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# Woodsmoke Extracts Cross-Link Proteins and Induce Cornified Envelope Formation without Stimulating Keratinocyte Terminal Differentiation

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## ABSTRACT

Air pollution poses a serious risk to human health. To help understand the contribution of smoke from wood burning to the harmfulness of air pollution toward the skin, we studied the effects of liquid smoke, aqueous extracts of wood smoke condensate, a commercially available food flavor additive, in cultured keratinocytes. We report that liquid smoke can react with and cross-link keratinocyte cellular proteins, leading to abnormal cross-linked envelope formation. Instead of inducing genes ordinarily involved in terminal differentiation, liquid smoke induced expression of genes associated with stress responses. When transglutaminase activity was inhibited, liquid smoke still promoted protein cross-linking and envelope formation in keratinocytes. This phenomenon likely results from oxidative stress and protein adducts from aldehydes as either preloading the cells with N-acetylcysteine or reducing the aldehyde content of liquid smoke decreased its ability to promote protein cross-linking and envelope formation. Finally, liquid smoke-induced envelopes were found to have elevated protein content, suggesting oxidative cross-linking and formation of protein adducts might impair barrier function by inducing abnormal incorporation of cellular proteins into envelopes. Since the cross-linked protein envelope provides structural stability to the stratum corneum and serves as a scaffold for the organization of the corneocyte lipid envelope (hydrophobic barrier to the environment), these findings provide new insight into the mechanism by which pro-oxidative air pollutants can impair epidermal function.

**Key words:** air pollution; aldehyde-induced protein adducts; liquid smoke; oxidative stress; transglutaminase.

Skin functions as a physical and permeability barrier that prevents the loss of water and the entry of environmental toxins and infectious microbes (Elias, 2005). This protective barrier is continuously regenerated by keratinocytes through terminal differentiation. During this highly organized process, keratinocytes move from the basal to the cornified layer, exiting a proliferative state to accumulate keratin filament proteins and to develop a specialized structure known as the cornified or cross-linked envelope (CE). The early stage of terminal differentiation involves cell cycle withdrawal and synthesis of differentiation marker proteins such as loricrin, keratins, filaggrin, and involucrin. Keratinocyte transglutaminase (TGM1), a calcium-dependent enzyme, is subsequently activated to cross-link many proteins beneath the plasma membrane of a terminally differentiating corneocyte. With the formation of very stable isopeptide cross-links, the CE is highly insoluble (chemically resistant to

denaturants) and functions as a scaffold for lipid attachment to help form the barrier to the environment (Karim et al., 2019; Meyer et al., 2021).

Skin barrier dysfunction, either through genetic deficits or environmental stressors, can lead to the development or exacerbation of various cutaneous diseases. Increasing numbers of studies indicate that prolonged or repetitive exposure to pro-oxidative air pollutants has profound deleterious effects on the skin, including promoting aging, inflammatory conditions (such as atopic dermatitis and psoriasis), and cancer (Araviiskaia et al., 2019; Krutmann et al., 2014; Mancebo and Wang, 2015; Parrado et al., 2019). Corneocyte barrier dysfunction can be mediated by effects on numerous components including skin lipids, specific proteins, and cornified envelopes (Lee, 2020), and the consequences often reflect the

propensity of the skin to exhibit an inflammatory response (Jiang et al., 2020).

Smoke from forest fires and residential wood burning is a major contributor to both indoor and outdoor harmful air quality (Naeher et al., 2007). During wood burning, as in cigarette smoke, smoldering combustion is responsible for most of the biomass consumption, and studying emissions from smoldering biomass has been recommended for assessing human health exposure (Chan et al., 2020). Liquid smoke (LS) is generated from a smoke condensate of smoldering wood chips or sawdust combusted under limited oxygen. The aqueous extract of crude condensate is fractionated, purified, and concentrated to yield the desired water-soluble LS product, a widely used food flavoring. Although the refining processes reduce the levels of polycyclic aromatic hydrocarbon considerably, LS still contains many potentially harmful compounds that can also be found in woodsmoke (eg, aldehydes and phenols) (Montazeri et al., 2013; Simon et al., 2005). In this way, LS becomes a convenient tool to study the biological effects of chemical mixtures from woodsmoke.

Here, we demonstrate that LS can react with and cross-link keratinocyte cellular proteins, leading to abnormal CE formation. Instead of reflecting normal differentiation, this phenomenon could arise from oxidative stress and protein adduct formation. Since the correct protein scaffolding is crucial for CE behavior, the abnormal incorporation of cellular proteins into CEs could disrupt barrier function, leading to the development or exacerbation of various skin conditions. These observations reveal an unanticipated way pro-oxidative air pollutants could adversely affect skin health.

## MATERIALS AND METHODS

**Cell culture.** Human epidermal keratinocytes were derived from a sample of ostensibly normal adult foreskin. The cells used in this study were from low passage (5–15) before they become spontaneously immortalized at higher passages (Rice et al., 1993). These cells were grown with 3T3 feeder layer support in a 2:1 mixture of Dulbecco Vogt Eagle's and Ham's F-12 media (Allen-Hoffmann and Rheinwald, 1984) supplemented with 5% fetal bovine serum, 0.4  $\mu$ l/mg hydrocortisone, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 0.18 mM adenine, 10 ng/ml epidermal growth factor (EGF), and 10  $\mu$ M Y-27632 (a Rho kinase inhibitor that increases proliferative capacity and lifespan of primary human keratinocytes while retaining their capacity to differentiate) (Chapman et al., 2010).

**Cornified envelope formation.** Keratinocytes grown to confluence were rinsed once with serum-free medium and treated with Mesquite or Hickory liquid smoke (Wright's) at indicated concentrations in serum-free medium. To collect CEs (which resist disaggregation by detergent and reducing agent) and dissolve soluble proteins, sodium dodecyl sulfate (SDS) and dithioerythritol (DTE) were added to the medium to a final concentration of 2% w/v and 20 mM, respectively. After over-night incubation at room temperature, CEs were harvested by centrifugation for 5 min at 10000 rpm. The collected CE pellets were rinsed 3 times with 0.1% SDS in water and resuspended in this solution for quantitation by counting with a hemocytometer. Alternatively, since CEs are insoluble protein particles that scatter light in suspension, light scattering at  $A^{340}$  is thereby used to reflect the quantity of CEs (Rice and Green, 1979).

**Ninhydrin assay.** The protein content of CEs was determined by a modified ninhydrin assay (Etzler, 1972). Equal aliquots of collected CE samples were hydrolyzed with 10% sulfuric acid in a sand bath at 110°C for at least 4 h. The residues were redissolved in water and reacted with ninhydrin reagent in a 90–100°C water bath for 20 min. The reaction products were diluted with 2 volumes of 50% ethanol and quantified at  $A^{595}$ . The amount of protein in each sample was calculated using serum albumin standards analyzed in parallel.

**Quantitative RT-PCR.** RNA samples were harvested in Trizol (Life Technologies) and isolated by phenol/chloroform extraction followed by ethanol precipitation. cDNA was prepared using a reverse transcription kit (Applied Biosystems) and analyzed by qPCR with Taqman assay (Applied Biosystems).  $\beta$ -Glucuronidase (GUSB) was used as a reference gene for normalization.

**Luciferase reporter assay.** Recombinant human, mouse, and rat hepatoma cells (HG2L6.1c1, H1L6.1c3, and H4L1.1c4, respectively) were grown in minimum essential medium  $\alpha$  containing 10% fetal bovine serum. These cells contain a stably transfected AhR-responsive firefly luciferase reporter gene plasmid (pGud-Luc6.1 or pGudLuc1.1) (Han et al., 2004). After 24 h incubation with Hickory or Mesquite LS at indicated concentrations at 37°C in 96-well plates, cells were rinsed twice with phosphate-buffered saline and lysed with Promega passive lysis buffer. Luciferase activity was measured using an Orin microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) with automatic injection of Promega stabilized luciferase reagent as previously described (He et al., 2014). Luciferase activity was corrected for background activity from DMSO-treated cells and expressed as a percent of the luciferase activity obtained with a maximally inducing concentration (10 nM) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

**In vitro cross-linking assay.** This method was based on an immunochemical assay for detecting transglutaminase activity (Hiiragi et al., 1999). Keratinocytes grown to confluence were rinsed twice with phosphate-buffered saline, harvested in 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), and lysed by freeze-thaw followed by brief sonication. The insoluble fraction (containing membrane-bound proteins, including TGM1) was collected by centrifugation at 1300 rpm for 5 min at 4°C. The pellets were resuspended in ice-cold HEPES (pH 7.4), homogenized by brief sonication, and evenly distributed into several tubes (~100 mg of protein per tube). 10 mM 5-(biotinamido)pentylamine (Alfa Chemicals) was added to each tube along with the indicated chemicals. After a 30 min incubation at 37°C, the reaction was stopped by the addition of SDS (2% w/v) and DTE (20 mM) and boiling at 100°C for 5 min. Samples (~10 mg of protein) were subjected to SDS-PAGE, and biotin incorporation was detected with an anti-biotin antibody conjugated to horseradish peroxidase (Cell Signaling).

**Statistical analysis.** Each figure shows results from 3 independent experiments except as noted. Unpaired t-test was used to compare differences between the 2 treatment groups. Comparisons of more than 2 groups were performed using one-way ANOVA and Dunnett's post hoc test to calculate individual differences. All statistical analyses were performed on GraphPad Prism8 with a significance set at  $p < .05$ .

## RESULTS

### Liquid Smoke Increased Cornified Envelope Formation and the Protein Content of Envelopes

CE formation was first measured to evaluate the biological effects of LS on keratinocytes. As shown in Figure 1A, the formation of CEs was increased after 12 h of incubation and plateaued at 48–72 h. Mesquite LS appeared to be more effective at inducing envelope formation than Hickory LS at the equal concentrations employed. In Figure 1B, the efficacy of LS on CE formation was seen to be concentration-dependent. Treatments with 1  $\mu$ l/ml or lower concentrations of LS did not induce CE formation within 24 h. The ionophore X537A is known to induce CE formation through permeabilizing the cell membrane to extracellular calcium and is thereby used as a positive control. The LS-induced CEs were as stable as the X537A-induced CEs. They were both resistant to detergent and reducing agent, and the quantity did not change significantly when remeasured up to 1 year later

(Supplementary Figure 1). Remarkably, treatments with 10  $\mu\text{l/ml}$  LS produced more light scattering than 100  $\mu\text{M}$  of X537A, which was found to induce CE formation from nearly all the cells in a previous study (Rice and Green, 1979). The possibility that LS promoted cell growth was excluded as cell growth was inhibited by treatments with LS at 0.5  $\mu\text{l/ml}$  or higher (Supplementary Figure 2). This LS-mediated increase in light scattering likely reflected more protein incorporation into the envelopes. As shown in Figure 1C, the calculated amount of protein per CE was significantly higher in LS-treated samples than X537A-treated samples, rationalizing the higher measures of light scattering.

### Liquid smoke induced Expression of Genes Associated with Stress Responses but Not Terminal Differentiation

We next investigated whether LS-induced CE formation by upregulating the expression of genes involved in terminal differentiation. Treatments with 4  $\mu\text{l/ml}$  or higher concentrations of LS were cytotoxic to keratinocytes and dramatically suppressed mRNA levels while promoting CE formation (Supplementary Figure 3). In Figures 2A and 2B, 2  $\mu\text{l/ml}$  of LS did not induce differentiation markers such as K1, K10, FLG, IVL, LOR, and TGM1. Instead, some expression was inhibited at 8 h, and at 24 h the expression of all the markers was significantly reduced. In contrast, treatment with LS upregulated genes related to oxidative stress response, proinflammatory response, and xenobiotic metabolism at both 8 h and 24 h (Figures 2C and 2D). GCLM, NQO1, TXNRD1, and HMOX1 are NRF2 target genes. The nuclear transcription factor erythroid 2-related factor 2 (NRF2) mediates the cellular defense system against oxidative stress by regulating the expression of numerous genes involved in glutathione production (eg, GCLM), ROS detoxification (eg, NQO1), the thioredoxin-based antioxidant system (eg, TXNRD1), and heme metabolism (eg, HMOX1) (Tonelli et al., 2018). CXCL8 and PTGS2 (also known as cyclooxygenase, COX2) are both inflammatory mediators that respond to oxidative stress (Yang et al., 2011). Finally, CYP1A1 and CYP1B1, genes of enzymes involved in phase I xenobiotic metabolism, were also induced by LS treatment. The upregulation of CYP1A1 at the enzyme level was confirmed by 7-ethoxyresorufin-O-deethylase activity (Supplementary Figure 4).

The induction of CYP1A1 and CYP1B1 suggested that LS activated the aryl hydrocarbon receptor (AhR) signaling pathway. AhR is a ligand-dependent transcription factor that mediates multiple cellular responses to xenobiotic chemicals by inducing the expression of CYP1A1, CYP1B1, and many other genes (Denison et al., 2011). To investigate the ability of LS to directly activate the AhR signaling pathway, we examined the effect of LS in recombinant human, rat, and mouse hepatoma cells containing a stably transfected AhR-responsive luciferase reporter gene. As shown in Figure 3, LS-induced luciferase reporter gene activity in human and rat hepatoma cells, with Mesquite LS being substantially more efficacious than Hickory LS. In contrast, little or no induction was observed in mouse hepatoma cells by either Mesquite or Hickory LS. Together, these findings indicated that, instead of stimulating genes related to terminal differentiation, LS constituents induced genes downstream of NRF2 and AhR signaling pathways, both being associated with cellular stress responses.

### Liquid smoke induced Protein Cross-Linking Independently of Transglutaminase Activity, Leading to Abnormal Cornified Envelope Formation

Transglutaminases (TGMs), especially TGM1 (1000-fold higher than TGM2 or TGM3, data of (Phillips et al., 2020)), are essential for CE assembly as TGM1 knockout mice die at birth due to impaired skin barrier function (Matsuki et al., 1998). The active site cysteine of TGMs can be readily alkylated by iodoacetamide (IA), preventing their catalytic activity (Kim et al., 1994). As shown in Figure 4A, X537A induced CE formation by promoting the entry of extracellular calcium that subsequently activates TGMs. When the cells were pretreated with 20 mM IA, the

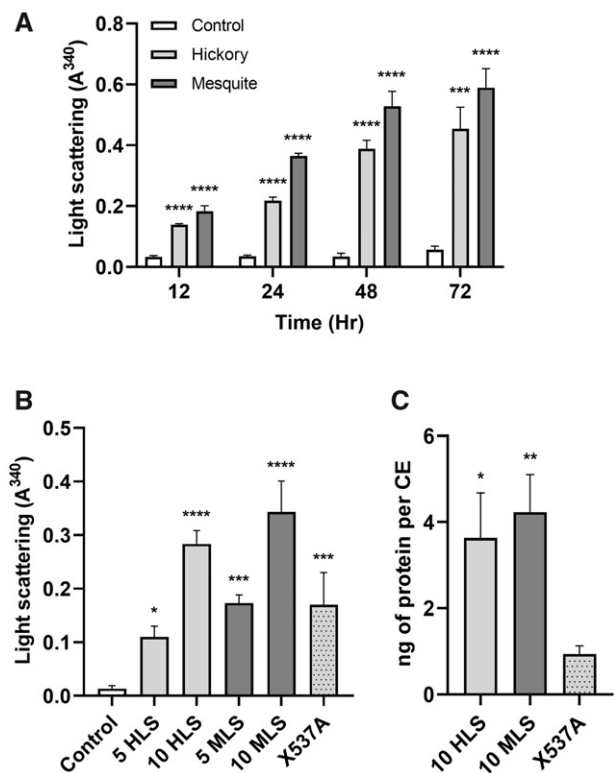


Figure 1. LS increased CE formation and the protein content of CEs. A, Keratinocytes were treated with 10  $\mu\text{l/ml}$  of Hickory or Mesquite LS (HLS and MLS, respectively) and harvested for CE quantitation at the indicated times. Since CEs are insoluble protein particles that scatter light in suspension, light scattering at A<sup>340</sup> measures the quantity of CEs. B, Keratinocytes were treated with 5 or 10  $\mu\text{l/ml}$  of LS for 24 h before harvest for CE quantitation. 100  $\mu\text{M}$  of X537A was used as a positive control for CE formation. C, CEs induced by 10  $\mu\text{l/ml}$  of LS or 100  $\mu\text{M}$  of X537A were counted visually with a hemocytometer, and the protein content of each sample was determined by ninhydrin assay. The ng of protein per envelope was calculated by dividing the total amount of protein by the total number of envelopes. Results are presented as the mean  $\pm$  SD. Significant differences from untreated control (A and B) or X537A positive control (C) are indicated (\* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ ).

promoting effect of X537A on CE formation was significantly reduced. In contrast, the CE induction capacity of Hickory or Mesquite LS was not inhibited by IA pretreatment. However, without TGM activity for proper CE assembly, the appearance of envelopes in IA-treated samples was abnormal, as the CEs were less transparent than CEs formed in the absence of IA (Figure 4B).

Similarly, in Figure 5, pretreatment with IA had very little effect on LS-mediated incorporation of 5-(biotinamido)pentylamine (BP). In this in vitro protein cross-linking assay, keratinocyte particulate extracts (the insoluble fraction of cell lysates containing membrane-bound TGM1) were incubated with LS or calcium in the presence of BP, a biotin-labeled aliphatic amine substrate of TGM1 (Ikura et al., 1998). Each reaction product was subjected to Western blotting, and the biotin cross-links were detected with an anti-biotin antibody. The cross-linking activity of TGM1 requires calcium as a cofactor. In the group treated with calcium, protein cross-linking was greatly stimulated due to the activation of TGM1, but when the cysteine active site of TGM1 was alkylated by IA, the incorporation of BP by calcium was completely inhibited. However, in groups treated with Hickory and Mesquite LS, only half the incorporation was lost with IA pretreatment, suggesting that some LS-induced protein cross-linking was mediated by a mechanism independent of TGM activity.

Human embryonic kidney 293 cells express very little TGM activity. The mRNA levels of TGM1 and TGM3 in 293 cells are  $\sim$ 1000-fold and 10-fold

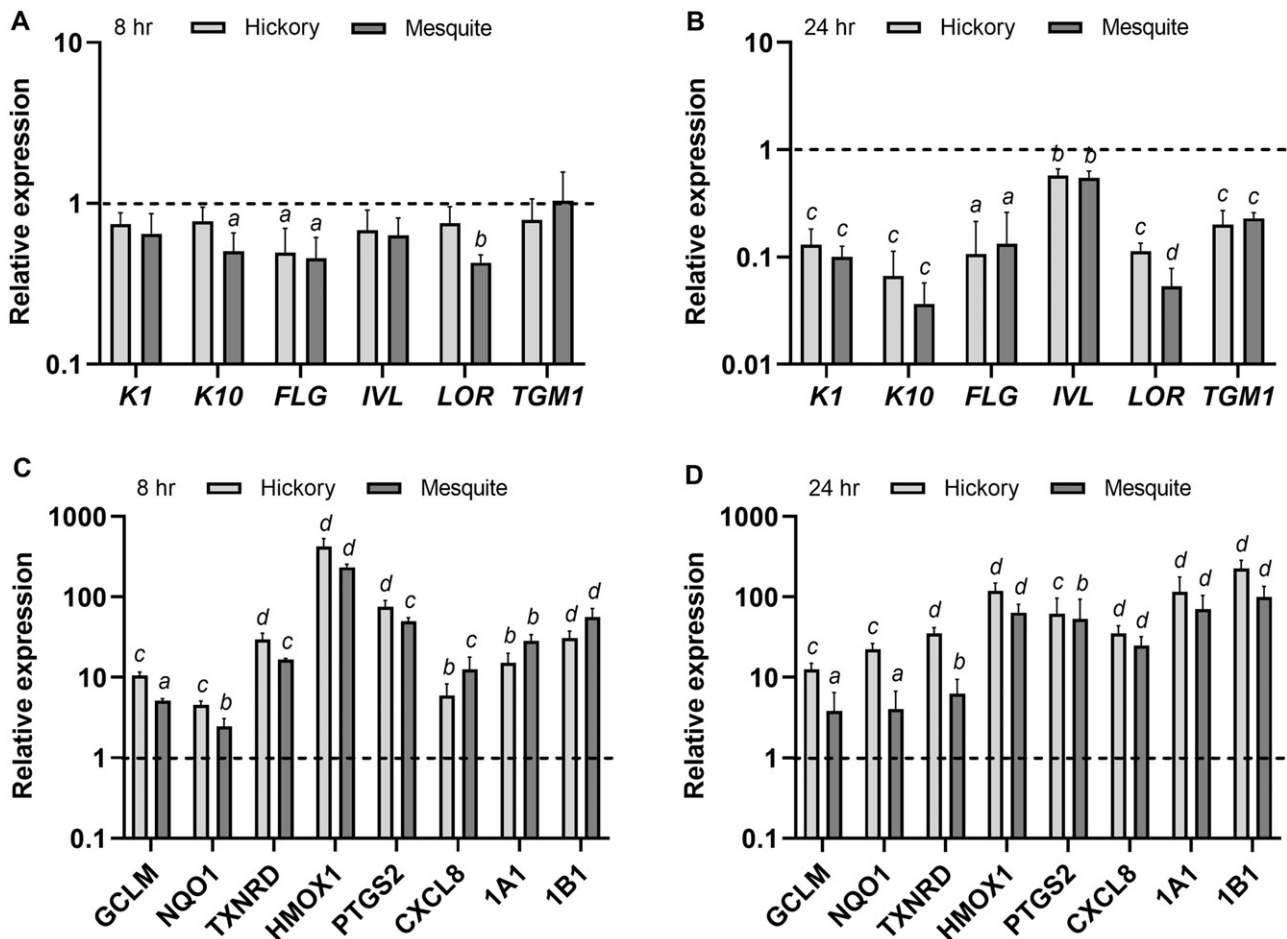


Figure 2. LS upregulated genes associated with oxidative stress, proinflammatory response, and xenobiotic metabolism. Keratinocytes were harvested after treatment with 2  $\mu$ l/ml of LS for 8 h (A and C) or 24 h (B and D), and the mRNA levels of target gene were measured using qPCR. Dashed line indicates untreated control value. Results are presented as the mean  $\pm$  SD. Significant differences from untreated control are indicated (a,  $p < .05$ ; b,  $p < .01$ ; c,  $p < .001$ ; d,  $p < .0001$ ).

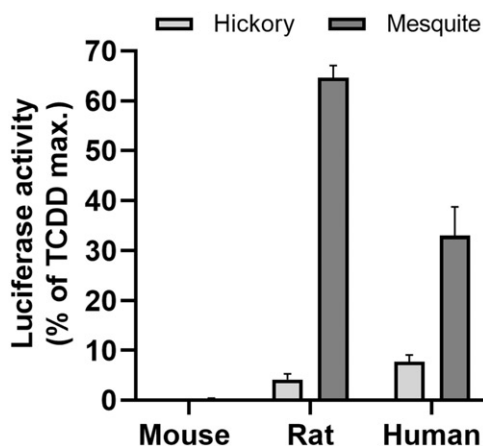


Figure 3. LS activated the AhR signaling pathway in human and rat hepatoma cells. Recombinant mouse, rat, and human hepatoma cells containing a stably transfected AhR-responsive luciferase reporter gene were incubated with 1  $\mu$ l/ml of LS for 24 h and luciferase activity was measured as described under Materials and Methods section. Values of luciferase activity was calculated by subtracting the background from cells treated with 0.1% DMSO and normalizing the response to that of cells incubated with a maximal inducing concentration of TCDD (set to 100%). Results are presented as the mean  $\pm$  SD of triplicates from a representative experiment.

lower, respectively, than the ones in keratinocyte cultures (Figure 6A). However, when 293 cells were exposed to LS, they formed a considerable number of CE-like particulates that were insoluble and resistant to detergent and reducing agent (Figure 6B). As 293 cells are not ordinarily CE-forming cells, the CE-like particulates were even more deformed, fragmented, and opaque than keratinocyte CEs (Figure 6C). In the in vitro protein cross-linking assay, high levels of biotin cross-links were also detected in 293 particulate extracts treated with LS but not calcium (Figures 6D and 6E). Together, these findings confirmed that LS can stimulate protein cross-linking through a mechanism independent of TGM activity, leading to abnormal CE formation.

#### Liquid Smoke-Induced Protein Cross-Linking and Cornified Envelope Formation Were Results of Oxidative Stress

Aldehydes are highly reactive electrophiles found in both wood smoke and LS (Lipari et al., 1984; Montazeri et al., 2013; Simon et al., 2005). Aldehydes exert cellular toxicity mainly through increased oxidative stress and the formation of a wide variety of intra- and intermolecular covalent protein adducts (Grimsrud et al., 2008). Sodium borohydride (SB) is an effective reagent for the reduction of aldehydes and ketones to the corresponding alcohols (Chaikin and Brown, 1949). As Hickory and Mesquite LS are both acidic solutions ( $\sim$ pH 2–3), to avoid the acid-catalyzed decomposition of borohydride, LS was neutralized with sodium

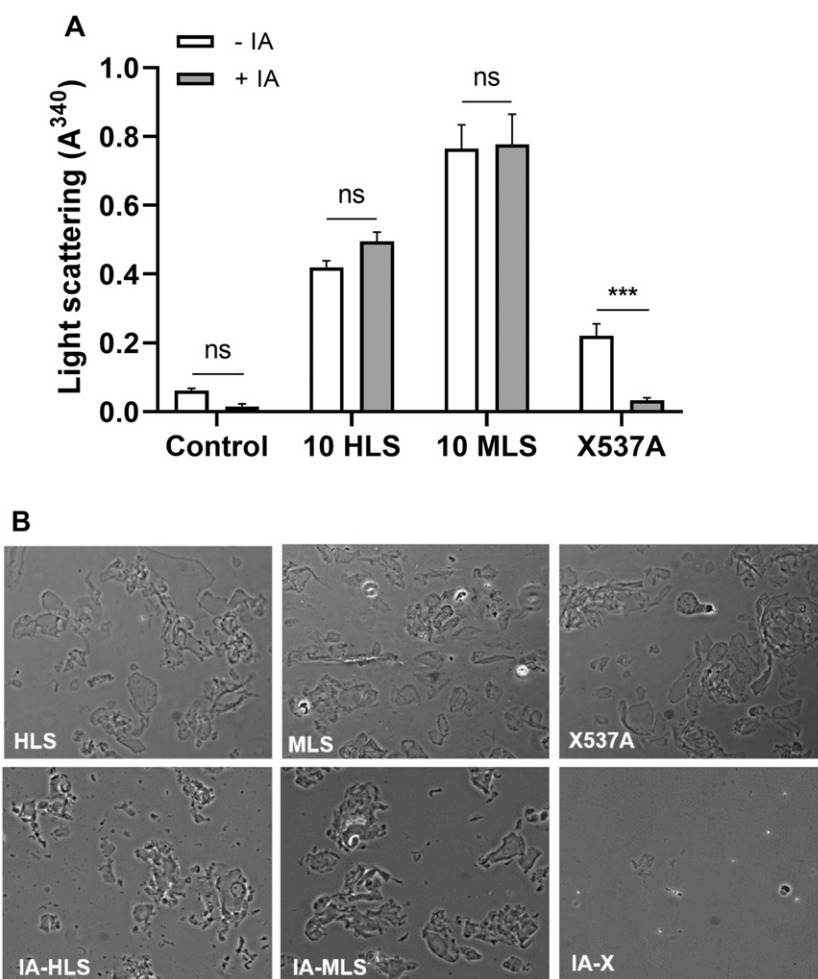


Figure 4. LS promoted CE formation in keratinocytes lacking TGM activity. A, Keratinocytes were pretreated with or without 20 mM IA for 1 h, rinsed, and switched to fresh serum-free medium containing 10  $\mu$ l/ml of LS or 100  $\mu$ M X537A. CEs were harvested and quantified after 24 h of incubation. Results are presented as the mean  $\pm$  SD. Significant differences between the presence and the absence of IA are indicated (ns, not significantly different; \*\*\* $p$  < .001). B, Representative images of CEs under indicated treatments were taken by phase contrast microscopy.

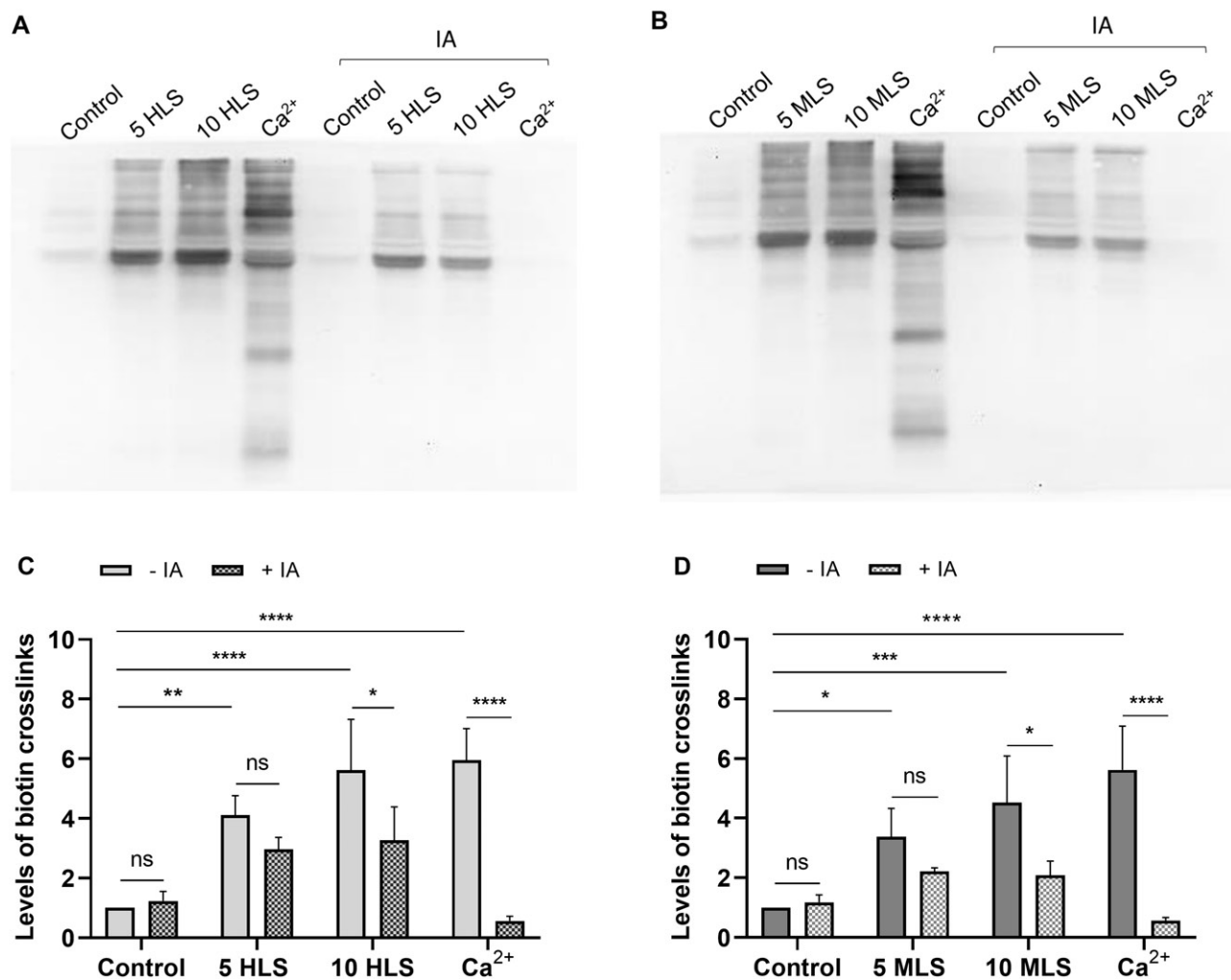
hydroxide prior to SB reduction. Aqueous solutions of SB were prepared for immediate addition to LS at the final concentrations of 0.02 M, 0.1 M, and 0.5 M. After over-night incubation, SB-LS solution was added to keratinocyte particulate extracts for the *in vitro* protein cross-linking assay. As shown in Figures 7A–7D, LS-stimulated protein cross-linking was dramatically decreased in an SB concentration-dependent manner. Similarly, when cultured keratinocytes were treated with SB-LS, the CE formation decreased as the concentrations of SB pretreatment increased (Figures 7E and 7F). Adjusting the pH of LS to neutral ( $\sim$ pH 7) did not significantly alter the activity of LS on protein cross-linking or CE formation.

N-Acetylcysteine (NAC) is a widely used antioxidant that protects cells against a variety of pro-oxidative insults (Zhitkovich, 2019), and treatment of cultured keratinocytes with NAC reduced the activity of LS on CE formation in a concentration-dependent manner (Figure 8). In contrast, NAC did not prevent X537A from inducing CE formation in keratinocytes. Pretreatment with NAC for 24 h produced a similar inhibitory effect on LS-induced CE formation as 1 h NAC pretreatment. Protein cysteine thiols are particularly vulnerable to oxidation by ROS and other electrophilic molecules due to their nucleophilicity. Oxidation of protein thiols and loss of free (or reduced) thiols could indicate increased oxidative stress in cells (Baba and Bhatnagar, 2018). When keratinocytes were exposed to LS, the presence of free thiols measured by

Ellman's reagent (5-5'-dithiobis-(2-nitrobenzoic acid)) decreased in a LS concentration-dependent manner (Supplementary Figure 5). Together, these findings suggested that LS-induced CE formation and protein cross-linking were results of oxidative stress and protein adducts from aldehydes.

## DISCUSSION

Oxidative stress, a state of elevated levels of ROS, occurs when the balance of oxidants within the cells exceeds the levels of antioxidants present (Sies, 2015). Exposure to environmental pollutants (such as pesticides, heavy metals, and cigarette smoke) can lead to excessive production of ROS, causing oxidative damage to cellular components, including DNA, lipids, and proteins (Al-Gubory, 2014). Woodsmoke contains free radicals and other components capable of generating ROS within cells. Substances in woodsmoke particulates such as water-soluble transition metals or organic compounds can generate hydroxyl and superoxide anion radicals through Fenton reaction and redox recycling (Valavanidis et al., 2005). In rural Nigeria, women who cook with biomass fuels were found to have increased systemic inflammation and oxidative stress (Oluwole et al., 2013). Firefighters occupationally exposed to woodsmoke also had an elevated body burden of oxidative stress (Adetona et al., 2013). Levels of DNA damage as well as malondialdehyde and



**Figure 5.** LS promoted protein cross-linking in keratinocyte particulate extracts lacking TGM activity. A and B, Keratinocytes were pretreated with or without 20 mM IA for 1 h before harvest. The particulate extracts were incubated with 5 or 10  $\mu$ l/ml of LS or 5 mM calcium in the presence of 10 mM BP for 30 min at 37°C. The biotin cross-links were visualized by Western blot. C and D, MYImageAnalysis was used to quantify relative biotin incorporation from each lane for statistical comparison. Results are presented as the mean  $\pm$  SD. ns, not significant different; \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ .

protein carbonyls (markers of lipid and protein oxidation, respectively) were strongly increased in patients with chronic inflammatory lung disease attributed to cigarette or biomass smoke exposure (Ceylan et al., 2006). In cultured cells, exposure to woodsmoke extracts has been associated with elevated ROS production, proinflammatory cytokine release, DNA strand breaks, and lipid peroxidation (Danielsen et al., 2011; Leonard et al., 2000). Concordantly, results from our qPCR analysis revealed increased expression of genes associated with NRF2 signaling and inflammatory responses, demonstrating the oxidative potential of LS.

LS clearly activated the AhR in human and rat hepatoma cultures, accounting for the CYP1A1 and CYP1B1 induction observed in LS-exposed human keratinocytes. In contrast, mouse hepatoma cells were virtually unresponsive to LS. This finding of species-specific AhR activation could help rationalize the discordance of LS-mediated toxicity in humans and rats versus mice (Braun et al., 1987; Ohshima et al., 1989). Although PAHs are commonly found in emissions from biomass burning, it is unlikely that the biological activities of LS in this study were caused by such compounds as the manufacturing process of LS greatly reduces their content (Montazeri et al., 2013; Simon et al., 2005), and they were found to be below the limit of detection in one experiment performed as in (Chan et al., 2020). The AhR is well-known for its species

differences in ligand specificity (Denison et al., 2011; Kikuchi et al., 1996). Accordingly, the activation of human and rat AhR, but not that of mouse AhR, by LS could result from the presence of human/rat-specific AhR agonists in LS, the presence of mouse-specific AhR antagonists in LS, or the presence of other substances that indirectly inhibited the mouse AhR signaling pathway. Nevertheless, the upregulation of AhR-targeted genes, CYP1A1 and CYP1B1, could further contribute to the LS-related ROS production as CYP enzymes are known to generate reactive intermediates or ROS during their catalytic cycle (Veith and Moorthy, 2018).

The oxidative potential of LS also arises from its aldehyde content. Aldehydes are strongly electrophilic, highly reactive, and relatively stable compounds (Singh et al., 2013). They are emitted into the atmosphere from the incomplete combustion of hydrocarbons and other organic materials. Due to their amphiphilic nature, aldehydes can easily diffuse across cell membranes to interact with macromolecules and generate a wide variety of intra- and intermolecular covalent adducts (Pizzimenti et al., 2013). In cultured cells, exposure to acetaldehyde (one of the common environmental aldehydes) has been associated with oxidative stress, membrane injury, and likely necrotic cell death (Elamin et al., 2014). In this study, membrane injury could plausibly be responsible for LS-induced CE formation as damaged cell membranes allow influx of

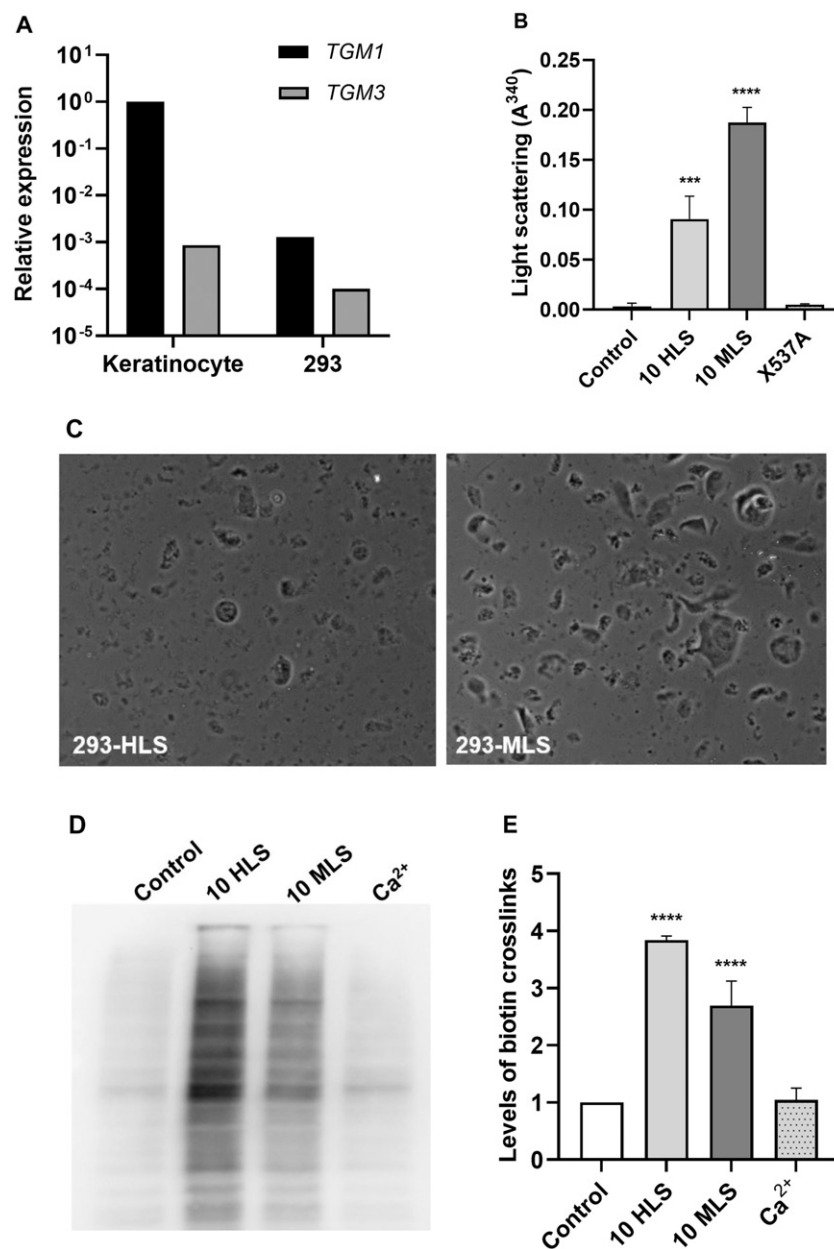


Figure 6. LS-induced protein cross-linking and CE formation in human embryonic kidney 293 cells. A, RNA samples from keratinocytes and 293 cells were collected at confluence. The mRNA levels of TGM1 and TGM3 were measured using qPCR. B, Cultured 293 cells were exposed to 10  $\mu\text{M}$  LS or 100  $\mu\text{M}$  X537A for 24 h before harvesting for CE quantitation. C, Representative images of CEs under indicated treatments were taken by phase contrast microscopy. D, 293 cell particulate extracts were incubated with 10  $\mu\text{M}$  LS or 5 mM calcium in the presence of BP for 30 min at 37°C. The biotin crosslinks were visualized by Western blot. E, MYImageAnalysis was used to quantify relative biotin incorporation from each lane for statistical comparison. Results are presented as the mean  $\pm$  SD. Significant differences from untreated control are indicated (\*\* $p < .001$ ; \*\*\*\* $p < .0001$ ).

extracellular calcium to activate TGMs for CE formation. However, inhibition of TGM activity did not prevent the formation of LS-induced protein cross-links. These cross-links can be formed initially with the adduction of a carbonyl to a protein side chain and, if there is a second reactive site, further reactions with another amino acid can produce a cross-linked species (Hagglund et al., 2018). The potential of LS oxidants to cross-link proteins thereby mimicked the terminal differentiation process, resulting in the formation of CE-like structures observed in IA-treated keratinocytes and nonkeratinocyte-derived cells, such as human embryonic kidney 293 cells. Supporting this interpretation, the LS-induced protein cross-linking and CE formation were attenuated when the aldehyde content was reduced by sodium borohydride. Similarly,

when the cells were preloaded with NAC, the formation of LS-induced CE was also reduced. Together, these findings support the idea that LS-induced protein cross-linking and CE formation were caused by elevated cellular ROS and electrophiles.

The phenomenon of chemical-induced protein cross-linking has been previously reported, where erbstatin and an analog did not induce differentiation but caused protein cross-linking into CE-like structures (Stanwell et al., 1995). Complementary to their findings, which focused on the therapeutic limitation of these chemicals at high concentrations due to their cross-linking activities, the LS results presented here have revealed an action through which air pollution could produce adverse effects on the skin. Since an intact CE is essential for skin barrier



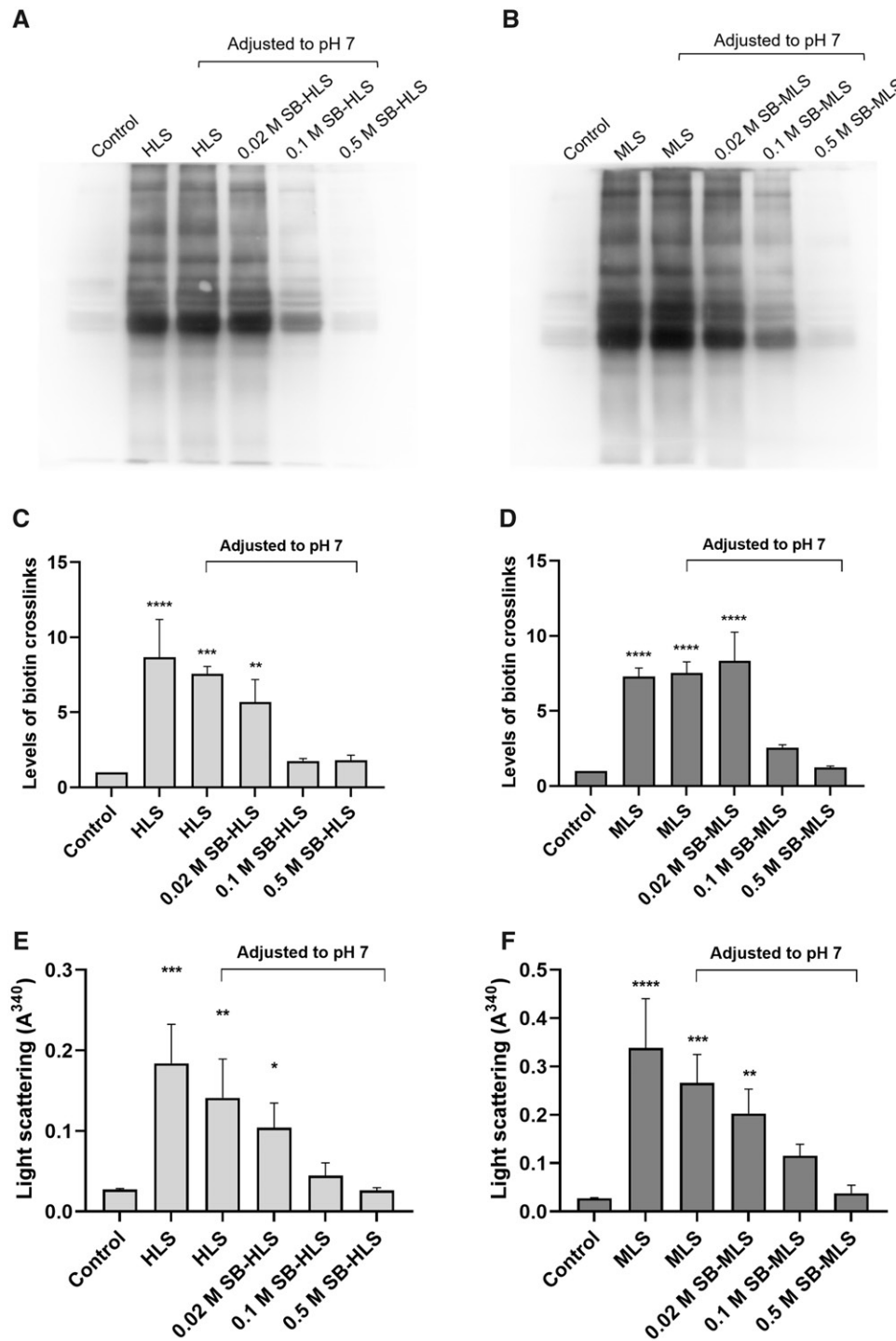


Figure 7. Pretreatment of LS with sodium borohydride (SB) reduced LS-induced protein cross-linking and CE formation. A and B, Hickory and Mesquite LS were pretreated with SB at indicated concentrations. 10  $\mu$ l/ml of the original LS, LS with pH adjusted to 7, and the SB-LS solutions were incubated with keratinocyte particulate extracts in the presence of BP for 30 min at 37°C. The biotin crosslinks were visualized by Western blot. C and D, MYImageAnalysis was used to quantify relative biotin incorporation from each lane for statistical comparison. E and F, Cultured keratinocytes were treated with 10  $\mu$ l/ml of the original LS, LS with pH adjusted to 7, and the SB-LS solutions for 24 h before harvest for CE quantitation. Results are presented as the mean  $\pm$  SD. Significant differences from untreated control are indicated (\* $p$  < .05; \*\* $p$  < .01; \*\*\* $p$  < .001; \*\*\*\* $p$  < .0001).

function, altered structural organization and protein composition of CE have been linked to barrier dysfunction in inherited skin disorders, inflammation, and aging (Agrawal and Woodfolk, 2014; Ishida-Yamamoto and Iizuka, 1998; Streubel et al., 2017). In our study, LS-induced CEs appeared to have higher protein content than calcium-

induced CEs. This could result from a highly oxidative cellular environment in which protein cross-linking is promoted not only between CE structural proteins but also widely among other cellular proteins. If proteins are incorporated abnormally into CEs, their protective function or desquamation might be impaired. Also, the incorporation of foreign

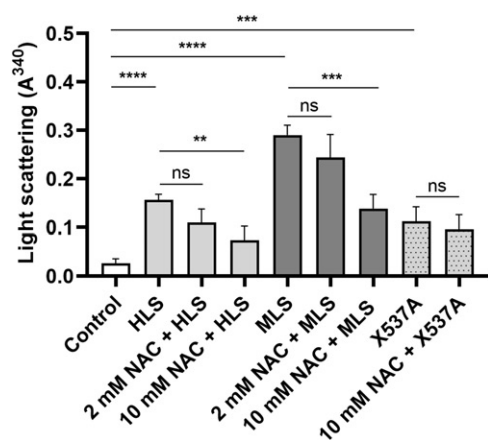


Figure 8. N-Acetylcysteine (NAC) reduced LS-induced CE formation. Cultured keratinocytes were preloaded with NAC for 1 h before cotreatment with 10  $\mu$ l/ml of LS or 100  $\mu$ M of X537A. After 24 h incubation, CEs were harvested for quantitation. Results are presented as the mean  $\pm$  SD. ns, not significant different; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ .

pollutant aldehydes could induce an immunological response, leading to skin inflammation (Natsch et al., 2012). As reported by several epidemiology studies, chronic exposure to traffic-related particulate matter is significantly associated with signs of extrinsic skin aging, including pigment spots and wrinkles (Vierkotter et al., 2010). Indoor air pollution generated from burning biomass fuels can also accelerate premature skin aging (Li et al., 2015). The prevalence of eczema is also high in children who live in areas with traffic-related air pollution (Kramer et al., 2009). Besides affecting healthy skin, air pollution is even more harmful to diseased human skin. In patients with atopic dermatitis or urticaria, air pollution aggravates the disease symptoms, leading to increased numbers of hospital visits (Kim et al., 2013; Kousha and Valacchi, 2015).

Several mechanisms have been proposed to explain the deleterious impacts of air pollution on the skin, including (1) activation of the AhR, (2) elevation of oxidative stress, and (3) induction of an inflammatory cascade (Araviiskaia et al., 2019; Mancebo and Wang, 2015; Parrado et al., 2019). As shown in this study, LS seemed to produce its effects through such mechanisms and could even include the receptor for advanced glycation products, active in epidermal keratinocytes (Iwamura et al., 2016). Furthermore, we have demonstrated that constituents in LS can cause abnormal CE formation through covalent cross-linking of cellular proteins. Future proteomic analysis of LS-induced CEs, including examination of differentially expressed and chemically modified (particularly oxidized/adducted) proteins will help characterize the effects of air pollutants on skin health.

## SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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## DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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