Direct Vasoconstriction Evoked by A₁-Adenosine Receptor Stimulation in the Cutaneous Microcirculation

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To determine whether the vasoconstriction evoked by A₁-adenosine receptor stimulation in the skin circulation caused the release of other substances or whether A₁ stimulation modulated the vasoconstriction evoked by other compounds, a potent A₁-selective, synthetic agonist, cyclohexyladenosine (CHA), was topically applied simultaneously with several different vasoconstrictor agonists or antagonists. CHA was chosen instead of adenosine because the parent compound is metabolized quickly and also does not discriminate between A₁ or A₂ receptors. Blood flow was calculated from measurements of arteriolar diameter (40–60 μm) and red blood cell velocity using intravital videomicroscopy. Responses were recorded only in a steady state. The dose-related vasoconstriction evoked by CHA (ED₉₀ 2.07±0.80 nM; half-minimal response, 93±1%) was not attenuated by antagonists to norepinephrine (phenolamine [11 μM] or prazosin [10 μM]), serotonin (methysergide [11 μM]), angiotensin II (saralasin [0.11 μM]), thromboxane (SK&F 88046 [13 μM]), or leukotrienes (SK&F 102922 [2.1 μM]). The vasoconstriction evoked by 2 nM CHA was attenuated by a subthreshold concentration (1 nM) of norepinephrine, whereas the vasoconstriction evoked by 0.1–1 μM norepinephrine was attenuated by a threshold concentration (1 nM) of CHA. Higher concentrations (10–100 nM) of CHA had no additional inhibitory effect. In contrast, CHA had no effect on the vasoconstrictions evoked by angiotensin II (10 nM or 1 μM) or serotonin (100 or 500 nM). Therefore, it is unlikely that A₁-receptor stimulation causes the release of norepinephrine, serotonin, angiotensin, thromboxane, or leukotrienes in the skin microcirculation. Because norepinephrine attenuated the vasoconstriction evoked by CHA while CHA attenuated that evoked by norepinephrine, there appears to be a negative interaction between α-adrenergic and A₁-adenosinergic receptors. (Circulation Research 1991;68:683–688)

The biological effects of adenosine (ADO) are mediated primarily by two types of cell surface receptors. A₂ receptors are found in almost every vascular bed of every species and mediate vasodilation. In contrast, A₁ receptors are relatively rare in vascular tissues and are localized primarily in neuronal tissue, where they have an important neuromodulatory role.¹

 Whereas A₂-receptor stimulation probably evokes a direct vasodilation linked to activation of adenylate cyclase,² the mechanism for the vascular effects evoked by A₁ stimulation is debatable. ADO-mediated vasoconstriction is attributed to the release of angiotensin II in the intact kidney,³–⁸ serotonin in peripheral arteries,⁹,¹⁰ or eicosanoids in the lung.¹¹ Furthermore, ADO can modulate adrenergic neurotransmission¹²–¹⁴ or potentiate the tonic vasoconstriction evoked by angiotensin.⁴ Thus, there is compelling evidence that intermediate factors contribute to the vascular responses evoked by A₁-receptor stimulation. Alternatively, in isolated renal tissues, A₁-receptor agonists evoke vasoconstriction either in single pass perfusion systems or in the absence of renin substrate and simultaneously inhibit renin secretion.¹⁵–¹⁸ These observations are consistent with direct activation of A₁ receptors on vascular smooth muscle.

We previously reported temperature-sensitive A₁ receptor-mediated vasoconstriction in the subcutaneous microcirculation. The purposes of this study were to determine whether A₁-receptor stimulation caused the release of other vasoactive substances or altered the response evoked by other agonists.

Materials and Methods

General

Male Syrian golden hamsters (100–120 g) were anesthetized with sodium pentobarbital (10–20 mg
per 100 g i.p.). The trachea was cannulated, and respiration was spontaneous on room air supplemented with O2. Catheters were placed in a femoral artery and vein. A solution of pentobarbital in saline (10 mg/ml) was administered continuously through the vein (0.5 ml/hr) to maintain anesthesia and to supplement surgical and evaporative fluid losses. Systemic blood pressure was measured continuously through the arterial catheter and ranged 80–120 mm Hg during the 2–4-hour duration of the typical experiment. An experiment was terminated if arterial blood pressure decreased below 70 mm Hg. Rectal temperature was monitored continuously and maintained at 37± 1°C with a servo-controlled heat lamp.

The skin microcirculation was prepared with a simple technique. Briefly, the animal was positioned laterally on a modified microscope stage. The dorsal skin was elevated from the back and secured with sutures. A 1.5-cm-diameter section of skin and underlying tissue was excised, which exposed the subcutaneous tissue under the intact epidermis of the opposite side. The connective tissue surrounding the blood vessels was dissected and removed from the exposed area. All bleeding points were ligated.

Throughout the surgical and experimental procedures, the tissue was suffused with a bicarbonate-buffered Ringer’s solution equilibrated with a gaseous mixture of 5% CO2–95% N2 (pH 7.4). Local skin temperature was monitored continuously in the vicinity of the observed arteriole and maintained at 32±1°C by varying the flow (3–7 ml/min) of the heated (37°C) suffusate.

The preparation was transilluminated with white light from a stable DC power supply (Hewlett-Packard 6247B, Palo Alto, Calif.) with a heat absorption filter in series with the light source. Observations were made with a long–working-distance objective (Leitz L-20X, 0.32 numerical aperture), a long–working-distance condensor (Leitz L-20, 0.45 numerical aperture), and a microscope equipped with a trinocular head (Leitz Laborlux II, Rockleigh, N.J.). The preparation was visualized with a video camera (model WV-5000, Panasonic, Secaucus, N.J.) that transmitted a signal to a video micrometer (model IV-550, FOR, A, Los Angeles) and color monitor (model 7280UM, JVC, Tokyo).

Arteriolar diameter was measured continuously from the analog voltage output of the video micrometer, which was calibrated to an accuracy of 1 μm. Red blood cell velocity was measured continuously with a velocimeter (model 100, Optech Instruments, Durham, N.C.) in series between the microscope and video camera. This instrument was calibrated to an accuracy of 1 mm/sec. Arteriolar blood pressure, arteriolar diameter, and red blood cell velocity were recorded continuously on a dynograph. Blood flow was calculated off-line from the product of arteriolar diameter and red blood cell velocity.

**Experimental Protocol**

After a 30–40-minute postsurgical equilibration, arterioles with resting diameters of 40–60 μm and resting blood flow of 15–35 nl/sec were tested for the level of spontaneous vasomotor tone with the topical application of 100 μM ADO. The typical response was more than a 50% increase in diameter and blood flow that was rapidly reversible on washout of ADO. Lack of reactivity indicated partial vascular occlusion, surgical damage, or systemic hypotension and was cause for rejection of approximately 10% of the preparations.

Two sets of experiments were performed. In the first set, ascending amounts of the synthetic A1 agonist cyclohexyladenosine (CHA) were added to a suffusate (final concentration, 0.0001–1 μM) that contained one of the following: phenolamine (non-selective α-adrenergic antagonist), prazosin (α1-selective adrenergic antagonist), saralasin (angiotensin antagonist), or methysergide (serotonin antagonist). The effective concentration was determined by comparing the vasoconstrictor response evoked by the appropriate agonist with and without the corresponding antagonist. In addition, two novel compounds were tested: SK&F 88046 (thromboxane antagonist) and SK&F 102922 (leukotriene antagonist). At the suffusate concentrations used in these experiments, none of the antagonists had an effect on resting arteriolar diameter per se.

In the second set of experiments, ascending amounts of norepinephrine, serotonin, or angiotensin were added to a suffusate (final concentrations, 0.01–1 μM, 0.01–0.1 μM, 0.0001–1 μM, respectively) that contained 1–100 nM CHA.

After 5–10 minutes of continuous topical application, data were collected. Previous work has established this interval for a steady-state response. The substance then was removed from the suffusate, and measurements were continued until the hemodynamics stabilized at a posttreatment baseline. Preparations were rejected if pretreatment and posttreatment baselines differed by more than 5% after 30 minutes of washout.

All drugs were purchased from Sigma Chemical Co., St. Louis, except SK&F 88046 (N,N'-bis[7-{3-chlorophenyl}amino]sulfonfonyl]-1,2,3,4-tetrahydroisquinolyl disulfonilylimide) and SK&F 102922 (4,6-dithia-5-{2-(8-phenylocetyl)-phenyl}-nonanediolic acid), which were gifts from Dr. David Brooks at SmithKline Beecham Pharmaceuticals, Swedeland, Pa., and methysergide, which was purchased from Sandoz, East Hanover, N.J.

**Statistical Analysis**

The order of application of the various drugs was randomized. The percent change is expressed relative to the steady-state baseline. Values of ED50 were calculated from the dose–response curves as the concentration required to evoke half-minimal decreases in diameter and blood flow. All data are expressed as mean±SEM. Treatment effects were compared with factorial analysis of variance and the Wilcoxon two-sample test at the 95% confidence level.
Results

Figure 1 shows the vascular caliber changes evoked by the topical application of a suffusate containing CHA alone or CHA plus various vasoconstrictor antagonists. The ED_{50} with CHA alone (n=6) was 2.07 \pm 0.80 nM at a half-minimal diameter decrease to 93 \pm 1\% of control. These results are virtually identical to those reported earlier.\textsuperscript{19,20}

CHA evoked similar vasoconstrictor responses when the suffusate contained phenolamine (3 \mu g/ml; 11 \mu M; n=7), saralasin (0.1 \mu g/ml; 0.11 \mu M; n=5), methysergide (4 \mu g/ml; 11 \mu M; n=7), SK&F 88046 (10 \mu g/ml; 13 \mu M; n=5), or SK&F 102922 (1 \mu g/ml; 2.1 \mu M; n=5). In contrast to the lack of effect of these antagonists, the vasoconstrictions evoked by CHA are attenuated by the methylxanthine 8-phenyl theophylline\textsuperscript{19,20} and by the A\textsubscript{1}-selective antagonist xanthine amine congener.\textsuperscript{21}

Effective antagonist concentrations in these conditions were verified in pilot experiments. Norepinephrine (100 nM and 1 \mu M) alone reduced diameter to 90\% \pm 5\% and 48\% \pm 11\% of control (n=5); with phenolamine (11 \mu M), the corresponding decreases were 96\% \pm 1\% and 85\% \pm 2\% of control. Serotonin (1 \mu M and 10 \mu M) alone reduced diameter to 88\% \pm 5\% and 82\% \pm 2\% of control (n=3); with methysergide (11 \mu M), the corresponding responses were 97\% \pm 2\% and 94\% \pm 3\% of control. Angiotensin alone (0.1 nM) reduced diameter to 59\% \pm 2\% of control (n=4); with saralasin (0.11 \mu M), the corresponding response was 95\% \pm 2\% (n=3).

Figure 2 shows the effect of CHA on the vasoconstriction produced by norepinephrine. At 0.1 and 1 \mu M, norepinephrine caused constrictrions averaging 20\% (n=20) and 60\% (n=20), with corresponding blood flow decreases of 40\% and 90\%. With 1 nM CHA (n=6) in the suffusate, the vasoconstriction evoked by both norepinephrine concentrations was significantly attenuated. There was no additional inhibitory effect of CHA at 10 nM (n=7) and 100 nM (n=6). It is conceivable that the direct vasoconstrictor response evoked by CHA at the higher concentrations might have obscured an inhibitory effect on the norepinephrine response.

![Figure 1: Effects of receptor antagonists on dose-related vasoconstriction evoked by cyclohexyladenosine (CHA). All substances were applied continuously via suffusate, and only steady-state responses were recorded. Suffusate concentrations of the antagonists blocked the effect of the corresponding agonist but had no effect on basal vasoconstrictor tone. There was no significant attenuation of the CHA-evoked response by any of the antagonists, which is consistent with the conclusion that A\textsubscript{1}-mediated vasoconstriction does not depend on the release of norepinephrine, serotonin, angiotensin, thromboxane, or leukotriene.](image)

![Figure 2: Effect of cyclohexyladenosine (CHA) on dose-related vasoconstriction evoked by norepinephrine. CHA (1 nM) significantly attenuated the reduction in diameter and blood flow caused by the topical application of norepinephrine. There was no additional inhibitory effect of 10–100 nM CHA, but the vascular response evoked by CHA per se could have obscured an inhibitory effect on the norepinephrine response. In addition, at higher concentrations, the selectivity of CHA for A\textsubscript{1} vs. A\textsubscript{2} receptors can be reduced, and CHA becomes a substrate for some metabolic processes. For details, see text.](image)
Figure 3: Effect of norepinephrine and prazosin on the vasoconstriction evoked by cyclohexyladenosine (CHA). Responses were significantly potentiated by prazosin and significantly attenuated by norepinephrine. In the absence of CHA, neither substance evoked a vascular response.

Figure 4: Effect of cyclohexyladenosine (CHA) on dose-related vasoconstriction evoked by angiotensin or serotonin. In contrast to data shown in Figure 2, vasoconstrictions evoked by antigen or serotonin were not altered by 1 nM CHA, which implies specificity to the interaction between CHA and the adrenergic system.

Discussion

We reported previously that exogenous ADO or its A2-selective synthetic analogue CHA evoked a temperature- and methylxanthine-sensitive, receptor-mediated vasoconstriction in the skin microcirculation whose magnitude was small relative to that of angiotensin or norepinephrine.\textsuperscript{19,20} The major new findings from this study are that 1) \(\alpha\)-adrenergic antagonists potentiate the vasoconstriction evoked by CHA, 2) CHA attenuates the vasoconstriction evoked by norepinephrine and vice versa, and 3) the vasoconstriction evoked by CHA is not attenuated by antagonists to norepinephrine, serotonin, angiotensin II, thromboxanes, or leukotrienes. These results are consistent with the conclusions that A2 stimulation causes a direct constriction not dependent on the release of other agonists and that there is a negative interaction between the adrenergic and adenosinergic receptor systems.

Critique

It is difficult to understand how two contractile agonists (i.e., norepinephrine and ADO) that apparently share adenylate cyclase as a second messenger can antagonize one another. Therefore, these present observations must be considered phenomenological until the mechanism is defined.

A1- and A2-ADO receptors have been defined operationally and distinguished by the potency order of a series of synthetic analogues, by the inhibitory effect of specific antagonists, and by opposing effects of the agonists on adenylate cyclase. In this present study, it was assumed responses evoked by CHA reflected A1-receptor stimulation, because our previous work\textsuperscript{19,20} showed that CHA satisfied several generally accepted criteria for evoking A1 receptor-mediated responses.\textsuperscript{22-24} Nevertheless, it should be emphasized that CHA is an A1-selective, not an A2-specific, agonist; its affinity is in the low nanomolar range, and its A1/A2 selectivity ratio is approximately 400.\textsuperscript{25}

In general, it is difficult to interpret responses evoked by ADO because it is rapidly metabolized and because it is a nonselective receptor agonist. For this reason, most investigators probe ADO receptor-mediated responses with synthetic agonists. Recently, it was suggested that CHA and several other synthetic agonists are poor candidates for stimulating ADO receptors in vivo because of potential metabolism by adenosine kinase; the IC\textsubscript{50} for inhibiting
Adenosine phosphorylation is 220 μM, and the EC\textsubscript{50} for stimulating phosphorylation is 30 μM.\textsuperscript{26} It is unlikely that metabolic effects of CHA interfered with these present observations, because the concentrations were more than 1,000-fold lower (1–2 nM, Figures 2 and 3). In addition, the vasoconstriction evoked by CHA and ADO in the skin microcirculation is inhibited by methylxanthines and is freely and rapidly reversible,\textsuperscript{19,20} which diminishes the likelihood of an unusual metabolic effect of CHA. Nevertheless, these present results provide no clues as to the underlying mechanism.

Another potential limitation was the interference caused by anesthesia. Some ADO-mediated responses are anesthetic-sensitive and some are not. For example, ADO-evoked hypotension and bradycardia in pentobarbital-anesthetized rats are diminished compared with the conscious state,\textsuperscript{27} whereas the renal and pulmonary vasoconstrictor responses and the renin inhibitory effect of ADO are not.\textsuperscript{5,6,11}

Other Studies

ADO-mediated vasoconstriction has been reported in the hamster skin microcirculation,\textsuperscript{19,20} rat femoral and tail artery,\textsuperscript{9,10} the sheep pulmonary artery,\textsuperscript{11} the cutaneous circulation of humans,\textsuperscript{28} and kidney vasculature of several species.\textsuperscript{3–8,15–18,29} The mechanism is most often attributed to the release of other vasoactive substances.

In the isolated perfused rat hind limb, single injections of ADO induced a long-lasting vasoconstriction preceded by a transient vasodilation.\textsuperscript{9} The vasoconstriction was methysergide-sensitive, which implicated serotonin. A similar, methysergide-sensitive response was observed in the isolated, perfused rat tail artery.\textsuperscript{10} However, two observations in these studies argue against direct involvement of A\textsubscript{1} receptors. First, tachyphylaxis was observed in both these preparations\textsuperscript{9,10} but not in the skin\textsuperscript{19,20} and renal\textsuperscript{3,4,7,15,18} vasculatures, where A\textsubscript{1} receptors have been well documented. Second, vasoconstrictor responses were observed at 100 μM to 1 mM in both these preparations\textsuperscript{9,10} but effects mediated by extracellular A\textsubscript{2} receptors or the intracellular P site would predominate at concentrations greater than 1 μM.\textsuperscript{22–25,30,31}

An increase in pulmonary pressure after ADO administration was reported in 1929\textsuperscript{32} but was interpreted as secondary to an increase in flow rather than to pulmonary vasoconstriction. Recently, it was observed that ADO-evoked vasoconstriction in the lung was abolished by cyclooxygenase inhibition,\textsuperscript{11} which implicated an eicosanoid. Because the increase in pulmonary pressure was blunted when ADO was injected into the left atrium, the authors concluded that ADO-induced vasoconstriction was generated in the lung via specific ADO receptors rather than from central or reflex mechanisms.\textsuperscript{11} However, tachyphylaxis was observed after ADO infusion, so one might question whether A\textsubscript{1} receptors per se were activated.

**Interpretation**

The role of angiotensin in the receptor-mediated ADO-evoked vasoconstriction in the kidney remains controversial after more than 20 years of active investigation.\textsuperscript{3–8,15–18,29} These present results argue against a role for angiotensin in the A\textsubscript{1} response in the skin, because saralasin did not attenuate the CHA-evoked vasoconstriction (Figure 1) and because CHA had no effect on the vasoconstriction evoked by angiotensin (Figure 4).

Because leukotriene, thromboxane, and serotonin antagonists did not alter the response evoked by CHA (Figure 1) and because CHA had no effect on the response evoked by serotonin (Figure 4), it is unlikely that ADO-induced vasoconstriction in the skin is caused by the release of an eicosanoid or serotonin, as in the lung\textsuperscript{11} or peripheral arteries.\textsuperscript{9,10}

Norepinephrine, but not the adrenergic antagonists phentolamine and prazosin, attenuated CHA-induced responses. Furthermore, CHA attenuated the dose-related vasoconstriction evoked by norepinephrine (Figures 1–3). These results are consistent with a negative interaction between α-adrenergic receptors and A\textsubscript{1}-adenosinergic receptors but are not consistent with an obligatory role for norepinephrine in ADO-induced vasoconstriction.

It is well established that ADO and adenine nucleotides modulate adrenergic neurotransmission\textsuperscript{33,34} and that nerve stimulation or exogenous norepinephrine releases physiological levels of ADO (0.1–1 μM) from subcutaneous adipose tissue.\textsuperscript{35} In the portal circulation, ADO can exert prejunctional inhibition at less than 10 μM or postjunctional enhancement of sympathetic activity at greater than 10 μM.\textsuperscript{1,14} In the mesentery,\textsuperscript{13} exogenous ADO antagonized vasoconstriction evoked by norepinephrine and sympathetic nerve stimulation, which suggests similar effects of ADO at the presynaptic and postsynaptic levels.

Because α\textsubscript{1}-adrenergic receptors are postsynaptic\textsuperscript{36} and because prazosin, an α\textsubscript{1}-antagonist, interfered with the response evoked by CHA (Figure 3), it is logical to speculate that ADO A\textsubscript{1} receptors are localized at a postsynaptic site in the cutaneous vasculature. In the kidney, A\textsubscript{1} receptors are probably postsynaptic, and their activation leads to vascular smooth muscle contraction linked to Ca\textsuperscript{2+} influx through potential-operated ion channels.\textsuperscript{16–18} The cellular mechanism underlying the interaction of adenosinergic and adrenergic systems in subcutaneous microcirculation is unclear from this present study, but there is evidence for a common effector mechanism of α- and A\textsubscript{1} receptors, that is, phosphatidylinositol turnover, adenylate cyclase inhibition, and mobilization of cytosolic calcium.\textsuperscript{36–38}

An additional significance of this study is that ADO evokes cutaneous vasoconstriction in humans\textsuperscript{28} and that vasoconstrictor responses of human subcutaneous resistance arteries are mediated by α-adrenoceptors.\textsuperscript{39}
In conclusion, activation of A1 receptors probably causes a direct receptor-mediated vasoconstriction, based on our previous work and on the present observations that antagonists to norepinephrine, angiotensin, serotonin, leukotrienes, or thromboxane did not prevent the response. In addition, there may be a negative interaction between A1 receptors and the adrenergic system, but not between A1 receptors and serotonin or angiotensin receptors.

References

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