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Hybrid malondialdehyde and acetaldehyde protein adducts form in the lungs of mice exposed to alcohol and cigarette smoke

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Abstract

Background—Most alcohol abusers smoke cigarettes and approximately half of all cigarette smokers consume alcohol. However, no animal models of cigarette and alcohol co-exposure exist to examine reactive aldehydes in the lungs. Cigarette smoking results in elevated lung acetaldehyde (AA) and malondialdehyde (MDA) levels. Likewise, alcohol metabolism produces AA via the action of alcohol dehydrogenase and MDA via lipid peroxidation. A high concentration of AA and MDA form stable hybrid protein adducts known as malondialdehyde-acetaldehyde (MAA) adducts. We hypothesized that chronic cigarette smoke and alcohol exposure in an *in vivo* mouse model would result in the *in vivo* formation of MAA adducts.

Methods—We fed C57BL/6 mice *ad libitum* ethanol (20%) in drinking water and exposed them to whole body cigarette smoke 2 hour/d, 5d/week for 6 weeks. Bronchoalveolar lavage fluid and lung homogenates were assayed for AA, MDA, and MAA adduct concentrations. MAA-adducted proteins were identified by Western blot and ELISA.

Results—Smoke and alcohol exposure alone elevated both AA and MDA, but only the combination of smoke+alcohol generated protein-adducting concentrations of AA and MDA. MAA-adducted protein (~500ng/ml) was significantly elevated in the smoke+alcohol-exposed mice. Of the five MAA-adducted proteins identified by Western blot, one protein band immunoprecipitated with antibodies to surfactant protein D. Similar to *in vitro* PKC stimulation by purified MAA-adducted protein, PKC epsilon was activated only in tracheal epithelial extracts from smoke and alcohol-exposed mice.

Conclusions—These data demonstrate that only the combination of cigarette smoke exposure and alcohol feeding in mice results in the generation of significant AA and MDA concentrations, the formation of MAA-adducted protein, and the activation of airway epithelial PKC epsilon in the lung.

Introduction

Cigarette smoke causes damage to proteins and other macromolecules (Reznick et al., 1992). Much of this damage is caused by aldehydes in cigarette smoke. Acetaldehyde forms from the pyrolysis of tobacco and is one of the most abundant aldehydes contained in cigarette smoke (Hoffman and Wunder, 1999; Freeman et al., 2005; Huber et al., 1991). Acetaldehyde has the propensity to create nucleophilic adducts with amine-containing compounds such as proteins and DNA (Schenk et al., 2002). This damage induces a robust inflammatory response mediated by pro-inflammatory cytokines such as IL-8 and TNF- α . Cigarette smoke has also been observed to reduce lung cell viability *in vitro* (Facchinetti et al., 2007; Moretto et al., 2009) and cause impaired lung function *in vivo* (Miller et al., 2002; Pauwels et al., 2001). As a result, acetaldehyde induces cilia dysfunction through direct cilia ATPase inactivation and adduct formation with cilia dynein and tubulin (Sisson et al., 1991). Acetaldehyde induces inhibition of DNA repair, potentiates N-nitrocompound-induced mutagenesis in cultured human cells (Grafstrom et al., 1986), and causes cancer in experimental animals (International Agency for Research on Cancer, 1985). Due to acetaldehyde's deleterious effect *in vivo*, the lowest observed adverse effect level (LOAEL) of inhaled acetaldehyde in humans is 134 ppm/30min with mild respiratory irritation reported (Lewis, 2004). Most of the exposure to acetaldehyde in humans is from cigarette and alcohol consumption (WHO, 1995).

In addition to acetaldehyde production, cigarette smoke has been observed to increase levels of malondialdehyde in the plasma and breast milk of human smokers (Ermis et al., 2005; Nielsen et al., 1997). Malondialdehyde is a by-product of cigarette smoke's interaction with cellular lipids (Church and Pryor, 1985). Malondialdehyde also can damage macromolecule such as DNA by creating adducts or indirectly via lipid peroxidation (Munnia et al., 2006).

Ethanol metabolism is also known to cause acetaldehyde and malondialdehyde production. Ethanol oxidation by phase one metabolizing enzymes, such as alcohol dehydrogenase and cytochrome P2E1, will lead to acetaldehyde production in ethanol-exposed tissue and produce, via lipid peroxidation, malondialdehyde (Church and Pryor, 1985; Kamimura et al., 1992). These aldehydes can cause the production of inflammatory mediators in lung epithelium (Wyatt et al., 2001). High levels of acetaldehyde and malondialdehyde due to ethanol metabolism have also been linked to liver injury (Israel et al., 1986; Tuma, 2002; Yang and Wender, 1964). Ethanol consumption and subsequent metabolism can lead to at least five types of protein adducts: acetaldehyde (AA), malondialdehyde (MDA), malondialdehyde-acetaldehyde hybrid complex (MAA), 4-hydroxynonenal (HNE), and Hydroxy ethyl radical (HDE) (Niemela, 1999; Niemela, 1995). Only MAA-adducted proteins have been shown to be highly stable and resistant to rapid degradation. Of the five protein adducts created by ethanol, MAA adducts have been documented to stimulate pro-inflammatory responses in airway epithelial cells, slow pulmonary epithelial cell wound healing *in vitro* and promote alcohol-related liver injury (Tuma, 2002; Tuma et al., 1996; Wyatt et al., 2005). The conjugation of MDA and AA with its target protein leads to MAA adduct formation as shown in Figure 1.

Reactive biologically-relevant aldehydes such as MAA will form adducts with many different nucleophilic proteins. The mammalian lung endogenously contains many accessible nucleophilic molecules. Pulmonary surfactants molecules are of special interest because of their ubiquity across mammalian species and general composition of 90% lipids and 10% proteins (Haagsman and Diemel, 2001; Johansson and Curstedt, 1997). Pulmonary surfactants are barrier materials of the lungs and have a dual role: firstly, as a true surfactant, lowering the surface tension; and secondly, participating in innate immune defense of the lung (Haagsman and Diemel, 2001). Pulmonary surfactant contains four non-serum proteins

SP-A, SP-B, SP-C and SP-D, named after their chronologic order of discovery (Brogden et al., 1996). Pulmonary surfactants are also very necessary for proper respiration. In fact SP-B deficient humans die shortly after birth, as do SP-B knockout mice (Cole et al., 2000; Tokieda et al., 1997).

MAA adducts have been shown to cause inflammation. This inflammation caused by MAA adducts are protein kinase C mediated (Kharbanda et al., 2002; Wyatt et al., 1999). Of the several isoforms of protein kinase C, protein kinase C epsilon (PKC ϵ) is the PKC isoform (Aksoy et al., 2004) that is associated with the Toll-like receptor 4, IL-12, and TNF- α mediated immune and inflammatory responses (Aksoy et al., 2004). PKC ϵ may also be associated with cigarette smoke and ethanol-induced MAA adduct effects.

This manuscript focuses on quantifying production and protein interaction of AA, MDA, and MAA adduct formation in the lungs of cigarette smoke and ethanol-exposed mice. We hypothesize that lung co-exposure to the combination of alcohol and cigarette smoke will uniquely result in lung concentrations of malondialdehyde and acetaldehyde necessary for the production of MAA-adducted protein. Elucidating the pathophysiology of MAA adduct formation in the lung will contribute to our understanding of one mechanism by which cigarette smoke and ethanol exert their combined deleterious effects.

Methods

Cigarette Smoke and Air Exposure

Female C57BL/6 mice were exposed to the smoke of 20 1R3F reference cigarettes (University of Kentucky, Lexington, KY) per day. Mice receiving cigarette smoke were gradually brought to their target exposure over a period of 2 wk and treated 6 d/wk for 6 wk. Cigarette smoke was administered using the Teague Small Animal smoke exposure chamber (Teague Industries, Davis, CA). The mice were exposed to smoke inhalation in the chamber via methods previously described (Elliott et al., 2007). Mice were exposed to 20 cigarettes total in one complete treatment. Sham treatments were conducted in the same manner in a similar apparatus for the same periods of time, but mice were exposed to filtered room air only. To ensure adequate cigarette smoke exposure, blood carboxyhemoglobin (COHb) levels were measured using an IL-682 CO-Oximeter (Instrumentation Laboratories, Lexington, MA). COHb levels did not differ between water- and alcohol-receiving groups. Cigarette smoke-exposed mice had COHb levels of $10.14 \pm 0.27\%$, whereas the air-exposed mice had COHb levels of $0.53 \pm 0.07\%$.

Alcohol Feeding

The alcohol-treated mice were given increasing concentrations of ethanol in water over 2 weeks until the target concentration of 20% was reached. Mice in the alcohol group were given 5% alcohol (wt/vol) to drink *ad libitum* for 2 days, 10% ethanol (wt/vol) for 5 d, 15% ethanol (wt/vol) for 7 days, and 20% ethanol (wt/vol) for 6 weeks as previously described (Song et al., 2002). Mice in the matched control group were given water from the same source without ethanol. All blood alcohol concentrations (BAC) were taken at the 20% target concentration during the nocturnal portion of the sleep/wake cycle. BAC were measured using a commercial assay kit (Pointe Scientific, Canton, MI).

Aldehyde Assay

At the end of the 8-week treatment, 3×1 mL aliquots of bronchoalveolar lavage fluid (BALF) were collected. Acetaldehyde and malondialdehyde levels were assayed by gas chromatography and thiobarbituric acid assay, respectively.

Vials containing the analytical solutions were incubated at 70°C for 1 hour to reach a thermodynamic equilibrium. Acetaldehyde was quantified by combining 0.3 ml BALF with 1 ml of Thiourea propanol. The admixture was then vortexed and subsequently centrifuged at 2060g for 10 minutes. Supernatant (1 mL) was transferred to a gas chromatograph tube and analyzed as previously described (Gramiccioni and Milana, 1986). Malondialdehyde (MDA) concentrations were quantified by thiobarbituric acid reactive substances (TBARS) assay reactivity. Under high temperatures, thiobarbituric acid will react with MDA to form an MDA-TBA adduct. This adduct was measured colorimetrically at 530nm. The TBARS assay is a widely documented and effectively utilized assay to quantify MDA presence (Armstrong 1994). To reduce the interference of non-MDA conjugation to thiobarbituric acid, we incorporated a 0.1% phosphoric acid precipitation step into our TBARS protocol.

Enzyme-linked Immunosorbent Assay (ELISA) for MAA-adducted protein

MAA-adducted proteins were quantified in five different treatment groups by indirect competitive ELISA as previously described (Xu et al., 1997). Briefly, polystyrene flat bottom plates (96-well) were coated overnight at 4°C in a humidified chamber with 2 µg/mL purified BSA-MAA antigen in a blocking agent consisting of 1% PBS/Tween (Blotto) for 2 hours at room temperature. Homogenized lung tissue samples and BSA/MAA standards were added to a 96-well PVC round bottom plate. Samples were then diluted (1:3) in Blotto and incubated with Biotinylated anti-BSA-MAA (1:100). The contents of the round-bottomed plate were then transferred to the flat-bottomed plate and incubated with a 1:200 dilution of Streptavidin-conjugated horseradish peroxidase (HRP) at room temperature for 45 min. Plates were developed with tetramethylbenzidine (TMB) substrate in the dark at room temperature before halting the color reaction with 8 M H₂SO₄. Plates were read at 450 nm using a Bio-Rad plate reader to determine optical density.

Western Blot for MAA-adducted protein

To determine specific size of the adducted proteins, Western blot for MAA-adducted protein was performed to detect the presence of MAA-adducted protein in mice lung tissue exposed to the five different treatment groups. Lungs were homogenized in a lysis buffer as described (Elliott et al., 2007). Homogenized lung tissue samples from each treatment group and purified BSA-MAA (positive control) were electrophoresed using a 10% Tris-HCL polyacrylamide gel (150 V for 75 min). Standard protein markers (Bio-Rad, Hercules, CA) were loaded for migratory distance controls. Gels were transferred to PVDF membrane (Millipore, Billerica, MA) using a Bio-Rad semi-dry transfer apparatus (15V for 15 min). To probe the transferred blots for MAA adduct, membranes were blocked overnight at 4°C in 3% BSA in Tris-buffered saline (20 mM Tris-HCL and 137 mM NaCl, pH 7.4), incubated for 1 hr at room temperature with rabbit anti-MAA antibodies (1:50,000) followed by HRP-conjugated goat anti-rabbit IgG (1:40,000) for 45 min at room temperature.

As a loading control, blots were incubated with rabbit anti-beta actin (1:5,000) for 1 hr at room temperature instead of the anti-MAA IgG. Blots were developed using a chemiluminescence kit (Pico West, Pierce, Rockford, IL) and exposed to x-ray film.

PKC Activity Assay

Mouse tracheal epithelial cell lysates were used to determine PKC activity. Both the cytosolic and particulate cell fractions were assayed. Activated PKC was translocated to the particulate fraction. PKC isoform activity was determined similar to methods described previously (Wyatt et al., 2003; Wyatt et al., 2000). Airway epithelium contains the α , β , δ , ϵ , and ζ PKC isoforms (Alpert et al., 1999). To measure specific PKC isoform activity, 24 µg/ml PMA, 30 mM dithiothreitol, 150 µM ATP, 45 mM Mg-acetate, PKC isoform-specific substrate peptide, and 10 µCi/mL [γ -³²P]-ATP were mixed in a Tris-HCl buffer (pH 7.5).

Chilled (4°C) cell lysate (cytosolic or particulate) samples (20 µl) were added to 40 µl of the reaction mix and incubated for 15 min at 30°C. This mixture (60 µl) was then spotted onto P-81 phosphocellulose papers (Whatman, Clinton, NJ) to halt incubation, and papers were subsequently washed 5 times in 75 mM phosphoric acid for 5 min, washed once in 100% ethanol for 1 min, dried, and counted in nonaqueous scintillant (National Diagnostics, Atlanta GA). PKC activity was expressed in relation to the total amount of cellular protein assayed as picomoles of phosphate incorporated per minutes per milligram.

Immunoprecipitation of MAA-adducted protein

To determine the identity of the MAA-adducted protein from mouse lung, surfactant protein-D (SP-D) was immunoprecipitated and the immunoprecipitated protein was probed by Western blot for MAA adduction. Mouse lung tissue from the smoke and alcohol treatment groups was homogenized in tissue lysis buffer, protein concentration was determined by Bradford assay (Kruger, 2002), and each sample was equilibrated by protein concentration. Samples were then continually rotated with 50 µg/ml rabbit anti-surfactant protein-D (Millipore) overnight at 4°C followed by the addition of 50 µl sepharose protein A (Amersham Pharmacia, Piscataway, NJ) for 4 hr at 4°C. Sepharose beads were then isolated by centrifugation and washed five times with 1 mL immunoprecipitation wash buffer consisting of 50 mM Tris (pH 7.5), 200 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.05% sodium dodecyl sulfate (SDS). Protein was extracted from the washed sepharose beads with 75 µL of SDS-2ME reducing buffer, vortexed, and boiled at 95°C for 15 min. A 12% Tris-HCL polyacrylamide gel was resolved from the reduced proteins and probed for MAA-adducted protein as stated above.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM of the indicated number of animals in each group. Statistical differences between the various group means were determined using the Mann-Whitney test and one-way ANOVA with Tukey's multiple comparison as a post-test (Graphpad Prism, San Diego, CA). A probability of less than 0.05 was accepted as significant.

Results

Effect of cigarette smoke and alcohol co-exposure on aldehyde levels in mouse lung

Mice were exposed to inhaled cigarette smoke, fed alcohol in their water, or given a combined exposure of both cigarette smoke and alcohol. Control animals were sham-exposed to air and only drank water. Following exposures, lung lavage fluids were assayed to determine the concentrations of acetaldehyde and malondialdehyde. Acetaldehyde levels were 37% higher in the smoke group as compared to the ethanol group (54.31 $\pm$ 8.6 µM and 34.12 $\pm$ 5.5 µM, respectively) and the acetaldehyde concentrations of the smoke and ethanol co-exposure group were 118.7 µM $\pm$ 14.0 (Figure 2a).

Malondialdehyde is a reactive metabolite of ethanol metabolism. The oxygen radicals resulting from Cyp2E1 metabolism of ethanol interact with lipids to create lipid peroxides such as malondialdehyde. The malondialdehyde levels were 51% higher in the ethanol group compared to the smoke-exposed group (8.04 $\pm$ 1.2 µM and 2.81 $\pm$ 0.65, respectively; Figure 2b). The malondialdehyde concentrations of the smoke and ethanol co-exposure group were 12.3 $\pm$ 0.92 (Figure 2b). These data display the propensity of cigarette smoke to produce more aldehydes than ethanol alone, and also show ethanol alone produced more malondialdehyde than cigarette smoke alone. Finally this data shows that malondialdehyde and acetaldehyde is produced in higher quantities in the co-exposed group.

Detection of MAA-Adducted Protein using Western blot

The co-exposure of ethanol and cigarette smoke resulted in the highest concentrations of acetaldehyde and malondialdehyde (Figure 2a & b). Because acetaldehyde and malondialdehyde have a well-documented interaction that creates the malondialdehyde-acetaldehyde reactive compound, resulting in the MAA adducts, we analyzed both the BALF and crude lung homogenates (0.76mg/ml) from exposed mice for the presence of MAA adducts by utilizing Western blotting. MAA-adducted proteins were detected only in the combination smoke and ethanol-exposed mice. Five adducted MAA proteins bands were observed with the strongest band at 43 kDa (Figure 3).

Quantification of MAA-Adducted Protein

After confirming the presence of MAA, we quantified the amount present in the exposure groups by ELISA. We observed that the concomitant smoke and ethanol group resulted in statistically significant concentrations of MAA-adducted proteins as compared to all treatment groups (Figure 4). MAA-adducted protein concentration (332.70 ± 92.9 ng/ml) in the concomitant group were nearly 9 times higher than the smoke-exposed group (37.37 ± 5.9 ng/ml), 4.5 times higher than the ethanol group (72.0 ± 14.65 ng/ml) and 33 times higher than the control (14.10 ± 6.6 ng/ml; Figure 4). When quantified by ELISA, MAA adducts in the co-exposed group were more than three times higher than the sum of the individual ethanol and cigarette smoke exposure generated MAA adducts.

Detection of MAA-adducted Surfactant Protein D

We detected the presence of MAA-adducted surfactant protein-D in the lungs of mice co-exposed to alcohol and cigarette smoke by protein immunoprecipitation using an antibody to surfactant protein D (SP-D) and subsequently probed by Western blot using rabbit anti-MAA antibodies. A MAA-adducted band observed at a molecular weight of 43 kDa was immunoprecipitated by antibodies to SP-D only in the concomitant alcohol and smoke exposure group (Figure 5). In addition, heavy and light chains of the anti-SPD IgG (Ab) were recognized by the secondary Western blot detection antibody, HRP-goat anti-rabbit (Figure 5). This identification of MAA-SPD only in the co-exposed group reinforces the previous presented data describing the high levels of acetaldehyde, malondialdehyde and MAA in this group. These data suggest that surfactant proteins are a target for MAA-adduction in the lung.

PKC epsilon Activity in Smoke and Alcohol exposed mice

Since we have observed a significantly increased level of acetaldehyde and malondialdehyde production resulting in higher levels of MAA-SPD adduct formation in the co-exposed groups, we wanted to determine whether elevated amounts of MAA adducted protein coincides with increased protein kinase C epsilon (PKC ϵ) activity as previously detected in vitro (Kharbanda et al., 2002; Wyatt et al., 2000). The exposure of ethanol and cigarette smoke led to a statistically significant 41% increase (103.86 ± 22.75 and 50.77 ± 3.87 , respectively; $p < 0.05$) in PKC ϵ activity in the trachea of co-exposed mice as compared to the control (Figure 6). These data demonstrate that tracheal epithelial cell PKC ϵ is activated under the same conditions of elevated MAA-adducted protein formation *in vivo*.

Discussion

In this study, we propose a model of malondialdehyde-acetaldehyde adducted (MAA) protein formation in lung airway (Figure 7). Ethanol is metabolized by alcohol dehydrogenase into acetaldehyde. Ethanol exposure also leads to lipid peroxidation products such as malondialdehyde. Burning tobacco results in the production of high concentrations

of acetaldehyde. Cigarette smoking produces lung inflammation and elevates lung malondialdehyde. Two molecules of malondialdehyde and one molecule of acetaldehyde covalently bind to nucleophilic sites of proteins to form a MAA-adducted protein. Acetaldehyde levels were 37% higher in the smoke group as compared to the ethanol group. Cigarette smoke contains high levels of acetaldehyde. The ethanol group also had statistically high levels of acetaldehyde as compared to the control group, but less acetaldehyde compared to the smoke group.

The malondialdehyde concentrations of the smoke and ethanol co-exposure group were also highest among all of the groups. Due mostly to the Cyp2E1 metabolism of alcohol, oxidative radicals are created. These oxidative radicals damage cellular lipids leading to lipid peroxide production such as malondialdehyde. As a result, the malondialdehyde levels were 51% higher in the ethanol group compared to the smoke-exposed group. Due to the well-established reaction of malondialdehyde and acetaldehyde to produce protein adducts, the presence of MAA was determined and quantified. MAA adducted proteins were detected and were statistically significantly higher in the co-exposed group as opposed to the other treatment groups. MAA in the co-exposed groups were determined by immunoprecipitation and Western blot to be adducted to surfactant protein D. Because surfactant protein D was immunoprecipitated from treated and homogenized mouse lungs, and subsequently probed for MAA protein via Western blot, the only adducted protein observed on the Western blot in Figure 5 is MAA-SPD. One of the results of the MAA-SPD presence in our co-exposed group was a statistically significant increase in activity of PKC ϵ which is associated with inflammatory cytokine production and release in similarly treated alcohol and smoke co-exposure groups (Elliott et al., 2007).

Acetaldehyde concentrations in the co-exposed group are 34% higher than the sum of the acetaldehyde concentrations in both the smoke-only exposed group and the ethanol-exposed group. This would suggest that cigarette smoke and ethanol exposure produce acetaldehyde concentrations higher than endogenous acetaldehyde dehydrogenase can metabolize. The additional acetaldehyde can be metabolized by CYP2E1. CYP2E1 is inducible and has a high affinity for acetaldehyde. However it is feasible to consider, due to the rate limiting nature in the acetaldehyde to acetate reaction (Weiner, 1987), that additional un-metabolized acetaldehyde could be contributing to lipid peroxidation and malondialdehyde production. Malondialdehyde (MDA) is a product of lipid peroxidation due to ethanol exposure and metabolism (Niemela, 1995; Nordmann et al., 1990; Tuma, 2002). We observed that ethanol exposure created 186% more BALF MDA compared to smoke exposed mice and the co-exposed mice had 15% more MDA present in their BALF as compared to the sum of the individual smoke and ethanol exposed mice groups combined. This elevated MDA production may be associated with the observed accumulation of acetaldehyde in the ethanol and cigarette exposed group.

The high concentrations of acetaldehyde and MDA in the co-exposure group are likely the reason for MAA-adducted protein to only be detected by Western blot and to be significantly higher than all other treatment groups as demonstrated by ELISA. The high levels of acetaldehydes in the co-exposed group could be potentiating the development of MAA outside of the direct aldehyde contribution. The potential for acetaldehyde to contribute to oxidative stress, thereby increasing MDA via the lipid peroxidation pathway, is reasonable and could be the explanation of the 15% increase in the co-exposure MDA levels as compared to the sum of the MDA produced by the smoke and ethanol exposed groups. Because MDA is the rate limiting reagent in the MDA + MAA reaction forming the most immunogenic moiety of MAA (Tuma 2002), it would be more efficient to attenuate MDA production to reduce MAA formation.

Elevated levels of acetaldehyde and malondialdehyde production, resulting in higher levels of MAA–surfactant D adduct formation in the co-exposed groups, could potentially lead to protein kinase C epsilon (PKC ϵ) activity. PKC ϵ activation is required for the production of certain inflammatory cytokine release from airway epithelium (Wyatt et al., 2010). Previously, we have reported that the in vitro stimulation of bronchial epithelial cells in culture with purified MAA-adducted protein results in the activation of PKC and the release of interleukin-8 (IL-8) (Kharbanda et al., 2002; Wyatt et al., 2000). Likewise, an enhanced release of the mouse IL-8 analog, KC, was observed in BALF of cigarette and alcohol co-exposed mice (Elliott et al., 2007). Thus, our finding that PKC ϵ activity is increased under conditions where SPD-MAA is present is consistent with previous in vitro and in vivo observations of elevated IL-8 in response to smoke and alcohol co-exposure. Surfactant Proteins (SP) are primarily a complex of lipids and proteins that line the alveolar surfaces to prevent alveolar collapse at end expiration, but also contain a COOH-terminal carbohydrate domain that binds to the carbohydrate surfaces of microorganisms enhancing uptake and killing by phagocytic cells (Madsen et al., 2000; Sastry and Ezekowitz, 1993). Surfactant Protein D enhances phagocytic killing by regulating phagocytic reactive oxygenated species (ROS) release to deactivate pathogens (Van Iwaarden et al., 1992). This is proposed to be accomplished by SP-D modulation of the surface expression of β -integrin receptors CD11b, 11c (Senft et al., 2007). As a result Ethanol and cigarette co-exposed mice should be more susceptible to respiratory infections. Data from Ackermann (Lazic et al., 2007) and another group, (Sozo et al., 2009) show that alcohol exposure in animals decreases surfactant protein in lung. If MAA-adducted surfactant proteins result in their binding to epithelial cell scavenger receptors and clearance from lung, this might be the mechanism. Both purified SP-D and SP-A readily form MAA adducts in vitro when exposed to malondialdehyde and acetaldehyde (data not shown). According to the data analyzed from mouse BALF as represented in Figure 4, it appears as if MAA preferentially adducts to SP-D. Current research is underway to determine if this adduct formation alters the function of SP-D. If this is the case, ethanol and cigarette co-exposed mice should be more susceptible to respiratory infections. In summary, when acetaldehyde levels increase due to cigarette smoke inhalation in combination with ethanol consumption, MDA creation may be potentiated. This additional acetaldehyde burden could be responsible for the statistically significantly higher levels of MAA adducted proteins observed in the co-exposed group. The differences in MAA-adducted proteins present in the ethanol and smoke groups as compared to the co-exposed group could be a function of a type of threshold synergism. Threshold synergism in this example would be the concentration of MDA and AA needed to overcome the metabolic and stoichiometric pressures against forming MAA. Thiele et al. in 2001 reported that MAA formation was first dependent on a FAAB moiety which is a MDA conjugated to a AA molecule. Secondly, a MDA-enamine must be created, after which MAA can spontaneously form. It is very plausible that the MDA and AA created by either ethanol or smoke exposure, allowed mostly for the FAAB (MDA-AA) moiety creation, but once a particular concentration of MDA was reached, a threshold was crossed leading to sufficient amounts of MDA-enamine to react with the FAAB moiety synergistically leading to non-additive concentrations of MAA. These MAA compounds preferentially adduct to Surfactant Protein D and which may interfere with proper expression or function of surfactant and lead to increased inflammatory response mediated partly through PKC ϵ activation.

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Figure 1.
Schematic of the conjugation of MDA and AA with its target protein leading to MAA adduct formation

Figure 2.

Aldehyde levels in mice exposed to cigarette smoke and alcohol. Mouse lungs were lavaged after exposure and assayed for acetaldehyde (Fig. 2A) and malondialdehyde (Fig. 2B). While smoke or alcohol alone elevated individual aldehyde levels, the combination of smoke+alcohol significantly increased the levels of AA vs. smoke only ($*P < 0.001$) and MDA vs. alcohol only ($*P < 0.01$). Bars represent the standard error of the mean of $n=6$ mice.

Figure 3.

The combination of alcohol and cigarette smoke exposure produces a malondialdehyde-acetaldehyde adducted (MAA) protein in mouse lung. Crude homogenates (0.76 mg/ml) of mouse lung were assayed using Western blot. Only the *in vivo* exposure of mice to both smoke and alcohol resulted in the detection of MAA-adducted protein by Western blot. Five distinct MAA-Adducted protein bands are resolved ranging from 25-27 kDa with a band observed at 43 kDa. Loading controls consisted of Western blot for beta-actin on the same blot.

Figure 4.

Quantization of malondialdehyde-acetaldehyde adducted (MAA) protein in mouse lung exposed to cigarette smoke and alcohol. Crude homogenates (0.76 mg/ml) of mouse lung were assayed using an indirect competitive ELISA. Only the *in vivo* exposure of mice to both smoke and alcohol resulted in the detection of ~400ng/ml MAA-adducted protein (* $P<0.05$ vs Control). Bars represent the standard error of the mean of $n=6$ mice.

Figure 5.

Protein that was immunoprecipitated using an antibody to surfactant protein D (SP-D) was subsequently probed by Western blot using Rabbit anti-MAA antibodies followed by HRP-goat anti IgG. MAA-adducted SP-D was only detected in immunoprecipitated protein from the combined alcohol + smoke treated mice. Heavy and Light chains of the anti-SPD IgG (Ab) were recognized by the secondary Western blot detection antibody.

Figure 6.

Activation of lung PKCε in mice exposed to cigarette smoke and alcohol. The exposure of ethanol and cigarette smoke lead to a statistically significant increase ($103.86 \pm 22.75/50.77 \pm 3.87$; $*P < 0.05$) in PKCε activity in the tracheal epithelium of co-exposed mice as compared to the control. Bars represent the standard error of the mean of $n=6$ mice.

Figure 7.

Proposed model of malondialdehyde-acetaldehyde adducted (MAA) protein formation in lung airway. Ethanol is metabolized by alcohol dehydrogenase into acetaldehyde. Ethanol exposure also leads to lipid peroxidation products such as malondialdehyde. Burning tobacco results in the production of high concentrations [mM] of acetaldehyde. Cigarette smoking produces lung inflammation and elevates lung malondialdehyde. Two molecules of malondialdehyde and one molecule of acetaldehyde covalently bind to nucleophilic sites of proteins to form a MAA-adducted protein.