in NHOK cells. We have previously determined that a concentration of 25 µg/ml of CSC is approximately equipotent to 1 nM of TCDD in its ability to activate the AHR signaling pathway (Puppala et al, manuscript submitted). CSC at this concentration was similar to TCDD in its ability to induce CYP1A1 mRNA levels, albeit at days 5 and 7, the CSC-induced fold induction was slightly less than that of TCDD (Figure 3). In addition, its ability to decrease the mRNA levels of p15^{INK4b}, p16^{INK4a} and p53 and were overall similar to that of TCDD where the extent to which CSC decreased the mRNA levels of p16^{INK4a} and p53 was greatest at day 7 and CSC was more effective in its ability to decrease the mRNA levels of p15^{INK4b} as compared to that of either p16^{INK4a} or p53.

We then confirmed that the changes observed in the mRNA levels of p16^{INK4a} and p53 led to corresponding changes in their protein levels by performing western blot analyses. As shown in Figure 4, the protein levels of p15^{INK4b}, p16^{INK4a} and p53 were increased in the DMSO control during the course of the experiment (i.e., compare day 2 to day 6). Activation of the AHR signaling pathway by either TCDD or CSC is indicated by the increase in CYP1A1 protein expression relative to the DMSO control at all time points examined. Treatment with either TCDD or CSC resulted in a decrease in protein expression of p15^{INK4b}, p16^{INK4a} and

p53 that was most clearly evident at the day 6 time point. The expression levels of p53 were also decreased by either TCDD or CSC at the day 2 time point. Interestingly, while CSC appeared to be equipotent in inducing CYP1A1 expression, its inhibition in the expression levels of p15^{INK4b}, p16^{INK4a} and p53 at this dose were greater than that of TCDD.

To determine whether the AHR played a role in the ability of TCDD and CSC to induce CYP1A1 expression and inhibit p15^{INK4b}, p16^{INK4a} and p53 expression, we utilized the AHR antagonist, MNF. As shown, MNF was capable of inhibiting TCDD's effects on CYP1A1 and p15^{INK4b} and p53, but not p16^{INK4a}, at days 2 and 6. With respect to CSC, MNF was only partially effective in inhibiting CSC's effects on CYP1A1 and p53 expression levels at day 2. At the day 6 time point, however, MNF was ineffective in blocking any of the CSC-dependent effects on protein expression. This result may be a consequence of using a concentration of MNF that is too low for sufficient blocking of AHR activities or may be due to the possibility that CSC activates a myriad of pathways, only one of which is the AHR. Nonetheless, the data shown in Figure 4 indicate that the ability of TCDD and CSC to decrease the expression levels of p15^{INK4b} and p53, but not p16^{INK4a} and induce the expression levels of CYP1A1 in NHOK involves the AHR.

Discussion

In this study we have shown that CSC, like TCDD, can inhibit premature senescence induced in NHOKs in a manner that involves the AHR and correlates with an inhibition of key regulators of senescence, p15^{INK4b}, p16^{INK4a} and p53. Previous studies have demonstrated that the onset of senescence in NHOK is accompanied by not only increased expression of these regulators of senescence, but also by an increase in the expression levels of other senescence-associated genes (i.e., matrix metalloproteinases) and the AHR target gene, CYP1B1.¹⁸ Although both replicative and premature senescence are thought to represent permanent states of cell cycle arrest, their underlying mechanisms are thought to be distinct.^{10, 11} Replicative senescence occurs after a finite number of population doublings and is triggered primarily by a critical loss of telomeres. Premature senescence, on the other hand, is independent of telomere shortening and involves elevated levels of p15^{INK4b}, p16^{INK4a} and p53. While these proteins all appear to be required for senescence, their actions within the senescence program vary with that of p16^{INK4a} appearing to be critical for the onset of the senescent state and that of p53 and likely others, are more important for the maintenance of senescence. Increasing evidence supports the idea that bypass of oncogene-induced premature senescence is an important step in the acquisition of the malignant tumor phenotype.¹⁹ What is currently unclear, however, is whether a cell becomes malignant due to its ability to escape from senescence or from its ability to resist and bypass the initiating senescent signal.

With respect to oral cancers, a number of studies support the idea that a disruption of the senescence process occurs as cells progress from a normal state to that of dysplasia and finally, squamous cell carcinoma.^{20–25} One of the most commonly inactivated tumor suppressor products thus far reported in primary oral squamous cell carcinomas is p16^{INK4a} that exhibits a decrease in protein expression that closely correlates with the transition from the hyperplastic to the dysplastic state. This loss of the expression of p16^{INK4a} expression occurs via a number of mechanisms including loss of heterozygosity, deletions, mutations or hypermethylation²⁴ and typically correlates with an increase in proliferation indices, such as that detected by PCNA labeling. The idea that a patient's continuous exposure to cigarette smoke may contribute to the loss in the expression of key regulators of senescence during the progression of oral cancers and in this manner facilitate the immortalization step of tumor development is supported by our observations that CSC is capable of downregulating the expression levels of the majority of these proteins (Figures 3 and 4). Further support for this idea is provided by a study performed in human subjects in which chronic smokers were found to have a significantly

higher frequency of p15^{INK4b} promoter methylation in their normal adjacent epithelia as compared to nonsmokers.²² Taken together, these observations in addition to a report that smoking may associated with an increase in telomerase activity of a patient's bronchial cells²⁶ indicates that both replicative and premature senescence may be involved in the progression of tobacco-smoke induced oral cancers.

While TCDD has been previously shown to inhibit both premature and replicative senescence in the neonatal human epidermal keratinocytes,^{15, 16} it appears to be capable of inhibiting only premature senescence in the NHOK cells (Figures 1 and 2). These disparate results may be due to either a cell-type specific effect or may be a function of the age of the patient. For example, the cells obtained from the neonate likely contain a higher proportion of stem or basal cells that may be more responsive to the effects of TCDD. Further research focused on determining the mechanisms by which TCDD exerts its effects on premature and replicative senescence will aid in clarifying this conundrum.

The results obtained following use of the AHR antagonist, MNF (Figure 4), together with the observations that the actions of CSC appear to be similar to that of TCDD in its ability to upregulate CYP1A1 (Figures 2A, 3A and 4) and downregulate p15^{INK4b}, p16^{INK4a} and p53 (Figures 2 and 3) indicate that the actions of CSC with respect to premature senescence require the AHR. Based on our previous findings that downregulation of p16^{INK4a} by TCDD is accompanied by changes in its methylation status,¹⁶ we propose that the actions of CSC on premature senescence of NHOK may involve similar events. In fact, a role of the AHR in the development of oral cancers is supported by the observations that progression of oral cancers is accompanied by changes in the expression levels of xenobiotic metabolizing genes, many of which are directly regulated by the AHR.^{8, 27, 28} A limitation of the current study, however, is that at the latter time points (i.e., day 6) the AHR antagonist failed to reverse the actions of CSC on expression of p15^{INK4b} and p16^{INK4a} (Figure 4). While this may associated with limited efficacy of the AHR antagonist used in this study, it may also be a consequence of the fact that CSC is a mixture of components that may act either synergistically to activate the AHR or may alter the expression of these gene products (and hence senescence) via both AHR dependent and independent mechanisms. For example, arsenic found in cigarette smoke²⁹ has been reported to act in a synergistic manner with AHR agonists to upregulate a number of AHR target genes.³⁰ Alternatively, the ability of CSC to inhibit senescence may involve both its activation of the AHR and additional activities such as those that involve inhibition of mitosis³¹.

Emerging studies indicate that the AHR may play important roles in several stages of tobacco smoke induced carcinogenesis. At the initiation stage, the AHR is a major determinate of the genotoxicity of tobacco smoke constituents as it is a major regulator of key enzymes involved in both phase I and Phase II metabolism.² In general, metabolic activation of carcinogenic substances is catalyzed by phase I enzymes like CYP 450 proteins. Many reactive intermediates that are formed are converted to polar conjugates for excretion by phase II enzymes such as glutathione-S-transferases and UDP-glucuronosyltransferases.^{2, 32} An interplay between these two types of enzymes appears to have an important role in determining an individual's susceptibility to tobacco smoke-induced cancer. A role of the AHR in mediating the events associated with the initiation of tobacco smoke-induced cancer is indicated by the fact that many CSC constituents have been shown to bind to the AHR, transforming it into an active transcription factor and subsequently inducing several of its target genes such as CYP1A1, CYP1B1 and COX-2.^{5, 6, 33-35} In these studies, the activation of these genes by CSC has also been shown to accompany CSC-induced genotoxicity. Further, the idea that cigarette smoke can induce AHR target genes in oral squamous carcinomas is supported by the recent observations that CSC induces expression of CYP1A1 and CYP1B1 in cultured oral squamous cell carcinoma-derived cell lines.³⁶ Finally, a recent epidemiological study has found that a

combination of mutations in both the AHR target gene, CYP1A1 and glutathione S-transferase M1 genes, is associated with a significant increase in a cigarette smoker's risk of developing lung cancer.³⁷ Thus, activation of the AHR by constituents of tobacco smoke upregulates AHR target genes that are known to be important in determining the genotoxicity and carcinogenicity of many constituents of tobacco smoke

The AHR appears to play a role in not only the initiation phase of tobacco smoke-induced carcinogenesis, but also the promotion and perhaps, progression phases². This idea is supported by increasing evidence that a number of pathways involved in regulating cell cycle progression, apoptosis and cell adhesion are altered by the AHR. For example, activation of the AHR results in an increase in the transcription of positive growth regulators such as K-RAS,³⁸ c-myc³⁹ and the ligands of EGFR (amphiregulin, TGF α and epiregulin)^{28, 40, 41} and altered cell cycle control.⁴² Further, the recent data demonstrating that the AHR is a negative regulator of TGF- β ⁴³ implies that activation of the AHR could relieve the growth inhibitory actions of TGF- β and thereby increase SCC tumor progression. Thus, use of an appropriate AHR antagonist would potentially alter a number of cell fate decisions and in this manner may serve as an effective approach in treating oral cancers

In conclusion, our findings that CSC inhibits expression of key regulators of senescence in an AHR dependent manner identifies a novel mechanism by which CSC may contribute to the development of oral cancers and the promise associated with use of the AHR as a target for cancer therapies.

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Figure 1. TCDD does not extend the life span of NHOK in culture

NHOK cells obtained from four individual donors were cultured in EpiLife medium containing 0.06 mM Ca^{2+} with either 0.01% DMSO or 1 nM TCDD in the presence or absence of 1 μM MNF. The cells were passaged when approximately 70% confluent until they ceased to divide. Cumulative population doublings (PD) were determined after each passage and plotted against total time in culture. Each point (n=4) represents means and standard error of means. $\text{PD} = \ln (\text{Number of cells at each subculture}/\text{Number of cell initially plated})/\ln 2$.

Figure 2. TCDD increases the mRNA levels of CYP1A1 (A) and decreases the mRNA levels of p15^{INK4b} (B), p16^{INK4a} (C), p53 (D) in NHOK cells

Upon reaching full confluence, the NHOK cells were treated with either DMSO (0.1%, vehicle control) or TCDD (1 nM) in DMEM medium containing 1.5mM Ca²⁺ and supplemented with 10% FBS. At the indicated time points, the cells were harvested and the mRNA levels relative to the reference RNA was determined using reverse transcription RT-PCR. Each error bar represents the means $\pm$ SEM for cells obtained from two individual donors with duplicates for each donor (n=2: * P <0.05, ** P <0.01, *** P <0.001, comparing TCDD to dimethyl sulfoxide (DMSO) at each time point; # P <0.05, ## P <0.01 comparing DMSO at each time point to DMSO at day 1).

Figure 3. CSC increases the mRNA levels of CYP1A1 (A) and decreases the mRNA levels of p15^{INK4b} (B), p16^{INK4a} (C), p53 (D)

Upon reaching full confluence, the NHOK cells were treated with either DMSO (0.1%, vehicle control) or CSC (25 $\mu\text{g/ml}$) in DMEM medium containing 1.5mM Ca^{2+} and supplemented with 10% FBS. At the indicated time points, the cells were harvested and the mRNA levels relative to the reference RNA was determined using reverse transcription RT-PCR. Each bar represents the means and SEM for cells obtained from two individual donors with duplicates for each donor ($n = 2$: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, comparing TCDD to dimethyl sulfoxide (DMSO) at each time point; # $P < 0.05$, ## $P < 0.01$, comparing DMSO at each time point to DMSO Day 1).

Figure 4. TCDD and CSC decrease the protein levels of p15^{INK4b}, p16^{INK4a} and p53 in NHOK cells

Upon reaching full confluence, the NHOK cells were treated with DMSO (0.1%, vehicle control), TCDD (1 nM) or CSC (25 μg/ml) in the presence or absence of 1 μM MNF in DMEM medium containing 1.5mM Ca²⁺ and supplemented with 10% FBS. At the indicated time points, the cells were harvested, total protein extracts were prepared and aliquots (100 μg) were subjected to SDS-PAGE analysis. *D* = DMSO-treated, *T* = TCDD-treated and *C* = CSC-treated cells.