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Effects of statin treatment and withdrawal on angiotensin II-induced phosphorylation of p38 MAPK and ERK1/2 in cultured vascular smooth muscle cells ☆

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Abstract

Abrupt discontinuation of 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase inhibitors (statins) is associated with increased cardiovascular risk. To investigate the molecular mechanisms determining the increased cardiovascular risk after statin withdrawal, we studied the effects of statin treatment and withdrawal on angiotensin II (AII) actions in rat aortic vascular smooth muscle cells (VSMC) in culture. In VSMC, AII stimulated the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), and of p38 mitogen-activated protein kinase (p38 MAPK), with an EC50% of 0.86 and 3 nM, respectively. Maximal stimulation was observed after 5–10 min of exposure to AII. Pretreatment with 1–3 μM simvastatin for 24 h inhibited AII-mediated stimulation of ERK1/2 and p38 MAPK phosphorylation; without affecting the levels on non-phosphorylated MAPK. Washout of simvastatin produced a rebound increase above control levels of AII-mediated phosphorylation of ERK1/2 and p38 MAPK. As previously reported for other agonists, the rebound increase of AII effects was observed from 1 to 3 h after statin withdrawal, and was lost at later times. The basal levels of phosphorylation and the amount of non-phosphorylated kinases were unaffected by statin withdrawal. Similar effects were observed with lovastatin. Our results suggest that statins modulate AII effects in VSMC, and that transient increases in AII effects mediated via the MAPK pathway may play a role in the vascular dysfunction associated with statin withdrawal.

Keywords: Angiotensin II; Statins; Statin withdrawal; Mitogen-activated protein kinases; Vascular smooth muscle cells

The 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA)-reductase inhibitors (statins) are the most commonly prescribed agents for the treatment of hypercholesterolemia, due to their efficacy in lowering LDL-cholesterol and ability to reduce clinical outcome in both primary and secondary prevention of coronary artery disease [1–6]. In addition to their serum lipid-lowering action, statins exert important direct effects on vascular cells (pleotropic actions) [7–11]. Acute discontinuation of statin treatment has been associated with an increased rate for adverse cardiovascular events in patients with acute coronary syndromes [12–16]. The mechanisms by which acute statin withdrawal is associated with greater cardiovascular events are under current investigation [16]. Acute removal of statins was associated with impaired nitric oxide production in endothelial cells, and with increased in NADPH oxidase activity, thrombin-induced phosphorylation of mitogen-activated protein kinases, and pro-coagulant activity in vascular smooth muscle cells (VSMC) in culture [17–21].

AII is a strong activator of the MAPK signaling pathway [22,23]. Enhanced activation of vascular MAPK has been demonstrated in hypertension, atherosclerosis, and diabetes, where AII plays a major role [24]. MAPK-dependent signaling pathways have been associated with cellular growth and apoptosis, cellular differentiation and transformation, and vascular contraction [25–27]. Because of the

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lack of information on the effects of statin withdrawal on AII actions, we investigated whether the stimulatory effects of AII on the MAPK pathways were affected following statin withdrawal. Specifically, we determined if the acute discontinuation of statin treatment was associated with increases in basal and/or in AII-mediated phosphorylation of ERK1/2 and p38 MAPK pathways in rat VSMC in culture.

Materials and methods

Materials. Statins were supplied by Calbiochem (La Jolla, CA) and AII from Bachem California Inc. (Torrance, CA). Rabbit polyclonal anti-phospho-p38 MAP kinase antibody (anti-phosho-p38), rabbit polyclonal anti-phospho-p44/42 MAP kinase (anti-phospho-ERK1/2), rabbit polyclonal anti-p44/42 MAP kinase antibody, rabbit polyclonal anti-p38 MAP kinase antibody, and anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling (Beverly, MA).

Cell culture. Adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 250–300 g were employed. VSMCs were isolated from the rats' aorta by the explant technique [28]. All experiments were performed from passages 3 to 15. The cells were seeded into 100×20 mm dishes and cultured in DMEM containing 20% fetal bovine serum (FBS), 5% antibiotic antimycotic solution (100× with 10,000 U penicillin, 10 mg streptomycin, and 25 μg amphotericin B/mL) at 37 °C in humidified atmosphere of 5% CO_2/95% air. For subsequent experiments, cells were seeded into dishes in DMEM containing 10% FBS and 5% antibiotic antimycotic solution. Once the cells reached confluency, the medium was exchanged for serum-free DMEM and the cells used for experiments after 48 h. The cultured cells were treated with different concentrations of simvastatin or lovastatin for 24 h. Where indicated, the cells were exposed to AII for different times and concentrations (i.e., 1 nM for 10 min). For withdrawal experiments, the cells were washed three times with serum-free DMEM with 0.1% of BSA and harvested at different times (immediately, at 1, 2, 3, 4, 6, and at 24 h after washing).

Western blot analysis. The cells were lysed with ice-cold lysis buffer at pH 7.4, containing 50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, a mixture of protease inhibitors, and 1 M sodium orthovanadate. Solubilized proteins were centrifuged at 10,000 rpm for 10 min at 4 °C, and supernatants were stored at −80 °C. Proteins (20 μg) were separated on 10% SDS–polyacrylamide gel electrophoresis (PAGE), and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μm; Amersham Biosciences). The membranes were first shaken for 2 h at room temperature in TBS, containing 0.4% Tween 20 and 5% non-fat dry milk (blocking solution), then incubated with anti-phospho-specific ERK1/2 antibodies (1:5000 dilution in blocking solution, overnight at 4 °C), anti-non-phosphorylated ERK1/2 antibodies (1:1000 dilution in blocking solution, overnight at 4 °C), and anti-phospho-specific p38 MAPK (1:1000 dilution in TBS/0.1% Tween/5% BSA, overnight at 4 °C), anti-non-phosphorylated p38 MAPK antibodies (1:1000 dilution in TBS/0.1% Tween/5% BSA, overnight at 4 °C), followed by incubation with an anti-rabbit IgG-HRP-linked (1:1000 dilution in blocking solution, 1 h at room temperature) or with an anti-mouse IgG-peroxidase (1:60,000 in blocking solution, 1 h at room temperature). The

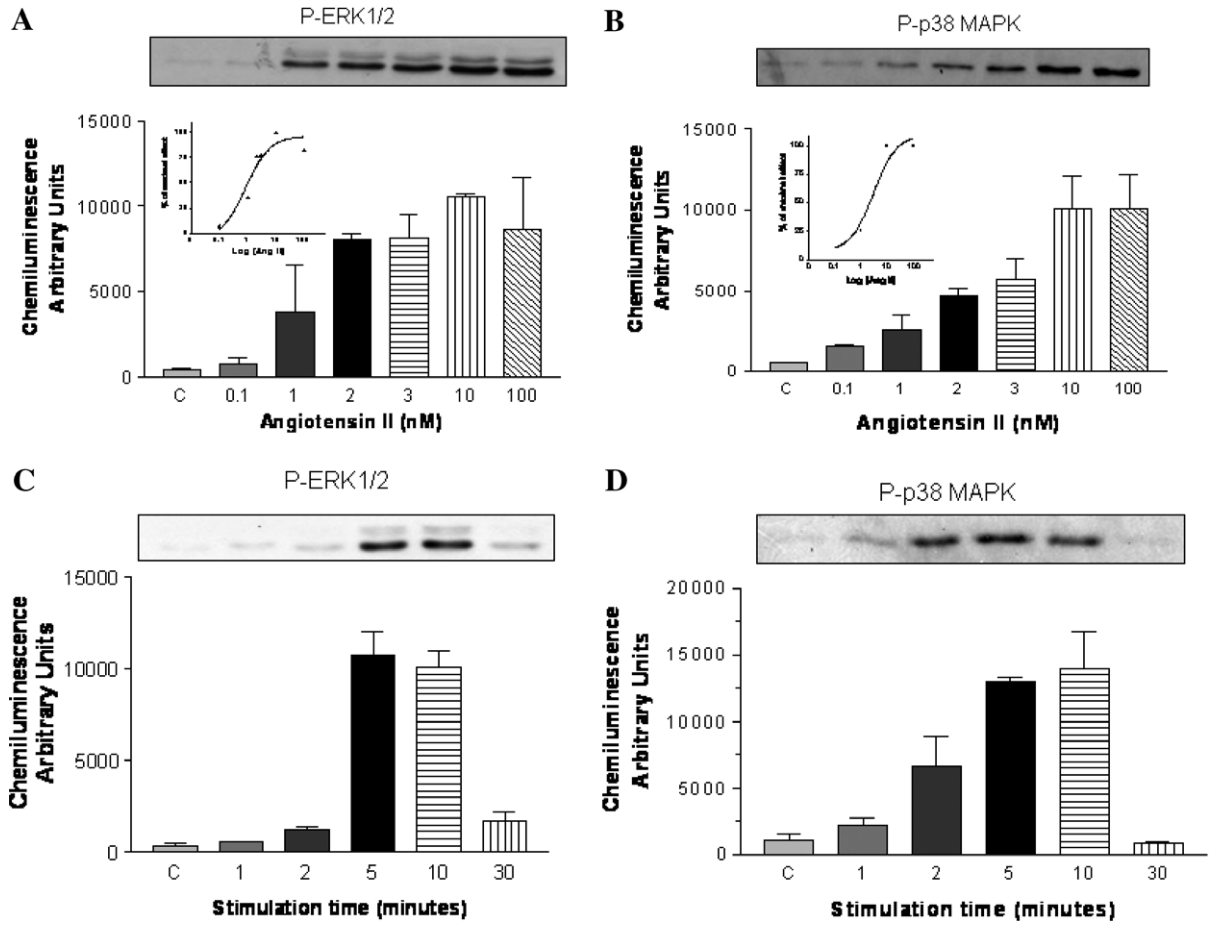

Fig. 1. AII-induced phosphorylation of ERK1/2 (A) and p38 MAPK (B) in rat VSMC in culture. Ordinates: chemiluminescence in arbitrary units. Abscissae: concentrations of AII (A,B) and time of exposure to AII (C,D). Shown are mean values ± SEM of three experiments. Insets: Western blot representative of a typical experiment.

immuno-reactive proteins were visualized by chemiluminescence using a peroxidase enzymatic reaction (ECL Western blot detection system, Amersham).

Statistics. Band densities were reported in chemiluminescence arbitrary units. Results were expressed either as absolute units, as change from baseline and/or as the ratio of phosphorylated to non-phosphorylated proteins. Data are expressed as mean values ± SEM. Two-sample comparison was carried out by unpaired Student's *t* test. ANOVA was employed for multiple sample comparison followed by a post hoc Duncan's test. A probability value of less than 0.05 was considered as statistically significant.

Results

AII-induced ERK1/2 and p38 MAPK phosphorylation

In VSMCs, AII produced a concentration and time-dependent increase in the phosphorylation of both ERK1/2 and p38 MAPK (Fig. 1). Maximal effects were obtained after 5–10 min of exposure to AII (Fig. 1). AII EC50% for ERK1/2 and p38 MAPK were 0.82 ± 0.1 and 3.0 ± 0.2 nM, respectively. A concentration of 1 nM AII close to the EC50% and an exposure time of 10 min were employed for subsequent experiments.

Effect of the statins on AII-induced phosphorylation of ERK1/2 and p38 MAPK

The effects of simvastatin (0.3, 1, and 3 μM) and lovastatin (0.5 and 5 μM) on AII-induced phosphorylation of ERK1/2 and p38 MAPK are shown in Fig. 2. Greater and more reproducible effects were observed with the higher concentrations tested. Both simvastatin and lovastatin inhibited AII-mediated phosphorylation of both ERK1/2 and p38 MAPK ($P < 0.01$). Lovastatin, however, did not significantly reduced phosphorylation of ERK1/2 stimulated by AII. Importantly, neither simvastatin (0.3–3 μM) nor lovastatin (0.5–5 μM) modified the levels of non-phosphorylated kinases in the presence and absence of AII.

Simvastatin also reduced the basal level of phosphorylation of ERK1/2, without significantly affecting that of p38

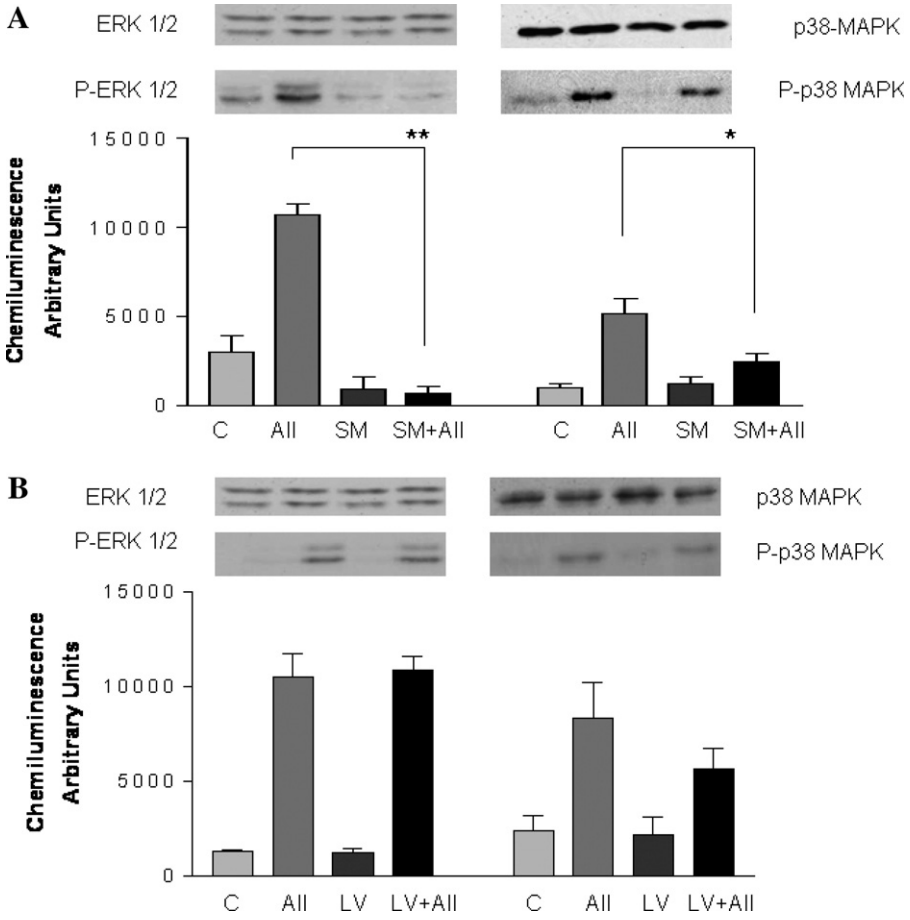

Fig. 2. Effects of simvastatin and lovastatin treatment on AII-induced phosphorylation of ERK1/2 and of p-38 ERK in rat VSMC in culture. Rat VSMC were incubated 3 μM simvastatin (A), 5 μM lovastatin (B), or vehicle (controls) for 24 h. Half of dishes were treated for 10 min with 1 nM AII, and the other half were left untreated to estimate basal levels of MAPK phosphorylation. The basal levels and the AII-stimulated levels of phosphorylated and non-phosphorylated ERK1/2 (left), and p38 MAPK (right) were studied at the end of the 24 h incubation period. Non-phosphorylated MAPK levels were measured to rule out non-specific effects of statins and of AII. C: control; AII: 1 nM AII for 10 min; SM: simvastatin; LV: lovastatin; SM+AII and LV+AII: effects of statins on AII actions. Ordinates: chemiluminescence in arbitrary units. Inset: Western blot representative of a typical experiment. Shown are mean values ± SEM of three experiments. $^{**}P < 0.01$ and $^{*}P < 0.05$.

MAPK. Basal levels of phosphorylation were not affected by lovastatin (Fig. 2).

Effect of the statin withdrawal on AII-induced ERK1/2 and p38 MAPK phosphorylation

The effects of removal of simvastatin and lovastatin from the incubation medium on basal and on AII-mediated stimulation of p38 MAPK and ERK1/2 phosphorylation were investigated. Experiments were carried out at 1, 2, 3, 4, 6, and 24 h after statin removal. Control cells, exposed to the statins' vehicle, were subjected to a similar protocol of washes as the statin-treated cells, and the effects of AII on control and statin-treated cells were compared at each time point. As for previous experiments, levels of non-phosphorylated kinases were assessed at all time points.

Interestingly, 2 and 3 h after removal of simvastatin AII-mediated phosphorylation of ERK1/2 and p38 MAPK was increased by $34 \pm 5\%$ and $49 \pm 7\%$, respectively, above the levels of stimulation induced by AII in control cells ($P < 0.05$) (Fig. 3). This overshoot effect was transient since it was not apparent at later times (Fig. 4). Basal levels of p38 MAPK phosphorylation were not affected by simvastatin withdrawal. The changes observed in phosphorylated ERK1/2 and p38 MAPK were not seen for the non-phosphorylated proteins (Fig. 3).

Similarly to simvastatin, withdrawal of 5 μM lovastatin was associated with a transient $30 \pm 7\%$ and $29 \pm 6\%$ increase in AII-mediated phosphorylation of both ERK1/2 and p38 MAPK, respectively, above that induced by AII in control cells (Fig. 3). Similarly to effects observed with simvastatin, no increases in AII-induced phosphorylation of MAPK were observed at later times after the removal of lovastatin (data not shown). There were no significant effects on basal levels of MAPK phosphorylation, or on the amount of non-phosphorylated MAPK after lovastatin washout (Fig. 3).

Discussion

Acute discontinuation of statin treatment has been associated with an increased rate for adverse cardiovascular events in patients with acute coronary syndromes [12–16]. The mechanisms by which acute statin withdrawal

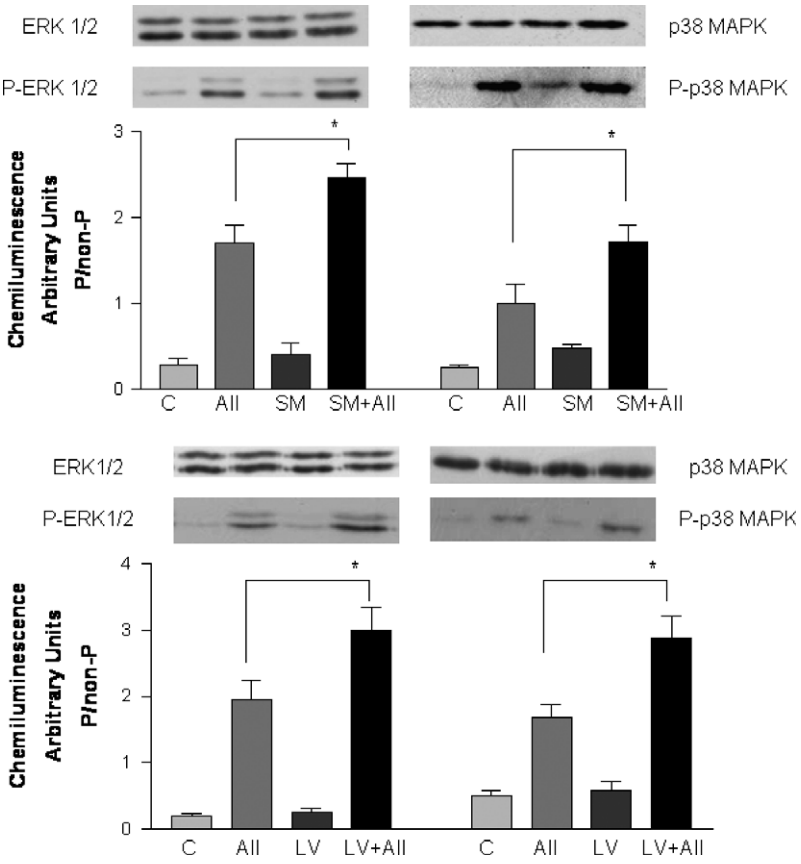

Fig. 3. Effects of simvastatin and lovastatin withdrawal on AII-induced phosphorylation of ERK1/2 and p38 MAPK in rat VSMC in culture. Rat VSMC were incubated with 3 μM simvastatin, 5 μM lovastatin, or vehicle (controls) for 24 h. At the end of 24 h treatment period, cells were washed three times with serum-free, statin-free DMEM containing 0.1% BSA, and harvested at several times thereafter. C: control; AII: 1 nM angiotensin II for 10 min; SM: simvastatin; LV: lovastatin; SM+AII, and LV+AII: effects of statins on AII actions. Ordinates: depicts the ratio of phosphorylated (P) to non-phosphorylated ERK1/2 (left) and p38 MAPK (right). Shown are results obtained at 2 and 3 h after statin withdrawal. Inset: Western blot for non-phosphorylated and phosphorylated MAPK representative of a typical experiment. Shown are mean values ± SEM of four experiments. *Significantly different at $P < 0.05$.

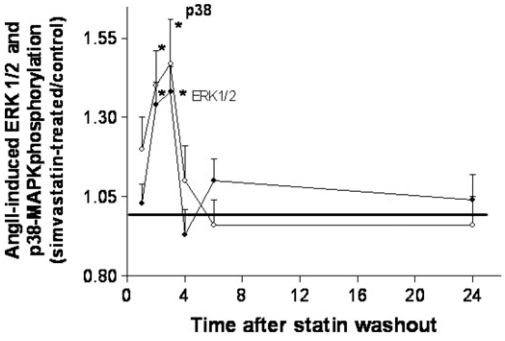

Fig. 4. Time-course of the effects of simvastatin withdrawal on AII-induced phosphorylation of ERK1/2 and p38 MAPK in rat VSMC in culture. Rat VSMC were incubated with 3 μM simvastatin or vehicle (controls) for 24 h. Half of dishes were treated for 10 min with 1 nM AII, and the other half were left untreated to estimate basal levels of phosphorylated and non-phosphorylated MAPK. The basal levels and the AII-stimulated levels of ERK1/2 and of p38 MAPK were studied at: 1, 2, 3, 4, 6, and 24 h following three washes with statin-free medium of cells treated with simvastatin or with vehicle (controls). Ordinates: ratio of chemiluminescence units for AII-induced phosphorylation of ERK1/2 or p38 MAPK in cells that have treated with statins/AII-induced phosphorylation of both kinases in control cells, not treated with statins. Shown are mean values ± SEM of four experiments. *Significantly different at $P < 0.05$.

is associated with greater cardiovascular events are under current investigation. Acute removal of statins has been shown to impair nitric oxide production in endothelial cells, and to increase NADPH oxidase activity, thrombin-induced phosphorylation of MAPK, and pro-coagulant activity in VSMC in culture [17–21]. All these effects were shown to be transient and to occur shortly after statin withdrawal. Although AII exerts numerous actions on vascular smooth muscle, including modulation of vasomotor tone, cell growth, vascular inflammation, production of growth factors, and of vasoconstrictors [20,29–31], prior to this work no studies had investigated a possible role of AII on the vascular dysfunction observed after statin withdrawal.

AII is known to stimulate MAPK signaling pathways, which are involved in vascular cell growth and apoptosis, differentiation and transformation, and vascular contraction [25–27,32,33]. Overstimulation of these pathways may lead to vascular dysfunction. In the present work we demonstrated that acute removal of simvastatin and lovastatin from VSMC enhanced AII-stimulated phosphorylations of both ERK1/2 and of p38 MAPK. Our findings support previous observations where two and three hours after cerivastatin withdrawal, thrombin-induced p38 MAPK phosphorylation rebounded to values that were above those induced by thrombin in control-untreated cells [21]. Interestingly, the time-courses for the increases in AII-induced MAPK phosphorylation observed in this study were similar to that reported for thrombin [21]. It thus appears that MAPK pathways can be transiently overstimulated by AII and thrombin shortly after removal of statin treatment, favoring cell growth, inflammation, prothrombotic, and pro-coagulant activity. In addition to the findings described for VSMC, endothelial cells in culture and aortas from rats and mice treated with statins, respond with inhibition of endothelial nitric oxide synthase expression, of nitric oxide production and availability, and with decreased endothelial-dependent vasodilation following removal or discontinuation of statin treatment [19,20,34]. It thus appears that following acute discontinuation of statin treatment, there is a transient period where vascular deleterious mechanisms are activated and vascular protective mechanisms are inhibited [16]. Although the clinical significance of these findings cannot be ascertain, our results suggest that a transient increase in AII and thrombin effects, combined with impairment of nitric oxide bioactivity, may play a role in the vascular dysfunction and the greater risk for cardiovascular events reported shortly after removal of statin [12–15].

Treatment with simvastatin or lovastatin inhibited the stimulation of MAPK phosphorylation induced by AII in vascular smooth muscle cells (present study). These results confirm previous observations where two different statins, cerivastatin and fluvastatin, were shown to inhibit AII-induced ERK1/2 phosphorylation [35–37]. The weaker inhibitory potency of lovastatin on HMGCoA reductase may account for the lesser inhibitory effect of this statin on AII-stimulated phosphorylation of MAPK. Inhibition of the 3-hydroxy-3-methylglutaryl-CoA reductase by statins with reduced formation of isoprenoid intermediates, may account for these findings [19,20,34]. Inhibition of ERK1/2 phosphorylation by statins is most likely the consequence of reduced Ras prenylation [27,38,39]; whereas, inhibition of AII-induced p38 MAPK phosphorylation by statins may be mediated via reduced prenylation of Rac. Removal of statins may disinhibit the mevalonate pathway leading to enhanced prenylation, membrane translocation and activation of small GTPases, which in turn would reduced NO production, and increase thrombin- and possibly AII-induced MAPK phosphorylation [19,20,34], present study.

Conclusion

Treatment with statins inhibited AII-induced phosphorylation of ERK1/2 and of p38 MAPK. The inhibitory effect of statins was not only rapidly lost upon removal of the statins from the culture medium, but it was followed by a rebound increase of AII-mediated phosphorylation of p38 MAPK and ERK1/2. Our findings support the view that the pleotropic effects of statins are rapidly lost and often reversed upon acute withdrawal of statin treatment [12,13,19,20,34], present study. The reported increase in AII signaling may lead to greater effects of AII, which together with impairment in NO production and increased thrombin actions, could play a role in the vascular dysfunction and increase in cardiovascular events reported after removal of statin. Experiments are in progress to determine it the increase in AII-stimulated phosphorylation of kinases translates in greater functional effects.

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