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Cigarette smoke extract-stimulated epithelial-mesenchymal transition through Src activation

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Abstract

Epithelial-mesenchymal transition (EMT) is implicated in the pathogenesis of lung fibrosis and cancer metastasis, two conditions associated with cigarette smoke (CS). CS has been reported to promote the EMT process. CS is the major cause of lung cancer and nearly half of lung cancer patients are active smokers. Nonetheless, the mechanism whereby CS induces EMT remains largely unknown. In this study we investigated the induction of EMT by CS and explored the underlying mechanisms in the human non-small cell lung carcinoma (H358) cell line. We demonstrate that exposure to an extract of CS (CSE) decreases E-cadherin and increases N-cadherin and vimentin, markers of EMT, in H358 cells cultured in RPMI-1640 medium with 1% fetal bovine serum. Pretreatment with N-acetylcysteine (NAC), a potent antioxidant and precursor of glutathione, abrogated changes in these EMT markers. In addition, CSE activated Src kinase (shown as increased phosphorylation of Src at Tyr418) and the Src kinase inhibitor, PP2, inhibited CS-stimulated EMT changes, suggesting that Src is critical in CSE-stimulated EMT induction. Furthermore, NAC treatment abrogated CSE-stimulated Src activation. However, co-incubation with catalase had no effect on CSE-mediated Src activation. Finally, acrolein, an unsaturated aldehyde present in CSE, caused Src activation. Taken together, these data suggest that CSE initiates EMT through Src, which is activated by CS through redox modification.

Keywords

epithelial-mesenchymal transition; Src; cigarette smoke; redox; N-acetylcysteine

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Introduction

Epithelial-mesenchymal transition (EMT) is a process in which cells lose epithelial phenotype and acquire mesenchymal features. EMT has been suggested to play important roles in the development of idiopathic pulmonary fibrosis (IPF) [1, 2] and metastasis of lung cancer [3, 4] although the involvement of EMT in fibrosis is far more controversial than in metastasis. Cigarette smoke (CS) is a major risk factor for lung fibrosis [5] and a major contributor to lung carcinogenesis [6]. Considering that second hand CS is a common environmental hazard [7, 8] and that nearly half of lung cancer patients remain active smokers [9–11], it is important to understand how CS affects the EMT process so that, given the less than complete success of smoking cessation efforts, effective strategies can be developed to prevent and treat these CS-induced EMT-related diseases. Some studies have shown that CS can promote EMT process in lung alveolar cells [12]. The induction of EMT by CS was also observed in a recent study reporting that the levels of vimentin and other EMT markers increased in smokers with COPD compared with normal non-smokers [13]. Nonetheless, the mechanisms underlying induction of EMT by CS remain unclear. In this study we used a water soluble extract of CS (CSE) in cell culture. Although there are obvious limitations to extrapolating to pathology in using CSE, it provides a convenient and reasonably reproducible method for studying signaling mechanisms.

EMT can be induced by a variety of growth factors and other external stressors such as transforming growth factor beta1 (TGFβ1) [14, 15], epidermal growth factor (EGF) [16], platelet-derived growth factor (PDGF) [17], and hypoxia [18]. These EMT inducers activate or repress various intracellular signaling pathways, such as Ras/Mitogen-activated protein kinase kinase 1 and 2 (MEK)/ extracellular signal-regulated kinases 1 and 2 (ERK), phosphatidylinositol 3-kinase (PI3K)/the non-specific serine/threonine-protein kinase Akt/ PKB, the non-receptor tyrosine kinase Src, and glycogen synthase kinase 3β (GSK3β), which sequentially regulate (activate or repress) transcription factors such as Mothers against decapentaplegic homologs 2 and 3 (Smad2/3), human snail homologs 1 and 2 (Snai1/2), and Twist, and cause the suppression of epithelial genes such as E-cadherin and zona occludens (ZO)-1, and increased expression of mesenchymal genes such as N-cadherin, vimentin, and smooth muscle actin A (SMA) [19, 20]. Interestingly, ERK, Akt, Src, GSK3β, and Smad2/3 have all been suggested as targets of redox activation [21–27].

Src is the prototypic member of a family of non-receptor membrane associated tyrosine kinases [28]. Studies have found that Src kinase is involved in cytoskeleton reorganization, cell migratory capacity, expression of mesenchymal proteins, and other EMT events [29–34]. Indeed, Src plays critical roles in EMT initiated by many stimulators [31–35]. Under resting conditions, most Src remains in an inactive form with phosphorylation at Tyr529 and intracellular interactions mediated by SH2 and SH3 domains. In response to agonists, Src undergoes dephosphorylation at Tyr529 and then conformation change that results in autophosphorylation at Tyr418 and its full activation [36]. Recently it has been found that Src activation by many stimuli is redox dependent [37–40], and can be activated directly by various oxidants such as peroxynitrite and H_2O_2 [24, 25, 41].

Accumulating evidence suggests that a diverse array of EMT inducers share a common redox dependent mechanism in initiating EMT [22, 27, 42–45]. N-acetylcysteine (NAC), a potent antioxidant and precursor of glutathione (GSH), inhibits EMT initiated by TGFβ and many other agonists [27, 42]. It has been reported that reactive oxygen species can directly induce or promote EMT [31, 46]. CS contains over 4000 compounds including several highly reactive α,β-unsaturated carbonyls, nitrogen oxides and H_2O_2. Given the facts that Src is critical for EMT initiation and redox regulated, and that CS contains strong oxidants

and electrophiles, in the current study we test the hypothesis that CS causes EMT through CS-induced activation of Src kinase.

Methods and materials

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). All antibodies were from Santa Cruz (Santa Cruz, CA). M-PER Mammalian Protein Extraction Reagent was from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL). All chemicals used were at least analytical grade.

Preparation of cigarette smoke extracts [47]

CSE was an extract of mainstream cigarette smoke. Briefly, the smoke from one filtered cigarette (Camel regular) containing 1.2 mg of nicotine and 18 mg of tar according to the manufacturer's report was drawn through an experimental apparatus with a constant airflow driven by vacuum. The smoke was bubbled through 25 ml of RPMI-1640 medium without fetal bovine serum (FBS) in 2 min and the solution was used as the stock (100%) for further dilutions. After adjusting the pH to 7.2, the obtained CSE was filtered through a 0.22-μm filter (Millipore, Bedford, MA) for sterilization and diluted for use within 20 min after the preparation.

Cell culture and treatment

A human non-small cell lung carcinoma cell line (H358) was used. H358 cells were cultured in RPMI-1640 medium with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5% CO_2 at 37°C. Cells were treated with CSE when about 70% confluent in RPMI-1640 medium with 1% FBS. NAC was added to culture medium 4 hours before cells being exposed to CSE, and the NAC medium was replaced every 24 hours thereafter. In an experiment to detemine the extent of H_2O_2 involvement, catalase (final concentration is 400 U/ml) was added to the culture medium immediately before H_2O_2 or CSE exposure. In another set of experiments, cells were exposed to 15 μM acrolein, a non-toxic dose.

Western Analysis

Briefly, cell lysate was extracted with M-PER and 15 μg protein was heated for 15 min at 95°C in 2X loading buffer containing SDS (Tris base, pH 6.5, glycerol, DTT, and pyronin Y), electrophoresed on a 4–20% Tris-glycine acrylamide gel (Invitrogen, Carlsbad, CA), and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore corporation, Bedford, MA). Membranes were blocked with 5% fat-free milk and then incubated overnight at 4°C with primary antibody in 5% milk in Tris-buffered saline (TBS). After being washed with TBS containing 0.05% Tween 20 (TTBS), membranes were incubated with secondary antibody at room temperature for 2 h. After TTBS washing, membranes were treated with an enhanced chemiluminescence reagent mixture (ECL Plus; Amersham, Arlington Heights, IL) for 5 min. The target bands were imaged on a Kodak Image Station 2000R (Kodak Company, Rochester, New York).

Statistical analysis

Densitometry data for Western Blots are expressed as mean ± standard error. SigmaStat 3.5 was used for statistical analysis and statistical significance was accepted when $p < 0.05$. The one-way ANOVA and Tukey test were used for comparison of protein levels.

Results

CSE induced EMT in lung epithelial H358 cells

EMT involves phenotypic changes such as shape change (from epithelial round-shaped to mesenchymal spindle-like), loss of epithelial marker proteins, including E-cadherin, and induction of mesenchymal marker proteins, including N-cadherin and vimentin [19, 20]. To examine whether CS induced EMT in human lung epithelial H358 cells, cell cultures at 70% confluence were exposed to variable concentrations of CSE for 72h. Using western Blots, cell lysates were then analyzed for the expression of epithelial and mesenchymal markers including E-cadherin, N-cadherin, and vimentin. As shown in Figure 1, treatment of cells with CSE caused a significant decrease in E-cadherin, and an increase in N-cadherin and vimentin proteins compared to untreated controls. The change of these proteins in response to CSE was dose-dependent and the effect was observed with 5% CSE exposure, and at a concentration of 20%, CSE caused a 40% decrease in E-cadherin, and a 60% and 50% increase in N-cadherin and vimentin respectively. Under the microscope, it is also observed that H358 cells became spindle-shaped and that some cells moved (detached) from the cell clusters upon CSE exposure, in contrast to rounded shape of the non-exposed cells (data not shown). This evidence suggests that CSE decreased epithelial proteins and increased mesenchymal changes in H358 cells, indicating the induction of EMT.

NAC inhibited CS-induced EMT markers

Some studies suggest that a redox mechanism is involved in EMT induction by various stimulators [22, 27, 42–45]. Since CS contains strong oxidants and other electrophiles and can produce additional oxidants in cells, we hypothesize that CS stimulated EMT via an oxidant-mediated mechanism. Generally, chemists consider oxidants as a subclass of electrophiles that remove electrons rather than share them with their target, as do other electrophiles. For biologists, oxidative stress also includes reactions in which electrophiles add to other molecules. Thus, Michael addition to compounds by α,β-unsaturated carbonyls, such as acrolein, a component of CSE that is formed by oxidative processes, are considered an oxidative mechanism. Therefore to test the hypothesis that this broadly defined oxidative mechanism was involved in CSE-induced EMT, H358 cells were pretreated with different concentrations of NAC for 4h and then exposed to 10% CSE for 72 h, followed by determining EMT marker proteins. As shown in Figure 2, 2 mM NAC pretreatment prevented the CSE-induced decrease in E-cadherin, and simultaneously suppressed up-regulation of vimentin by CSE. But, when cells were washed after the incubation with NAC and then CSE was added, the NAC was ineffective. These data suggest that CSE induced EMT via an oxidative mechanism (see above for the meaning of this oxidative mechanism in this context) that was prevented when NAC was present during the addition of CSE. NAC can supply cysteine, which is a limiting component for the synthesis of glutathione, a major antioxidant *in vivo*. Nonetheless, as NAC was only effective when in the solution to which CSE was added, it is more likely that NAC reacted directly with the oxidants in the extracellular medium. NAC, unlike antioxidants such as vitamin E, is a two electron donor that can either reduce oxidants or add to electrophiles by Michael addition. Although these reactions are slow compared with enzymatic removal of oxidants, in the extracellular environment they can be quite significant.

Src is involved in CS-induced EMT

The tyrosine kinase Src has been reported to be important in EMT initiation by many stimulators [31–35]. To investigate whether Src was involved in CSE-caused EMT, we first studied Src activation by determining its autophosphorylation at Tyr418 (pTyr418-Src). H358 cells were treated with or without 10% CSE for various times and the cell lysates were analyzed for pTyr418-Src. Src phosphorylation at Tyr418 was increased at as early as 5 min

and reached a maximum at 15 min of CSE treatment. By 1 h after CSE treatment, phosphorylation at Tyr418 had started to decrease to baseline levels (Fig. 3).

To examine if Src was involved in CSE-induced EMT, H358 cells were pretreated with or without PP2 for 1 h, followed by treatment with 10% CSE for 72 h, and then EMT markers were determined. As shown in Fig. 4, treatment with 2 μM PP2 could suppress the E-cadherin decrease caused by CSE and inhibit the increase in vimentin, two characteristics of the EMT process. These data indicate that Src kinase plays a critical role in the CSE-induced EMT process.

NAC abrogated Src activation by CS

Previous reports have shown that Src activity could be oxidatively regulated [37–40]. As we observed that CSE induction of EMT was NAC-inhibitable and that Src was involved, we investigated whether Src activation by CSE might also be inhibited by NAC. H358 cells were pretreated with 2 mM NAC for 4h and then exposed to 10% CSE for 15 min. As shown in Fig. 5, NAC pretreatment inhibited phosphorylation of Src at Tyr418, suggesting that Src activation by CSE was dependent upon some component of CSE that was antagonized by NAC.

Effect of catalase on CSE-stimulated Src activation

Cigarette smoke is a mixture that contains various oxidative compounds including H_2O_2 and unsaturated aldehydes. To examine if H_2O_2 is involved in CSE-mediated Src activation, catalase, which rapidly converts H_2O_2 to O_2 and H_2O, was added to the medium before the cells were exposed to CSE. Fig. 6 shows that both H_2O_2 (200 μM) and CSE activated Src (phosphorylation of Src at Tyr418) at 15 min of exposure. Catalase (final concentration 400 U/ml) completely abrogated H_2O_2-stimulated Src activation. However, catalase had no significant effect on Src activation by CSE exposure, suggesting that oxidants/electrophiles other than H_2O_2 in the CSE might be responsible for CSE-mediated Src activation.

Acrolein activated Src

Cells were exposed to acrolein, a potent electrophilic α,β- unsaturated aldehyde present in CSE. As shown in Fig. 7, 15 μM acrolein increased Src phosphorylation at Tyr418 at 5 min and the phosphorylation remained as late as 2 hours after exposure. These data indicate that acrolein and other electrohiles in CSE are at least partly involved in CSE-mediated Src activation.

Discussion

EMT has been suggested to play important roles in the initiation of pulmonary fibrosis and metastasis of lung cancers, two pulmonary diseases associated with cigarette smoke [5, 6]. In this paper we found that CS induced EMT in human pulmonary epithelial cells (H358), as evidenced by a decrease in epithelial proteins such as E-cadherin and a concomitant increase in mesenchymal markers such as N-cadherin and vimentin. To explore the underlying mechanism(s), we examined whether NAC, which can directly react with many CSE components as well as potentially increase intracellular glutathione, and PP2 (4-amino-5-(4-chloro- phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), a relatively specific Src inhibitor [48, 49], abrogated the changes of these EMT markers. Furthermore, it was found that Src activation by CSE was also prevented by NAC. Although NAC inhibited EMT extracellularly, it was important to explore whether NAC also inhibited Src activation as some other component of CSE that did not activate EMT and did not react with NAC might still activate Src; however, that was not found to occur. These findings suggest that smoking could cause EMT through an oxidative pathway in which Src plays a critical role. It should

be noted that the composition of cell culture medium used in the current study is not the same as the extracellular fluid surrounding the alveolar epithelial cells *in vivo*. Physiological components that are missing include mucins, while glutathione, ascorbate, and urate differ in concentrations from those present in the cell medium. Further studies using *in vivo* models are obviously required to confirm current findings. Nevetheless, as the lung epithelial cells cultured in the cell culture medium maintain the epithelial phenotype and change to mesenchymal phenotype upon exposure to CSE, the current cell culture model is a useful model system study EMT at cell and molecular levels with the caveat that additional components of CS as well as differences in timing and presence of other cells and components of extracellular fluids would also affect pathophysiologic processes.

EMT is characterized by changes of cell phenotype and protein expression profile [19, 20]. During this process, cells lose epithelial phenotypic characteristics such as stable cell-cell junctions and apical-basolateral polarity, and acquire mesenchymal features such as increased matrix degradation, the ability to migrate, and a lack of cellular polarity. In addition, down regulation of epithelial proteins such as E-cadherin and β-catenin, and up regulation of the mesenchymal proteins such as fibronectin, N-cadherin and vimentin, usually occur in EMT and thus these proteins are often used as EMT markers. The current findings that CSE decreased epithelial protein E-cadherin and increased mesenchymal proteins N-cadherin and vimentin suggest that CSE induced EMT in the examined cell model. This result is in agreement with previous reports that cigarette smoking or a component (nicotine) could cause EMT changes in lung cancer cells [12, 50]. Taken together, these findings reveal that cigarette smoke is a potent EMT inducer, as are other sources of oxidative stress [1, 51]. This may partly explain a mechanism through which smoking contributes to the development of IPF, a disease in which EMT appears to play a role [1, 2], and the reason why smoking enhances metastasis and phenotypic changes of cancer cells, which also has been suggested to involve EMT [3, 4]. CSE used in this study contained about 20 μM acrolein [52]. Therefore, 10% CSE exposure contained 2 μM acrolein. Considering that acrolein in the pulmonary tract lining fluid can reach as high as 80 μM during smoking of 1 cigarette [53], the CSE concentration used in current study is within the range relevant to CS exposure. Although the lining fluid contains mucins and small molecular weight compounds that can react with acrolein, there are also many other components of CS that would also react with the lining fluid components.

In the context of the extracellular milieu, NAC may be an effective antioxidant. We demonstrated that extracellular NAC abrogated CSE-induced EMT changes (Fig. 2). CS can also induce lipid peroxidation products *in vivo* [54–56]. We thus conclude that CS induced EMT through a redox dependent mechanism. Many other EMT agonists, including TGFβ, EGF, and others also share this common redox dependent mechanism in EMT initiation [22, 27, 42–45], as evidenced by the fact that NAC inhibited EMT initiation by these inducers [27, 42], and that oxidants could directly induce or promote EMT [31, 46]. The results here may suggest to some that NAC administration could be a potential strategy for treatment and/or prevention of CS-induced EMT; however, the concentration and conditions here while demonstrating the ability of extracellular NAC to neutralize the EMT-inducing and Src-activating compounds in CSE are far greater than one would reasonably be able to give individuals by inhalation.

Src kinase is involved in cytoskeleton reorganization, cell migratory capacity, expression of mesenchymal proteins, and other aspects of EMT [29–34]. It is a common and critical downstream signaling target of a variety of EMT agonists, including TGFβ [33], EGF [35], endoplasmic reticulum (ER) stress [34], hepatitis B virus × protein (HBx) [32], and arachidonic acid [31, 32]. Src inhibition allows recovery of E-cadherin and suppresses expression of vimentin [57] and other proteins of the mesenchymal phenotype [30, 32]. In

line with these reports, our data showed that CS activated Src and its inhibition restored E-cadherin and abrogated vimentin (Fig. 3 and Fig. 4), indicating that Src activation is essential for CS-induced EMT. Although the exact mechanisms remains to be elucidated, CS might initiate EMT through additional oxidative mechanisms and indeed, pathways contributing to EMT, such as Ras/ERK, PI3K/Akt, and GSK3β, are also redox regulated [26, 58]. However, what is most notable is that inhibition of Src activation by PP2 also abrogated EMT and that Src or other members of the Src family are modulators of the ERK, Akt and GSK3β pathways [25, 35, 59–66].

Accumulating evidence suggests that Src activity could be regulated through a redox mechanism [37–40]. Consistent with this, this study showed that CS activation of Src is also redox dependent, since NAC, a powerful antioxidant inhibited Src activation by CS (Fig.5). The redox activation of Src seems to be mediated through direct modification of cysteine residues in Src protein [67–69]. Indeed, mutation of selected cysteine residues in the C-terminus makes Src insensitive to redox regulation [70]. NAC can reverse Src activation when inactivated by disulfide formation [69] but as NAC cannot reduce an alkylated cysteine, it would not reverse that type of modification. The initial activation of Src by CSE does not appear to involve H_2O_2, since catalase did not significantly reduce CSE-mediated Src activation while it abrogated the positive control of H_2O_2-caused Src activation (Fig. 6). Whole CS contains various oxidative/electrophilic compounds, including H_2O_2 and α,β-unsaturated aldehydes, which can modify cysteine residues in signaling proteins including Src. Although CSE can activate NADPH oxidase and producing H_2O_2 [47, 71], the current data shows that oxidants other than H_2O_2 in the CSE are responsible for CSE-mediated Src activation. The identification of the components involved requires analysis of the adducts formed with Src using mass spectrometry, which is beyond the scope of the current study.

Finally, it remains unclear whether the redox regulation of Src by CS is by directly modifying the cysteine residues in Src protein or indirectly by regulating kinases or phosphatases involved in the dephosphorylation of Tyr529 of Src. The phosphorylation status of Tyr529 is determined by the balance between C-terminal Src kinase (CSK), a constitutively expressed kinase that phosphorylates Tyr529, and protein tyrosine phosphatases (PTPs) such as PTP1B, CD45, SHP-2, and PTPα, which mediate the dephosphorylation of Tyr529 [36]. Both CSK and aforementioned PTPs are also redox regulated and their activity can be inhibited by oxidation of their active site cysteines [72–76]. These observations suggest that CS might also activate Src by regulating Tyr529 dephosphorylation in addition to being directly activated by modification of its cysteines. Thus, determining whether there are multiple mechanisms whereby Src is redox regulated by CS need further study.

Acknowledgments

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Highlights

- Cigarette smoke extract (CSE) activates c-Src in H358 cells.
- CSE-activated Src causes epithelial-mesenchymal transition (EMT).
- N-acetylcysteine inhibits CSE-induced Src activation and EMT.
- The Src kinase inhibitor, PP2, also inhibits CSE-induced Src activation and EMT.

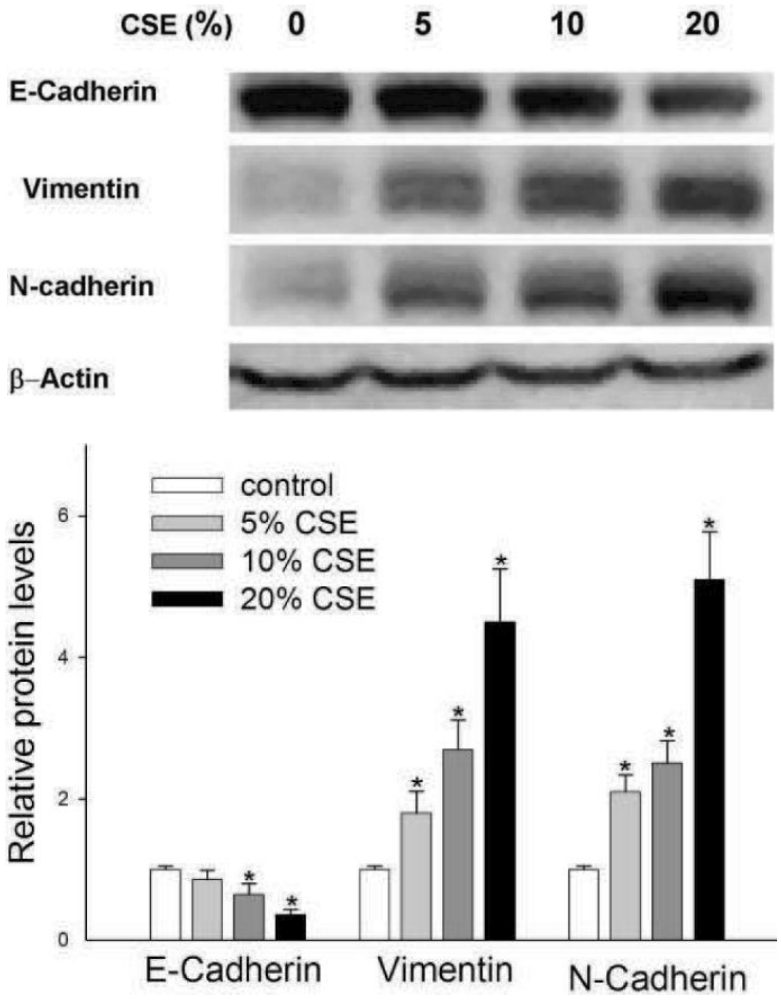

Figure 1.

CSE induced changes in epithelial and mesenchymal proteins in H358 cells. H358 cells were treated with different concentrations of CSE in 1% FBS medium for 72 h, and cell lysates were assayed for EMT proteins by western analysis. *, P<0.05 compared with control, N=3.

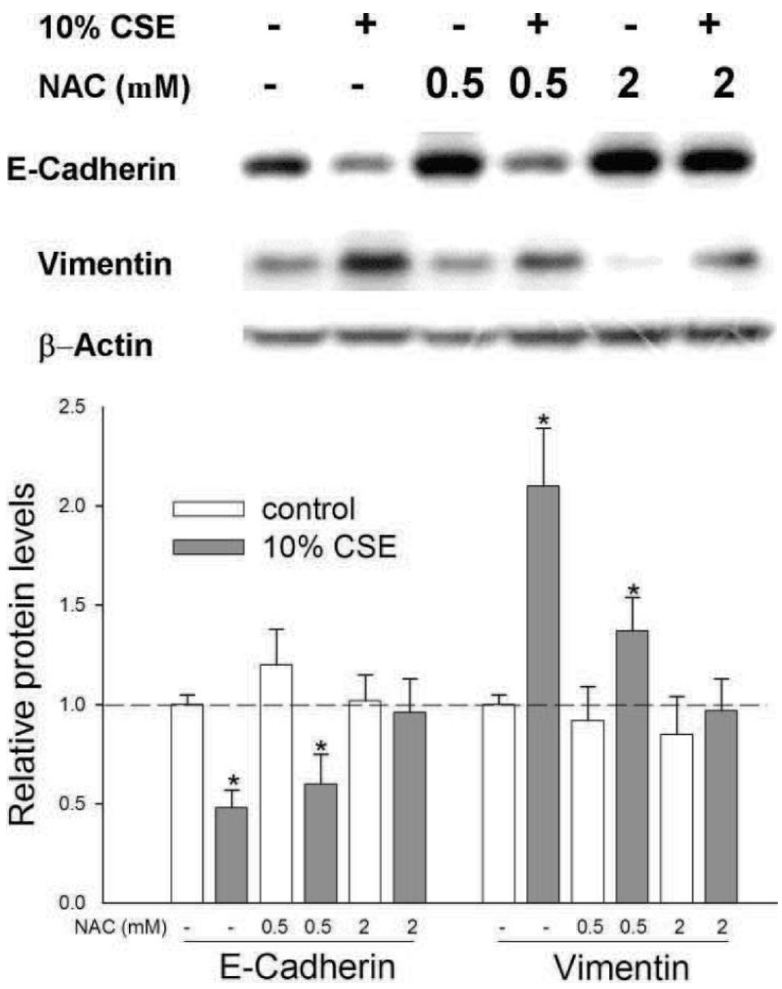

Figure 2.
NAC pretreatment abrogated changes in epithelial and mesenchymal proteins caused by CSE. *, P<0.05 compared with control, N=3.

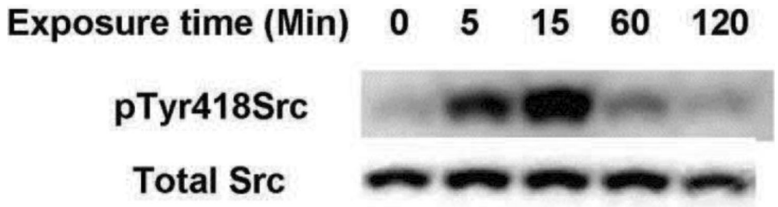

Figure 3.
Src activation by CSE. H358 cells were exposed to 10% CSE for different times. Phosphorylation of Src at Tyr418 was determined by western analysis.

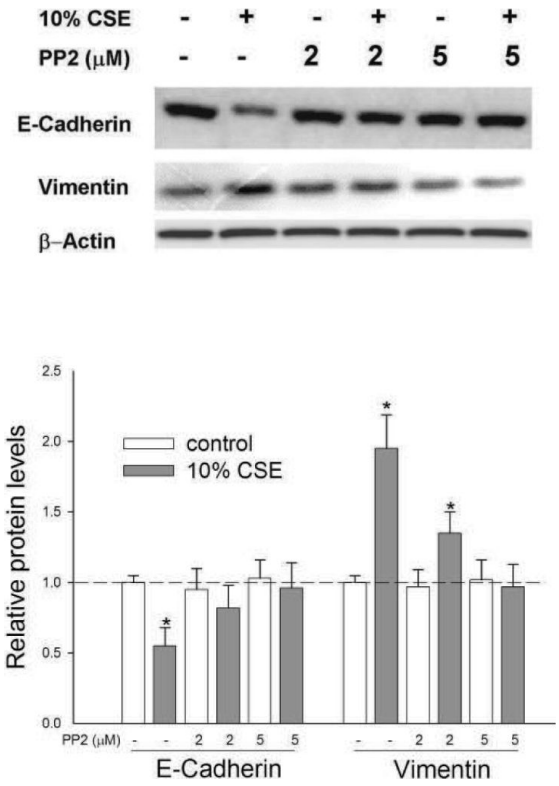

Figure 4.

Inhibition of Src suppressed changes in epithelial and mesenchymal proteins by CSE. H358 cells were pretreated with or without PP2 for 1 h and then exposed to 10% CSE for 72 h. The cell lysates were assayed for EMT-related proteins by western analysis. *, $P<0.05$ compared with control, N=3.

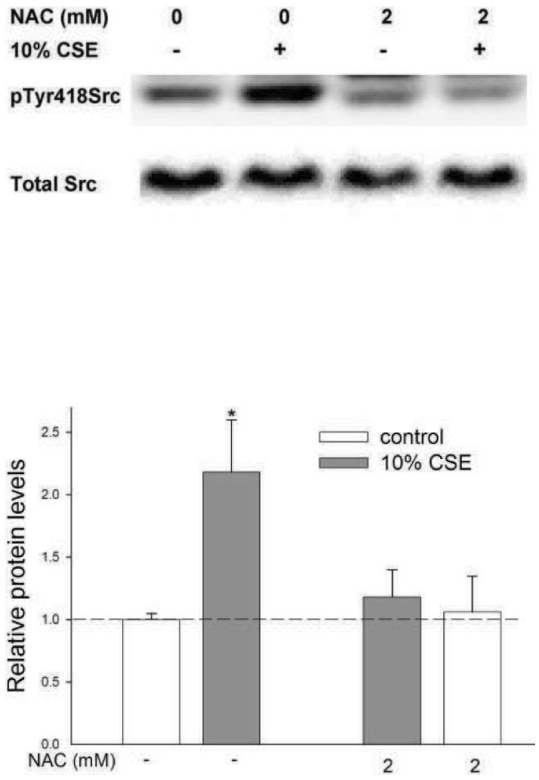

Figure 5.
NAC inhibited CSE-mediated Src activation. H358 cells were pretreated with/without 2mM NAC for 4 h and then exposed to 10% CSE for 15 min. Phosphorylation of Src at Tyr418 and total Src were determined with Western blots. *, P<0.05 compared with control, N=3.

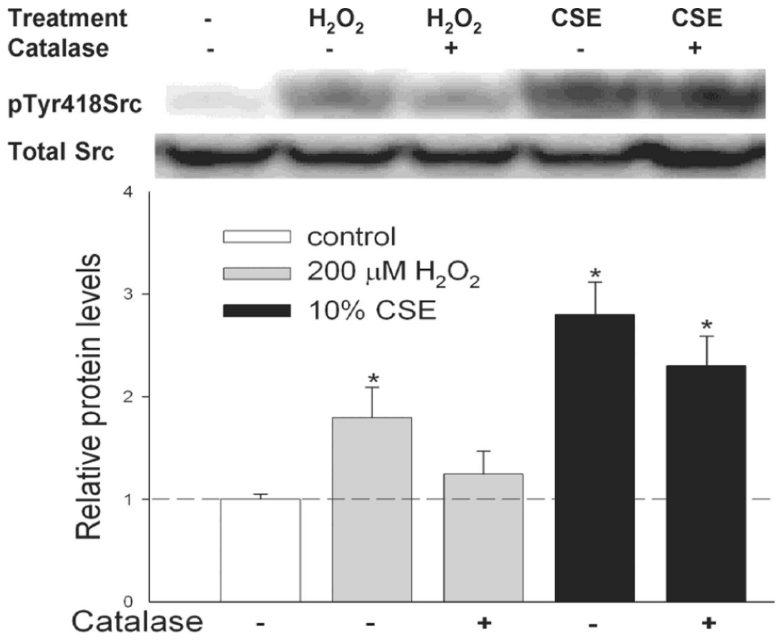

Figure 6.

Catalase effect on CSE-mediated Src activation. catalase (final concentration 400 U/ml) was added to culture medium immediately before H358 cells were exposed to 10% CSE. Phosphorylation of Src at Tyr418 and total Src were determined with Western blots. *, $P<0.05$ compared with control, N=3.

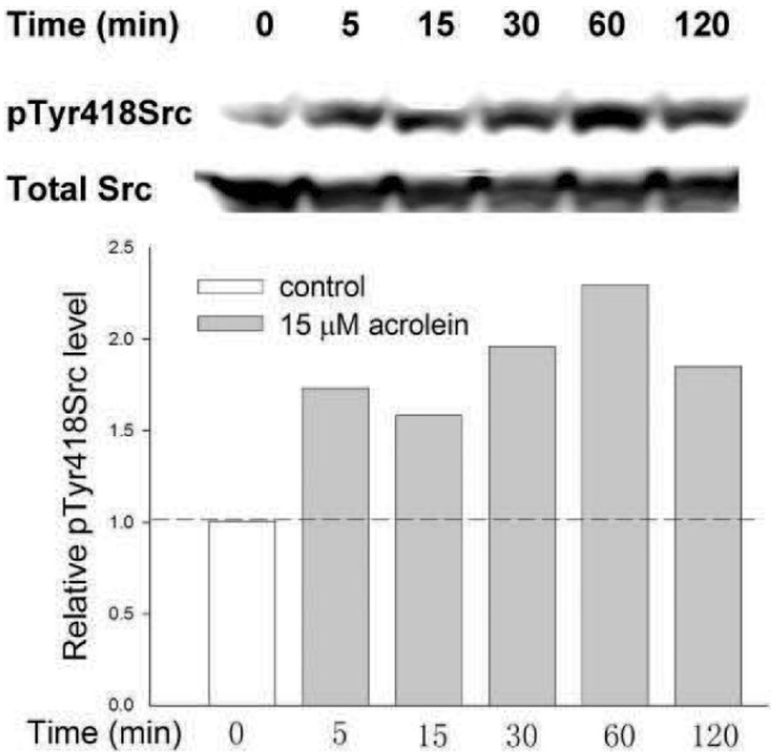

Figure 7.
Src activation by acrolein. H358 cells were exposed to acrolein (15 PM) for indicated time and the phosphorylation of Src at Tyr418 and total Src were determined with Western blots.