Published in final edited form as:

Biochem Pharmacol. 2017 December 01; 145: 81–93. doi:10.1016/j.bcp.2017.08.022.

Statin therapy exacerbates alcohol-induced constriction of cerebral arteries via modulation of ethanol-induced BK channel inhibition in vascular smooth muscle

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Abstract

Statins constitute the most commonly prescribed drugs to decrease cholesterol (CLR). CLR is an important modulator of alcohol-induced cerebral artery constriction (AICAC). Using rats on a high CLR diet (2% CLR) we set to determine whether atorvastatin administration (10 mg/kg daily for 18–23 weeks) modified AICAC. Middle cerebral arteries were pressurized *in vitro* at 60 mmHg and AICAC was evoked by 50 mM ethanol, that is within the range of blood alcohol detected in humans following moderate-to-heavy drinking. AICAC was evident in high CLR + atorvastatin group but not in high CLR diet + placebo. Statin exacerbation of AICAC persisted in de-endothelialized arteries, and was blunted by CLR enrichment *in vitro*. Fluorescence imaging of filipin-stained arteries showed that atorvastatin decreased vascular smooth muscle (VSM) CLR when compared to placebo, this difference being reduced by CLR enrichment *in vitro*. Voltage- and calcium-gated potassium channels of large conductance (BK) are known VSM targets of ethanol, with their beta1 subunit being necessary for ethanol-induced channel inhibition and resulting AICAC. Ethanol-induced BK inhibition in excised membrane patches from freshly isolated myocytes was exacerbated in the high CLR diet + atorvastatin group when compared to high CLR diet + placebo. Unexpectedly, atorvastatin decreased the amount and function of BK beta1 subunit as documented by immunofluorescence imaging and functional patch-clamp studies. Atorvastatin exacerbation of ethanol-induced BK inhibition disappeared upon artery CLR enrichment *in vitro*. Our study demonstrates for the first time statin's ability to exacerbate the vascular effect of a widely consumed drug of abuse, this exacerbation being driven by statin modulation of ethanol-induced BK channel inhibition in the VSM via CLR-mediated mechanism.

Keywords

Patch-clamp; MaxiK channel; Atorvastatin

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Conflict of interest

None.

1. Introduction

Cholesterol (CLR) is one of the major structural lipid components of animal cell membranes, a key mediator in lipid homeostasis, and precursor for the biosynthesis of steroid hormones, bile acids and vitamin D [26,17,22]. However, high levels of CLR in humans represent a risk factor for life-threatening cardio/cerebrovascular conditions, including heart attack and stroke [30]. It is estimated that nearly 94.6 million, or 40 percent of American adults, have total blood CLR levels above 200 mg/dL, with approximately 12 percent of such population reaching over 240 mg/dL (American Heart Association, news release, April 10, 2017). The latter is recognized as one of the criteria for clinically defined hypercholesterolemia [53]. With such high prevalence of elevated CLR and the severe risks it poses on human health, it comes as not a surprise that CLR-lowering pharmacological agents are widely prescribed. In particular, statins are the most prescribed group of medications in the US to decrease CLR levels in humans (http://www.forbes.com/2008/10/29/cholesterol-pharmaceuticals-statin-biz-cx_mh_1030cholesterol.html).

Statins inhibit the enzyme HMG-CoA (or 3-hydroxy-3-methyl glutaryl-coenzyme A) reductase, which plays a central role in the production of CLR [33]. Remarkably, the overall health benefits observed with statins include effects beyond CLR lowering [43,25,50,64,2]. Thus, statins exert CLR-independent or “pleiotropic” effects. The extent to which particular mechanism (CLR-dependent versus independent) plays role in a given effect of statin treatment remains the subject of discussion.

Episodic moderate-to-heavy alcohol (ethyl alcohol, ethanol) consumption that results in blood alcohol levels of 18–80 mM gives rise to an increased risk for cerebral ischemia, stroke and death from cerebrovascular disease [51,52]. These pathologies may result or be accompanied by an abnormal constriction of cerebral arteries. It has been shown that alcohol-induced cerebral artery constriction (AICAC) is mediated by ethanol inhibition of voltage/Ca²⁺-gated K⁺ channels of large conductance (BK) in vascular smooth muscle (VSM) [41]. BK channels control numerous physiological processes, including smooth arterial contractility [48,13]. Upon depolarization-induced calcium entry into the myocyte, BK channels generate outward K⁺ currents, which hyperpolarize the membrane and promote smooth muscle relaxation [48]. In smooth muscle (including VSM), BK channel complexes include channel-forming alpha and small, accessory beta 1 subunits [48]. The latter represents one of the major protein targets for ethanol, as ethanol-induced BK channel inhibition and resulting vasoconstriction are drastically diminished in *KCNMB1* knock-out e.g. beta 1 protein-lacking) mouse arteries [10].

Several factors beyond the presence of BK beta 1 subunit contribute into the degree of ethanol-induced BK channel inhibition and resulting AICAC. CLR buildup in cerebral artery tissue arising from high CLR food intake *in vivo* protects against AICAC [13]. While the mechanisms behind CLR control of AICAC are unraveled [3,13], the consequences of statin therapy on AICAC and underlying mechanisms remain fully unknown.

In the current work, we set to determine the effect of statin therapy on AICAC and to identify the mechanisms that would enable statin-driven modification of AICAC. We used

atorvastatin administration to rats on a high CLR diet, evaluation of *in vitro* pressurized cerebral artery diameter, fluorescence imaging of VSM BK channel subunit and CLR, and patch-clamp electrophysiology on native VSM BK channels in cerebral artery myocytes. Thus, we tested the hypothesis that statins exacerbated AICAC by removing excessive CLR from cerebral artery tissue and shifting VSM CLR to the optimal level for ethanol inhibition of BK channels. Our work reports for the first time statin-driven modulation of a vascular effect exerted by a commonly used and abused substance.

2. Material and methods

2.1. Ethical aspects of research

The care of animals and experimental protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, which is an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. High CLR diet and atorvastatin administration

Three groups of male Sprague-Dawley rats (≈ 50 g; Harlan) were enrolled into the study. The first group was fed by regular Teklad rodent food (Indianapolis, IN). The second group was fed by high CLR food (2% CLR *ad libitum* for 18–22 weeks) supplemented by daily administration of atorvastatin (10 mg/kg, suspension of atorvastatin calcium powder in 500 ml of distilled water) via steel gavage. The third group was fed by high CLR food (2% CLR *ad libitum* for 18–22 weeks) supplemented by daily administration of placebo (500 μ L of distilled water). High CLR food was purchased from Tek-lad (Indianapolis, IN).

2.3. Determination of blood CLR level

Adult male Sprague-Dawley rats were decapitated under isoflurane anesthesia using a guillotine. Blood samples were collected, incubated at room temperature for 10 min, and spun at 10³ rpm, 4 °C, using Mikro 200R centrifuge (Hettich GmbH & Co., Tuttlingen, Germany). Serum was collected, and total CLR level was determined using Cobas Mira biochemistry analyzer (Roche, Basel, Switzerland) at the University of Tennessee HSC Endocrinology laboratory on a fee-for-service basis.

2.4. Modification of CLR levels in myocytes and arteries

For CLR enrichment, bath solution and PSS contained 5 mM MbetaCD + 0.625 mM CLR (8:1 M ratio). To ensure MbetaCD saturation with CLR, the solution was vortexed and sonicated for 30 min at room temperature, then shaken at 37 °C overnight and filtered on the morning of the experiment [68,3].

2.5. Cerebral artery diameter monitoring

Middle cerebral arteries (MCA) were dissected out on ice under the Nikon SMZ645 microscope (Nikon, Tokyo, Japan) from rat brain and cut into 1 to 2 mm-long segments. A segment was cannulated at each end in a temperature-controlled, custom-made perfusion chamber. Using a Dynamax RP-1 peristaltic pump (Rainin Instr., Oakland, CA), the chamber

was continuously perfused at a rate of 3.75 ml/min with PSS (mM): 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.6 CaCl₂, 1.2 MgSO₄, 0.023 EDTA, 11 glucose, 24 NaHCO₃. PSS was equilibrated at pH 7.4 with a 21/5/74% mix of O₂/CO₂/N₂ and maintained at 35–37 °C. Arteries were monitored with a Sanyo VCB-3512T camera (Sanyo, Osaka, Japan) attached to an inverted Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). The artery external wall diameter was measured using the automatic edge-detection function of IonWizard software (IonOptics, Waltham, MA) and digitized at 1 Hz using a personal computer. Steady-state changes in intravascular pressure were achieved by elevating an attached reservoir filled with PSS and were monitored using a pressure transducer (Living Systems Instr., St. Albans City, VT). Arteries were first incubated at an intravascular pressure of 10 mmHg for 10 min. Then, intravascular pressure was increased to 60 mmHg and held steady throughout the experiment to evoke development and maintenance of arterial myogenic tone. Alcohol (ethanol ultra-pure, 200 proof; American Bioanalytical, Natick, MA) was diluted in PSS to final concentration, and applied to the artery via chamber perfusion. The effect of drug applications was evaluated at the time it reached a maximal, steady level. For experiments with de-endothelialized arteries, endothelium was removed by passing an air bubble into the vessel lumen for 90 s prior to vessel cannulation. This method is highly effective for removing the endothelial layer [11,12]. As previously established in our lab, de-endothelialized vessels failed to dilate in presence of endothelium-dependent vasodilators (acetylcholine and carbachol), yet dilated in response to the endothelium-independent nitric oxide donor sodium nitroprusside [11,13].

2.6. Rat cerebral artery myocyte isolation

Middle cerebral arteries were dissected out and placed into ice-cold dissociation medium (DM) with the following composition (mM): 0.16 CaCl₂, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH₂PO₄, 2 MgCl₂, 110 NaCl, 0.5 NaH₂PO₄, 10 NaHCO₃, 0.02 phenol red, 10 taurine, 10 glucose. Each artery was cut into 1- to 2-mm long rings (up to 30 rings/experiment). Individual myocytes were enzymatically isolated. For this purpose, rat arterial rings were put in 3 ml DM containing 0.03% papain, 0.05% bovine serum albumin (BSA), and 0.004% dithiothreitol at 37 °C for 15 min in a polypropylene tube and incubated in a shaking water bath at 37 °C and 60 oscillations/min for 15 min. Then, the supernatant was discarded, and the tissue was transferred to a polypropylene tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor, 0.05% BSA, and 2% collagenase (26.6 units/ml). The tube was incubated again in a shaking water bath at 37 °C and 60 oscillations/min for 15 min. Finally, the artery tissue pellet was transferred into a tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor. Tissue-containing DM was pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. The procedure rendered a cell suspension containing relaxed, individual myocytes (5 myocytes/field using a 20× objective) that could be identified under an Olympus IX-70 microscope (Tokyo, Japan). The cell suspension was stored in ice-cold DM containing 0.06% soybean trypsin inhibitor and 0.06% BSA. Myocytes were used for electrophysiology up to 4 h after isolation.

2.7. Immunocytochemistry and confocal fluorescence imaging

Staining procedures were performed on several independent occasions. However, to avoid the high variability in fluorescence signal, staining on each occasion was performed in parallel on specimens from all the experimental groups. Arteries were harvested from several rat donors, staining was repeated on two different occasions. Middle cerebral arteries were fixed in 3% paraformaldehyde at room temperature for 30 min and permeabilized with 0.1% Triton-100 in phosphate buffered saline at room temperature for 30 min.

For filipin staining, arteries were incubated with the CLR-sensitive dye filipin (Sigma, St. Louis, MO) at room temperature for 2 h. For immunostaining, arteries were incubated at room temperature for 2 h in a mixture of the following primary antibodies: mouse monoclonal antibody against BK alpha subunit (clone L6/60, UC Davis/NIH NeuroMab Facility, Davis, CA) and rabbit polyclonal antibody against BK beta 1 subunit (PA 1-924, Invitrogen, Carlsbad, CA). After primary antibody washout, arteries were incubated in anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa488 (A11001, Invitrogen, Carlsbad, CA) and Cy5 (ab6564, Abcam, Cambridge, MA) dyes, respectively. Cellular nuclei were stained with DAPI (Life Technologies Corporation, Willow Creek Road Eugene, OR). Slips were mounted using ProLong AntiFade kit (Invitrogen, Carlsbad, CA) and sealed using clear nail polish.

Filipin-stained specimens were imaged using a 40× objective and the 405 laser line of an Olympus FV-1000 laser scanning confocal system (Center Valley, PA). The acquisition settings of the confocal microscope system remained unchanged throughout imaging of all filipin-stained specimens. Specimens that were stained with antibodies, were imaged using 40x objective and 405 (DAPI), 488 (Alexa488), and 635 (Cy5) laser lines of Olympus FV-1000 laser scanning confocal system (Center Valley, PA). Immunostained specimens were imaged using sequential line acquisition to minimize the probability of fluorescence emission crossover. The acquisition settings of the confocal microscope system remained unchanged throughout imaging of all immunostained specimens.

2.8. Antibody validation

Rat middle cerebral arteries were dissected out and subjected to surface protein biotinylation using the Pierce™ Cell Surface Protein Isolation Kit (Thermo Fisher Scientific, Waltham, MA) and following manufacturer's instructions. Surface protein was analyzed by a Western blotting using standard methodology that confirmed presence of bands corresponding to ≈130 kDa BK alpha and ≈24 kDa BK beta1 proteins in the membrane protein fraction.

Additional validation was performed in the course of immunofluorescence labeling: specificity of binding for anti-BK beta1 antibody was confirmed by the lack of fluorescence signal when incubation with primary antibody was performed in presence of the immunogenic peptide (Thermo Fisher Scientific, Waltham, MA) [13]. Performance of secondary antibodies was confirmed by the lack of fluorescence signal in cerebral artery myocyte specimens that were stained following the omission of primary antibodies [13].

2.9. Electrophysiology data acquisition and analysis

BK currents at single channel resolution were recorded from excised, inside-out (I/O) membrane patches. Both bath and electrode solutions contained (mM): 130 KCl, 5 EGTA, 2.44 MgCl₂, 15 HEPES, 1.6 HEDTA, 5.59 CaCl₂ ([Ca²⁺]_{free} ≈ 30 μM), pH 7.35. Nominal free Ca²⁺ was calculated with MaxChelator Sliders (C. Patton, Stanford University, CA) and validated experimentally using Ca²⁺-selective electrodes (Corning Incorporated Science Products Division, Corning, NY) [19]. Patch-clamp electrodes were pulled from glass capillaries (Drummond Scientific Co., Broomall, PA). Immediately before recording, the tip of each electrode was fire-polished on a microforge WPI MF-200 (World Precision Instruments, Sarasota, FL) to give resistances of 5–9 MΩ when filled with electrode solution. An agar bridge with Cl[−] as the main anion was used as a ground electrode. Solutions were applied onto the patch cytosolic side using a pressurized, automated Octaflow system (ALA Scientific Instruments, Farmingdale, NY) via a micropipette tip with an internal diameter of 100 μm. Experiments were performed at room temperature (20–22 °C). The ionic current was recorded using an EPC8 amplifier (HEKA, Lambrecht, Germany) at 1 kHz. Data were digitized at 5 kHz using a Digidata 1320A A/D converter and pCLAMP 8.0 (Molecular Devices, Sunnyvale, CA).

2.10. Reagents

Cholesterol was purchased from Avanti Polar Lipids (Albaster, AL). Ethanol ultra-pure, 200 proof was purchased from American Bioanalytical (Natick, MA). With the exception of antibodies see above), all other chemicals including atorvastatin were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol was freshly diluted in bath solution immediately before application to the artery or myocyte. Unless otherwise specified, each pressurized artery or myocyte membrane patch was exposed to ethanol only once to avoid reduced responsiveness of BK current to multiple applications of ethanol [20].

2.11. Data analysis

Artery diameter data were analyzed using IonWizard 4.4 software (IonOptics, Waltham, MA). Fluorescence was quantified using built-in function in FV10-ASW 3.1 software (Olympus American Inc., Center Valley, PA). No more than three artery segments of equal size were imaged from each artery. Fluorescence signals were quantified along three randomly-picked individual myocyte plasma membranes within each segment and visually defined upon the superposition of fluorescence with visible light images. The data from individual myocytes within each artery segment were averaged to represent one data point.

For patch-clamp data, the product of number of channels in the patch (N) and channel open probability (Po) was used as an index of channel steady-state activity. NPo was obtained using a built-in option in Clampfit 9.2 (Molecular Devices, Sunnyvale, CA) from 30 s of gap-free recording under each condition. For determination of V_{half}, NPo/NPo_{max}-V plots were fitted using the built-in Boltzmann fitting function in Origin 7.0 (Originlab Corp, Northampton, MA). To obtain best fitting results, open probabilities were assumed equal zero and 1 at −250 and +150 mV, respectively, as expected at 30 μM [Ca²⁺]_{free} [28,60].

Final plotting, fitting, and statistical analysis of the data were conducted using Origin 8.5 (OriginLab, Northampton, MA) and InS-tat 3.0 (GraphPad, La Jolla, CA). Statistical analysis was conducted using either one-way analysis of variance and Tukey's multiple comparison test or Student's *t* test, according to experimental design. Patch-clamp data showing decrease in BK channel activity upon ethanol administration were analyzed using repeated measures ANOVA. In all cases, $P < 0.05$ was considered statistically significant. Data are expressed as the mean $\pm$ S.E.M. In pressurized artery experiments and in patch-clamping, "n" represents the number of arteries and membrane patches, respectively. In these experiments, each artery or membrane patch being obtained from a separate animal. In fluorescence staining, "n" refers to the number of artery segments, with no more than three arterial segments being imaged from one animal donor.

3. Results

3.1. Statin therapy exacerbates AICAC

Consumption of a high CLR diet supplemented by daily administration of placebo resulted in a significant increase in blood serum total CLR when compared to rats on normal chow (Fig. 1A). A third group received daily supplementation with 10 mg/kg atorvastatin; this dose has been used in similar experimental protocols in rodent models by other groups [59,65]. Daily administration of atorvastatin partially blunted the increase in blood CLR levels introduced by the high CLR diet, as blood CLR levels in the statin-treated rats reached blood CLR levels that were intermediate between those from rats on normal chow and rats receiving high CLR diet with placebo (Fig. 1A).

A comparison of the effects of high CLR diet+placebo vs high CLR diet+atorvastatin on AICAC was conducted by measuring middle cerebral artery (MCA) diameter in pressurized arteries *in vitro*. This approach replicates the *in vivo* response of cerebral vessels to alcohol, as was consistently demonstrated by our group [13,15]. Arteries were dissected out, pressurized at 60 mmHg and probed with 50 mM ethanol. This concentration is reached in systemic circulation during moderate-to-heavy drinking and represents an EC_{50} for AICAC [18,41]. MCAs from rats on high CLR diet with placebo did not constrict in presence of alcohol (Fig. 1B). The absence of AICAC after high CLR diet does not represent a general impairment of vasomotion, as degree of myogenic tone and artery constriction in response to 60 mM KCl were not altered by high CLR diet [13]. Thus, the effect of high CLR diet on AICAC is rather selective toward alcohol. Remarkably, MCAs from rats that were subjected to high CLR diet supplemented by daily atorvastatin administration consistently constricted in presence of 50 mM ethanol (Fig. 1C-D).

3.2. Statin therapy exacerbates AICAC by preventing CLR buildup in the vascular smooth muscle

Considering that CLR buildup in the vascular smooth muscle protects against AICAC and this protection is independent of endothelial function [13,3], statin-induced exacerbation of AICAC could be explained by possible prevention of high CLR diet-driven CLR buildup in VSM. To test this hypothesis, we first established whether statin-induced exacerbation of AICAC required the presence of a functional endothelium. We repeated the experiments

described in the previous section, but this time the endothelial layer was removed prior to artery cannulation by passing an air bubble through arterial lumen. This method has been used effectively to deprive cerebral arteries of a functional endothelium [11,12]. Results in de-endothelialized vessels were remarkably similar to arteries with intact endothelium: 50 mM ethanol evoked vasoconstriction in rats subjected to daily administration of atorvastatin whereas remained ineffective in rats subjected to high CLR diet+placebo (Fig. 2A-B, D). Thus, statin exacerbation of AICAC did not require a functional endothelium.

To determine whether atorvastatin exacerbated AICAC by preventing CLR buildup in cerebral artery tissue as opposed to possible CLR-independent effects, we harvested arteries from rats on high CLR diet that were supplemented by daily atorvastatin and subjected these vessels to *in vitro* CLR-enrichment as was previously done by our group [3]. AICAC disappeared upon *in vitro* CLR enrichment of de-endothelialized arteries from rats subjected to high CLR diet-atorvastatin (Fig. 2C-D). Thus, statins exacerbated AICAC decreasing CLR buildup in the de-endothelialized arterial wall. Considering that upon endothelial removal, the vast majority of remaining cellular elements in the cerebral artery wall are myocytes [39], it is highly conceivable that statins exacerbated AICAC by preventing CLR buildup in VSM itself.

To determine whether CLR level in MCA smooth muscle indeed differs between experimental groups, we evaluated artery smooth muscle CLR levels using fluorescence staining of arteries with the CLR-sensitive dye filipin [27,45]. MCAs were stained with filipin using standard methods [8,9]. Upon superposition of fluorescent images with visible light images that defined the silhouettes of individual myocytes, it became apparent that the majority of cellular CLR was present in the plasma membrane. This observation is consistent with previous reports pointing at the preferential localization of CLR in cellular plasma membranes [4,31]. Remarkably, results revealed a significant decrease in CLR levels within MCA myocytes of rats on high CLR diet supplemented by atorvastatin when compared to the high CLR diet group that was treated with placebo (Fig. 3). Interestingly, CLR enrichment *in vitro* resulted in a mild but consistent increase in MCA CLR level. This increase eliminated the statistically significant difference between atorvastatin versus placebo-supplemented groups of arteries (Fig. 3).

To ensure the sensitivity of filipin staining to modification in cerebral artery CLR levels *in vitro*, we performed filipin staining of cerebral arteries harvested from animals that were fed normal rodent chaw. Following CLR enrichment *in vitro*, filipin-associated fluorescence signal from this group was significantly increased (Fig. 3C-D). Thus, lack of statistically significant increase in filipin-associated fluorescence upon CLR *in vitro* enrichment of rat arteries following daily atorvastatin administration is a result of changes in intrinsic properties of cerebral artery myocyte membranes rather than of filipin's lack of sensitivity to variations in CLR levels (see Discussion).

3.3. Statin exacerbation of AICAC is underlied by statin-driven amplification of ethanol-induced BK channel inhibition

BK channels are major targets of ethanol within VSM, their inhibition by ethanol underlying AICAC [41]. Thus, we studied the effect of atorvastatin administration on the ethanol

sensitivity of native BK channels in freshly isolated myocytes from rat cerebral arteries. Channel activity was recorded in excised, I/O patches, at -40 mV and $[Ca^{2+}]_{free} = 30$ μ M. These values of transmembrane voltage and intracellular calcium correspond to conditions that are faced by BK channels in the contracting myocyte [36,49]. One-two minutes following membrane patch excision, steady-state BK channel activity was recorded for 1 min to obtain pre-perfusion values of NPo. After pre-perfusion recording, BK channel activity was recorded under perfusion with the bath solution for a total of 16 min. Perfusion with ethanol lasted for 10 min and was immediately followed by a 5 min-long perfusion with bath solution (washout). This 10 min-long perfusion with 50 mM ethanol fits the time-frame of AICAC previously reported both *in vivo* and *in vitro* (Fig. 1B-C) [13]. Channel activity was sampled every 1 min, repeated measures ANOVA statistical analysis was applied to evaluate the ethanol effect.

Perfusion with bath solution rendered stochastic fluctuations of channel activity with a very mild overall trend of diminished BK NPo over time (Fig. 4A-B). This trend is consistent with previous reports, and may be explained by the depletion of physiologically relevant modulators that sustain BK channel activity, such as phosphatidylinositol 4,5-bisphosphate [62].

Consistent with the diminished AICAC during the course of high CLR diet (Fig. 1B-C) [13], we did not observe significant ethanol-induced inhibition of native BK channels in membrane patches that were excited from arterial myocytes from the high CLR diet group (Fig. 4). In contrast, BK channel open probability in myocyte patches from the high CLR diet+atorvastatin was progressively reduced in a time-dependent manner, until reaching values that were ~35% lower than those obtained in myocytes from the high CLR+placebo group. This robust decrease in activity was fully reversible upon ethanol washout (Fig. 4B), with BK channel activity returning to control levels 2 min after ethanol-containing solution was switched to bath washout (Fig. 4B). Thus, statin administration exacerbates ethanol-induced BK channel inhibition when compared to high CLR diet.

3.4. Atorvastatin diminishes the amount and function of BK beta 1 subunits

Our earlier work showed that AICAC and ethanol-induced BK channel inhibition was drastically ablated in *KCNMB1* beta1-lacking knockout mouse arteries/myocytes [10]. Thus, the exacerbation of AICAC and underlying ethanol-induced BK channel inhibition could potentially arise from an atorvastatin-driven increase in the amount and/or function of BK beta 1 subunits. To test this possibility, we performed immunofluorescence staining of MCA segments followed by confocal microscopy imaging of the BK channel-associated signal in plasma membranes of individual myocytes within the smooth muscle layer. Anti-BK alpha and anti-BK beta 1 antibodies were validated by Western blotting of cerebral artery samples following surface protein biotinylation (Fig. 5E-F). Additional validation was obtained for anti-BK beta 1 subunit antibody by performing immunofluorescence labeling of cerebral arteries in presence of beta 1 subunit-corresponding immunogenic peptide as described elsewhere [13].

As determined by the fluorescence intensity, the amount of BK channel-forming alpha subunit protein remained unchanged when the high CLR diet+atorvastatin samples were

compared to those from the high CLR diet+placebo group (Fig. 5A-B). Unexpectedly, the amount of BK beta1 subunit in atorvastatin-treated group was not increased, but diminished (Fig. 5A, C). Accordingly, the amount of BK beta1 protein per BK alpha was significantly lower in high CLR diet+atorvastatin group when compared to high CLR diet+placebo (Fig. 5A, D). Therefore, atorvastatin did not increase ethanol-induced BK channel inhibition by increasing the amount of ethanol target in the vascular smooth muscle, that is, the BK beta1 subunit.

Next, we used patch-clamp recordings to determine whether the functionality of BK beta1 within the BK channel complex was actually reduced in atorvastatin-treated group. BK channel activity in excised I/O patches was recorded at $[Ca^{2+}]_{free} = 30 \mu M$ at trans-membrane voltages ranging from -80 mV to $+40$ mV in a 20 mV increment (Fig. 6A). At this level of Ca^{2+} , maximal channel activation was observed at $+20$ or $+40$ mV, thus, NPo/NPo_{max} values at each transmembrane voltage could be accurately determined (Fig. 6B). V_{half} in high CLR diet+atorvastatin was shifted to more depolarized values when compared to high CLR+placebo group (Fig. 6C). Therefore, atorvastatin treatment resulted in decreased BK channel activity as more membrane depolarization was needed to reach half-maximal BK current when compared to placebo. The result is consistent with a diminished functionality of BK channel beta1 subunits [6,38]. Thus, while atorvastatin exacerbates ethanol-induced BK channel inhibition, statin treatment diminishes basal channel function.

3.5. Atorvastatin exacerbation of ethanol-induced BK channel inhibition is removed upon artery in vitro enrichment with CLR

Our recent work using CLR manipulation *in vitro*, demonstrated that excessive CLR in cerebral artery vasculature blunted ethanol-induced BK channel inhibition [3]. To test whether suppression of ethanol-induced BK channel inhibition in high CLR diet +atorvastatin group was due to removal of excessive CLR from arterial wall, we incubated dissected MCAs in PSS that contained MbetaCD complex with CLR. Then arteries were subjected to enzymatic digestion to render individual myocytes for patch-clamp recordings. Native BK channels in excised I/O patches were challenged with 50 mM ethanol as described above (Fig. 7). CLR enrichment prevented development of ethanol-induced BK channel inhibition, and rendered BK NPo undistinguishable from that observed upon ethanol perfusion in high CLR diet + placebo group (Fig. 7A-B). Thus, atorvastatin exacerbation of ethanol-induced BK channel inhibition was achieved via statin removal of excessive CLR from the artery.

4. Discussion

In the current work, we demonstrate for the first time that statin therapy exacerbates AICAC. Mechanistically, we established that statin exacerbation of AICAC was underlied by statin-induced potentiation of VSM BK channel inhibition by ethanol. Statin overall effects, however, involved a dissociation between a reduction in the amount and function of an alcohol target within the BK channel complex, i.e., the BK beta1 subunit, and an apparent increase in the sensitivity of the BK channel complex to ethanol-induced inhibition. Our

study clearly demonstrate that the latter is driven by statin-induced decrease in membrane CLR level within the cerebral artery VSM, as ethanol-induced BK channel inhibition could be restored by CLR enrichment of cerebral artery myocytes *in vitro*.

The therapeutic value of statins stems from their ability to inhibit HMG-CoA reductase, the first enzyme in the mevalonate pathway leading to CLR biosynthesis [7]. Besides CLR lowering, statins exert a plethora of effects. The widespread pleiotropic effects of statins reasonably arise from the statin-induced inhibition of the HMG-CoA, a central enzyme in the mevalonate pathway that results in synthesis of isoprenoids – building blocks for the diverse group of chemical products, including cholesterol and derivatives [67,58]. As a result, statin physiological effects have numerous and diverse mechanisms [40]. For example, protection against amyloid β -peptide production and Alzheimer's disease was proposed to arise from modulating amyloid precursor protein maturation and phosphorylation *in vitro* [29]. Anti-metastatic activity of statins seems to arise from that activation of the AMPK-TOR signaling pathway and resulting statin-induced autophagy [66].

Beneficial effects of statin administration include major improvement in cardiovascular health [16], improved recurrence-free survival in breast cancer [44], protection against amyloid β -peptide production and Alzheimer's disease [35,29]. On the other hand, unwanted consequences of statins such as new onsets of diabetes, myopathy, elevation of plasma cortisol levels have all been reported [5,46,54]. To the best of our knowledge, present data on statin therapy-induced exacerbation of AICAC (Fig. 1) represents the first report on statin modulation of vascular effect of a commonly abused substance, i.e., alcohol. This finding may contribute to explain previously reported, rather paradoxical data: although statin therapy usually reduces the risk for cerebrovascular events [47], data that included drinkers demonstrated that statin therapy in hyperCLRemic patients had little benefit and even increased risk for cerebral ischemia and stroke [61]. Both cerebrovascular events list excessive alcohol consumption as an independent risk factor [51,52].

In the cardiovascular system, statins modulate apolipoprotein E levels, exert anti-inflammatory properties and stabilize atherosclerotic plaques [35,37]. It also has been shown that statins improve endothelial function and vasomotor reactivity by upregulating endothelial nitric oxide synthase, and reducing angiotensin II type 1 receptor gene expression [67,1,32]. Thereby, it was concluded that one of the major effects of statin regimen in cardiovascular system stems is CLR synthesis-independent, and can be categorized as pleiotropic effects via endothelial function. Thus, another novel aspect of our study lies in the fact that atorvastatin exacerbated AICAC independent of functional endothelium, as statin effect was observed in de-endothelialized cerebral arteries (Fig. 2). Moreover, we showed that statin administration effectively decreased CLR level in cerebral artery smooth muscle when compared to high CLR diet supplemented by placebo (Fig. 3). This result might arise from the reduced influx of CLR from bloodstream following statin-induced reduction in total CLR blood level (Fig. 1A). In addition, statin's ability to modulate CLR content in cerebral artery vasculature could arise from the statin-induced inhibition of artery smooth muscle HMG-CoA. In this case, statin should have penetrated the blood-brain barrier. This penetration is expected, as the atorvastatin used in current work belongs to the

group of lipophilic statins and thus, is expected to easily cross cellular membranes [55,34]. Whether HMG-CoA is present and contributes to the CLR pool in rat cerebral artery tissue, is currently under investigation in our lab.

Another interesting observation in our work was a limited ability of MbetaCD complexed with CLR to enrich arterial smooth muscle (Fig. 3). Indeed, our studies and work of others consistently point at the ability of MbetaCD-CLR complex to increase tissue CLR level up to $\times 1.5$ -fold [68,3]. In contrast, treatment of cerebral arteries that were harvested from rats subjected to high CLR diet supplemented by atorvastatin only resulted in $\times 1.1$ -fold increase in tissue CLR level (Fig. 3B). CLR is an important structural component of cellular membranes and exerts profound effects on membrane physical properties [21,23,42]. It is conceivable that tissue's ability to accumulate CLR may be limited by the membrane physical properties required for life as we know it. In this context, we speculate that atorvastatin might modify artery smooth muscle membrane physical properties, which may limit the drug's ability to increase membrane CLR further. This hypothesis is in line with several reports documenting statins' ability to modify membrane lipid composition in numerous organs and tissue types, such as brain and erythrocytes [24,14,63]. In particular, statins decreased plasma membrane CLR while amount of sphingolipids was increased [63]. Acyl chain composition is also modulated by statins, with statin treatment being able to decrease the content of saturated and increase the content of polyunsaturated fatty acids within membrane lipid fraction [24,63].

Another novel finding in our work is the ability of atorvastatin to restore ethanol-induced BK channel inhibition which is suppressed by high CLR diet (Fig. 4). Thus, statin treatment is able to change pharmacological properties of BK channel, this result being consistent with previous reports. In particular, simvastatin was reported to markedly attenuated isopimaric acid-induced enhancement of the BK whole-cell current and cromakalim-induced K_{ATP} current in porcine coronary artery smooth muscle [56,57]. However, the effect was observed upon acute simvastatin application, while our data mimic clinically relevant scenario when statins are administered persistently over an extended period of time. Moreover, the statin-mediated changes in BK pharmacological profile we report here persist in absence of statin, as myocytes were isolated and thus deprived of circulating statin. This fact means that atorvastatin does not modulate ethanol sensitivity of the BK channel directly, but rather pre-conditions the channel to enhanced responses to ethanol or induces long-term changes in the channel's immediate lipid and protein environment.

The mechanistic understanding of such "pre-conditioning" may arise from the statin-driven change in BK channel basal characteristics. In the current work, we documented a decrease in the amount of BK channel beta1 subunit and, therefore, decrease in BK channel open probability when compared to BK channels from high CLR diet group receiving placebo (Figs. 5 and 6). Our findings are in line with previous reports documenting hydrophobic statin-driven inhibition of BK and K_{ATP} currents in vascular myocytes [56,57]. However, the distinct aspect of our work resides on the physiological route of atorvastatin application (protracted oral consumption) when compared to acute application of statin on cellular preparation [56,57]. Moreover, while previous reports on statin inhibition of K^+ channel function concluded on the involvement of several intracellular pathways [56,57], we

documented changes in BK channel basal activity in excised patches, that is, in absence of freely diffusible intracellular messengers and complex cytoarchitecture. The mechanisms of statin-driven decrease in BK channel $\beta 1$ protein level are currently under investigation and may include both genomic and post-translational regulatory pathways that result in lasting, rather than transient, changes in BK channel composition and activity. Remarkably, while statin decreased the amount and function of alcohol target within BK channel complex (Figs. 5 and 6), the ethanol effect was exacerbated (Fig. 4). Thus, atorvastatin dissociated increase in ethanol sensitivity from the decrease in channel $\beta 1$ subunit amount and function.

A rather surprising but straightforward conclusion from our work is that atorvastatin did not display any pleiotropic effects in exacerbating AICAC and ethanol-induced BK channel inhibition, as statin effect was robustly reversed by CLR enrichment *in vitro*. Notably, CLR level upon enrichment *in vitro* with MbetaCD/CLR complex was only increased by 10–15% (Fig. 3B). Yet, this subtle change was sufficient to reverse atorvastatin exacerbation of alcohol effect. High sensitivity of AICAC to elevation of CLR level is in unison with our previous findings: artery wall CLR elevation by 15% via incubation with low density lipoprotein significantly diminished AICAC [3]. The fact that two CLR delivery systems (low density lipoprotein and MbetaCD/CLR complex) rendered a similar outcome, i.e., robust blunting of AICAC, argues that CLR, a common component to both systems, modifies the sensitivity of cerebral artery to alcohol. The mechanism(s) behind CLR tuning of alcohol effect remains elusive. However, the fact that increase in CLR level by 10–15% drove notable pharmacological consequences, argues in favor of a mechanism that is highly sensitive to CLR levels and/or being able to amplify CLR effect on alcohol-induced BK channel inhibition and resulting vasoconstriction. Whether it is a CLR-sensing site on a protein structure, or a physical property of the membrane that works as a switch upon CLR membrane levels reaching a particular threshold, remains to be determined.

In conclusion, in the present work we for the first time report novel effect of atorvastatin, e.g. exacerbation of AICAC and underlying ethanol-induced inhibition of the vascular smooth muscle BK channel. The newly discovered effect is mediated by statin-driven removal of excessive CLR level in cerebral artery VSM and dissociated from statin-induced modification of BK channel basal function. In light of our findings, it may be advisable to remind cardiovascular patients about the danger of excessive alcohol drinking after adjusting CLR levels with statins.

Acknowledgments

Authors deeply thank Ms. Kelsey Cleland for excellent technical assistance. Current work was supported by NIH grants R01 AA-023764 (AB), R01 HL-104631 and R37 AA-11560 (AD).

Abbreviations

AICAC	alcohol-induced cerebral artery constriction
ANOVA	analysis of variance
BK	voltage-/calcium-gated potassium channel of large conductance

CLR	cholesterol
EtOH	ethanol (ethyl alcohol)
HMG-CoA	3-hydrox y-3-methylglutaryl-coenzyme A
I/O	inside-out (patch)
MbetaCD	methyl-beta-cyclodextrin
MCA	middle cerebral artery
NPo	open probability (N-number of channel, Po-probability of single channel opening)
VSM	vascular smooth muscle
Vhalf	voltage that is needed to reach half-maximal current

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Fig. 1.

Atorvastatin exacerbates AICAC. A. Averaged data showing that consumption of high CLR diet + placebo ($n = 10$) resulted in significant increase in blood serum total CLR when compared to rats on normal chaw ($n = 11$). Consumption of high CLR diet + atorvastatin ($n = 8$) partially decreased blood serum total CLR, the difference from normal chaw consumption is not statistically significant. Here and in Fig. 2, (n) is the number of experimental observations, each data point being obtained from a separate animal.

**Statistically significant difference; $P < 0.01$. B. Original trace showing lack of AICAC in response to 50 mM ethanol in arteries with intact endothelium from rats subjected to high CLR diet + placebo. C. Original trace showing decrease in artery diameter (constriction) in response to 50 mM ethanol in arteries with intact endothelium from rats subjected to high CLR diet + atorvastatin. D. Averaged data showing changes in artery diameter in response to 50 mM ethanol in arteries with intact endothelium from rats subjected to high CLR diet + placebo ($n = 3$) versus high CLR diet + atorvastatin ($n = 5$). **Statistically significance difference; $P < 0.01$.

Fig. 2.

Statin exacerbation of AICAC persists in absence of functional endothelium and is removed upon CLR enrichment *in vitro*. Original trace showing robust AICAC in de-endothelialized MCA from high CLR diet + atorvastatin group (B) when compared to lack of AICAC in de-endothelialized MCAs from high CLR diet + placebo group (A) and in high CLR diet + atorvastatin arteries subjected to CLR enrichment *in vitro* (C). D. Averaged data showing changes in artery diameter in response to 50 mM ethanol in de-endothelialized arteries from rats subjected to high CLR diet + placebo (n = 4), high CLR diet + atorvastatin (n = 4), and high CLR diet + atorvastatin subjected to CLR enrichment *in vitro* (n = 3). *Statistically significant difference, P < 0.05.

Fig. 3.

Fluorescence imaging confirms differences in MCA smooth muscle CLR levels among experimental groups. A. Original snapshots showing vascular smooth muscle CLR levels via fluorescence staining of arteries with CLR-sensitive dye filipin. Arteries were collected from rats on high CLR diet + placebo, and high CLR diet + atorvastatin. Several arteries from rats on high CLR diet + atorvastatin were subjected to *in vitro* CLR enrichment. Artery staining from all experimental groups was performed in parallel to minimize the experimental error and justify the direct comparison of the fluorescence signal intensity. Snapshots on the right show smooth muscle layer in visible light. These images were superposed with fluorescence ones to define the individual myocyte plasma membranes for filipin intensity quantification.

In the right top panel, arrows point at the example of individual myocyte's silhouette (light grey) within vasculature layer. B. Averaged data showing filipin fluorescence signal from high CLR diet + placebo (n = 7), high CLR diet + atorvastatin (n = 10), and high CLR diet + atorvastatin subjected to CLR enrichment *in vitro* (n = 8). ("n" refers to number of artery segments). Here and in C, *statistically significant difference; $P < 0.05$. C. Original snapshots showing increase in filipin-associated fluorescence upon CLR *in vitro* enrichment of rat cerebral arteries that were harvested from rats fed by normal rodent chaw. D. Averaged data showing filipin fluorescence signal from arteries of rats on normal rodent chaw with naïve (n = 31) and enriched CLR (n = 32).

Fig. 4.

Ethanol-induced BK channel inhibition in cerebral artery myocytes is increased by atorvastatin. **A.** Original records of vascular smooth muscle BK channel activity during perfusion with the bath versus 50 mM ethanol-containing solution in high CLR diet (left) versus high CLR diet + atorvastatin group (right). Arrows point at the baseline (all channels closed); dashed lines highlight individual channel opening levels. $V_m = 40$ mV; $[Ca^{2+}]_{free} = 30$ μ M. **B.** Changes in BK channel NPo as a function of time from the start of perfusion with either bath or 50 mM ethanol-containing solution in high CLR diet ($n = 5$) versus high CLR diet + atorvastatin ($n = 3$) group. **C.** Averaged ethanol-induced decrease in BK channel NPo during maximal ethanol-induced effect (9 and 10 min) compared to NPo during perfusion with the bath solution at matching time-points (9 and 10 min) in high CLR diet ($n = 5$)

versus high CLR diet + atorvastatin (n = 3) group. Dashed line highlights the lack of effect.

*Statistically significant difference; $P < 0.05$.

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Fig. 5.

Atorvastatin diminishes the amount BK beta1 subunit in the vascular smooth muscle. A. Original snapshots showing fluorescence signal associated with BK alpha and BK beta1 subunit protein in the vascular smooth muscle of arteries from animals on high CLR diet + placebo versus high CLR diet supplemented by atorvastatin. Myocyte nuclei are shown in blue (DAPI); BK alpha protein is in green; BK beta1 protein is in red. Right panels show the surface of the vascular smooth muscle layer in visible light; the image was used for the superposition with fluorescence data to define the individual myocyte plasma membrane

areas. Averaged data showing BK alpha- (B) and BK beta1-associated fluorescence signals in the plasma membrane areas of the myocytes (C) in high CLR diet + placebo (n = 4) versus high CLR diet + atorvastatin (n = 8) group. *Statistically significant difference; $P < 0.05$. D. Averaged ratio of beta1 over alpha subunit-associated signals. *Statistically significant difference; $P < 0.05$. E, F. Western blots showing validation of anti-BK alpha (E) and anti-BK beta 1 (F) subunit antibodies following surface protein biotinylation of rat cerebral arteries. Antibodies against BK alpha and beta1 subunits detect a single protein band of ≈ 130 kDa and an apparent protein band at ≈ 24 kDa, respectively. Arrows point at the bands of the expected molecular weight. Sham procedures were performed following the biotinylation protocol, but biotin was omitted from biotinylation solution.

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Fig. 6.

A. Atorvastatin diminishes basal activity of vascular smooth muscle BK channel. A. Original records of vascular smooth muscle BK channel at varying transmembrane voltages. Arrows point at the baseline (all channels closed); dashed lines highlight individual channel opening levels. $[Ca^{2+}]_{free} = 30 \mu M$. B. NPo/NPo_{max} -V curves show rightward shift along V-axis during atorvastatin supplementation of high CLR diet. C. Averaged V_{halves} (e.g. voltages that are needed to reach half-maximal BK current) for BK channels in cerebral

artery myocytes from rats on high CLR diet + placebo (n = 4) versus high CLR diet + atorvastatin (n = 3). *Statistically significant difference; $P < 0.05$.

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Fig. 7.

Atorvastatin exacerbation of ethanol-induced BK channel inhibition is removed upon *in vitro* enrichment of cerebral artery with CLR. A. Original records of vascular smooth muscle BK channel activity during perfusion with 50 mM ethanol-containing solution in high CLR diet + atorvastatin group following artery *in vitro* enrichment with CLR. Arrows point at the baseline (all channels closed); dashed lines highlight individual channel opening levels. $V_m = -40$ mV; $[Ca^{2+}]_{free} = 30$ μ M. Dashed lines highlight individual channel opening levels. B. Changes in BK channel NPo as a function of time from the start of perfusion with 50 mM ethanol-containing solution in high CLR diet + placebo group ($n = 5$) and in high CLR diet + atorvastatin arteries following *in vitro* enrichment with CLR ($n = 3$).