Dysregulation of Extracellular Adenosine Levels by Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

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Abstract—The objective of this investigation was to determine whether the regulation of extracellular adenosine levels by smooth muscle cells (SMCs) from conduit arteries (aorta) and resistance microvessels (renal arterioles) is different in spontaneously hypertensive rats (SHR) versus normotensive Wistar-Kyoto (WKY) rats. Basal extracellular adenosine levels were decreased in cultured aortic and arteriolar SHR SMCs, and the increase in extracellular adenosine levels induced by stimulation of the cAMP-adenosine pathway was less in aortic and arteriolar SHR SMCs. Extracellular adenosine levels were lower in SHR SMCs, however, even when the cAMP-adenosine pathway was inhibited with 3-isobutyl-1-methylxanthine. Inhibition of adenosine kinase with iodotubercidin and inhibition of adenosine deaminase with erythro-9-(2-hydroxy-3-nonyl) adenine increased extracellular adenosine; however, only inhibition of adenosine deaminase equalized extracellular adenosine levels in SHR versus WKY SMCs. Membrane-disrupted SHR SMCs metabolized exogenous adenosine faster than WKY SMCs did, and this difference was abolished by inhibition of adenosine deaminase but not adenosine kinase. SHR SMCs demonstrated a greater proliferative response than WKY SMCs but was blocked by inhibition of adenosine deaminase and by 2-chloroadenosine (adenosine deaminase–resistant adenosine analogue). We conclude that dysregulation of extracellular adenosine levels exists in SHR SMCs, that this dysregulation is not due to a defect in the cAMP-adenosine pathway but rather to enhanced activity of adenosine deaminase, and that the dysregulation of extracellular adenosine mediates the enhanced proliferative response of SHR SMCs. (Arterioscler Thromb Vasc Biol. 2001;21:249-254.)

Key Words: adenosine deaminase ■ adenosine kinase ■ hypertension ■ proliferation ■ vascular disease

Smooth muscle cells (SMCs) within conduit and resistance arteries are importantly involved in the pathophysiology of vascular remodeling induced by hypertension. In this regard, SMC growth, both hypertrophic and hyperplastic, contributes to pathological structural changes within the vessel wall in spontaneously hypertensive rats (SHR).

SMC growth is regulated by multiple factors. Under normal conditions, vascular SMC quiescence is maintained by a balance between vessel wall–derived and circulating growth inhibitors and growth promoters. Disruption of the balanced generation of growth promoters and growth inhibitors under pathological conditions triggers a cascade of events leading to increased proliferation of SMCs and enhanced deposition of extracellular matrix proteins by SMCs. Therefore, endogenous factors that are generated by cells within the vessel wall and that inhibit SMC growth may play a major vasoprotective role, and decreased vascular levels of growth-inhibitory factors may contribute to vascular disease in hypertension.

In this regard, it is conceivable that dysregulation of vascular adenosine levels in hypertension may be important. Adenosine induces vasodilation, inhibits platelet aggregation, prevents neutrophil adhesion to vascular and cardiac endothelial cells, attenuates neutrophil-induced endothelial cell damage, stimulates nitric oxide release from vascular endothelial cells and SMCs, activates cellular antioxidant defense systems, prevents oxygen free radical–induced injury, and blocks the synthesis of potent mitogenic factors such as angiotensin II and norepinephrine by inhibiting renin release and noradrenergic neurotransmission. The local role of adenosine within the cardiovascular system is evident from the findings that adenosine is synthesized by vascular fibroblasts, cardiomyocytes, and both vascular and cardiac endothelial cells. Moreover, we recently showed that vascular SMCs and cardiac fibroblasts also synthesize adenosine and that SMC-derived as well as cardiac fibroblast–derived adenosine inhibits serum-induced SMC and cardiac fibroblast growth. Thus, if the vascular levels of adenosine are decreased in hypertension, this could contribute importantly to vascular sequelae.

The first aim of the present study was to determine whether basal extracellular levels of adenosine are decreased in aortic
and renal arteriolar SMCs cultured from SHR compared with cells obtained from normotensive Wistar-Kyoto (WKY) rats. Because our results indicated that extracellular adenosine levels are dysregulated by SHR SMCs, a second aim of this investigation was to determine why extracellular adenosine levels are reduced in SHR SMCs. Finally, a third aim was to determine whether the diminished extracellular levels of adenosine contribute to the enhanced proliferative response of SHR SMCs.

Methods

Materials

DMEM, DMEM/F12 medium, HBSS, penicillin, streptomycin, 0.25% trypsin-EDTA solution, and all tissue culture ware were purchased from Gibco Laboratories. FCS was obtained from HyClone Laboratories Inc. cAMP, 3-isobutyl-1-methylxanthine (IBMX), erythro-9-(2-hydroxy-3-nonyl) adenosine hydrochloride (EHNA), 2-chloroadenosine, and adenosine were purchased from Sigma Chemical Co. Idoxutubercidin (IDO) was purchased from Research Biochemicals International.

Culture of Aortic and Renal Arteriolar SMCs

Aortic and renal arteriolar SMCs from SHR and normotensive WKY male rats. Aortic SMCs were cultured by the explant method as we previously described. Renal arteriolar (primarily afferent arteriolar and interlobular) SMCs were cultured as explants from preglomerular arterioles magnetically isolated after infusion of a ferrosoferric oxide suspension into the aorta proximal to the renal arteries as we described in detail. The purity of aortic and arteriolar SMCs was characterized as we described in detail previously.

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Protocol for Basal and cAMP-Mediated Synthesis of Adenosine by SMCs

Once confluent, the culture medium was removed, and the culture dish was washed twice with HEPES (25 mmol/L)-buffered HBSS. SMCs then were treated with 0.5 mL of PBS buffered with HEPES (25 mmol/L) and NaHCO3 (13 mmol/L). To evaluate basal adenosine levels achieved by SHR and WKY SMCs, we assessed the levels of adenosine in the medium of confluent monolayers of aortic and renal arteriolar SMCs treated for 12 to 24 hours with buffered PBS containing or lacking EHNA (10 μmol/L; adenosine deaminase inhibitor), IDO (0.1 μmol/L; adenosine kinase inhibitor), or EHNA plus IDO. To determine adenosine levels achieved by SHR and WKY SMCs when the cAMP-adenosine pathway was activated, we assessed the levels of adenosine in the medium of aortic and renal arteriolar SMCs incubated for various lengths of time with or without various concentrations of exogenous cAMP. In addition, exogenous cAMP was stimulated by treating cells with isoproterenol (1 μmol/L; β-adrenergic receptor agonist) for 20 minutes, and the levels of extracellular adenosine were measured. To determine whether IBMX (1 μmol/L) inhibits the cAMP-adenosine pathway and equalizes extracellular adenosine levels in SHR versus WKY SMCs, cells were incubated in buffered PBS in the absence or presence of cAMP (30 μmol/L). IBMX (a phosphodiesterase inhibitor), or IBMX plus cAMP. After 1 hour of incubation under standard tissue culture conditions, the medium was collected. In all of the aforementioned studies, medium was transferred immediately into ice-cold tubes and frozen at −70°C until adenosine levels were estimated. In all of the above-described experiments, after collection of medium, cells were trypsinized and counted on a Coulter counter to normalize the levels of adenosine to cell number. To ensure that the various treatments did not cause cell death, trypan blue exclusion assays were used to evaluate the viability of SMCs treated in parallel.

Protocols for the Catabolism of Exogenous Adenosine by SHR and WKY Aortic SMCs

Aortic and renal arteriolar SMCs from SHR and WKY rats were grown to confluence in 75-cm2 culture flasks and then trypsinized to obtain cell suspensions. The cell suspensions were washed once with DMEM supplemented with 10% FCS and subsequently with PBS buffered with HEPES. The cells were suspended in HEPES-buffered PBS and disrupted by mild sonication. Aliquots of disrupted cells were transferred into microfuge tubes and incubated with 1 mmol/L adenosine for 0.25, 0.5, 1, 2, and 4 hours in the presence and absence of EHNA (10 μmol/L) or IDO (1 μmol/L). After incubation, the tubes were centrifuged, and 500-μL aliquots of the supernatant were collected and stored at −70°C until adenosine levels were analyzed. Total protein was assayed in parallel in aliquots of disrupted cells, and adenosine levels were normalized to the amount of protein. Adenosine deaminase activity was calculated by subtracting the rate of loss of adenosine in the presence of EHNA from the rate of loss of adenosine in the absence of EHNA. Adenosine kinase activity was calculated by subtracting the rate of loss of adenosine in the presence of IDO from the rate of loss of adenosine in the absence of IDO.

Adenosine Analysis

Adenosine levels in the samples were analyzed by high-pressure liquid chromatography with either fluorescence or ultraviolet detection via our previously described methods. Adenosine levels were quantified as the area under the chromatographic peak, the absolute amount in each sample was calculated from a standard curve of adenosine analyzed in parallel, and the values were normalized to cell number and presented as nmol/L or μmol/L per 10^6 cells.

Cell Proliferation Studies

SMCs were suspended in complete culture medium containing 10% FCS and plated in a 24-well culture dish at a density of 5×10^4 cells/well. After 5 hours of incubation, the cells were fed complete culture medium containing 0.25% albumin for 48 hours to growth-arrest the cells. To study the effects of exogenous and endogenous (SMC-derived) adenosine on FCS-induced cytokinesis, growth-arrested SMCs were treated every 24 hours for 4 days with complete culture medium containing 2.5% FCS un-supplemented or supplemented with EHNA (10 μmol/L), adenosine (10 μmol/L), adenosine plus EHNA, IDO (0.1 μmol/L), adenosine plus IDO, or 2-chloroadenosine (10 μmol/L). On day 5, the cells were dislodged with trypsin-EDTA, diluted in Isoton-II, and counted with a hemocytometer-calibrated Coulter counter. Aliquots from 3 to 4 wells were counted for each group. Three independent experiments were performed with separate cultures.

Statistics

Results are presented as mean±SEM of separate SMC preparations. Statistical analysis was performed with ANOVA, Student’s t test, or Fisher’s least significant difference test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Significant basal levels of adenosine were detected in the medium obtained from cultures of renal arteriolar and aortic SMCs incubated for 12 hours (Figure 1, top and middle, respectively; labeled Basal). Compared with WKY SMCs, the basal levels of extracellular adenosine generated by SHR SMCs were decreased by ~50% in both renal arteriolar and aortic SMCs (Figure 1, top and middle, respectively; labeled Basal). Treatment of renal arteriolar and aortic SMCs for 12 hours with IDO (Figure 1, top and middle, respectively; labeled IDO) increased extracellular levels of adenosine; even in the presence of IDO, however, extracellular adenosine levels remained depressed in SHR compared with WKY SMCs. Treatment of renal arteriolar and aortic SMCs with EHNA or EHNA plus IDO also increased extracellular adenosine levels (Figure 1, top and middle, respectively; labeled EHNA and EHNA+IDO, respectively). Moreover, in the presence of EHNA or EHNA plus IDO, extracellular...
Adenosine levels were similar in SHR compared with WKY SMCs, regardless of whether the incubation time was 12 or 24 hours (Figure 1, bottom).

Activation of the extracellular cAMP-adenosine pathway in renal arteriolar SMCs (Figure 2) by addition of exogenous cAMP caused a concentration-dependent (Figure 2, top) and time-dependent (Figure 2, bottom) increase in extracellular levels of adenosine. As in renal arteriolar SMCs, activation of the extracellular cAMP-adenosine pathway in aortic SMCs by addition of exogenous cAMP caused a concentration-dependent and time-dependent increase in extracellular levels of adenosine (data not shown). At all concentrations of cAMP and at all time points after the addition of cAMP, however, extracellular adenosine levels generated by SHR SMCs were ∼50% lower than the levels generated by WKY SMCs.

Activation of the extracellular cAMP-adenosine pathway by the addition of isoproterenol (20 minutes) also increased extracellular levels of adenosine in renal arteriolar and aortic SMCs (Figure 3, top and bottom, respectively). Adenosine levels remained depressed in SHR SMCs, however, even in the presence of isoproterenol. In renal arteriolar SMCs (Figure 4), IBMX (1 mmol/L) attenuated the conversion of exogenous cAMP to adenosine but did not equalize extracellular levels of adenosine in SHR versus WKY SMCs. Similar modulatory effects of IBMX on the conversion of cAMP to adenosine were also observed in aortic SMCs (data not shown).

A time-dependent decrease in adenosine levels was observed when disrupted SHR and WKY aortic SMCs were incubated with exogenous adenosine (Figure 5, bottom). SHR

**Figure 1.** Extracellular adenosine levels generated by renal arteriolar SMCs (RASMCs, top) and aortic SMCs (middle and bottom) from SHR and WKY rats. Aortic SMCs or RASMCs were treated for 12 hours (all panels) or 24 hours (bottom only) with PBS (Basal) containing or lacking IDO (0.1 μmol/L; adenosine kinase inhibitor), EHNA (10 μmol/L; adenosine deaminase inhibitor), or EHNA plus IDO. Results represent mean±SEM of 6 to 9 separate cultures of SMCs. *P<0.01 vs WKY or levels at time 0.

**Figure 2.** Concentration- and time-dependent metabolism of cAMP to adenosine by renal arteriolar SHR and WKY SMCs. For concentration-dependent metabolism of cAMP to adenosine, SMCs were incubated for 1 hour with PBS containing or lacking 0.01 to 30 μmol/L cAMP. For time-dependent metabolism of cAMP to adenosine, cells were treated with cAMP (30 μmol/L) for 2.5 to 120 minutes. Results represent mean±SEM of 6 to 9 separate cultures of renal arteriolar SMCs. †P<0.01 vs SHR; *P<0.05 vs PBS or levels at time 0 minutes in same strain. Similar results were observed in aortic SMCs.

**Figure 3.** Effects of isoproterenol (ISO; 1 μmol/L for 20 minutes) on extracellular adenosine levels generated by renal arteriolar (top) and aortic (bottom) SMCs cultured from WKY and SHR. Results represent mean±SEM of 6 to 9 separate cultures of aortic and renal arteriolar SMCs. †P<0.01 vs SHR; *P<0.05 vs PBS in same strain.

**Figure 4.** Metabolism of cAMP (30 μmol/L for 1 hour) to adenosine in the presence and absence of IBMX (phosphodiesterase inhibitor; 1 mmol/L) by renal arteriolar SMCs cultured from WKY rats and SHR. Results represent mean±SEM of 6 to 9 separate cultures of renal arteriolar SMCs. †P<0.01 vs SHR; *P<0.05 vs PBS or IBMX in same strain. Similar results were observed in aortic SMCs.
aortic SMCs catabolized exogenous adenosine at approximately twice the rate of WKY aortic SMCs (Figure 5, left). The increased rate of adenosine metabolism by SHR aortic SMCs was blocked by EHNA (Figure 5, middle) but not by IDO (Figure 5, right). Like aortic SMCs, renal arteriolar SMCs from SHR catabolized adenosine at a higher rate, and these effects were abolished by EHNA but not IDO (data not shown). Consistent with these observations, compared with WKY, the adenosine deaminase but not adenosine kinase activity was increased by ≈3-fold in aortic SMCs from SHR (Figure 5, bottom).

Renal arteriolar and aortic SHR SMCs proliferated at a significantly faster rate in response to FCS compared with WKY SMCs (Figure 6, top and bottom, respectively). Treatment with adenosine, 2-chloroadenosine, IDO, EHNA, IDO plus adenosine, EHNA plus adenosine, and EHNA plus IDO inhibited FCS-induced growth of renal arteriolar and aortic SMCs. Although adenosine, IDO, and adenosine plus IDO inhibited FCS-induced proliferation of SMCs, the growth of SMCs remained significantly higher in SHR cells than in WKY cells. In contrast, the increased proliferation of SHR SMCs in response to FCS was equalized by EHNA, 2-chloroadenosine, adenosine plus EHNA, and EHNA plus IDO.

**Discussion**

The key observation in the present study is that compared with WKY SMCs, SHR SMCs, whether aortic or renal arteriolar, generate lower extracellular levels of adenosine. An important question is what is the cause of the lower extracellular adenosine levels in SHR SMCs. Theoretically, the lower levels of extracellular adenosine could be due to a decrease in adenosine biosynthesis, an increase in adenosine catabolism, and/or an alteration in adenosine transport.

With regard to biosynthesis, adenosine is synthesized via 4 pathways: (1) the intracellular ATP pathway involves sequential dephosphorylation of ATP to adenosine within the cell; (2) the extracellular ATP pathway is mediated by extracellular conversion of released ATP to adenosine by ectoenzymes; (3) the transmethylation pathway entails the hydrolysis of 5′-adenosyl-L-homocysteine to L-homocysteine and adenosine; and (4) the cAMP-adenosine pathway involves the egress of cAMP to the cell surface, followed by conversion of cAMP to AMP by ectophosphodiesterase and conversion of AMP to adenosine by ecto-5′-nucleotidase.

The first pathway is triggered when energy demand exceeds energy supply; the second pathway is activated when cells are injured and release ATP/ADP; the rate of the third pathway is determined by the methylation requirements of the cell; and the fourth pathway is stimulated by activation of adenyl cyclase. Because in these experiments the cells were not exposed to hypoxia, injury, or conditions that would alter methylation reactions, we focused our attention on the cAMP-adenosine pathway as a possible site of dysregulation of extracellular adenosine levels.

In the present study, the cAMP-adenosine pathway was activated by either addition of exogenous cAMP to the SMCs or by stimulation of endogenous cAMP production with the β-adrenergic receptor agonist isoproterenol. These experiments demonstrate that the ability of SHR SMCs to generate extracellular levels of adenosine is attenuated when the cAMP-adenosine pathway is activated by either exogenous or endogenous (isoproterenol-induced) cAMP. One interpretation of these results is that the cAMP-adenosine pathway is defective in SHR SMCs. However, this interpretation is not supported by the observation that a concentration of IBMX that blocks the conversion of cAMP to adenosine does not equalize extracellular levels of adenosine in SHR versus WKY SMCs. Also, we previously demonstrated that formation of cAMP in response to β-adrenergic receptor activation with isoproterenol is not attenuated in SHR SMCs.
fore, a defect in the β-adrenergic response does not contribute to the decreased formation of extracellular adenosine in SHR SMCs.

Another interpretation is that the cAMP-adenosine pathway is normal in SHR SMCs, but the adenosine is more rapidly eliminated from the extracellular compartment. The elimination of adenosine from the extracellular compartment is mediated by facilitated transport of adenosine into cells followed by metabolism of adenosine to inosine by adenosine deaminase. Hence, it is possible that increased activity of adenosine deaminase and/or adenosine kinase contributes to the decrease in the levels of extracellular adenosine. To test this hypothesis, the levels of extracellular adenosine were measured in the presence of EHNA (an adenosine deaminase inhibitor), IDO (an adenosine kinase inhibitor), or EHNA plus IDO. Importantly, EHNA in either the presence or absence of IDO, but not IDO alone, equalized extracellular adenosine levels in SHR versus WKY SMCs. These findings are consistent with the conclusion that the decreased extracellular levels of adenosine in SHR SMCs is mediated by an increased activity of adenosine deaminase, but not adenosine kinase. This conclusion is further supported by our observations that SHR SMCs catalyze adenosine more rapidly than do WKY SMCs, and this enhanced catalysis is blocked by EHNA but not by IDO.

The conclusion that increased activity of adenosine deaminase contributes to the decrease in extracellular levels of adenosine in SHR SMCs is also supported by recent findings from our laboratory that plasma adenosine deaminase activity is increased with age in SHR but not in WKY rats. Moreover, administration of the adenosine deaminase inhibitor EHNA to aging SHR reduces mean arterial blood pressure from 159±2 to 115±8 mm Hg but does not affect mean arterial blood pressure in aged-matched WKY rats.

Because adenosine deaminase is mostly a cytosolic enzyme, it is conceivable that enhanced adenosine transport, not the activity of adenosine deaminase per se, is the cause of the increased catalysis of adenosine. If this were the case, however, then extracellular levels of adenosine would not be normalized by EHNA alone, because increased transport would deliver adenosine to the highly efficient enzyme adenosine kinase. Also, the fact that differential catalysis of adenosine is observed in cells with disrupted membranes rules out the possibility that differences in adenosine metabolism are due to alterations in adenosine transport.

In previous studies, we demonstrated that SMC-derived adenosine inhibits SMC growth. Because it is well known that SHR SMCs proliferate more rapidly than do WKY SMCs, we evaluated whether the decreased generation of extracellular adenosine by SHR SMCs is responsible for the increased proliferation of SHR SMCs. These experiments demonstrated that (1) compared with WKY SMCs, FCS-induced proliferation of aortic and renal arteriolar SHR SMCs is enhanced; (2) the effect of FCS on WKY versus SHR cell growth is equalized by blockade of adenosine deaminase with EHNA and by administration of 2-chloroadenosine (an adenosine analogue that is less susceptible to adenosine deaminase); and (3) the effect of FCS on WKY versus SHR cell growth is not equalized by adenosine (highly susceptible to adenosine deaminase) or IDO (an adenosine kinase, not adenosine deaminase, inhibitor). These findings suggest that the increased proliferation of SMCs in SHR is due to decreased availability of active adenosine caused by increased SMC adenosine deaminase activity.

Although it was not the purpose of the present study, we observed that adenosine levels tended to be higher at baseline and during various treatments in SMCs from conduit arteries than in SMCs from resistance arteries. This difference could potentially be due to the existence of different modes of cell growth, i.e., hypertrophy versus hyperplasia, between the 2 SMC types, as proposed by Owens. Whether endogenous adenosine plays a role in determining whether SMCs grow by hypertrophy versus hyperplasia is an important issue worthy of further exploration.

In conclusion, we provide the first evidence that (1) compared with normotensive WKY rats, extracellular adenosine levels generated by aortic and renal afferent arteriolar SHR SMCs are decreased; (2) increased adenosine deaminase activity mediates the decreased extracellular levels of adenosine in both aortic and renal arteriolar SHR SMCs; and (3) treatment with an adenosine deaminase inhibitor normalizes the differences in extracellular adenosine levels and cell proliferation observed between SHR and WKY SMCs. Our findings suggest that adenosine produced by SMCs may play a vital role as a local antigrowth factor, that dysregulation of extracellular adenosine levels in genetic hypertension may contribute to vascular disease, and that adenosimetic drugs may prove beneficial in preventing vascular sequelae in hypertension.

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References


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