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Dimethylarginine dimethylaminohydrolase (DDAH) overexpression enhances wound repair in airway epithelial cells exposed to agricultural organic dust

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Abstract

Objective: Workers exposed to dusts from concentrated animal feeding operations have a high prevalence of pulmonary diseases. These exposures lead to chronic inflammation and aberrant airway remodeling. Previous work shows that activating cAMP-dependent protein kinase (PKA) enhances airway epithelial wound repair while activating protein kinase C (PKC) inhibits wound repair. Hog barn dust extracts slow cell migration and wound repair via a PKC-dependent mechanism. Further, blocking nitric oxide (NO) production in bronchial epithelial cells prevents PKA activation. We hypothesized that blocking an endogenous NO inhibitor, asymmetric dimethylarginine, by overexpressing dimethylarginine dimethylaminohydrolase mitigates the effects of hog dust extract on airway epithelial would repair.

Materials/methods: We cultured primary tracheal epithelial cells in monolayers from both wild-type (WT) and dimethylarginine dimethylaminohydrolase overexpressing C57Bl/6 ($DDAH_1$ transgenic) mice and measured wound repair using the electric cell impedance sensing system.

Results: Wound closure in epithelial cells from WT mice occurred within 24 hr *in* vitro. In contrast, treatment of the WT cell monolayers with 5% hog dust extract prevented significant NO-stimulated wound closure. In cells from $DDAH_1$ transgenic mice, control wounds were repaired up to 8 hours earlier than seen in WT mice. A significant enhancement of wound repair was observed

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Disclosure of interest

The authors have no conflicts of interest to disclose.

in DDAH cells compared to WT cells treated with hog dust extract for 24 hr. Likewise, cells from $DDAH_1$ transgenic mice demonstrated increased NO and PKA activity and decreased hog dust extract-stimulated PKC.

Discussion/conclusion: Preserving the NO signal through endogenous inhibition of asymmetric dimethylarginine enhances wound repair even in the presence of dust exposure.

Keywords

epithelial wound repair; nitric oxide; protein kinase C; dimethylarginine dimethylaminohydrolase

Introduction

Inhalation of organic dust from concentrated animal feeding operations (CAFO) is known to cause airway inflammation in exposed agricultural workers (Wunschel and Poole 2016). This likely accounts for the high prevalence of pulmonary disorders such as asthma, bronchitis, organic dust toxic syndrome and chronic obstructive pulmonary disease (COPD) in workers of such agricultural facilities (May, Romberger, Poole 2012). Multiple exposures in these closed environments, including gases, allergens, pesticides, inorganic dusts and airborne organic dusts, which are rich in Gram-positive and Gram-negative bacteria contribute to chronic inflammation, affecting airway epithelial healing that results in aberrant lung remodeling (Sethi et al. 2017).

Hog barn dust slows wound repair, stimulates cytokine production, slows ciliary beat frequency and impairs mucus clearance (Slager et al. 2007; Wyatt et al. 2008). Hog barn dust can induce airway epithelial cells to release tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), and interleukin-8 (IL-8) (Wyatt et al. 2010). *In vitro* studies show that hog barn dust peptidoglycan stimulates airway epithelial cells to release TNF-α, which in turn can induce bronchial epithelial cells to release IL-6 and IL-8 through a protein kinase C-dependent manner (Wyatt et al. 2010). Previous studies show that hog barn dust-mediated PKC activation decreases bronchial epithelial cell migration, thereby slowing wound repair (Slager et al. 2007). In contrast, activation of the cAMP-dependent protein kinase A (PKA) enhances airway epithelial wound repair (Spurzem et al. 2002). In bronchial epithelial cells, nitric oxide (NO) is required for downstream PKA activation (Sisson, May, Wyatt 1999). Likewise, we demonstrated that the endogenous inhibitor of nitric oxide, asymmetric dimethylarginine (ADMA), blocks NO production and prevents full activation of PKA (Wyatt et al. 2013). This inhibition of the NO pathway can be reversed with dimethylarginine dimethylaminohydrolase (DDAH), an inhibitor of ADMA (Tran, Leiper, Vallance 2003). Thus, transgenic mice that overexpress DDAH demonstrate a decreased *in vivo* inflammatory response to hog barn dust (K. Bailey et al. 2014).

Because blocking ADMA with elevated DDAH levels results in diminished airway inflammation, we hypothesized that promoting the NO pathway through ADMA inhibition would enhance airway epithelial wound repair in response to organic dust-mediated injury. We quantitatively assayed tracheal epithelial wound repair as a function of electrical resistance across a monolayer after addition of hog barn dust extract (HDE) in cells obtained from both wild type and DDAH transgenic mice. Our data reveal enhanced wound repair in

the DDAH transgenic mice as compared to wild type cells, even after HDE exposure. DDAH mice demonstrated an enhanced epithelial NO level and PKA activity as well as decreased PKC activity, leading to a mitigation of the negative HDE effects on wound repair. These findings indicate that activating the NO pathway may be an important therapeutic target for airway epithelial repair in agricultural workers exposed to harmful organic dust.

Methods

Hog barn dust extract preparation

Organic dust that had settled onto horizontal surfaces in a moderately-sized (approximately 500–700 head) swine confinement facility was collected as previously described (K. Bailey et al. 2014). Aqueous extracts of this dust were prepared in a previously published manner (Wyatt et al. 2010) whereby 1 g of dry dust was extracted in 10 ml of Hank's balanced salt solution at room temperature for 1 hr, cleared of particles by centrifugation, and sterile filtered. The resulting supernate was used to treat cells at a final concentration of 5% v/v dilution. The composition of HDE has been extensively characterized in previous reports (Boissy et al. 2014; Poole et al. 2010).

Mouse model

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center and done in compliance with our institution's and the National Research Council's criteria for humane care as outlined in the "Guide for the Care and Use of Laboratory Animals."

The $DDAH_1$ transgenic (Tg) mouse colony was established from a breeding pair of $DDAH_1$ Tg mice on a C57BL/6 background. The specific strain was C57BL/6J-Tg(ACTB-DDAH1)1Jpck (Jackson Laboratories, Bar Harbor, ME, (Klein et al. 2010)). Mice were bred and maintained in micro-isolator cages where they were allowed rodent chow and water *ad libitum*. Mice were genotyped to confirm their $DDAH_1$ Tg status (Klein et al. 2010), and wild-type C57BL/6 littermates were used as controls. The mice were used for experiments at the age of 6–12 weeks.

Cell culture

Tracheal epithelial cells were obtained from wild-type mice (WT) and DDAH overexpressing mice using a previously reported cell isolation method (Berger et al. 2014) and cultured at air-liquid interface until confluent, as monitored by transepithelial resistance. Cells were cultured in Ham's modified F-12 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotic/antimycotic solution (Media Tech, Herndon, VA) at 37°C in a humidified atmosphere with 5% CO2–95% air. Cells were seeded at 50% confluence and used when they reached 95–100% confluence. Cell viability was maintained at ≥95% throughout all procedures as determined by lactate dehydrogenase activity assay (Sigma, St. Louis, MO; data not shown). In all cellular studies, epithelial cells derived from WT and DDAH overexpressing mice were treated with and without 5% HDE in the presence or absence of 100 μM ADMA.

Wound repair assays

The rate of wound repair was measured as a function of cell monolayer resistance using the Electric Cell-substrate Impedance Sensing (ECIS) device (Applied BioPhysics, Troy, NY) (Tian et al. 2017). Mouse tracheal epithelial cells were cultured to confluence onto ECIS electrode slides (8W1E; Applied Biophysics) and baseline impedance values recorded for the purpose of calculating both resistance and capacitance. Identical wounds were made in each confluent monolayer with an elevated field pulse of 1400 μA at 32,000 Hz applied for 20 sec, producing a uniform circular lesion 250 μm in diameter. The wounds were tracked for 24 hr, impedance measured at 4000 Hz, and transepithelial resistance (TEER; ohms) normalized relative to baseline, and plotted as a function of time. Because a larger number of cells are required for protein kinase and nitric oxide assays, some cell cultures were wounded mechanically using the scraping method and quantified as described (Slager et al. 2007). No differences in wound repair responses were observed between the two wounding methods.

Kinase activity assays

Catalytic transfer of phosphate from radiolabeled ^{32}P-ATP onto specific substrate peptides was used to determine enzyme activity. Direct measurement of kinase activity was assayed for the cyclic AMP-dependent protein kinase (PKA) (Spurzem et al. 2002) and protein kinase C (PKC) α and ε (Wyatt et al. 2010) as previously reported.

Nitric Oxide measurement

Non-ciliated monolayers of mouse tracheal epithelial cells were cultured, treated with dust extract, and wounded as described above. NO levels released into the media supernatant were measured using the Sievers Nitric Oxide Analyzer (Model 280i; GE Healthcare Systems) as previously described (Wyatt, Forget, Sisson 2003).

Statistical analysis

All data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and presented as mean with error bars representing standard error of the mean (SEM). Statistical significance was determined using ANOVA with post-hoc Bonferroni test, with $p<0.05$ accepted as significant.

Results

DDAH enhances epithelial wound repair.

Tracheal epithelial cell monolayers from WT and DDAH transgenic mice were cultured to confluence onto ECIS electrode slides in control media (Ham's F12) and treated with or without 5% HDE. A rapid decrease in resistance was immediately observed after each wound was created electrically by the ECIS device (Figure 1A). No change in resistance was observed in monolayers not wounded during the same time period. In cells from wild-type mice, cell migration restored resistance by 20–24 hr after the wound injury. In cells from DDAH mice, an enhanced recovery of resistance was observed to occur by 8–10 hr after wounding. Although having no effect on resistance in unwounded monolayers, treatment of

WT cells with 5% HDE completely abrogated any restoration of resistance after wounding (Figure 1B). Remarkably in DDAH cells, a significant increase in resistance was restored after wounding in the presence of HDE, although at a much later time point (24 hr) than observed under control media conditions.

DDAH promotes wound-mediated PKA activity.

Wounded epithelial cells demonstrate an increased level of PKA activity compared to unwounded resting cells (Spurzem et al. 2002). We confirmed that there is a significant increase in PKA activity in WT wounded cells as compared to unwounded cells (Figure 2). Moreover, wounded cells from DDAH mice showed significantly increased PKA activity over those of wounded WT cells (Figure 2). In agreement with previous reports (Slager et al. 2007), cells from wild type mice treated with 5% HDE 1 hr prior to wounding failed to increase PKA activity. Cells from DDAH mice, however, continued to display wound-induced increased PKA activity even when pretreated with HDE. No increases in PKA were observed under any treatment in unwounded cells.

DDAH enhances wound-mediated NO production.

DDAH activity promotes the conversion of arginine into L-citruline and promotes the production of nitric oxide. We measured NO production in response to mechanical scratch wounds made in both WT and DDAH mouse tracheal epithelium treated with control culture medium with or without 5% HDE. Wounding significantly increased NO in both cell types, but NO levels were higher in DDAH cells as compared to WT cells under both wounded and non-wounded conditions (Figure 3). A further significant increase in NO production was observed in response to wounding in cells from DDAH Tg mice. In contrast to control media, wounds from HDE-treated cells from control mice produced no significant increase in NO. However, similar to control media, DDAH Tg cells retained a significant elevation of NO, even in the presence of HDE. As expected, ADMA treatment prevented wound-mediated increases in NO in both WT and DDAH cells (data not shown).

DDAH decreases HDE-stimulated PKC activity.

HDE activates PKCα, which impairs cell migration into a wound (Slager et al. 2007), and PKCα is an upstream activator required for HDE-stimulated PKCε (Wyatt et al. 2010). PKCα and ε activity were assayed at their optimal activation times (1 and 6 hr, respectively) in WT cells and DDAH cells treated with 100 μM ADMA, 5% HDE, or both. HDE stimulated PKCα in both WT and DDAH at 1 hr and stimulated PKCε in WT cells at 6 hr. However, HDE failed to activate PKCε in DDAH cells at 6 hr (Figure 4). The addition of exogenous ADMA reversed the DDAH cell effect and restored HDE-mediated PKCε activity. By itself, ADMA had no effect on PKC activity.

Discussion

Exposure of cells *in vitro* and mice *in vivo* to organic dust extracts from swine CAFOs result in profoundly increased lung inflammatory mediators and cell recruitment, decreased ciliary-mediated clearance, and impaired migration of epithelial cells to close a wound [reviewed in (Sethi et al. 2017)]. In support of our earlier findings using an irregular scratch

assay (Slager et al. 2007), we now confirm the migratory stasis effect of HDE through a quantitative measurement of restored cell monolayer resistance after precision wounds using the ECIS system. For the first time, we show that airway epithelial cells from over-producing DDAH transgenic mice have an enhanced capability to repair a monolayer wound more rapidly than those cells from WT mice. Importantly, such over-expressing DDAH cells even demonstrate migration repair responses to HDE treatment. Under wounding conditions, tracheal epithelial cells from DDAH mice demonstrate enhanced NO/PKA activity and decreased PKC activity when treated with HDE. Such findings establish the potential for exploiting the NO pathway to modulate dust-injured airway epithelial wound repair.

Airway wound injury repair, as in other sites, is a systematic process consisting of airway epithelial cell migration, proliferation, and differentiation. The regulation of the epithelial cell migration response involves a coordinated response of several protein kinases. Protein kinase A activation enhances wound repair by accelerating epithelial cell migration (Spurzem et al. 2002). In contrast, some protein kinase C activating agents slow cell migration, thereby delaying wound repair (Slager et al. 2007; Wyatt et al. 2005). Previously, we have shown that HDE stimulates release of pro-inflammatory cytokines from airway epithelial cells via a sequential PKCα- and PKCε-dependent mechanism (Wyatt et al. 2010). Interestingly, beta-agonist activation of PKA can inhibit HDE-stimulated cytokine production (Romberger et al. 2016). This apparent bi-directional kinase regulation of dust-mediated cytokine release led us to explore other epithelial cell functional responses to dust exposure injury.

Epithelial cell migration in response to wound repair is by no means a universally characterized mechanism (Tesfaigzi 2003). Indeed, regulation of the migratory response appears to be subject to the tissue source of the epithelial cell in question and the specific agent used to promote enhanced migration. For certain tissues, PKC activation has been reported to enhance cell migration into a wound area, while inhibitors of PKC activity demonstrate the opposite (van der Vliet and Bove 2011). In the case of bronchial epithelium, PKC regulation has been implicated for treatments that both enhance (Zhou et al. 2013) and impair (Jozic et al. 2017) cell migration into a wound. Previously, we found that TNF-α stimulates bronchial epithelial wound repair in a PKC-dependent manner (Wyatt et al. 1997). Counterintuitively, agents that stimulate the production and release of high levels of lung TNF-α, such as cigarette smoke (Cantral et al. 1995) or hog barn dust extracts (Slager et al. 2007), significantly inhibit bronchial cell migration into a wounded area. This can most likely be explained by the observation that HDE slows wound repair by a PKCα mechanism, while TNF-α activates PKCε (Wyatt et al. 2010). Conversely, agents that stimulate cAMP production and activate PKA increase bronchial epithelial cell migration into a wounded area (Spurzem et al. 2002; Tan et al. 2006). Singh et al. (Singh et al. 2003) report that cigarette smoke (a potent activator of PKC) results in a PDE4-mediated lowering of lung cAMP levels and reduction in epithelial migration. PKA-mediated activation of cAMP Response Element Binding Protein (CREB) might also regulate this pathway (Guan et al. 2009). Indeed, activation of the cAMP-PKA pathway can block dust-stimulated subsequent PKC activation (Wyatt et al. 2014). Thus, in the specific case of hog barn dust extract, a bidirectional kinase control system appears to exist involving PKCα-mediated inhibition and PKA-mediated enhancement of epithelial cell wound repair (Figure 5).

Such a bidirectional control hypothesis predicts that functional inhibition of PKA would result in the enhancement of dust-stimulated PKC responses. Indeed, that may be the case as we have observed an enhanced HDE-mediated TNF-α response in mice that were fed a chronic alcohol diet (McCaskill et al. 2012). Chronic alcohol feeding desensitizes airway epithelial PKA activity by uncoupling the NO-PKA pathway (Wyatt and Sisson 2001). This is supported by previous observations that NO inhibitors can block PKA activation (Sisson et al. 1999). These findings led us to observe that a soluble cyclase-generated cAMP-PKA pathway is dependent upon the action of NO to inhibit dust-stimulated ADAM17 activation and TNF-α release (Gerald et al. 2016; Wyatt et al. 2014). Because TNF-α has been shown to potentiate an accumulation of ADMA (Eid et al. 2007), the action of NO on decreasing TNF-α could potentially enhance NO through negative feedback regulation of ADMA. Thus, in the absence of a functional inhibitory or “anti-inflammatory” PKA pathway, dust inhalation would favor a “pro-inflammatory” PKC pathway leading to greater injury and the balance between the two potentially governed by NO.

Our findings support those of Bove et al. whereby NO was shown to promote bronchial epithelial wound repair (Bove et al. 2007). In this study, while an important process of repair occurred through PKG-dependent activation of MMP-9, a PKG-independent component of NO-mediated wound repair was also observed. NO levels can be regulated through ADMA and DDAH action in the human body (Teerlink et al. 2009). ADMA inhibits nitric oxide synthase (NOS), thereby blocking the conversion of L-arginine to NO while DDAH hydrolyzes ADMA into L-citrulline, resulting in production of more NO via NOS (Tran et al. 2003). For our study, we utilized an over-producing DDAH transgenic mouse model to produce increased levels of NO through ADMA inhibition. We previously demonstrated that DDAH mice nasally instilled with HDE produce less lung inflammation (K. L. Bailey et al. 2014). Our data now supports that increased NO production in airway epithelial cells from DDAH transgenic mice result in enhanced wound repair by stimulation of the PKA pathway. We cannot, however, rule out that elevated DDAH and subsequently lowered ADMA levels led to a decrease in peroxynitrite and superoxide formation, reactive oxygen species known to activate PKC and downstream inflammatory pathways (Wells and Holian 2007).

Clearly, the study at hand is limited to those observations made in cultured epithelial cell monolayers. Inhalation of organic dusts results in a substantial cumulative lung inflammatory response (McClendon, Gerald, Waterman 2015). Pro-inflammatory cytokines are generated from the epithelium and resident macrophages, resulting in the recruitment of neutrophils and lymphocytes to the lung (Charavaryamath and Singh 2006). Such cytokines and inflammatory cells have a profound impact on modulating the re-epithelialization process involved in wound repair (Beers and Morrisey 2011). Furthermore, our current study does not take into account the likely role of repair cytokines, such as interleukin-10, in the response to dust mediated injury. Such future studies will require more complex cell co-culture, isolated tissue, and animal models.

Regardless of the precise mechanism of action, ADMA in the lung could be potentially targeted through the use of existing pharmacotherapeutics, some of which are currently approved for clinical use. The anti-hypertensive, nebivolol, decreases ADMA levels by increasing the expression of DDAH2 (Garbin et al. 2007). Fenofibrate, used to lower

cholesterol, has also been shown to decrease ADMA (Yang et al. 2005). Because such studies have focused on endothelial action, it remains to be established if such drugs effectively decrease epithelial cell ADMA or whether ADMA-targeted therapies could prove beneficial in reducing organic dust-mediated lung injury.

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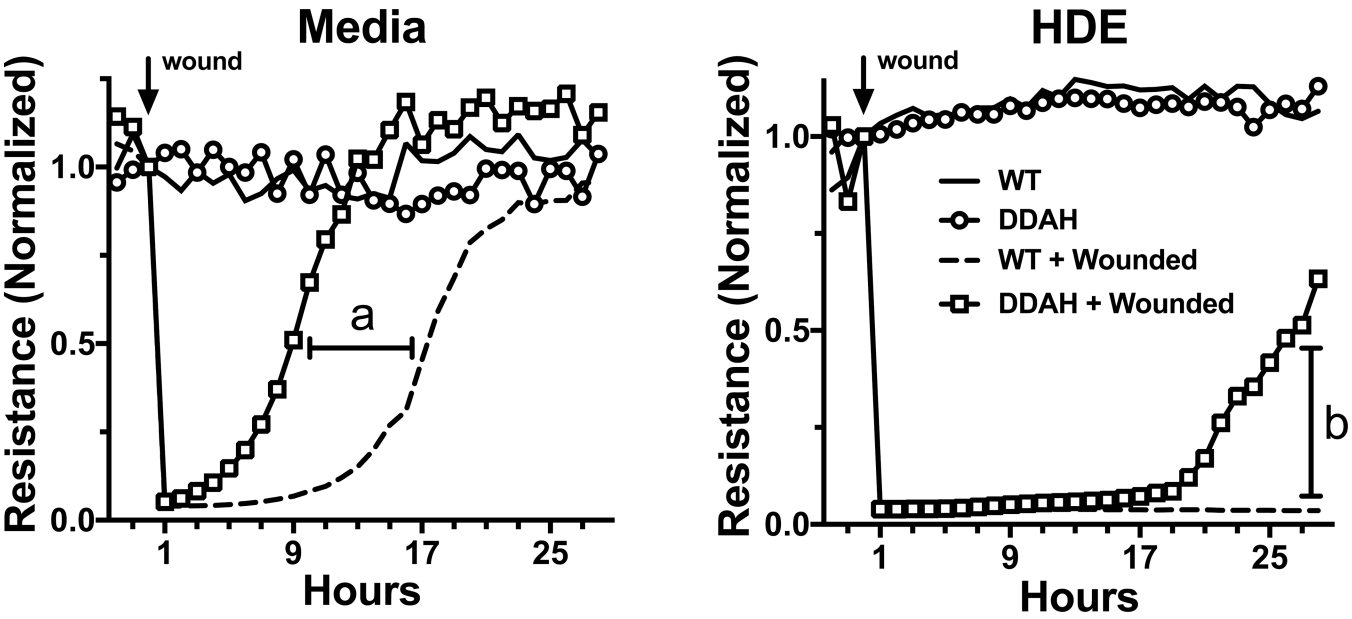

Figure 1. Rate of wound repair is faster in wounded DDAH overexpressing cells with and without 5% HDE:

A. Intact monolayers of wild type (WT) mouse cells and DDAH overexpressing mouse cells, respectively, display similar electrical resistance as measured by Electric Cell-substrate Impedance Sensing system (ECIS) under control conditions. Wounded WT cells regain resistance in about 24 hours. A significantly decreased time of half-maximal resistance is noted in wounded DDAH overexpressing cells ($^{a}p<0.01$ vs. WT). B. 5% HDE treatment of WT cells results in complete loss of repair response as measured by resistance. In contrast, DDAH overexpressing cells treated under the same conditions demonstrate a delayed, but significant ($^{b}p<0.05$ vs HDE-treated WT) recovery of resistance. Data shown is representative of three independent experiments; n=6 mice per each unique condition.

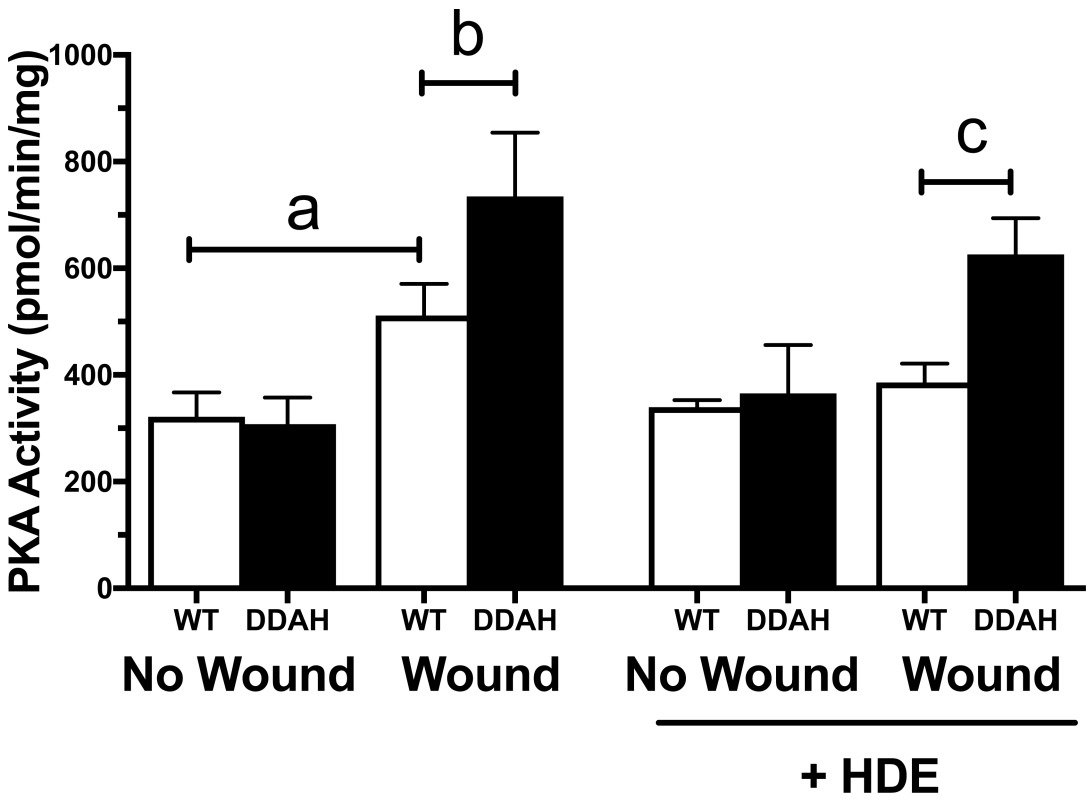

Figure 2. Protein Kinase A activity elevated after wounding:

Wounding results in significantly elevated PKA activity in WT cells ([a]$p<0.001$ vs. WT No wound) and DDAH overexpressing cells ([b]$p<0.0001$ vs. WT wounded). This response is absent in WT cells after HDE treatment; however, wounded DDAH overexpressing cells demonstrated a significant elevation in PKA activity after HDE treatment ([c]$p<0.001$). Bars represent SEM (n=6).

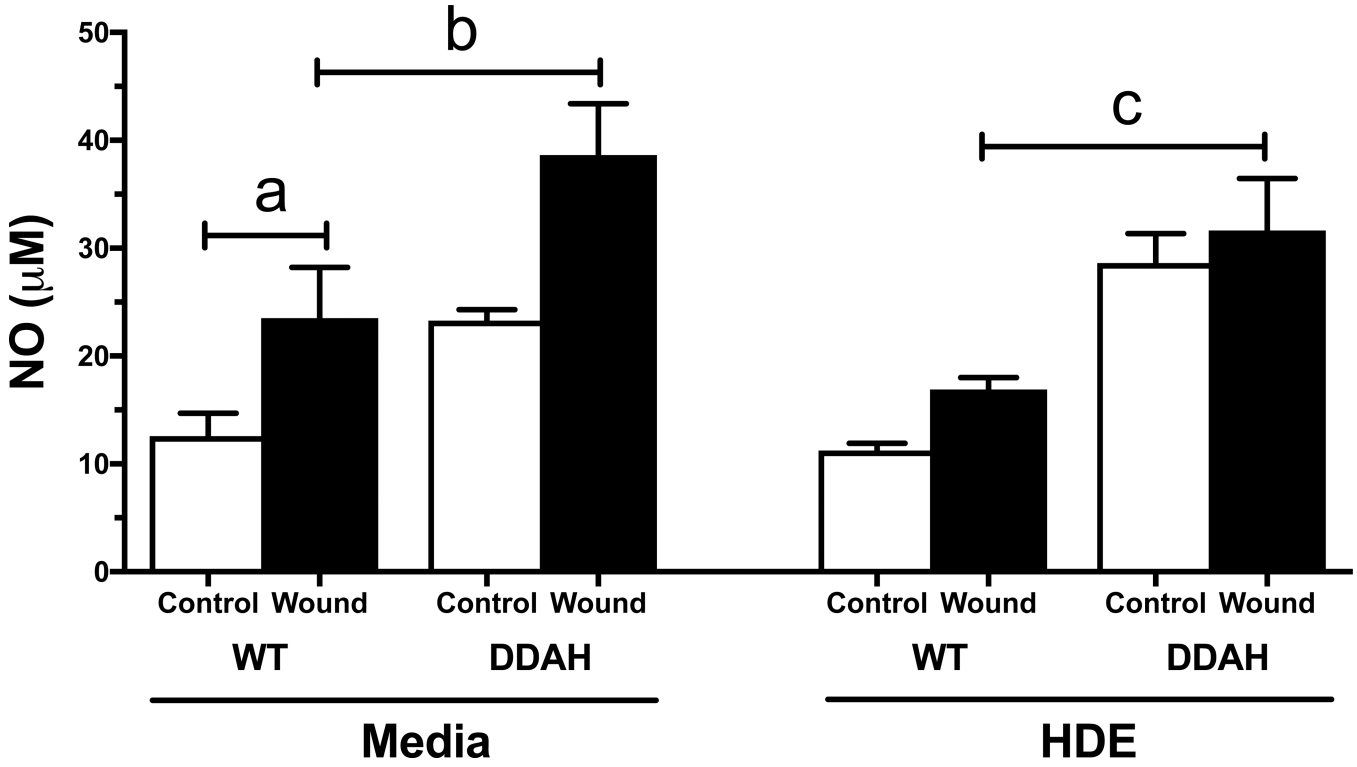

Figure 3. Nitric Oxide (NO) production elevated in DDAH cells in response to wounding:
Wounding of WT cells results in elevated NO ($^{a}p<0.05$ vs. WT no wound). NO production is significantly enhanced in DDAH overexpressing cells in response to wounding, when compared to WT cells ($^{b}p<0.01$). Even though no wound-induced increase in NO is observed in HDE-treated WT cells, DDAH cells produce a significantly enhanced amount of NO when treated with HDE ($^{c}p < 0.05$ WT vs. DDAH). Bars represent SEM (n=6).

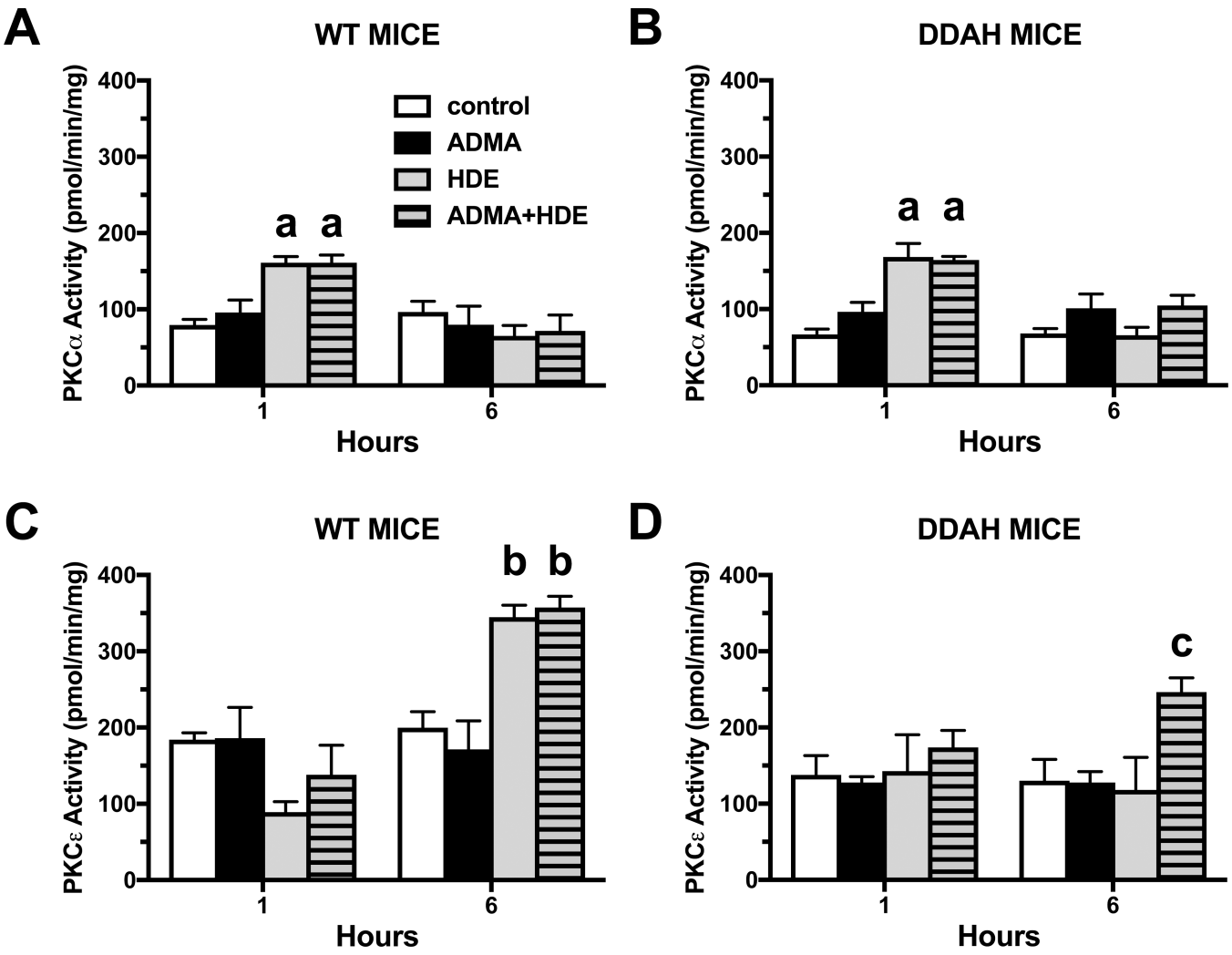

Figure 4. Protein Kinase C activity after wounding:

WT cells and DDAH cells were treated with ADMA, HDE, and ADMA with HDE and PKCα and ε activites were measured.

A) Both PKCα ([a]p<0.05 vs. matched 1 hr controls without HDE) and C) PKCε ([b]p<0.05 vs. matched 6 hr controls without HDE) activity were significantly elevated in 5% HDE-treated WT cells. The addition of 100 μM ADMA had no effect on either kinase in the presence or absence of HDE. B) In DDAH cells, HDE activated PKCα ([a]p<0.05 vs. matched 1 hr controls without HDE), but failed to activate PKCε. Pretreatment with ADMA before and during HDE treatment restored HDE-stimulated PKCε ([c]p<0.01 vs. HDE alone) activity. Bars represent SEM (n=6).

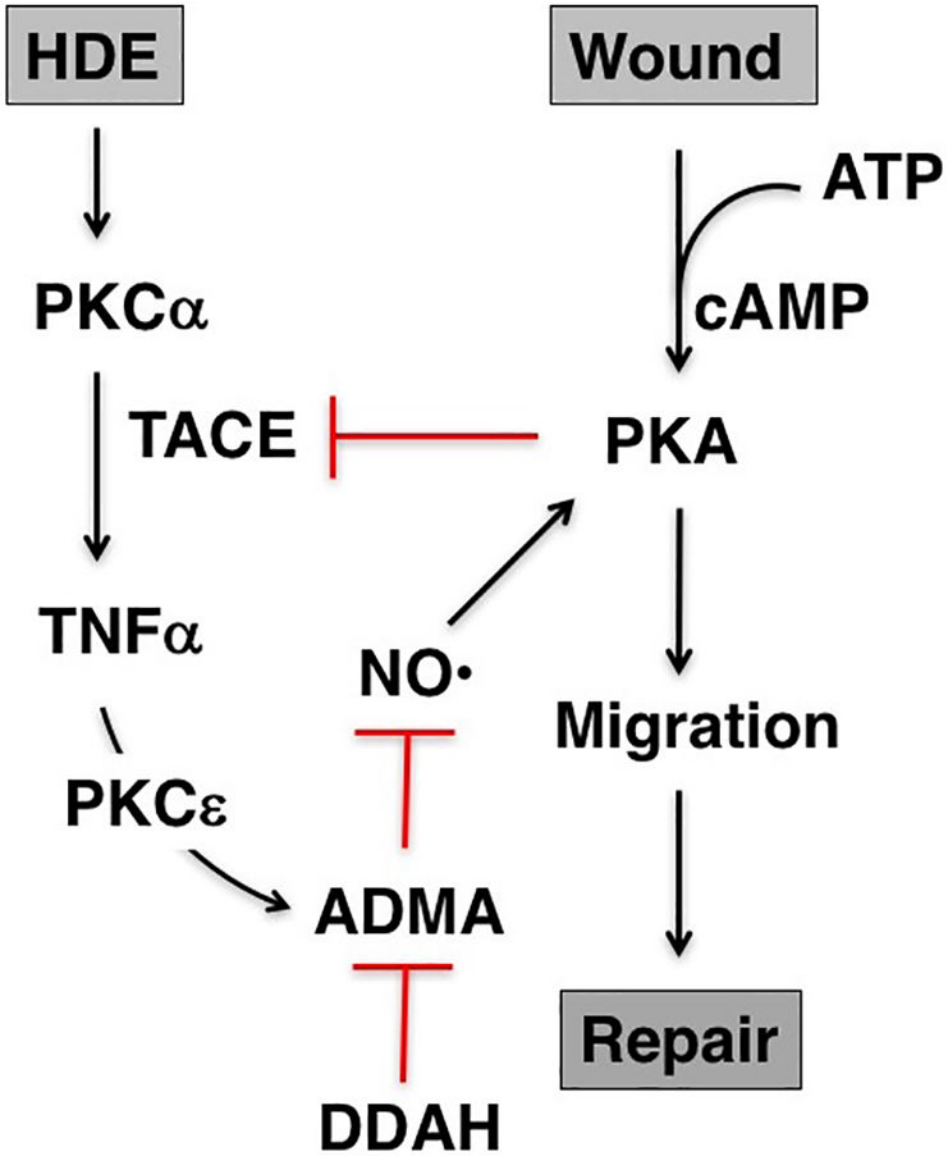

Figure 5. Proposed model of bidirectional wound repair regulation in HDE-treated airway epithelium:

A wound to airway epithelium initiates cell migration to close the wound area. Migration is enhanced by activators of the cAMP-dependent protein kinase (PKA). Extracts from hog barn dust (HDE), which blunt wound repair, increase epithelial-derived tumor necrosis factor alpha (TNF-α) release via the protein kinase C alpha (PKCα)-mediated activation of TNF-alpha converting enzyme (TACE; ADAM-17). Nitric oxide (NO) enhances PKA activity, but NO is decreased by the action of asymmetric dimethylarginine (ADMA). Increased TNF-α activates PKCε and also leads to increased ADMA, but PKA activation blocks TACE-mediated release of TNF-α. The inhibition of ADMA by dimethylarginine dimethylaminohydrolase (DDAH) results in accumulated NO, increased PKA activity, and wound repair even in the presence of HDE.