Cell-Signaling Evidence for Adenosine Stimulation of Coronary Smooth Muscle Proliferation via the A1 Adenosine Receptor

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Abstract—For decades, it has been thought that adenosine is exclusively antimitogenic on vascular smooth muscles via the A2-type adenosine receptor. Recently, we have demonstrated that adenosine stimulates proliferation of porcine coronary artery smooth muscle cells (CASM C) through the A1 adenosine receptor. However, the cell-signaling mechanisms underlying A1 receptor–mediated CASMC proliferation in response to adenosine have not been defined. Here, we show that in cultured CASMC, adenosine stimulates phosphorylation of extracellular signal–regulated kinase (ERK), Jun N-terminal kinase (JNK), and AKT in a concentration- and time-dependent manner. This effect is fully mimicked by NECA (nonselective agonist), largely mimicked by CCPA (A1-selective agonist), weakly mimicked by 2-Cl-IB-MECA (A1-selective agonist), but not by CGS21680 (A2A-selective agonist), indicating that adenosine signals strongly via the A1 receptor to these mitogenic signaling pathways. This interpretation is supported by the finding that adenosine- and CCPA-induced phosphorylation of ERK, JNK, and AKT are inhibited by pertussis toxin (inactivator of Gi proteins) and by DPCPX (A1-selective antagonist), but not by SCH58261, MRS1706, and VUF5574 (A2A-, A2B-, and A3-selective antagonists, respectively). In addition, adenosine- and CCPA-induced phosphorylation of ERK, JNK, and AKT is inhibited, respectively, by U0126, PD98059 (mitogen-activated protein kinase kinase inhibitors), SP600125 (JNK kinase inhibitor), and wortmannin (phosphatidylinositol 3-kinase inhibitor). Furthermore, these kinase inhibitors abolish or diminish adenosine- and CCPA-induced increases in the rate of cellular DNA synthesis, bromodeoxyuridine incorporation, protein synthesis, and cell number. We conclude that adenosine activates the ERK, JNK, and phosphatidylinositol 3-kinase/AKT pathways primarily through the A1 receptor, leading to CASMC mitogenesis. (Circ Res. 2005;97:574-582.)

Key Words: porcine protein kinase phosphorylation extracellular signal–regulated kinase Jun N-terminal kinase AKT G protein

The abnormal growth and migration of vascular smooth muscle cells (VSMC) are thought to play important roles in the development of occlusive vascular diseases such as hypertension, atherosclerosis, and restenosis after angioplasty.\(^1,2\) To date, numerous factors, produced within the vascular wall or from the circulating blood, were demonstrated to stimulate proliferation and/or migration of VSMC through their cognate receptors, which are either receptor tyrosine kinases or G protein–coupled receptors.\(^3\) Among these vasoactive factors, extracellular nucleotides such as ATP and UTP are relatively newly defined mitogens in VSMC.\(^4,6\) The receptors responsible for ATP/UTP-induced mitogenesis in VSMC have been thought to be primarily the P2Y nucleotide receptors, of which 8 subtypes have been cloned and pharmacologically characterized.\(^5,7\) However, we recently demonstrated that P2Y receptors are only partially involved in ATP-induced proliferation of porcine coronary artery smooth muscle cells (CASM C).\(^6\) In exploring the P2Y-independent–receptor mechanism underlying ATP-induced CASMC proliferation, we unexpectedly found that adenosine, a degradation product of ATP, stimulates CASMC proliferation in vitro and in situ.\(^8\) Using pharmacological and molecular biological approaches, we demonstrated that the trophic effect of adenosine on CASMC is predominantly mediated by the A1 adenosine receptor (A1R),\(^8\) which is 1 of the 4 G protein–coupled adenosine receptors (ARs) thus far cloned: A1R, A2AR, A2BR, and A3R.\(^9\) This original finding challenged the historical understanding of the vascular protective effect of adenosine because adenosine had been shown to be exclusively antimitogenic in aortic VSMC through the A3R.\(^5,10,11\) Although compelling data from our study had shown that adenosine-induced CASMC mitogenesis is mediated by the
A, R, postreceptor mechanisms coupling the A, R stimulation to increased proliferation of these CASMC have not been investigated. Mitogen-activated protein kinases (MAPKs) are essential in regulating cell growth in response to various extracellular stimuli, including G protein–coupled receptor agonists. There are 3 major subfamilies of structurally related MAPKs identified in mammalian cells, which are termed p44/42 MAPK (extracellular signal–regulated kinase 1/2 [ERK1/2]), p38 MAPK, and Jun N-terminal kinase (JNK)/stress-activated protein kinases. Among them, ERK1/2 and JNK have been shown to be critical for VSMC proliferation in vitro and neointimal formation in vivo. However, evidence for the role of p38 in VSMC mitogenesis is scarce and has not been conclusively demonstrated. In addition to the MAPK signaling pathways, recent studies have also implicated the phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt pathway in VSMC proliferation induced by various mitogens. Interestingly, all the MAPKs and the PI3K/Akt pathways have been shown to be activated by 1 or more of the AR subtypes expressed in transfectant cell lines or in nonvascular smooth muscle cell lines such as DDT, MF-2 cells. However, nothing is known about adenosine stimulation of these signaling pathways in VSMC and their potential linkage to adenosine regulation of VSMC mitogenesis.

One purpose of the current study was to determine whether adenosine can activate the ERK1/2, JNK, and PI3K/Akt pathways in CASMC, and, if so, which AR subtype(s) is responsible for adenosine-induced activation of these pathways. Second, we sought to examine which signaling pathway(s) is involved in adenosine-induced CASMC mitogenesis. Our findings demonstrate that in porcine CASMC, adenosine stimulates the phosphorylation of ERK1/2, JNK, and Akt predominantly through the A. Furthermore, all 3 kinase pathways are involved in adenosine-induced CASMC proliferation.

Materials and Methods

CASMC Isolation and Culture

CASMC were isolated from the right coronary arteries of farm pigs as previously detailed in our laboratory (Jennings Premium Meats, New Franklin, Mo). Briefly, the conduit segments of the right coronary arteries were dissected from the pig hearts and denuded of endothelium using aseptic techniques. CASMC were dispersed by enzymatic digestion of the connective tissue in a solution containing 294 U/mL collagenase, 0.2% BSA, 0.1% soybean trypsin inhibitor, and 0.04% DNase. The artery segments were incubated for 3 hours in a shaking water bath at 37°C, the collagenase solution being replaced hourly. Dispersed CASMC were grown in DMEM/high-glucose medium that contained 10% FBS at 37°C with 5% CO2. The quiescent CASMC were pretreated with different kinase inhibitors for 40 minutes before being stimulated with adenosine or CCPA for 4 hours at 37°C, the cells were washed 3 times in PBS and solubilized in 0.1% SDS. Trichloroacetic acid (10%) was then added, and the precipitates were collected by filtration on glass-fiber disks for determination of radioactivity by liquid scintillation counting.

Immunocytochemistry for Bromodeoxyuridine Staining

Bromodeoxyuridine (BrdUrd) staining was performed using a BrdUrd staining kit-II (Roche) according to the instructions of the manufacturer, with a minor modification in the detection system. Briefly, ~1x10^6 CASMC were seeded in 8-well chamber slides. Cells were serum starved for 48 hours, stimulated with adenosine or platelet-derived growth factor (PDGF) for 24 hours in the absence or presence of various inhibitors, pulsed with 30 μCi/mL BrdUrd for 3 hours, and, finally, fixed in -20°C methanol. Bound mouse anti-BrdUrd antibody was detected by anti-mouse IgG conjugated with Oregon Green-488 (1:200 dilutions; Molecular Probes). The nuclei were counterstained with 4',6-diamidino-2-phenylindole. Immunofluorescence was detected using a fluorescent microscope (>200 magnification; Nikon Eclipse E600). The experiment was performed 3 times, each with duplicate. At least 500 cells were counted per chamber, and data were collected in a blinded manner. The BrdUrd-incorporation index, defined as the number of cells with green nuclei divided by the total number of cells counted and expressed as a percentage, was calculated.

Protein Synthesis Assay

[3H]Leucine incorporation was used to measure cellular total protein synthesis as described previously in our laboratory. Briefly, the method is identical to that described above, under Measurement of DNA Synthesis, with the exception that 1 μCi of [3H]-leucine instead of radioactive thymidine was added.

Cell Number Quantification

Cell number was determined using the MTT Cell Growth Assay Kit (CHEMICON), as described previously in our laboratory. Briefly, it is known that the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is bioreduced by viable cells into colored formazan that is soluble in tissue-culture medium. The amount of formazan can then be determined by measuring absorbance at 490 nm in an ELISA plate reader. The quantity of formazan product, and thus the amount of 490-nm absorbance, is directly proportional to the number of living cells. Based on this principle, cultured CASMC were suspended by trypsinization, counted, replated into 96-well plates at a density of 2000 cells/well in medium containing 10% FBS for 48 hours, and then starved for 48 hours to induce quiescence, after which the cells were pretreated with or without different inhibitors for 40 minutes and then stimulated with adenosine or CCPA for 4 days. At the end of stimulation, medium was removed and 20 μL of MTT reagent in 100 μL of DMEM was added. After an additional 4 hours at 37°C, absorbance at 490 nm was measured.

Western Blotting for p-ERK, p-JNK, and p-AKT

CASMC were seeded in 6-well plates for 48 hours in DMEM medium containing 10% FBS, after which the cells were serum starved (0.1% BSA in DMEM) for 24 hours at ~70% confluence. The quiescent CASMC were pretreated with different kinase inhibitors or AR antagonists for 40 minutes before being stimulated with different AR agonists at the indicated concentration. The pretreatment time for pertussis toxin (PTX) was 24 hours, and the stimulation time for ERK, JNK, and AKT was 5 minutes (unless otherwise indicated). After stimulation, cells were washed with ice-cold PBS, solubilized in Laemmli sample buffer with 200 mmol/L dithiothreitol, and boiled for 2 minutes. Lysates were sonicated to disrupt DNA, and proteins were separated on SDS-PAGE gels (Bio-Rad). The proteins were electrophoretically transferred to nitrocellulose in 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, and 0.02%
The nitrocellulose was blocked with 5% nonfat dry milk in 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 0.01% Tween 20. The membranes were probed with the individual primary antibody (anti–p-ERK1/2, anti–p-JNK, and anti–p-AKT; 1:1000 dilutions; Cell Signaling) overnight in 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 3% BSA, and 0.01% Tween 20. The blots were washed in 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 0.01% Tween 20, and bound antibody was detected by a horseradish peroxidase–conjugated anti-rabbit IgG and enhanced chemiluminescence (Pierce). Of note, because the commercially available antibodies for total ERK1/2 and JNK did not work in these porcine CASMC, equal protein loading was verified by stripping off the original antibodies, and the membranes were reprobed with the primary antibody anti-actin (1:1000; CYTOSKELETON) or anti–total AKT (1:1000; Cell Signaling).

Materials

All cell culture media were from Invitrogen. The following compounds were purchased from Tocris: NECA, CCPA, CGS21680, 2-Cl-IB-MECA, DPCPX, MRS1706, VUF5574, and PD98059. Adenosine, SCH58261, and wortmannin were purchased from Sigma (St Louis, Mo). U0126 was from Cell Signaling. SP600125 and PTX were from Calbiochem.

Data Analysis

Data are expressed as means±SEM. Means of 2 groups were compared using Student’s $t$ test (unpaired, 2 tailed), and 1-way ANOVA was used for comparison of more than 2 groups, with $P<0.05$ considered to be statistically significant. Unless indicated in the figure legends, all the experiments were performed at least 3 times in triplicate with similar results.

Results

Activation of ERK1/2, JNK, and AKT by Adenosine in CASMC

To define the potential cell-signaling pathways relevant to adenosine-induced CASMC mitogenesis, we stimulated CASMC with adenosine and found that the agonist dose-dependently increased the phosphorylation levels of ERK1/2 and JNK with similar potency and efficacy (Figure 1A and 1B). Specifically, significant phosphorylations of both ERK1/2 and JNK were induced by 1 μmol/L adenosine and maximal phosphorylations ($\sim7$-fold) were observed at 100 μmol/L (Figure 1A and 1B). Interestingly, adenosine also increased the phosphorylation level of AKT, but the effect was biphasic, with peak activation at 1 μmol/L ($\sim3$-fold) and lesser activation at 100 μmol/L (Figure 1C). Because phosphorylation of ERK, JNK, and AKT is strongly correlated with their kinase activity, these data indicate that adenosine can activate the ERK, JNK, and AKT signaling pathways.
Differential Effects of AR Agonists on ERK1/2, JNK, and AKT Activation

To determine the AR subtype(s) responsible for adenosine stimulation of these signaling pathways, we stimulated CASMC with different AR agonists. As shown in Figure 1A and 1B, the effects of adenosine on ERK and JNK phosphorylation was largely mimicked by NECA (nonselective AR agonist) and CCPA (A1R-selective agonist), and very weakly mimicked by 2-Cl-IB-MECA (A3R-selective agonist), but not by CGS21680 (A2AR-selective agonist). In addition, NECA fully mimicked the biphasic effects of adenosine on AKT phosphorylation with peak activation (3-fold) at 1 μmol/L and much less activation at 10 μmol/L (Figure 1C). In contrast, CCPA dose-dependently increased AKT phosphorylation more potently and efficaciously than adenosine or NECA, with detectable activation at nanomolar concentrations, whereas 2-Cl-IB-MECA was active only at micromolar concentrations, and CGS21680 was totally inactive (Figure 1C). Because our previous study had shown that porcine CASMC primarily express the A1R, A2AR, and A3R,8 the above data indicate a differential role of the AR subtypes on ERK, JNK, and AKT activation, with the A1R as the major mediator of adenosine activation of these mitogenic signaling pathways.

Similar Time Course for Adenosine and CCPA Activation of ERK1/2, JNK, and AKT

To assess whether the A1R agonist shares the same time course with adenosine in activating ERK, JNK, and AKT pathways, we stimulated CASMC with different AR agonists. As shown in Figure 1A and 1B, the effects of adenosine on ERK and JNK phosphorylation was largely mimicked by NECA (nonselective AR agonist) and CCPA (A1R-selective agonist), and very weakly mimicked by 2-Cl-IB-MECA (A3R-selective agonist), but not by CGS21680 (A2AR-selective agonist). In addition, NECA fully mimicked the biphasic effects of adenosine on AKT phosphorylation with peak activation (3-fold) at 1 μmol/L and much less activation at 10 μmol/L (Figure 1C). In contrast, CCPA dose-dependently increased AKT phosphorylation more potently and efficaciously than adenosine or NECA, with detectable activation at nanomolar concentrations, and CGS21680 was totally inactive (Figure 1C). Because our previous study had shown that porcine CASMC primarily express the A1R, A2AR, and A3R,8 the above data indicate a differential role of the AR subtypes on ERK, JNK, and AKT activation, with the A1R as the major mediator of adenosine activation of these mitogenic signaling pathways.

Differential Effects of AR Antagonists on ERK1/2, JNK, and AKT Activation

To further examine the role of the individual AR subtype in adenosine activation of ERK, JNK, and AKT, we used AR subtype-selective antagonists. As shown in Figure 3A, pretreatment of CASMC with DPCPX (0.1 μmol/L, A1R-selective antagonist), but not with SCH58261, MRS1706, or VUF5574 (A2AR-, A2BR-, and A3R-selective antagonists, respectively; each 0.1 μmol/L), significantly decreased adenosine-induced phosphorylation of ERK1/2, suggesting a prominent role for the A1R. Consistent with this result, DPCPX also dose-dependently inhibited CCPA-induced ERK phosphorylation (Figure 3B). In addition, a similar pattern of inhibition by DPCPX on adenosine- and CCPA-induced JNK activation was also observed, whereas all of the other AR antagonists were inactive (Figure 3C and 3D), indicating that the A1R mediates adenosine-induced JNK activation. Furthermore, DPCPX pretreatment also abolished adenosine- and CCPA-induced AKT activation (Figure 3E and 3F). Although SCH58261 and MRS1706 tended to increase adenosine-induced AKT phosphorylation, and VUF5574 pretreatment appeared to decrease adenosine activation of AKT, these changes were not statistically significant (Figure 3E and 3F). Of note, none of the treatments affected the expression level of AKT protein (Figure 3E and 3F). Together, data in this set of experiments strongly support the notion that adenosine predominantly uses the A1R to transduce cell proliferation-related signals in CASMC.

PTX Inhibition of Adenosine- and CCPA-Induced ERK, JNK, and AKT Activation

Because our previous study demonstrated that the A1R in CASMC is coupled to the Gi protein,8 which can be inactivated by PTX-catalyzed ADP ribosylation, we took advantage of this reagent to further investigate adenosine signaling. As shown in Figure 4A through 4C, pretreatment of CASMC with PTX (100 ng/mL, 24 hours) alone had no significant effect on the basal phosphorylation of ERK, JNK, and AKT.
However, it eliminated the effects of CCPA and also dramatically decreased or abolished adenosine-induced activation of ERK, JNK, and AKT. Of note, PTX treatment did not change the total protein level of AKT (Figure 4C). These results provide another piece of evidence in support of a major role for the A1R in adenosine activation of ERK, JNK, and AKT pathways.

Inhibition of Adenosine- and CCPA-Induced ERK, JNK, and AKT Activation by Protein Kinase Inhibitors
To determine whether adenosine- and CCPA-activated mitogenic signaling pathways could be similarly inhibited by the corresponding protein kinase inhibitors, we used a set of well-documented inhibitors that target the protein kinases upstream of ERK1/2, JNK, or AKT. Figure 5A shows that pretreatment of CASMC with U0126, a selective MAPK kinase (MEK) inhibitor, abolished both adenosine- and CCPA-induced ERK1/2 phosphorylation. However, PD98059, another selective MEK inhibitor at the same concentration, only partially inhibited adenosine- and CCPA-induced ERK1/2 phosphorylation (Figure 5B). This apparent discrepancy could be attributable to the different potencies of U0126 and PD98059 for MEK inhibition. These data suggest that both adenosine and CCPA induce ERK1/2 phosphorylation by activating the upstream kinase MEK. In addition, adenosine- and CCPA-induced JNK phosphorylation could be partially, but similarly, inhibited by SP600125 (Figure 5C), a selective inhibitor of JNK kinase, indicating that adenosine and CCPA share the same signaling pathway in activation of JNK. Furthermore, as expected, pretreatment of CASMC with wortmannin, a selective inhibitor of PI3K
upstream of AKT, abolished adenosine- and CCPA-induced AKT phosphorylation (Figure 5D), suggesting that both adenosine and CCPA can activate the PI3K/AKT signaling pathway.

**Inhibition of Adenosine- and CCPA-Induced CASMC Proliferation by Protein Kinase Inhibitors**

Because ERK1/2, JNK, and AKT have all been implicated in VSMC proliferation, we further tested whether activation of these pathways by adenosine and CCPA are essential for adenosine- and CCPA-induced CASMC growth. As shown in Figure 6, both adenosine- and CCPA-induced increases in the rate of cellular DNA synthesis (Figure 6A), protein synthesis (Figure 6B), and cell number (Figure 6C) were either abolished by U0126 and SP600125 or dramatically inhibited by PD98059 and wortmannin. Of note, although the magnitudes of inhibition by the 4 inhibitors were variable on the rate of cellular DNA synthesis, protein synthesis, and cell number, the overall inhibition pattern on the effects of adenosine and CCPA was virtually identical (Figure 6A through 6C). In addition, stimulation of CASMC with adenosine also increased BrdUrd-incorporation indices, which was approximately half of that induced by a maximal concentration of PDGF and was significantly inhibited, respectively, by

![Figure 4. Effect of PTX on adenosine and CCPA stimulation of ERK1/2, JNK, and AKT.](image-url)
DPCPX, U0126, SP600125, and wortmannin (Figure 7). Together, these data suggest that adenosine and CCPA may activate the same cellular signaling pathways such as ERK1/2, JNK, and PI3K/AKT to trigger CASMC proliferation.

**Discussion**

In the present study, we have shown for the first time that adenosine can activate the MAPKs (ERK1/2 and JNK) and the PI3K/AKT signaling pathways in cultured porcine CASMC. We also have demonstrated that activation of these signaling pathways by adenosine is predominantly mediated by the A1R. In addition, our data show that activation of these pathways is necessary for adenosine stimulation of CASMC mitogenesis. These new findings, focused on cell signaling, extend and strengthen our original observation that adenosine functions as a mitogen in CASMC, a unique feature not shared by other smooth muscle vasodilators.

It is known that VSMC proliferation is pivotal for the development of the occlusive vascular diseases such as atherosclerosis and angioplasty-induced restenosis. A conceptual view culminating from numerous studies in the past several decades in this field is that virtually all vasoconstrictors are mitogenic and smooth muscle dilators are antimitogenic in VSMC. However, we recently challenged this conventional view by showing that adenosine, a well-known vasodilator, stimulates the proliferation of porcine CASMC in cultured cells and in an ex vivo organ culture model. Although we demonstrated previously that the mitogenic action of adenosine in CASMC is predominantly mediated by the A1R, supportive evidence for the post–receptor signaling mechanisms was lacking. In the present study, we found that stimulation of CASMC with adenosine caused phosphorylation of ERK1/2, which was inhibited by U0126 and PD98059, 2 specific inhibitors of the upstream kinase MEK. These results, together with our previous finding that ERK1/2 phosphorylation level is highly correlated with the ERK1/2 activity in these CASMC, led us to conclude that adenosine activates the MEK/ERK signaling pathway in CASMC, a well-known signaling mechanism required for VSMC proliferation induced by many mitogens. In addition, we showed that among all the tested AR agonists, only NECA and CCPA largely mimicked the stimulatory effect of adenosine on ERK1/2 phosphorylation. Conversely, among all the tested AR antagonists, only DPCPX significantly inhibited adenosine-induced ERK1/2 phosphorylation. These results...
strongly suggest that the A₁R, but not other ARs, is predominantly involved in adenosine-induced ERK1/2 activation. Given the central importance of the ERK signaling pathway in cell proliferation, it can be inferred that adenosine activation of the MEK/ERK pathway via the A₁R could be 1 of the key signaling mechanisms leading to CASMC mitogenesis. Indeed, this notion is supported by our finding that inhibition of the MEK/ERK pathway by U0126 and PD98059 abolished or dramatically decreased adenosine- and CCPA-induced CASMC mitogenesis. It should be noted, however, that all of the 4 ARs in transfected cell lines can activate the ERK1/2 pathway, although the role of the A₂ßR and A₂BR could also be inhibitory, depending on the type of cells. In this regard, it is not clear why adenosine preferentially uses the A₁R to signal the mitogenic ERK1/2 pathway in CASMC. One possible explanation is that the A₁R may be expressed at high level and the A₂R is low in cultured CASMC. In addition, both the A₂ßR and A₂BR may be inhibitory on the ERK pathway in CASMC, as reported in other cell types. Indeed, our previous study had shown that there is highly expressed A₁R and negligible A₂ßR in these CASMC.

Historically, studies on AR signaling in vascular smooth muscles have been limited to the cAMP/protein kinase A pathway, which is both positively (A₂ßR and A₂BR) and negatively (A₁R and A₁R) regulated. This is because of the well-documented role of the cAMP/protein kinase A pathway in smooth muscle relaxation, which fits well with the vaso-dilator property of adenosine. It is also because adenosine had been described as exclusively antimitogenic in VSMC; no study so far has been reported to show a stimulatory effect of adenosine on the cell growth–promoting pathways in VSMC.

In the present study, we found that besides ERK1/2, adenosine also activates the JNK pathway, which was reported to be crucial for VSMC proliferation. The fact that among all of the selective agonists, only CCPA largely mimicked the effect of adenosine and had a similar time course as adenosine also activates the JNK pathway. This notion was strongly supported by the following findings: (1) among the many tested nucleotides/nucleosides, ADP, AMP, and adenosine all largely mimicked the effect of ATP on cellular DNA synthesis, and the effects of ADP, AMP, and adenosine were virtually identical (see Figure I in the online data supplement available at http://circres.ahajournals.org); (2) more importantly, ATP-induced cellular DNA synthesis was similarly decreased by DPCPX and the antisense oligonucleotide specific to the A₁R (~40% to 50% inhibition), whereas the effect of UTP was not affected (see online Figure II).

Thus, it can be inferred that approximately half of the ATP-induced CASMC proliferation is attributable to adenosine-mediated A₁R activation. However, one might wonder why the increase in cell number induced by adenosine is so small (~30% over basal), especially when compared with that induced by ATP reported earlier (~140% over basal). A precise reason for this apparent discrepancy is unknown. However, it should be noted that the current study used the MTT assay for the measurement of cell proliferation, which is convenient but may not be as sensitive as other methods, such as cell counting used in our early study. It is possible that the MTT assay may underestimate the true increase in cell number induced by adenosine. For example, by direct counting of cell number, we previously found that ATP increased CASMC number by ~140%, whereas the MTT assay showed that the same concentration of ATP increased cell number only by ~50%. Thus, different experimental approaches could explain the apparent discrepancy.

In summary, we report the first evidence that in porcine CASMC, adenosine activates the ERK, JNK, and PI3K/AKT pathways, all of which are involved in adenosine-induced CASMC mitogenesis. Also, judging by any of the 3 pathways, the A₁R is primarily responsible for adenosine stimulation of mitogenic signaling and cell mitogenesis. Identification of the A₁R as the key receptor responsible for adenosine stimulation of the mitogenic pathways provides a convincing explanation at the molecular level of how adenosine functions as a mitogen in CASMC. Our results may have significant implications for the role of the A₁R in vascular smooth muscle biology and diseases. Future studies
focused on the up-/down-stream signaling mechanisms and the nuclear events on adenosine stimulation would provide a more complete picture for a better understanding of the roles of adenosine and its receptors in CASMC.

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**References**

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Supplemental figure 1. Differential effects of nucleotides/nucleosides on cellular DNA synthesis of cultured CASMC. Porcine coronary artery smooth muscle cells (CASMC) in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with indicated nucleotides or nucleosides at 10 µM for 48 h, after which the rate of cellular DNA synthesis was determined by [³H]-thymidine incorporation assay. Data shown are the means ± S.E.M. from four independent experiments performed in triplicate. *, p < 0.05; **, p < 0.01 relative to the control.
Supplemental figure 2. Inhibition of DPCPX and A₁R anti-sense oligonucleotide (OGN) on ATP-induced DNA synthesis of cultured CASMC. Porcine CASMC in standard density (35,000 cells/well) were serum-starved for 24 h and then stimulated with ATP or UTP (each 10 µM) for 48 h in the absence (-) or presence (+) of DPCPX (0.1 µM, 40 min pretreatment, A) or sense/anti-sense OGN specific to the porcine A₁R (each 0.3 µg, 24 h pretreatment, B), after which the rate of cellular DNA synthesis was determined by [³H]-thymidine incorporation assay. Data shown are the means ± S.E.M. from three independent experiments performed in triplicate. *, p < 0.05 relative to the ATP alone.