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RESEARCH ARTICLE | *Vascular Biology and Microcirculation*

Differential effects of alcohol and its metabolite acetaldehyde on vascular smooth muscle cell Notch signaling and growth

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Hatch E, Morrow D, Liu W, Cahill PA, Redmond EM. Differential effects of alcohol and its metabolite acetaldehyde on vascular smooth muscle cell Notch signaling and growth. *Am J Physiol Heart Circ Physiol* 314: H131–H137, 2018. First published December 6, 2017; doi:10.1152/ajpheart.00586.2017.—Alcohol (EtOH) consumption can variously affect cardiovascular disease. Our aim was to compare the effects of EtOH and its primary metabolite acetaldehyde (ACT) on vascular smooth muscle Notch signaling and cell growth, which are important for atherogenesis. Human coronary artery smooth muscle cells (HCASMCs) were treated with EtOH (25 mM) or ACT (10 or 25 μM). As previously reported, EtOH inhibited Notch signaling and growth of HCASMCs. In contrast, ACT treatment stimulated HCASMC proliferation (cell counts) and increased proliferating cell nuclear antigen expression, concomitant with stimulation of Notch signaling, as determined by increased Notch receptor (N1 and N3) and target gene (Hairy-related transcription factor 1–3) mRNA levels. Interaction of the ligand with the Notch receptor initiates proteolytic cleavage by α- and γ-secretase, resulting in the release of the active Notch intracellular domain. Neither EtOH nor ACT had any significant effect on α-secretase activity. A fluorogenic peptide cleavage assay demonstrated almost complete inhibition by EtOH of Delta-like ligand 4-stimulated γ-secretase activity in solubilized HCASMCs (similar to the effect of the control inhibitor DAPT) but no effect of ACT treatment. EtOH, but not ACT, affected the association and distribution of the γ-secretase catalytic subunit presenilin-1 with lipid rafts, as determined by dual fluorescent labeling and confocal microscopic visualization. In conclusion, ACT stimulates vascular smooth muscle cell Notch signaling and growth, effects opposite to those of EtOH. These differential actions on vascular smooth muscle cells of EtOH and its metabolite ACT may be important in mediating the ultimate effects of drinking on cardiovascular disease.

NEW & NOTEWORTHY Acetaldehyde stimulates, in a Notch-dependent manner, the vascular smooth muscle cell growth that contributes to atherogenesis; effects opposite to those of ethanol. These data suggest that in addition to ethanol itself, its metabolite acetaldehyde may also mediate some of the effects of alcohol consumption on vascular cells and, thus, cardiovascular health.

acetaldehyde; alcohol; atherosclerosis; cardiovascular disease; ethanol; Notch; vascular smooth muscle

INTRODUCTION

Alcohol (EtOH) consumption is a factor affecting the incidence and severity of cardiovascular disease (CVD) (4, 15, 23, 24). Meta analysis suggests that, compared with abstinence, "light to moderate" daily consumption of EtOH is associated with the lowest risk for CVD incidence and mortality (2, 5, 30). Light to moderate EtOH consumption is generally considered to be in the range 1–3 drinks/day, giving rise to blood alcohol levels (BAC) of ~5–25 mM (4, 31, 34). On the other hand, episodic binge drinking (i.e., 5 or more drinks in <2 h) and excessive EtOH use resulting in a BAC up to 50 mM are associated with a higher incidence of cardiovascular disorders and increased mortality (25, 31, 33, 34). These studies point to a complex influence of different patterns of EtOH consumption on CVD and health.

The cellular signaling mechanisms whereby moderate and excessive EtOH consumption elicits beneficial and deleterious effects, respectively, on CVD have not been fully elucidated, and it is unclear if these effects are due to the direct actions of EtOH itself and/or caused by its primary metabolite, acetaldehyde (ACT). It is possible that the balance between levels of EtOH and ACT may dictate the ultimate health consequences of EtOH consumption. There is evidence that in binge drinkers and alcoholics, the EtOH-ACT balance may more frequently tip in favor of higher ACT levels (16, 17, 19, 35). We have previously demonstrated in a controlled study protective and exacerbatory effects of daily moderate and 2-day binge EtOH consumption, respectively, on injury-induced atherosclerotic plaque development in mice (20). Moreover, we have reported opposite effects of EtOH and ACT in monocytes: anti- and proatherogenic, respectively (6, 29). In general, however, the contribution of ACT in causing EtOH's various biological effects is still controversial. While the local production of ACT mediated the EtOH-evoked pressor response in hypertensive rats (9), other studies have argued against the involvement of ACT in mediating EtOH's effects, e.g., its neurobehavioral effects (27).

Vascular smooth muscle cells (VSMCs) play a key role in the pathogenesis of CVD-associated atherosclerosis and arteriosclerosis. Their growth and migration, in response to a variety of injurious stimuli, contribute to pathophysiological arterial remodeling and plaque formation, resulting in thickened vessels with a narrowed lumen that may result in blood flow blockage leading to heart attack or stroke. Activation of the Notch signaling pathway has been implicated in regulating VSMC differentiation in the context of vessel remodeling and atherogenesis (1, 18, 22, 28). While we have previously reported that EtOH inhibits VSMC proliferation in a Notch-dependent manner (21), any effect of ACT had not been determined. We report here that in contrast to the effects of

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EtOH, ACT stimulates Notch signaling and VSMC proliferation.

METHODS

Cell culture. Human coronary artery SMCs (HCASMCs; Clonetics primary cells) were cultured in optimized SMC medium supplemented with epidermal growth factor, insulin, fibroblast growth factor, and 5% FCS (Clonetics). Cells were characterized by staining positively for SMC α-actin and used in experiments between *passages 4 and 11*. HCASMCs were treated (24 h) with ACT (10 or 25 μM or as indicated) or with EtOH for comparison (25 mM). ACT was obtained from Sigma (St. Louis, MO). A stock concentration of 100 mM ACT was made up in PBS and diluted in culture medium to the desired concentration. EtOH (200 proof, American Chemical Society/United States Pharmacopeia grade, Ultrapure, Darien, CT) was diluted in media to achieve the desired concentration before being added to cultures.

Western blot analysis. Proteins from cell lysates (12–15 μg) were resolved on precast SDS-PAGE gels (12% resolving, 5% stacking) before transfer onto nitrocellulose membrane (Bio-Rad, Carlsbad, CA). Membranes were stained with Ponceau S to assess protein loading and transfer and probed using commercially available antibodies from Abcam (Cambridge, MA) essentially as we have previously described (21). Blots were reprobed for GADPH as a loading control.

HCASMC proliferation. Changes in HCASMC number were determined by cell counting. Cells were serum starved for 24 h and then seeded at 1×10^4 cells/well onto six-well plates in 5% growth media with or without ACT or EtOH at the indicated concentrations. Cells were counted each day, and the average of three wells was quantified using a hemocytometer. In parallel experiments, proliferating cell nuclear antigen (PCNA) protein expression in the different experimental groups was determined by Western blot analysis.

Quantitative real-time RT-PCR. Total RNA (1–2 μg) isolated from cells using a Qiagen RNeasy kit (Valencia, CA) was reverse transcribed using an iScript cDNA Synthesis kit from Bio-Rad. The gene-specific [Notch 1, Notch 3, Hairy-related transcription factor (Hrt)-1, Hrt-2, and Hrt-3] oligonucleotide sequences were as previously described (21, 22). Real-Time RT-PCR was performed using the Stratagene MX3005 machine and the SYBER green jumpstart PCR kit (Sigma) as described by the manufacturer.

γ-Secretase assay. γ-Secretase activity was measured using an intramolecularly quenched fluorogenic peptide substrate from Calbiochem (catalog no. 565764) essentially as previously described by Farmery et al. (10). This substrate contains the COOH-terminal amyloid β-peptide precursor protein amino acid sequence that is cleaved by γ-secretase. The ReadyPrep (membrane II) protein extraction kit (catalog no. 163-2084, Bio-Rad) was used to isolate HCASMC cellular membranes and then to extract the associated integral membrane proteins into a solution. Protein concentration in membrane preparations was determined by the Bradford assay. Activity of γ-secretase was measured by preincubating solubilized membranes [10 μg total protein in assay buffer with 0.25% (wt/vol) CHAPSO] with or without Delta-like ligand 4 (DLL4) in the absence or presence of ACT (10 μM) or EtOH (25 mM) or the γ-secretase inhibitor DAPT (20 μM, Tocris Bioscience) for 1 h. The fluorescent peptide probe (8 μM) was then added, and tubes were incubated at 37°C overnight. Fluorescence was measured using a plate reader (POLARstar OPTIMA) with an excitation wavelength of 355 nm and emission wavelength of 440 nm. Background fluorescence of the peptide probe was subtracted from all readings.

α-Secretase (TACE/ADAM17) assay. α-Secretase activity in SMC lysates stimulated with or without DLL4 in the absence or presence of ACT or EtOH was measured using a SensoLyte 520 TACE (α-secretase) activity kit (fluorometric assay, AnaSpec, Freemont, CA) according to the manufacturer's instructions. This kit uses a 5-FAM (fluorophore)- and QXL 520 (quencher)-labeled FRET peptide substrate for continuous measurement of enzyme activity. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL 520. Upon cleavage of the FRET peptide by the active enzyme, the fluorescence of 5-FAM is recovered and can be continuously monitored at excitation/emission wavelengths of 490/520 nm. Tumor necrosis factor-α protease inhibitor (TAPI), a broad-spectrum metalloprotease inhibitor, was used as a control α-secretase inhibitor.

Lipid rafts. Lipid rafts in HCASMCs were labeled using Alexa 488-conjugated cholera toxin B subunit (CTB; catalog no. V-34403, Vybrant Lipid raft labeling kit, Molecular Probes, Invitrogen). This CTB conjugate binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into lipid rafts. Alexa 594-conjugated anti-presenilin-1 antibody was used for immunohistochemical detection of this γ-secretase catalytic subunit.

Data analysis. Results are expressed as means ± SE. Experimental points were performed in duplicate with a minimum of three independent experiments. ANOVA was performed on cell count data, and a Wilcoxan signed-rank test was used for comparison of two groups compared with normalized control. Values of $P \leq 0.05$ were considered significant.

RESULTS

ACT stimulates VSMC proliferation. The effect of ACT on the proliferation of VSMCs was determined by cell counts and PCNA expression as an index of cell growth. Daily exposure of HCASMCs to ACT (10 μM) significantly increased their proliferation over time (Fig. 1*A*) Stimulation of HCASMCs proliferation by ACT was prevented by cotreatment with the γ-secretase/Notch inhibitor DAPT (20 μM; Fig. 1*B*). PCNA expression, as assessed by Western blot analysis, was increased after ACT treatment, as indicated (Fig. 1*C*). In comparison, EtOH inhibited HCASMC growth, as determined by cell counts (Fig. 1, *A* and *B*), and reduced PCNA expression (Fig. 1*C*), in agreement with our previous study (21).

ACT stimulates Notch signaling in HCASMCs. HCASMCs were treated with ACT, and Notch receptors and target gene mRNA levels were determined by quantitative RT-PCR. ACT treatment (10 μM for 24 h) significantly increased (2- to 3-fold) Notch 1 and Notch 3 receptor mRNA expression in these cells (Fig. 2*A*) and also stimulated expression of the Notch target genes Hrt-1, Hrt-2, and Hrt-3 (3- to 8-fold increase; Fig. 2*B*). Protein expression of Notch 1 and Notch 3 intracellular domains in HCASMCs was also significantly increased after ACT exposure (10 and 25 μM; Fig. 2*C*), as determined by Western blot analysis.

ACT has no effect on γ-secretase activity, whereas EtOH inhibits it. The effect of ACT on γ-secretase protease activity, a rate-limiting step for Notch signaling transduction, in HCASMC membranes was determined using a fluorogenic peptide substrate. In this assay, increased fluorescence indicates increased γ-secretase-mediated cleavage activity. EtOH, and the control inhibitor DAPT, inhibited basal γ-secretase activity in HCASMCs (Fig. 3). Treatment of HCASMCs with the Notch ligand DLL4 stimulated γ-secretase activity above basal levels (Fig. 3). This activity was inhibited by DAPT and by EtOH treatment. There was no significant effect of ACT treatment on basal or DLL4-stimulated γ-secretase activity (Fig. 3).

Neither ACT nor EtOH has an effect on α-secretase (TACE/ADAM17) activity. As determined using the Sensolyte 520 TACE (α-Secretase Activity Assay kit), DLL4 treatment stim-

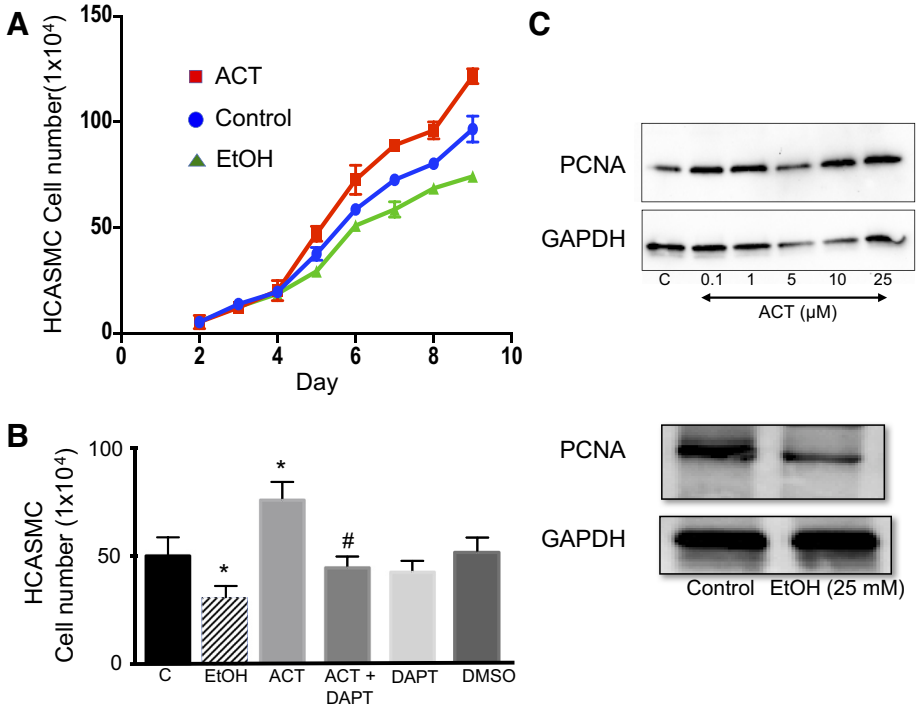

Fig. 1. Acetaldehyde (ACT) stimulates, and ethanol (EtOH) inhibits, human coronary artery smooth muscle cell (HCASMC) proliferation. *A*: growth curves of HCASMCs treated with or without ACT (10 μM) or EtOH (25 mM). Cell counts of parallel triplicate wells were performed daily over 9 days. Data are means ± SE; $n = 3$. *B*: ACT-stimulated HCASMC proliferation was prevented by cotreatment with the γ-secretase inhibitor DAPT (20 μM). HCASMCs were treated as indicated for 5 days, and cells were then counted. EtOH was used at 25 mM; ACT was used at 10 μM. DMSO was the vehicle control for DAPT. Data are means ± SE; $n = 3$. $^*P < 0.05$ vs. control (C); $\#P < 0.05$ vs. ACT alone. *C*: representative Western blots of proliferating cell nuclear antigen (PCNA) protein expression in HCASMCs treated (24 h) with or without ACT at the indicated concentrations (*top*) or with or without EtOH (25 mM; *bottom*). Blots were reprobed for GAPDH as a loading control.

ulated α-secretase activity above basal levels (Fig. 4). Basal and DLL4-stimulated α-secretase activity were inhibited by the metalloprotease control inhibitor TAPI. Neither ACT nor EtOH treatment had any significant effect on either basal or DLL4-stimulated α-secretase activity (Fig. 4).

Lipid raft distribution and presenilin-1 association. Lipid rafts were visualized by fluorescent labeling with the Alexa 488-conjugated CTB subunit for lipid rafts. The catalytic subunit of γ-secretase was identified using an Alexa 594-conjugated anti-presenilin-1 antibody. In control HCASMCs and in ACT-treated cells, lipid rafts and presenilin-1 were distributed more obviously along the cell membrane/edge, whereas in EtOH-treated cells fluorescent-tagged lipid rafts and presenilin-1 had a more punctate distribution throughout

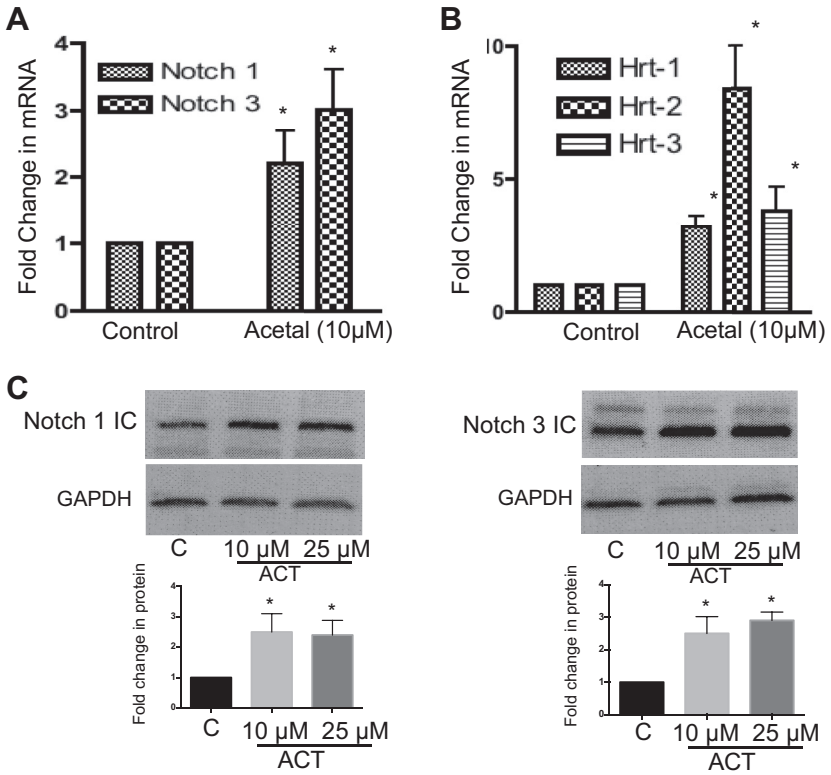

Fig. 2. Acetaldehyde (Acetal or ACT) stimulates Notch signaling in human coronary artery smooth muscle cells (HCASMCs). *A* and *B*: quantitative RT-PCR analysis of Notch 1 and Notch 3 receptors (*A*) as well as Notch target gene Hairy-related transcription factor (Hrt)-1, Hrt-2, and Hrt-3 mRNA (*B*) after ACT treatment for 24 h. Data were normalized to GAPDH and are means ± SE; $n = 3$. $^*P < 0.05$ vs. control. *C*: representative Western blots together with cumulative data]means ± SD, $n = 3$, $^*P < 0.05$ vs. control (C)] showing an increase in N1 intracellular (IC) domain and N3 IC domain protein expression with ACT (10 and 25 μM for 24 h) treatment. Blots were reprobed for GAPDH as a loading control.

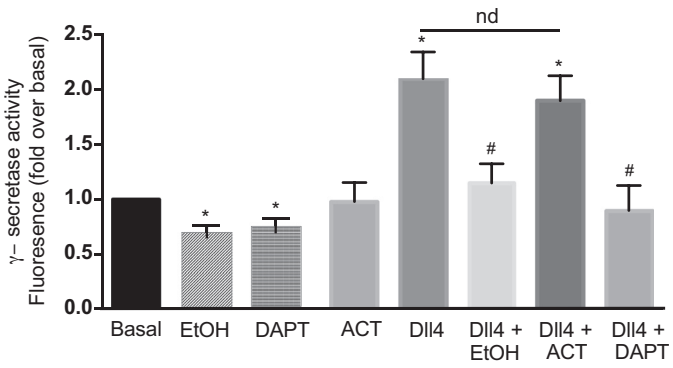

Fig. 3. Acetaldehyde (ACT) has no effect on γ-secretase activity, whereas ethanol (EtOH) inhibits it. γ-Secretase activity in solubilized human coronary artery smooth muscle cell membranes was measured using a fluorogenic peptide substrate (COOH-terminal fragment of amyloid β-peptide precursor protein). Cells were stimulated with EtOH (25 mM), DAPT (control γ-secretase inhibitor, 20 μM), ACT (10 μM), Notch ligand Delta-like ligand 4 (Dll4; 2 μg/ml), or combinations as indicated. Data are means ± SE; $n = 3$. *$P <$ 0.05 vs. basal; #$P < 0.05$ vs. Dll4 stimulated. nd, no difference.

the cell (Fig. 5). These data suggest that EtOH treatment, but not ACT, affects the association and distribution of presenilin-1 with lipid rafts.

DISCUSSION

Here, we report that ACT, the primary metabolite of EtOH, stimulates Notch signaling and cell proliferation in arterial SMCs. These effects are opposite to those caused by EtOH itself and may be responsible, at least in part, for the effects of drinking on CVD.

The relationship between EtOH consumption and CVD is complex. Depending not only on the amount consumed but also the pattern of drinking, it can have either beneficial or detrimental effects on cardiovascular health. Habitual light to moderate EtOH intake is associated with decreased risk for coronary artery disease, congestive heart failure, and stroke (24). However, binge drinking and excessive EtOH consumption are associated with increased cardiovascular risk and is the third leading cause, behind smoking and obesity, of premature death in the United States (24).

EtOH in the body is mainly metabolized by alcohol dehydrogenase (ADH), resulting in ACT, the cause of facial flushing and hangover symptoms (8). ACT, a known carcinogen, is thought to play a role in the pathology of EtOH by forming the protein adducts that are found in the blood after heavy drinking and may be used as biomarkers in alcoholism (11). While ACT is relatively short lived before being further metabolized to the less active byproduct acetate, by aldehyde dehydrogenase (ALDH) (8), it has the potential to cause significant effects. The amount of EtOH metabolized over time can differ widely depending on factors including body mass, sex, and liver size as well as genetic variants of the ADH and ALDH enzymes that are more or less efficient (8, 37). With respect to the latter, a fast ADH enzyme or a slow ALDH enzyme can cause ACT to build up in the body. There is also evidence that in binge drinkers and/or after excessive EtOH consumption that the EtOH-ACT balance tips in favor of ACT (16, 17, 35). Thus, elucidation of possible vascular effects of ACT itself, in addition to those of EtOH, warranted investigation and provoked this study.

A key role for Notch signaling in regulating VSMC fate and pathological vessel remodeling relevant to the etiology of CVD has been established (18, 22, 28). Inhibition of Notch 1 signaling attenuates HCASMC proliferation (21), and perivascular Notch 1 siRNA inhibits arterial remodeling in response to injury (28). While we have previously demonstrated that EtOH inhibits VSMC proliferation in vitro and in vivo in a Notch-dependent manner (13, 21), any effect of ACT was unknown. Here, we report that in contrast to the inhibitory effects of EtOH, ACT stimulates Notch signaling and proliferation in HCASMCs. The anticipated functional significance of this ACT effect in vivo would be greater arterial SMC proliferation resulting in increased intimal medial thickening, with the latter used clinically as a measure of atherosclerosis. While EtOH inhibits Notch signaling by inhibiting γ-secretase cleavage activity, in the absence of any effect on α-secretase activity (12), ACT's stimulatory effect on the Notch pathway does not appear to be related to any proteolytic stimulatory effects as both α- and γ-secretase activity remained unchanged after ACT treatment. Moreover, the distribution of lipid rafts and presenilin-1 in HCASMCs was not affected by ACT treatment, suggesting that ACT does not affect lipid raft trafficking, whereas EtOH appears to do so. Reported blood levels of ACT in vivo vary considerably (14, 16, 35), as do the levels used experimentally in vitro (μM to mM range) (7, 29, 36). Blood ACT levels of 2–5 μM were recorded in subjects with a moderate blood alcohol content of 15–17 mM (35), whereas blood ACT levels of ~4 and 27 μM were recorded in subjects with a high blood alcohol content of 46 and 124 mM, respectively (14). We report here effects of ACT on Notch and VSMC proliferation at concentrations of 10 and 25 μM, which are achievable after binge drinking or chronic abuse and are considered to be clinically relevant (14, 35).

In terms of ACT's "mechanism of action," apart from its ability to bind to protein and DNA, forming adducts that inhibit DNA repair and methylation and potentiate liver cancer (26), to increase superoxide levels (in cardiomyoctyes) (3), and to modulate cytokine (TNF-α and IL-6) production (in astrocytes) (32), not much is known to date with respect to it affecting specific cell signaling pathways, especially in the vasculature. Our study provides evidence that the Notch pathway in VSMCs is a novel target for ACT. The Notch signal trans-

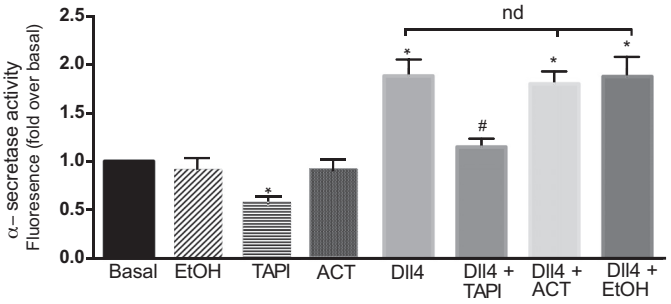

Fig. 4. Neither acetaldehyde (ACT) nor ethanol (EtOH) affect α-secretase (TACE/ADAM17) activity. α-Secretase activity in human coronary artery smooth muscle cell lysates stimulated with or without Delta-like ligand 4 (Dll4; 2 μg/ml) in the absence or presence of ACT (10 μM) or EtOH (25 mM) was determined using a Sensolyte 520 TACE (α-secretase) fluorometric assay. Tumor necrosis factor-α protease inhibitor (TAPI) was used as a control α-secretase inhibitor. Data are means ± SE; $n = 3$. *$P <$ 0.05 vs. basal; #$P <$ 0.05 vs. Dll4 stimulated. nd, no difference.

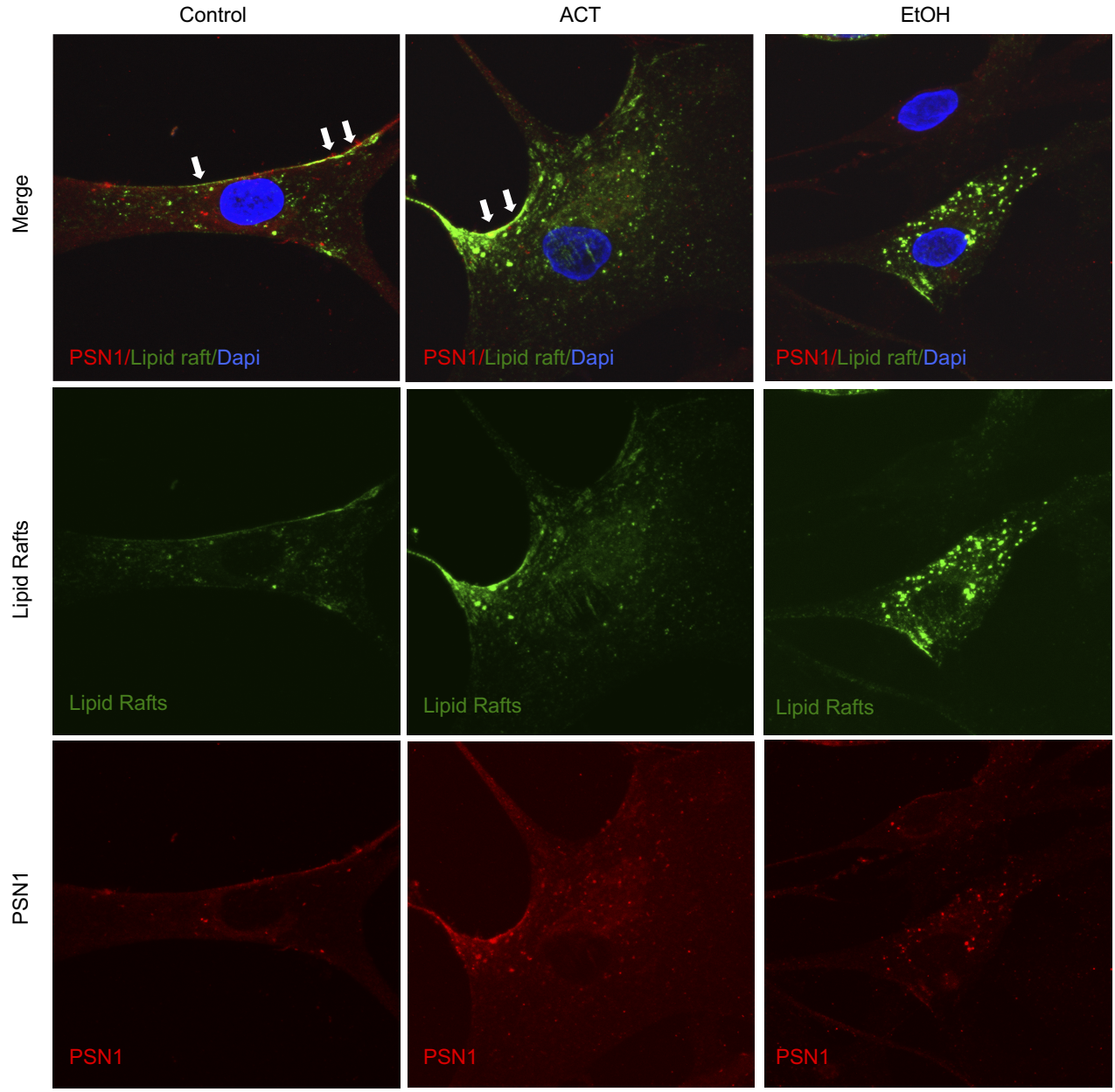

Fig. 5. Effects of acetaldehyde (ACT; 10 μM) and ethanol (EtOH; 25 mM) on the distribution of lipid rafts and presenilin-1 (PSN1) in human coronary artery smooth muscle cell lysates. Lipid rafts were labeled using Alexa 488-conjugated (green) cholera toxin B subunit (Vybrant Lipid raft labeling kit, Molecular Probes). PSN1 was detected using Alexa 594-conjugated (red) anti-PSN1 antibody. Arrows indicate PSN1 that colocalized with lipid rafts at the membrane in control and ACT-treated groups.

duction pathway is multistepped. Interaction of a ligand with the Notch receptor instigates cleavage events at two sites by distinct proteases, resulting in the release of the Notch intracellular domain from the cytoplasmic side of the cell membrane. This intracellular receptor fragment moves to the nucleus, where it interacts with coactivators to form a transcription activating complex. Thus, there are several points in the pathway that might be regulated. Our data rule out activation of α- or γ-secretase proteolytic activity as the mechanism involved. ACT might affect ligand-receptor binding, Notch intracellular domain translocation, or gene transcription regulation. Further investigation will be required to establish precisely the mechanism whereby ACT stimulates Notch signaling in these cells.

Of interest, with respect to "opposite effects" of EtOH and its metabolite ACT, relevant to CVD, we have previously shown that while EtOH inhibits monocyte chemotactic protein-1 expression and monocyte adhesion to activated endothelium (6) (considered antiatherogenic effects), ACT treatment increases monocyte adhesion in a P-selectin- and TNF-α-dependent manner (29) (considered proatherogenic). Taken together, the stimulatory effects of ACT on monocyte adhesion, and on SMCs (as reported here), may synergistically act to exacerbate atherosclerosis and CVD, especially in people in whom ACT blood levels become elevated relevant to EtOH levels.

In conclusion, this study demonstrates that ACT, a primary metabolite of EtOH, stimulates the proliferation of arterial SMCs in a Notch-dependent manner. These effects are oppo-

site to those elicited by EtOH itself, which is inhibitory. It may be that the balance of EtOH/ACT levels after different drinking patterns is key in dictating the subsequent consequences, good or bad, on CVD development. Further experimentation will be required, however, to definitively prove this hypothesis.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

E.H., D.M., and W.L. performed experiments; E.H., D.M., W.L., and E.M.R. analyzed data; E.H., D.M., W.L., and E.M.R. prepared figures; E.H., D.M., W.L., P.A.C., and E.M.R. approved final version of manuscript; D.M., P.A.C., and E.M.R., conceived and designed research; D.M. and E.M.R. interpreted results of experiments; P.A.C. and E.M.R. edited and revised manuscript; E.M.R. drafted manuscript.

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