The central role of adenosine in statin-induced ERK1/2, Akt, and eNOS phosphorylation

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Merla R, Ye Y, Lin Y, Manickavasagam S, Huang M-H, Perez-Polo RJ, Uretsky BF, Birnbaum Y. The central role of adenosine in statin-induced ERK1/2, Akt, and eNOS phosphorylation. Am J Physiol Heart Circ Physiol 293: H1918–H1928, 2007. First published July 6, 2007; doi:10.1152/ajpheart.00416.2007.—Statins activate phosphatidylinositol-3-kinase, which activates ecto-5′-nucleotidase and phosphorylates 3-phosphoinositide-dependent kinase-1 (PDK-1). Phosphorylated (P-)PDK-1 phosphorylates Akt, which phosphorylates endothelial nitric oxide synthase (eNOS). We asked if the blockade of adenosine receptors (A1, A2A, A2B, or A3 receptors) could attenuate the induction of Akt and eNOS by atorvastatin (ATV) and whether ERK1/2 is involved in the ATP regulation of Akt and eNOS. In protocol 1, mice received intraperitoneal ATV, theophylline (TH), and/or vehicle. In protocol 2, mice received intraperitoneal injections of ATV, U0126 (an ERK1/2 inhibitor), TH, and/or vehicle; 8 h after injection, hearts were assessed by immunoblot analysis. In protocol 3, mice received intraperitoneal ATV alone or with 8-sulfophenyltheophylline (SPT); 1, 3, and 6 h after injection, hearts were assessed by immunoblot analysis. In protocol 4, mice received intraperitoneal ATV alone or with SPT, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC), alloxazine, or MRS-1523; 3 h after injection, hearts were assessed by immunoblot analysis. ATV increased P-ERK, P-PDK-1, Ser473 P-Akt, Thr308 P-Akt, and P-eNOS levels. TH blocked ERK1/2-induced increases in P-ERK, Ser473 P-Akt, Thr308 P-Akt, and P-eNOS levels without affecting the induction of P-PDK-1 by ATV. U0126 blocked the ATP induction of Ser473 P-Akt and Thr308 P-Akt while attenuating the induction of P-eNOS. A detectable increase in P-ERK, Ser473 P-Akt and P-eNOS was seen 3 and 6 h after injection but not at 1 h. DPCPX, CSC, and alloxazine partially blocked the ATP induction of P-ERK, Ser473 P-Akt, and P-eNOS. In conclusion, blockade of adenosine A1, A2A, and A2B receptors but not A3 receptors inhibited the induction of Akt and eNOS by statins. Adenosine was required for ERK1/2 activation by statins, which resulted in Akt and eNOS phosphorylation.

extracellular signal-regulated kinase 1/2; endothelial nitric oxide synthase

the 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) protect against ischemia-reperfusion injury and reduce myocardial infarct size in mice (7, 20, 30, 31, 61, 67), rats (3, 9, 12, 15, 41, 55, 56, 60, 62, 63, 68), rabbits (57), dogs (48), and pigs (16, 35). It has been reported that the mechanism of protection involves the activation of phosphatidylinositol 3-kinase (PI3K) (7, 20, 27, 33, 59, 62), leading to parallel activation of both Akt (7, 8, 14, 20, 26, 27, 33, 41, 48, 51, 62, 68) and eNOS (11, 48, 57). Wortmannin, a PI3K inhibitor, blocks both E5N and Akt activation (48). Akt activates endothelial nitric oxide (NO) synthase (eNOS) by phosphorylation at Ser1177 (7, 26, 33, 59), and E5N releases adenosine into the interstitial space (11, 36, 48, 57). Both E5N and E5N are essential for mediating the protective effect of statins, as statins do not limit infarct size in eNOS−/− mice (7, 30, 67) and adenosine receptor blockers abolish the protective effect of statins (37, 48). Adenosine has an established role in mediating ischemic preconditioning (25) and postconditioning (25, 40). We (11) have recently shown that atorvastatin (ATV) upregulates E5N activity in wild-type mice but not eNOS−/− mice; however, ATV limits infarct size only in wild-type mice, suggesting that E5N is either upstream to eNOS or that the two pathways are independent and needed in parallel. Several investigators (23, 38, 52, 53, 58) have suggested that adenosine augments NO release by increasing eNOS expression and/or activity; however, the role of adenosine in mediating statin-induced E5N phosphorylation has not been described. The activation of PI3K leads to the generation of phosphatidylinositol (3,4,5)-trisphosphate (P3IP), which interacts with the pleckstrin homology domain of Akt, mobilizing it to the plasma membrane and leading to Akt phosphorylation at Thr308 and Ser473 (4, 21, 24). Phosphorylation of Akt at both residues is needed for the full activation of Akt. Akt phosphorylation at Thr308 is catalyzed by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (21, 54). PDK-1 itself is activated by phosphorylation at Ser241 by an as yet unknown kinase (17, 24). Initially, it was thought that another kinase phosphorylates Akt at Ser473 (PDK-2) (1); however, it was later shown that PDK-1 phosphorylates Akt at Ser473 after being modified by PKC-related kinase-2 (4). More recently, it has been shown that Ser473 Akt phosphorylation is mediated by the rictor-mammalian target of rapamycin (mTOR) complex (29, 49). Statins have been reported to activate mTOR (47). As mentioned above, phosphorylated (P-)Akt activates eNOS by phosphorylation at Ser1177 (7, 26, 33, 59).

Others (64) have suggested that adenosine activates eNOS via p42/p44 MAPK (ERK1/2) phosphorylation (64). ERK1/2 has been shown to be activated by statins (18) and to be involved in the infarct-size limiting effects of statins (20). It has been suggested that the activation of ERK1/2 at reperfusion limits infarct size, independent of eNOS (27, 28); however, statins administered at reperfusion failed to limit infarct size in eNOS−/− mice (7), suggesting that eNOS is essential for the

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protection from ischemia-reperfusion injury.

We asked whether the three reported signaling pathways (PI3K/Akt/eNOS, ERK1/2, and E5N) are interdependent by blocking four adenosine receptors and measuring the statin-induced augmentation of eNOS upregulation via increases in expression or phosphorylation at Ser1177 and, if so, whether adenosine was necessary for ERK1/2, PDK-1, or Akt expression and/or phosphorylation. In addition, we asked if ERK1/2 is involved in statin-induced Akt and eNOS increases and which adenosine receptor was involved.

METHODS

Animal Care

All animals received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). The protocol was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. Experiments were conducted on male C57Bl/6 mice (17–20 g body wt).

Treatment and Western Immunoblot Analysis

**Protocol 1.** Mice received an intraperitoneal injection of 1) ATV (20 mg/kg); 2) theophylline (TH; 50 mg/kg); 3) ATV + TH; or 4) vehicle alone. There were four mice in each group. Eight hours after injection, mice were anesthetized and euthanized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg) and euthanized. Hearts were rapidly explanted, rinsed in cold PBS (pH 7.4) containing 0.16 mg/ml heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at −70°C.

**Protocol 2.** Mice received an intraperitoneal injection of 1) ATV (20 mg/kg); 2) 1,4-diamino-2,3-dicyano-1,4-bis(2-iminophenylthio)butadiene (U0126; a specific MEK1 and MEK2 inhibitor, 40 μg/kg); 3) ATV + U0126; or 4) vehicle alone. There were four mice in each group. Eight hours after injection, mice were anesthetized and euthanized, and hearts were explanted and stored as described above.

**Protocol 3.** Mice received an intraperitoneal injection of 1) ATV (5 mg/kg) alone or in combination with 8-sulfophenyltheophylline (SPT; a nonselective adenosine receptor inhibitor, 20 mg/kg). One, three, or six hours after injection, mice were anesthetized and euthanized, and hearts were explanted and stored as described above. There were four mice in each group.

**Protocol 4.** Mice received an intraperitoneal injection of 1) ATV (5 mg/kg) alone; 2) ATV + SPT (20 mg/kg); 3) ATV + 1,3-dipropyl-8-cyclopentanylxanthine (DPCPX; a selective A1 antagonist, 100 μg/kg); 4) ATV + 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC; a selective A2a antagonist, 5 mg/kg); 5) ATV + benzo(g)pteridine-2,4-[1H,3H]-dione isothioxafoxalazine (alloxazine; a selective A3 antagonist, 3.0 mg/kg); or 6) ATV + 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carbonyl (MRS-1523; a selective A1 antagonist, 2 mg/kg). Three hours after injection, mice were anesthetized and euthanized, and hearts were explanted and stored as described above. There were four mice in each group. The doses of DPCPX and MRS-1523 are based on those in a report by Lasely et al. (34). The dose of CSC is based on that in a report by Bove et al. (13). The dose of alloxazine is based on that in a report by Shin et al. (50).

ATV and U0126 were dissolved in DMSO (final concentration: 0.05%). Adenosine receptor antagonists were dissolved in water.

Myocardial samples from the anterior left ventricular wall were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and the total protein concentration was determined using the Lowry protein assay. Protein samples with loading buffer were run in 4–20% Tris·HCl Ready Gels at a 100 V for 2 h until the desired molecular weight bands were separated. After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 193 mM glycine, 0.1% SDS, and 20% methanol), and proteins were transferred to a nitrocellulose membrane. Protein expression was assayed by standard SDS-PAGE Western immunoblot analysis. Fifty micrograms of protein were loaded for each sample. Protein signals were quantified by an image scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin stain signal. Data are expressed as percentages of expression in the control group.

Materials

Monoclonal anti-eNOS antibodies were purchased from BD Bioscience (San Jose, CA), and monoclonal anti-β-actin antibody was from Sigma (St. Louis, MO). Anti-PDK-1 antibodies, anti-Ser177 P-PDK-1 antibodies, anti-Akt antibodies, anti-Thr180 P-Akt antibodies, anti-Ser473 P-Akt antibodies, anti-Thr202/Thr204 P-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). U0126 was purchased from Calbiochem (San Diego, CA). TH, SPT, DPCPX, CSC, alloxazine, and MRS-1523 were purchased from Sigma.

Statistical Analyses

Data are expressed as means ± SE. Comparisons among groups were performed by one-way ANOVA with the Sidak correction for multiple comparisons (SPSS version 14.0). Values of P < 0.05 were considered statistically significant. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

**Statin Stimulation of PDK-1 Phosphorylation Is Independent of Adenosine Activity**

Myocardial PDK-1 levels were not affected by ATV, TH, or their combination (Fig. 1, A and B). In contrast, ATV increased myocardial levels of P-PDK1 (Fig. 1, C and D). TH alone had no effect on the P-PDK1 level and did not block the effect of ATV on PDK-1 phosphorylation (Fig. 1, C and D).

**Statin Stimulation of Akt Phosphorylation Is Adenosine Dependent**

ATV and TH alone or in combination did not affect total Akt levels (Fig. 2, A and B). ATV increased myocardial levels of both Ser473 P-Akt and Thr308 P-Akt. TH alone had no effect on Ser473 and Thr308 P-AKT levels; however, it blocked the ATV-induced increase in both Ser473 P-Akt (Fig. 2, C and D) and Thr308 P-Akt (Fig. 2, E and F).

**Statin Stimulation of eNOS Phosphorylation Is Dependent on Adenosine Activity**

ATV and TH alone or in combination did not affect total eNOS levels (Fig. 3, A and B). However, ATV did increase myocardial phosphorylation of eNOS, and blockade of adenosine activity prevented statin-induced phosphorylation (Fig. 3, C and D).

**Statin Stimulation of ERK1/2 Phosphorylation Is Dependent on Adenosine Activity**

ATV also increased myocardial ERK1/2 phosphorylation, and blockade of adenosine activity prevented statin-induced phosphorylation (Fig. 4).
In summary, ATV increased phosphorylation of PDK-1, Akt (at both Ser473 and Thr308), eNOS, and ERK1/2. Since the increase in Ser473 P-Akt, Thr308 P-Akt, P-eNOS, and P-ERK1/2, but not PDK-1, induced by ATV was attenuated by the coadministration of TH, we conclude that statin stimulation of PDK-1 is adenosine independent. Statin treatment, alone or in combination with adenosine blockade, had no effect on the myocardial content of total PDK-1, Akt, or eNOS.

Statin Stimulation of ERK1/2 Phosphorylation

ATV treatment increased myocardial levels of P-ERK1/2. The ERK1/2 inhibitor U0126 blocked ATV stimulation (Fig. 5).

Statin Stimulation of Akt Phosphorylation Is Partially ERK1/2 Dependent

Treatment with ATV alone increased myocardial levels of Ser473 P-Akt and Thr308 P-Akt, and the ERK1/2 inhibitor U0126 abolished this effect. U0126 alone had no effect on P-Akt levels but partially blocked the ATV effect on Ser473 Akt phosphorylation (Fig. 6, A and B) and Thr308 Akt phosphorylation (Fig. 6, C and D).

Statin Stimulation of eNOS Phosphorylation Is Partially ERK1/2 Dependent

Treatment with ATV alone increased myocardial levels of P-eNOS. The ATV effect was partially blocked by the coadministration of ERK1/2 inhibitor U0126, which had no effect when administered alone (Fig. 7).

The Dependence of Statin Stimulation of ERK1/2 Phosphorylation on Adenosine Activity Is Transient

ATV significantly increased myocardial P-ERK1/2 levels 3 and 6 h after intraperitoneal injection (Fig. 8, A and B). Myocardial levels of P-ERK1/2 1 h after ATV injection were identical to the control group. P-ERK1/2 levels were significantly lower in ATV + SPT-treated mice 1 and 3 h after injection than in the control group. In contrast, 6 h after
injection of ATV + SPT, P-ERK1/2 levels were higher than in the control group, although they were significantly lower than those in the ATV alone group after 6 h.

Statins Had a Delayed Stimulatory Effect on Myocardial P-Akt Levels

One hour after intraperitoneal injection, ATV did not have a significant effect on myocardial P-Akt levels; however, at 3 and 6 h after injection, myocardial levels were significantly higher in ATV-treated mice (Fig. 8, C and D). ATV + SPT-treated mice showed a transient inhibition of myocardial P-Akt down to negligible levels. By 6 h after injection, myocardial P-Akt returned to basal levels.

Statins Had a Delayed Stimulatory Effect on Myocardial P-eNOS Levels

P-eNOS levels did not significantly change 1 h after intraperitoneal injection of ATV (Fig. 8, E and F). However, at 3 and 6 h, myocardial levels were significantly higher than in the control group. At 1 and 3 h after injection of ATV + SPT, myocardial levels of P-eNOS were comparable with those in the
control group, suggesting complete block of the ATV effect at 3 h. In contrast, 6 h after injection, P-eNOS levels were increased compared with the control group, although they were still significantly lower than in ATV alone-treated mice after 6 h.

Adenosine-Dependent Stimulation of Myocardial P-ERK1/2 by Statins Is Via A1, A2A, and A2B Receptors

ATV treatment increased P-ERK1/2 levels (Fig. 9, A and B). P-ERK1/2 levels in the ATV + H11001 SPT group were significantly lower than in the ATV alone group and control group. DPCPX, CSC, and alloxazine attenuated the ATV effect, suggesting that A1, A2A, and A2B receptors participate in augmenting the ERK1/2 activation by adenosine. In contrast, MRS-1523 had no effect, suggesting that A3 receptors are not involved in the ERK1/2 activation.

Adenosine-Dependent Stimulation of Myocardial P-Akt by Statins Is Via A1, A2A, and A2B Receptors

ATV increased Ser473 P-Akt levels (Fig. 9, C and D). Myocardial levels of P-Akt were significantly lower in the ATV + SPT group compared with both the control group and ATV alone group. DPCPX, CSC, and alloxazine attenuated the ATV effect on P-Akt levels, suggesting that A1, A2A, and A2B receptors participate in augmenting the Akt activation by adenosine. In contrast, MRS-1523 had no effect, suggesting that A3 receptors are not involved in Akt phosphorylation.

Adenosine-Dependent Stimulation of Myocardial P-eNOS by Statins Is Via A1, A2A, and A2B Receptors

ATV alone increased myocardial levels of P-eNOS (Fig. 9, E and F). Myocardial levels of P-eNOS in the ATV + SPT group were comparable with those in the control group and significantly lower than in the ATV alone group. DPCPX, CSC, and alloxazine partially blocked the ATV effect on P-eNOS levels, suggesting that A1, A2A, and A2B receptors participate in augmenting the eNOS activation by adenosine, which is generated by ATV. In contrast, MRS-1523 had no effect, suggesting that A3 receptors are not involved in eNOS phosphorylation.

DISCUSSION

The main findings of our study include the following: first, ATV augments PDK-1 phosphorylation; however, this effect is independent of adenosine receptor activation. Second, phosphorylation of Akt at both Ser473 and Thr308 and the downstream phosphorylation of eNOS at Ser1177 were completely blocked by TH and SPT, nonspecific adenosine receptor blockers, strongly suggesting that adenosine is involved in Akt phosphorylation. Our results from inhibitor blocking experiments showed that A1, A2A, and A2B adenosine receptors are
involved in mediating these effects, whereas A3 receptors are not. Third, ATV augments ERK1/2 phosphorylation. Fourth, ERK1/2 phosphorylation was completely blocked by TH and SPT and partially blocked by specific A1, A2A, and A2B adenosine receptor antagonists, suggesting that adenosine receptor activation is mediating the activation of ERK1/2 by ATV. Finally, ERK1/2 facilitates Akt phosphorylation at both Thr308 and Ser473 and, hence, eNOS phosphorylation (Fig. 10).

**ESN and Adenosine**

Adenosine has an established role in mediating ischemic preconditioning (25, 28) and postconditioning (25, 28, 40). It has been shown that PI3K activation by statins leads to the activation of ESN (11, 48, 57) with increasing release of adenosine to the interstitial space (11, 36, 48, 57). Wortmannin, a PI3K inhibitor, blocks the ATV-induced ESN activation (48). Adenosine receptor blockers abrogate the protective effect of statins (37, 48). We (11) have recently shown that ATV upregulates ESN activity in wild-type as well as eNOS−/− mice; however, ATV limits infarct size only in wild-type mice, suggesting that ESN is either upstream to eNOS or that, although the two pathways are independent, they are needed in parallel for statin-mediated protection. Moreover, we (69) have shown that dipyridamole, an adenosine reuptake inhibitor,

**Fig. 8.** Samples of immunoblots (A, C, and E) and densitometric analyses (B, D, and F) of myocardial levels of P-ERK1/2 (A and B), Ser473 P-Akt (C and D), and P-eNOS (E and F) 1, 3, and 6 h after intraperitoneal injection of ATV alone or ATV + 8-sulfophenyltheophylline (SPT). *P < 0.05 vs. control; #P < 0.05, ATV vs. ATV + SPT.

**Fig. 9.** Samples of immunoblots (A, C, and E) and densitometric analyses (B, D, and F) of myocardial levels of P-ERK1/2 (A and B), Ser473 P-Akt (C and D), and P-eNOS (E and F) 3 after intraperitoneal injection of ATV alone (No), ATV + SPT (a nonspecific adenosine receptor inhibitor), ATV + 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; an A1 inhibitor), ATV + 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC; an A1A inhibitor), ATV + alloxazine (an A1B inhibitor), or ATV + MRS-1523 (an A3 inhibitor). *P < 0.05 vs. control; #P < 0.05, ATV + inhibitor vs. ATV alone.
increases tissue levels of adenosine and augments the infarct size-limiting effect of ATV.

One study (32) has suggested that adenosine increases Akt phosphorylation at Ser473 and PI3K activity in isolated rabbit hearts. However, our findings suggest that the adenosine involvement is downstream of PI3K, as PDK-1 phosphorylation was not affected, whereas Akt phosphorylation was completely blocked, by TH and SPT. This may explain the finding of Xu et al. (66) in that PI3K inhibitors only partially blocked the upregulation of NOS activity by adenosine. Moreover, Sanada et al. (48) showed that both E5N and Akt activation by statins were blocked by wortmannin, a PI3K inhibitor, placing PI3K upstream to E5N.

Several investigators (23, 38, 52, 53, 58) have suggested that adenosine and adenosine receptor agonists augment NO release by increasing eNOS expression and/or activity; however, the role of adenosine in mediating statin-induced eNOS phosphorylation has not been described. Here, we have provided more detail to our understanding of the statin activation pathway.

**PDK-1**

It is well established that statins activate PI3K, leading to increased membranous levels of PI3P (7, 20, 27, 33, 59, 62). From reading the literature on myocardial protection, one may get the impression that PI3P augments Akt phosphorylation, leading to the activation of prosurvival kinases and eNOS and, thus, to myocardial protection against ischemia-reperfusion injury (7, 8, 14, 20, 26, 27, 33, 41, 48, 51, 62, 68). However, it has been shown in other models that PI3P does not have a direct catalytic effect on Akt phosphorylation. PI3P interacts with the pleckstrin homology domain of Akt, mobilizing it to the plasma membrane and thus facilitating Akt phosphorylation at Thr308 and Ser473 (4, 21, 24). Akt phosphorylation at Thr308 is catalyzed by PDK-1 (21, 54). PDK-1 itself is activated by phosphorylation at Ser473, by an as yet unknown kinase (17, 24). It has also been shown that Ser473 Akt phosphorylation is mediated by the rictor-mTOR complex (29, 49), and statins have been reported to activate mTOR by an as yet unknown mechanism (47). Here, we have shown that ATV increased PDK-1 phosphorylation and that this effect was independent of adenosine receptor activation. On the other hand, the phosphorylation of Akt at both Thr308 and Ser473 was dependent on the combined activation of adenosine A1, A2A, and A2B receptors and on ERK1/2, suggesting that the activation of adenosine receptors (with downstream activation of ERK1/2) is needed for enabling P-PDK-1 and probably the rictor-mTOR complex to phosphorylate Akt at Thr308 and Ser473, respectively.

**ERK1/2**

Most studies support an essential role for ERK1/2 in ischemic preconditioning and postconditioning (27, 28). Ischemic preconditioning induces two phases of ERK1/2 activation, the first occurring immediately following the ischemic preconditioning stimulus with a second phase taking place at the time of myocardial reperfusion (28). In ischemic preconditioning, ERK1/2 has been reported to be activated by PKCe and reactive oxygen species and, hence, is thought to be downstream to Akt (28). For example, Reid et al. (45) also showed that adenosine administered before ischemia acts through both A1 and A2A receptors to reduce infarct size in the rat via the activation of ERK1/2, but they did not report on eNOS activation.

Although the exact mechanism through which ERK1/2 is activated in postconditioned hearts is currently unknown, some investigators (28) have suggested that adenosine may be involved. However, it is commonly believed that the protective effect of ERK1/2 against reperfusion injury is independent of eNOS (28).

ERK1/2, among other members of the MAPK family, has been shown to be activated by statins (18, 20, 27) and to be involved in the infarct size-limiting effects of statins (20). However, using the same experimental model, it has been shown that ATV, administered at reperfusion, failed to limit infarct size in eNOS−/− hearts, suggesting that eNOS is involved in the protective effect of ERK1/2 (7). In our experiments, we found that intraperitoneal ATV treatment increased myocardial P-ERK1/2 levels at 3 and 6 h after administration. ERK1/2 was dependent on the activation of three adenosine receptors (A1, A2A, and A2B) but not on adenosine A3 receptors. Inhibition of ERK1/2 phosphorylation with U0126 attenuated Akt phosphorylation at both Thr308 and Ser473 as well as eNOS phosphorylation, suggesting that in statin preconditioning, ERK1/2 is upstream to Akt and eNOS activation. As mentioned above, Wyatt et al. (64) reported that adenosine-induced increases in NO release in human umbilical venous endothelial cells (HUVECs) is dependent on P-ERK1/2, in support of our findings. It is not clear how ERK1/2 affects Akt phosphorylation. As adenosine receptor inhibition prevented ERK1/2 phosphorylation without affecting PDK-1 phosphorylation, it may be that ERK1/2 does not phosphorylates PDK-1 but rather facilitates directly or by downstream effects the
phosphorylation of Akt by P-PDK-1 and probably the rictor-mTOR complex. We did not inhibit Akt in our in vivo model; therefore, we do not know whether ERK1/2 affects Ser1177 eNOS phosphorylation by kinases other than Akt (26).

**Adenosine Augments NO Production**

There are several studies of the effects of adenosine on NO production with contradictory results. Bell et al. (6) reported that 24 h after the intravenous administration of 2-chloro-N6,cyclopentyl adenosine (CCPA), an adenosine A1 receptor agonist, there was an increase in the concentrations of NOx in the coronary sinus effluent of wild-type and inducible NO synthase (iNOS) knockout mice (6). They also reported that 24 h after CCPA administration, eNOS and iNOS protein concentrations increase in wild-type mice while eNOS protein levels increase in iNOS−/− mice (6). Gabazza et al. (22) showed that adenosine increases eNOS but not iNOS mRNA levels in HUVECs. Furthermore, Wyatt et al. (64) reported that adenosine-induced increases in NO release in HUVECs are mediated by the activation of adenosine A2A receptors and are dependent on protein tyrosine kinases and ERK1/2 phosphorylation but not on Akt phosphorylation. Xu et al. (65) studied adult rat cardiomyocytes and reported that adenosine-induced NO production is mediated by adenosine A2 receptors and associated with Ser1177 phosphorylation of eNOS. This effect could be blocked by 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine, a selective Src tyrosine kinase inhibitor (66). In their experiments, PI3K inhibitors (wortmannin and LY-294002) and the Akt inhibitor partially blocked the increase in NOS activity (66). Moreover, the Akt inhibitor did not completely block eNOS phosphorylation at Ser1177, consistent with a partial blockade of the PI3K/Akt pathway or the activation of other enzymes, such as PKA or MAPK, that are activated by Src tyrosine kinase and phosphorylate eNOS (66). Ansari et al. (2) also found that the adenosine-induced increases in NO release in the mouse aorta are mediated via adenosine A2B receptors. On the other hand, in a model of subarachnoid hemorrhage in rats, Lin et al. (39) found that N6-cyclopentyl adenosine, an adenosine A1 receptor antagonist, prevents the suppression of eNOS mRNA and protein expression in the basilar artery and ameliorates subarachnoid hemorrhage-induced vasospasm. Ray et al. (43, 44) reported that both adenosine A1 and A2A receptors are involved in the induction of NO release from the endothelium of the rat aorta by adenosine and suggested that the K+ efflux resulting from A1 receptor-coupled ATP-sensitve K+ channel activation facilitates Ca2+ influx, which stimulates eNOS. The increase in Ca2+ influx also stimulates phospholipase A2 and cyclooxygenase to generate prostacyclin (PGI2). PGI2 acts on its endothelial receptors to increase cAMP, leading to the activation of PKA, which in turn activates eNOS by phosphorylation. In contrast, the K+ efflux resulting from A2A receptor-coupled Ca2+ activated K+ channels facilitates Ca2+ influx, thereby activating eNOS and NO release (44). We, on the other hand, have reported that ATV fails to augment cyclooxygenase-2 activity and increase 6-keto-PGF1a( the stable metabolite of PGI2) in eNOS−/− mice hearts, as it does in wild-type mice, suggesting that eNOS is upstream to PGI2 production and not vice versa (10).

Thus, different mechanisms for the augmentation of NO release by adenosine in vitro have been reported. The results of the present study are in agreement with some of these, as we found that selective individual blockade of adenosine A1, A2A, and A2B receptors partially inhibited Akt and eNOS phosphorylation, whereas blockade of all adenosine receptors with TH or SPT completely inhibited their phosphorylation. In agreement with Wyatt et al. (64) and Reid et al. (45), we found that adenosine receptor activation leads to the activation of ERK1/2 and that inhibition of ERK1/2 prevented the increase in eNOS phosphorylation. It is as yet unclear which of the secondary messengers activated by the adenosine receptor subtypes mediate the activation of ERK1/2 with the subsequent phosphorylation of Akt and eNOS.

Since there was no detectable increase in P-ERK, P-Akt, and P-eNOS 1 h after ATV administration, and myocardial levels of P-ERK1/2 and P-Akt were lower than controls at 1 and 3 h after ATV + SPT injection, it seems that a baseline level of adenosine receptor activation is needed to maintain myocardial levels of P-ERK1/2 and P-Akt. On the other hand, as levels of Ser1177 P-eNOS did not decrease in ATV + SPT-treated mice at 1 and 3 h despite a significant decrease in P-Akt, it seems that under basal conditions, kinases other than Akt are phosphorylating eNOS, as suggested by Harris et al. (26).

TH is a potent inhibitor of adenosine receptors at therapeutic concentrations (5). In addition, TH is a weak and nonselective inhibitor of phosphodiesterases, which act on cellular cyclic nucleotides, leading to an increase in intracellular cAMP and cGMP (5). However, the inhibition of phosphodiesterases should augment eNOS phosphorylation, as sildenafil, a phosphodiesterase IV inhibitor, increases eNOS phosphorylation at Ser1177 and sildenafil and ATV have synergistic effects on eNOS phosphorylation (46). Furthermore, cilostazol, a phosphodiesterase III inhibitor, also has synergistic effects with ATV in augmenting Akt and eNOS phosphorylation (40a). We confirmed the essential role of adenosine receptors in mediating the phosphorylation of these enzymes by using SPT, a nonspecific adenosine receptor inhibitor that does not penetrate cells and therefore does not have phosphodiesterase activity (protocols 3 and 4). Furthermore, we showed that the inhibition of A1, A2A, and A2B receptors by specific blocking agents caused a partial attenuation of phosphorylation of ERK1/2, Akt, and eNOS, suggesting that all three receptors are needed to mediate the effect.

The activation of Akt and eNOS occurs rapidly after statins are added to cell cultures (26, 33). Similarly, the infarct size limitation and activation of Akt and eNOS by statins occur rapidly in isolated heart models (7, 19). When administered intravenously before myocardial ischemia, statins reduce infarct size when administered 60 min (60), 20 min (56), and 10 min (48) before coronary occlusion and even when activated simvastatin is administered just before reperfusion (62). However, when administered intraperitoneally, simvastatin limits infarct size in the mouse only when given 3–18 h before coronary occlusion but not 1 h before ischemia (31). Similarly, intraperitoneal rosvastatin reduces infarct size in the mouse only when given 6–18 h before coronary artery occlusion but not 3 h before ischemia (30). These differences are probably related to the time needed to achieve effective myocardial cellular levels of statins. Indeed, our results confirm the results of Jones et al.’s (30), as increases in P-ERK1/2, P-Akt, and P-eNOS were not detected at 1 h after intraperitoneal injection of ATV. The pharmacokinetics of adenosine receptor inhibi-
tors in animal models are not clear. Our data show that the inhibitory effects of SPT on ERK1/2, Akt, and eNOS phosphorylation start to wane between 3 and 6 h after a single intraperitoneal injection. Therefore, we chose to continue with a 3-h time point in protocol 4 to assess the role of the four specific adenosine receptor subtype blocking agents. As statins are currently not available for parenteral use in the clinical setting, the administration of statins before reperfusion is still not feasible. The myocardial protective effects of statins, seen early in patients with acute coronary syndromes or those undergoing percutaneous coronary interventions or surgery (42), are therefore more compatible with the experimental models of pretreatment before ischemia, as we used in the present experiments, than the models of prereperfusion administration.

As we found no significant differences in myocardial levels of P-Akt and P-eNOS 8 h after the administration of ATV (5 or 20 mg/kg; unpublished data), we proceeded with a dose of 5 mg/kg in protocols 3 and 4. Indeed, no there were no major differences in myocardial levels of P-ERK, P-Akt, and P-eNOS 8 h after intraperitoneal ATV at 20 mg/kg and 6 h after intraperitoneal ATV at 5 mg/kg.

Limitations

We used a pharmacological approach to block adenosine receptors and inhibit ERK1/2 phosphorylation. The various agents may have unexpected additional effects. For example, we cannot exclude that U0126 has a direct effect on P-PDK-1 or the rictor-mTOR complex in addition to blockade of ERK 1/2 phosphorylation. In addition, specific adenosine receptor inhibitors may have an effect on the other adenosine receptor subtypes. Therefore, further studies using a genetic approach (knockout mice models and/or silencing of genes with short interfering RNA in vitro) should be conducted to confirm our findings.

Conclusions

The results of our study suggest that ATV-induced eNOS phosphorylation is mediated by the activation of adenosine A1, A2A, and A2B receptors, whereas A3 receptors are not involved in mediating the effect. The inhibition of A1, A2A, or A2B receptors by specific blocking agents caused a partial attenuation of phosphorylation of eNOS, suggesting that all three receptors are needed for mediating the effect. The activation of the three adenosine receptor subtypes leads to ERK1/2 and Akt phosphorylation with subsequent eNOS phosphorylation.

REFERENCES


