The inhibitory effect of simvastatin and aspirin on histamine responsiveness in human vascular endothelial cells

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Absi M, Bruce JI, Ward DT. The inhibitory effect of simvastatin and aspirin on histamine responsiveness in human vascular endothelial cells. Am J Physiol Cell Physiol 306: C679–C686, 2014. First published January 29, 2014; doi:10.1152/ajpcell.00304.2013.—Statins and aspirin deliver well-established cardiovascular benefits resulting in their increased use as combined polypills to decrease risk of stroke and heart disease. However, the direct endothelial effect of combined statin/aspirin cotreatment remains unclear. Histamine is an inflammatory mediator that increases vascular permeability, and so we examined the effect of treating human umbilical vein endothelial cells (HUVECs) for 24 h with 1 μM simvastatin and 100 μM aspirin on histamine responsiveness. Subsequent histamine (1 μM) challenge increased intracellular calcium (Ca2+) concentration, an effect that was significantly inhibited by combined simvastatin/aspirin pretreatment but not when the compounds were given separately, even at 10-fold higher concentrations. In contrast, the Ca2+ mobilization response to ATP challenge (10 μM) was not inhibited by combined simvastatin/aspirin pretreatment. The H1 receptor antagonist pyrilamine significantly inhibited both histamine-induced Ca2+ mobilization and extracellular signal-regulated kinase (ERK) activation, whereas ranitidine (H2 receptor antagonist) was without effect. However, combined simvastatin/aspirin pretreatment failed to decrease H1 receptor protein expression ruling out receptor downregulation as the mechanism of action. Histamine-induced ERK activation was also inhibited by atorvastatin pretreatment, while simvastatin further inhibited histamine-induced vascular endothelial cadherin phosphorylation as well as altered HUVEC morphology and inhibited actin polymerization. Therefore, in addition to the known therapeutic benefits of statins and aspirin, here we provide initial cellular evidence that combined statin/aspirin treatment inhibits histamine responsiveness in HUVECs.

endothelium; histamine; intracellular calcium; cadherin; HUVEC

CARDIOVASCULAR DISEASE REMAINS the major worldwide cause of morbidity and mortality, and statins are considered drugs of choice in both primary and secondary prevention of coronary artery disease and stroke (19, 34). In addition to lowering plasma cholesterol levels, there is increasing evidence that statins also deliver other cardiovascular benefits (32, 35, 37) including the following: 1) inhibiting vascular smooth muscle cell proliferation, 2) improving endothelial function by increasing levels of nitric oxide (NO), 3) decreasing the synthesis and function of endothelin-1 and angiotensin II, and 4) decreasing oxidative stress and LDL oxidation (22, 24, 27, 29, 50).

The endothelium represents a barrier that controls the passage of cells, macromolecules, and fluid between the blood and the adjacent tissue interstitium (7, 20, 39). Disruption of the endothelial barrier can lead to microvascular leakage, a common and important consequence of inflammation seen in isch-
factors including von Willebrand factor (9). Human umbilical vein endothelial cells (HUVECs) were used as they represent a well-established model to investigate endothelial cell integrity and signaling (10, 111, 43). We believe that such information may assist the development of novel statin/aspirin polypill pharmacotherapy for the prevention and treatment of cardiovascular disease.

EXPERIMENTAL PROCEDURES

Cell culture. HUVECs (Millipore) were grown in EndoGRO-LS medium in the absence of antibiotics or antimycotics at 37°C in a humidified 5% CO₂ incubator. HUVECs, used between passages 3 and 8, were grown to ~80% confluence on glass coverslips and then treated with or without simvastatin or aspirin for 24 h (unless otherwise stated). All chemicals were from Sigma unless otherwise stated.

Ca²⁺-i assay. HUVECs cultured on glass coverslips were loaded with fura-2/AM (5 μM for 30 min to 1 h) at room temperature in the dark in Ca²⁺-i assay buffer (comprising in mM: 125 NaCl, 4 KCl, 1.2 CaCl₂, 0.5 MgCl₂, 5.5 glucose, and 20 HEPES pH 7.4) supplemented with 0.1% bovine serum albumin. At the beginning of the experiment, cells were transferred into a perfusion chamber and washed with Ca²⁺-i assay buffer (containing 0.5 mM CaCl₂) before treatment. Cells were observed through a ×40 oil-immersion objective. Dual-excitation wavelength microfluorometry was performed using a Cairn monochromator-based illumination system and a Nikon Diaphot inverted microscope. To estimate thapsigargin-induced release of Ca²⁺-i, and Ca²⁺ influx rate, cells incubated in extracellular Ca²⁺-i (Ca²⁺-i)-free buffer (+2 mM EGTA) were exposed to 2 μM thapsigargin, followed by exposure to 20 mM Ca²⁺-i in the presence of thapsigargin.

Extracellular signal-regulated kinase phosphorolyis assay. Cells were grown to ~80% confluent in 35-mm culture dishes and rinsed in PBS for 5 min before equilibration for 25 min in experimental buffer at 37°C. Following culture in the absence or presence of aspirin or simvastatin, cells were then incubated for up to 10 min in buffer supplemented with various experimental treatments and then lysed on ice in the following RIPA buffer: 12 mM HEPES (pH 7.6), 300 mM mannitol, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium dodecyl sulfate supplemented with 1.25 mM peptatin, 4 μM leupeptin, 4.8 μM PMSF, 1 mM EDTA, 1 mM EGTA, 100 μM vanadate, 1 mM NaF, and 250 μM sodium pyrophosphate. Lysate was then mixed with 5× Laemmli buffer and boiled for 5 min before immunoblotting using phospho-specific anti-extracellular signal-regulated kinase (ERK) monoclonal antibody. Lysates were also probed with antibodies against signaling molecules as illustrated below. Total ERK levels were demonstrated using anti-ERK2 antibody (Santa Cruz Biotechnology).

Immunoblotting. Immunoblotting was performed as previously described (44). Briefly, cell lysates were prepared using RIPA buffer and boiled for 5 min in Laemmli buffer. Protein samples were then resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose. Nonspecific binding sites were blocked using 2% (wt/vol) bovine serum albumin or 5% nonfat milk solutions (according to antibody protocol), followed by a 1-h incubation in either anti-histamine H1 receptor (H1R; 1:1,000; Santa Cruz Biotechnology), anti-perkunclonal (1:3,000 dilution; Chemicon), anti-β-actin polyclonal (1:4,000 dilution; Cell Signaling), anti-VE-CadY731 rabbit polyclonal (1:3,000; Invitrogen), or anti-phospho-myosin light chain-2 phosphorylation (MLC2T18S19; 1:500; Invitrogen) antibodies. After being washed, blots were exposed to horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5,000) for 1 h and then developed using ECL reagents (Geneflow). All washes and incubations used Tween/TBS solution [15 mM Tris (pH 8), 150 mM NaCl, and 0.1% (vol/vol) Tween 20]. Immunoreactivity was detected using light-sensitive film and quantified by densitometry.

RESULTS

Combined simvastatin/aspirin treatment inhibits histamine-induced Ca²⁺-i mobilization in human umbilical vein endothelial cells. Acute stimulation of fura-2-loaded HUVEC cells with 1 μM histamine elicited a rapid increase in Ca²⁺-i concentration, as assessed by fura-2 that was generally sustained for several minutes (Fig. 1A). Separate pretreatment of the cells for 24 h with either 1 μM simvastatin or 100 μM aspirin failed to inhibit subsequent histamine responsiveness. However, when the cells were cotreated for 24 h with simvastatin (1 μM) and aspirin (100 μM), the resulting histamine-induced increases in Ca²⁺-i concentration were significantly inhibited (0.9 ± 0.2 vs. 3.6 ± 0.6 control, area under the curve ratio units/min; P < 0.01; Fig. 1Bii). The initial peak response, which is due primarily to inositol 1,4,5-trisphosphate-mediated Ca²⁺-i release as opposed to subsequent store-operated calcium (Ca²⁺-i) entry (SOCE), was also significantly inhibited by combined simvastatin/aspirin cotreatment (0.5 ± 0.1 vs. 1.1 ± 0.2 control, fura-2 ratio; P < 0.05; Fig. 1Bii) but not affected at all by treatment with the same compounds when given separately. In addition, the simvastatin/aspirin cotreatment significantly increased the latency of the response (217 vs. 17 s for control, median; P < 0.001; Fig. 1Biii) as well as increasing the proportion of cells that failed to respond at all to the histamine (31.7 ± 10.5 vs. 0.3 ± 0.2% control; P < 0.05; Fig. 1Biv). Collectively, these data suggest that the initiation of the histamine-induced Ca²⁺-i response is inhibited by combined pre-treatment with simvastatin and aspirin.

To determine whether the inhibitory effect of the combined simvastatin/aspirin treatment on histamine responsiveness could be mimicked by either compound used individually but at higher concentrations, the experiments were repeated using 10 μM simvastatin and 1 μM aspirin. However, neither pretreatment significantly inhibited the histamine-induced increase in Ca²⁺-i concentration (fura-2 ratio area under the curve) when given independently (Fig. 2A). Next, we examined whether the inhibitory effect of combined simvastatin/aspirin pretreatment occurred with other membrane receptor activators G protein-coupled receptors (GPCRs), such as ATP, which triggers P2Y receptor-mediated release of Ca²⁺-i from intracellular stores and P2X receptor-mediated Ca²⁺-i influx (45). However, 100 nM to 10 μM ATP-induced increases in Ca²⁺-i concentration were unaffected by combined simvastatin (1 μM)/aspirin (100 μM) pretreatment (Fig. 2B), suggesting that the effect of simvastatin/aspirin pretreatment was relatively specific for histamine.

Identification of the receptor subtype mediating histamine-induced Ca²⁺-i mobilization and the possible role of Ca²⁺-i influx in the statin/aspirin effect. Pyrilamine (a selective blocker of histamine H1R; 1 μM) substantially inhibited (~84 ± 10%; P < 0.01) the 1 μM histamine-induced increase...
in Ca^{2+} concentration in HUVECs (Fig. 3A). In contrast, ranitidine (a selective blocker of histamine H2R; 1 μM) was without significant effect (−17 ± 9%; NS) on the histamine response (Fig. 3B). In both sets of experiments, combined simvastatin/aspirin pretreatment again significantly inhibited histamine responsiveness.

The requirement for extracellular calcium (Ca^{2+},o) in the histamine response was confirmed in Fig. 4A, with Ca^{2+},o-free buffer substantially inhibiting the histamine response. When instead the cells were treated in the presence of 1.2 mM Ca^{2+},o, histamine responses similar to those seen in Fig. 1 were observed and 24 h combined pretreatment with simvastatin (1 μM) and aspirin (100 μM) inhibited these responses as before (Fig. 4A).

SOCE in response to Ca^{2+},i store depletion has been reported previously in HUVECs (1, 31). To determine the effect of simvastatin and aspirin pretreatment on Ca^{2+},i stores, HUVECs were pretreated as before and the thapsigargin-mobilizable
stored Ca\(^{2+}\) pool was quantified. The total amount of stored Ca\(^{2+}\) in HUVECs estimated using thapsigargin treatment (in the absence of Ca\(^{2+}\)o) was similar in control and treated cells (Fig. 4Bi). In addition, following replacement of the thapsigargin-containing buffer with 20 mM Ca\(^{2+}\)o-containing buffer, the initial rate of Ca\(^{2+}\) entry was not significantly different between control cells and those pretreated with aspirin and simvastatin (Fig. 4Biv). These data suggest that neither ER Ca\(^{2+}\) stores nor SOCE was responsible for the diminished histamine-induced responsiveness with simvastatin/aspirin co-treatment.

Effect of simvastatin/aspirin pretreatment on H1R expression and ERK activation. Having identified H1R as the principle mediator of histamine responsiveness in the HUVECs, the protein abundance of H1R was determined in HUVECs pretreated with and without simvastatin and aspirin to determine whether the impaired histamine responsiveness is due to H1R downregulation. However, there was no significant change in H1R abundance observed followed either separate or combined simvastatin/aspirin pretreatment (Fig. 5A).

Furthermore, to determine whether other signaling readouts of histamine responsiveness were affected by simvastatin/aspirin pretreatment, cells were treated as before but then lysed and their phospho-ERK content assayed as a measure of ERK activation. Acute histamine exposure (5 min) elicited an increase (+34.4 ± 111%; \(P < 0.01\); Fig. 5B) in ERK phosphorylation, and this effect was substantially reduced in cells preexposed to simvastatin/aspirin (−92 ± 26%; \(P < 0.01\)).
However, unlike for Ca\textsuperscript{2+}, mobilization, simvastatin pretreatment alone was also sufficient to significantly inhibit histamine-induced ERK phosphorylation (−62 ± 38%; \( P < 0.05 \)). This inhibitory effect was not statin compound specific as atorvastatin elicited a similar reduction in histamine-induced ERK activation as simvastatin (\( P < 0.05 \); Fig. 5C). Furthermore, histamine-induced ERK activation was shown to be mediated via H1R as the response was substantially attenuated by pyrilamine (−90.2 ± 4.3%; \( P < 0.01 \); Fig. 5D) but was completely unaffected by ranitidine (−0.9 ± 21.1%).

Effect of simvastatin/aspirin pretreatment on cytoskeletal signaling and morphology. Given the importance of endothelial permeability in the development of atherosclerosis, we next examined the relative effects of simvastatin and aspirin on other histamine-induced cytoskeletal signals in the HUVECs.

Next, 24-h histamine pretreatment significantly increased VE-cadherin\textsuperscript{Y731} phosphorylation, an effect that was prevented by cotreatment with simvastatin either with or without aspirin (Fig. 6A). In contrast, neither simvastatin nor aspirin altered baseline levels of VE-cadherin phosphorylation in the absence of histamine challenge. Finally, acute histamine treatment did not affect ML\textsubscript{C2}\textsuperscript{T18/S19} in HUVECs in either control cells or in those pretreated with either aspirin or simvastatin for 24 h (Fig. 6B). However, a combined aspirin/simvastatin pretreatment for 24 h did increase ML\textsubscript{C2} phosphorylation in response to histamine challenge (1 \( \mu \text{M}, \text{5 min})

We also observed that simvastatin pretreatment in either the presence or absence of aspirin also affected HUVEC cell morphology, with the cells appearing smaller/less spread on the coverslips (Fig. 6C). Therefore, we investigated the effects of simvastatin (1 \( \mu \text{M} \)) and aspirin (100 \( \mu \text{M} \)) pretreatment, either alone or in combination, on the HUVEC F-actin cytoskeletal network using fluorophore-labeled phalloidin. While control endothelial cells exhibited long, cell-spanning stress fibres, these were significantly disrupted by both aspirin and simvastatin (24 h) with the greatest effect occurring in response to combined simvastatin/aspirin pretreatment indicating significant cytoskeletal reorganization. Histamine itself failed to elicit gross changes in the actin cytoskeleton.

**DISCUSSION**

Acute histamine challenge induced a rapid increase in Ca\textsuperscript{2+}, concentration in fura-2-loaded HUVECs. These histamine responses were substantially inhibited (−84%) by pyrilamine indicating the involvement of type-1 histamine receptors in the response, confirming previous studies showing H1R-induced Ca\textsuperscript{2+}, mobilization in HUVECs (9, 18). The histamine concentration used here, 1 \( \mu \text{M} \), is equivalent to the circulating histamine levels observed in a study of myocardial ischemia (2 days after an acute myocardial event) compared with \( \sim400 \text{ nM} \) in healthy controls (49). Indeed, the local histamine levels at sites of inflammation or atherosclerotic plaques may be even higher, and thus the concentration used here is of pathophysiological relevance. Interestingly, there is increasing evidence of a role for histamine in vascular disease. For example,
Histamine inducible intimal hyperplasia (26) 2) increases the expression and activity of tissue factor TF (a key initiator of blood coagulation (14); and 3) increases vascular permeability for LDL permitting atherosclerotic lesion development (38). Furthermore, histamine levels are significantly increased in patients suffering from different types of ischemic heart diseases (49). Finally, selective H1R activation in human endothelial cells elicits not only Ca\textsuperscript{2+} mobilization (from acidic organelles in addition to the endoplasmic reticulum) but also stimulates the exocytosis of von Willebrand factor-containing Weibel-Palade bodies, which may then elicit a range of other vascular effects (9). Therefore, the present study focused on investigating whether aspirin and simvastatin, two drugs widely prescribed in the treatment and prevention of cardiovascular disease, may also attenuate histamine signaling in HUVECs, as this might in turn contribute to vascular protection in the endothelium.

The suppression of histamine-induced Ca\textsuperscript{2+} mobilization in the HUVECs resulting from combined simvastatin (1 \textmu M)/aspirin (100 \textmu M) pretreatment was not achieved when the pretreatments were applied separately and not even when 10-fold higher aspirin or simvastatin concentrations were employed. Therefore, the inhibitory effect on Ca\textsuperscript{2+}i that we observed appears specific to the specific cotreatment, at least at the concentrations tested. This is consistent with a previous study in which acute cotreatment with either cerivastatin or fluvastatin alone also failed to inhibit the increase in Ca\textsuperscript{2+}i concentration in histamine-stimulated HUVECs (16). However, the current study is, to our knowledge, the first study to report that combined pretreatment with simvastatin plus aspirin significantly attenuates subsequent histamine-induced Ca\textsuperscript{2+}i mobilization in HUVECs. If this effect could be replicated in animal models, then it might indicate an additional, direct endothelial benefit for combined simvastatin plus aspirin treatment in humans.

The combined simvastatin/aspirin-induced decrease in histamine-mediated response was not a general effect on GPCR signaling since the Ca\textsuperscript{2+} mobilization induced by ATP (45) was unaffected by the combined simvastatin/aspirin pretreatment. This also suggests that any morphological changes in the cells induced by simvastatin/aspirin pretreatment cannot solely explain the apparent suppression of Ca\textsuperscript{2+}i mobilization, as otherwise the ATP responses should have been similarly affected. It was then confirmed that the histamine response is largely dependent on the presence of Ca\textsuperscript{2+}o and that simvastatin/aspirin pretreatment does not significantly alter either the total amount of stored Ca\textsuperscript{2+} in HUVECs, estimated using ATP-evoked Ca\textsuperscript{2+}i release and thapsigargin treatment, or SOCE. This suggests that neither store content nor SOCE was responsible for the attenuated Ca\textsuperscript{2+} responses. Since the peak Ca\textsuperscript{2+} response of histamine was markedly reduced and latency was markedly increased, this suggests that the inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+}i release mediated specifically through H1R was the major target of combined simvastatin/aspirin treatment.

With the use of H1-selective (pyrilamine) and H2-selective (ranitidine) antagonists, it was found that the histamine-induced Ca\textsuperscript{2+}i mobilization (Fig. 3) and ERK activation (Fig. 5D) occurs largely via H1R, with no significant ranitidine effect detected. In the absence of ranitidine-inhibitable Ca\textsuperscript{2+}i mobilization or ERK activation, we cannot therefore say whether the statin/aspirin cotreatments may have also affected
H2R signaling. However, having identified the contribution of H1R to histamine responsiveness in the HUVECs, combined simvastatin/aspirin failed to alter H2R protein abundance ruling out H1R downregulation as the mechanism of action of the simvastatin/aspirin combined treatment. To date therefore we have been unable to identify the specific mechanism of action by which simvastatin/aspirin suppresses Ca^{2+}; concentration in HUVECs. That said, we can at least rule out 1) a general suppression of GPCR signaling, 2) gross changes in Ca^{2+}; store capacity/refilling, or 3) H1R downregulation.

Although HUVECs can synthesize and release PGI_{2} in response to histamine (2), here we saw no inhibitory effect of aspirin alone on Ca^{2+}; mobilization, even when used at 1 mM, suggesting that COX inhibition alone is insufficient to explain the suppressive effect of simvastatin/aspirin. It should be noted that the concentration of aspirin used was relatively moderate with low millimolar plasma aspirin concentrations used to treat chronic inflammatory diseases (36). Similarly, the concentration of simvastatin employed throughout (1 \mu M) is also relatively modest with regards in vitro cell analyses (33, 41, 42, 46). However, in two recent human studies, the peak plasma concentration of simvastatin acid after a 20-mg simvastatin dose was reportedly ~11 nM (6), while the combined peak plasma concentration of simvastatin lactone and simvastatin acid after a 40-mg simvastatin dose was ~36 nM with concentrations up to 70 nM observed (21). Therefore, the concentration employed in the current in vitro study is just over an order of magnitude higher than the simvastatin blood concentrations reported in the human studies; however, here we are exposing cells for 24 h, whereas, clinically, statin use may continue for many years.

Histamine exposure in the HUVECs also elicited H1R-mediated ERK phosphorylation/activation, which was significantly inhibited by combined simvastatin/aspirin treatment, although in this case simvastatin alone significantly inhibited the histamine response albeit to a slightly lesser extent. Similarly, VE-cadherin\textsuperscript{\gamma731} phosphorylation was also reduced by simvastatin pretreatment in either the absence or presence of aspirin. Interestingly, increased VE-cadherin phosphorylation is associated with increased dissociation from \beta-catenin, resulting in destabilization of the VE-cadherin/\beta-catenin/cytoskeleton and thus impaired cell contact with increased vascular permeability (7). In this regard, Haidari et al. (12) recently reported that exposure of HUVECs to proinflammatory stimuli such as monocyte adhesion or IL-1\beta treatment results in elevated VE-cadherin\textsuperscript{\gamma731} phosphorylation, an effect partly mediated via ERK. They further showed that by inhibiting VE-cadherin\textsuperscript{\gamma731} phosphorylation, atorvastatin protected the adherens junction and the integrity of endothelial cells, with possible therapeutic potential in vascular diseases that involve adherens junction disruption, such as atherosclerosis (15). Thus, together, these studies could suggest that statins might improve the integrity of endothelial cells when disrupted by inflammatory stimuli.

Regarding the HUVEC cytoskeleton, simvastatin pretreatment had a marked effect on cell morphology with significant disruption to the actin filaments. Another morphological feature of simvastatin pretreatment in the HUVECs was endothelial cell process retraction resulting in cells with a more rounded appearance. Endothelial cell retraction involves the interaction between actin and myosin under the control of MLC phosphorylation (7, 20, 39), and interestingly combined simvastatin/aspirin pretreatment did indeed significantly increase phosphorylation of MLC\textsuperscript{\gamma18/19}. Our data showed a significant increase in pMLC\textsuperscript{\gamma} in simvastatin/aspirin-cotreated cells, which may explain the rounding of HUVECs, but whether this has a significant effect on endothelial permeability to LDL will require further investigation.

In an animal model of inflammation, a synergistic protective role of combined treatment with simvastatin and aspirin has been shown previously (13). This was based on the finding that combined therapy with simvastatin and aspirin lowered serum concentrations of the proinflammatory cytokines TNF-\alpha and IL-6 while raising serum concentrations of the anti-inflammatory cytokine IL-4. Furthermore, a significant increase in serum NO concentration was reported followed combined therapy suggesting an improved antioxidant activity as well. Whether combined statin/aspirin treatment has sustained functional effects on the endothelium in vivo thus representing an additional, potential vasculoprotective mechanism remains to be determined. However, the current data do at least provide cellular signaling evidence, suggesting that statins interfere with histamine responsiveness in HUVECs and that when cotreated with aspirin, histamine-induced Ca^{2+}; mobilization is significantly inhibited.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.A., J.I.B., and D.T.W. conception and design of research; M.A. performed experiments; M.A. analyzed data; M.A. and D.T.W. interpreted results of experiments; M.A. drafted manuscript; M.A., J.I.B., and D.T.W. approved final version of manuscript; J.I.B. and D.T.W. edited and revised manuscript; D.T.W. prepared figures.

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