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Adenosine A_{2A} receptor modulation of juvenile female rat skeletal muscle microvessel permeability

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Abstract

Little is known of the regulation of skeletal muscle microvascular exchange under resting or stimulating conditions. Adenosine (ADO) levels in skeletal muscle increase during physiological (exercise) and pathological (hypoxia, inflammation, and ischemia) conditions. Later stages of these pathologies are characterized by the loss of vascular barrier integrity. This study focused on determining which ADO receptor mediates the robust reduction in microvessel permeability to rat serum albumin (P_s^{RSA}) observed in juvenile female rats. In microvessels isolated from abdominal skeletal muscle, ADO suffusion induced a concentration-dependent reduction in arteriolar [$\log(IC_{50}) = -9.8 \pm 0.2$ M] and venular [$\log(IC_{50}) = -8.4 \pm 0.2$ M] P_s^{RSA} . RT-PCR and immunoblot analysis demonstrated mRNA and protein expression of ADO A₁, A_{2A}, A_{2B}, and A₃ receptors in both vessel types, and immunofluorescence assay revealed expression of the four subtype receptors in the microvascular walls (endothelium and smooth muscle). P_s^{RSA} responses of arterioles and venules to ADO were blocked by 8-(*p*-sulphophenyl)theophylline, a nonselective A₁ and A₂ antagonist. An A_{2A} agonist, CGS21680, was more potent than the A₁ agonist, cyclopentyladenosine, or the most-selective A_{2B} agonist, 5'-(*N*-ethylcarboxamido)adenosine. The ability of CGS21680 or ADO to reduce P_s^{RSA} was abolished by the A_{2A} antagonist, ZM241385. An adenylyl cyclase inhibitor, SQ22536, blocked the permeability response to ADO. In aggregate, these results demonstrate that, in juvenile females (before the production of the reproductive hormones), ADO enhances skeletal muscle arteriole and venule barrier function predominantly via A_{2A} receptors using activation of adenylyl cyclase-signaling mechanisms.

Keywords

adenosine receptors; albumin; microvascular exchange; arteriole; venule

Skeletal Muscle Microvessels have a large capacity to vasodilate and increase blood flow, thereby increasing oxygen and nutrient supply during exercise (7). During whole body dynamic exercise at maximal oxygen consumption, skeletal muscle receives 85–90% of

cardiac output (11) compared with 20% at rest. Adenosine (ADO), a ubiquitous adenine nucleoside, was shown to be a key vasodilator in skeletal muscles under physiological and pathophysiological conditions including hypoxia (5), ischemia, and muscle contraction (8, 22, 23, 34). Furthermore, ADO was found, from measures of permeability-surface area product (13), to increase skeletal muscle microvasculature blood-tissue exchange, an increase believed to be secondary to the increased exchange surface area downstream from the arterioles dilating in response to ADO (1, 6, 7, 14). It is unclear, however, whether ADO itself could modulate skeletal muscle permeability. The feedback mechanism of skeletal muscle myocyte contraction resulting in ADO production, which can regulate blood flow through smooth muscle dilatation thereby enhancing substrate delivery, led us to hypothesize that ADO could also regulate substrate transport by altering directly exchange skeletal muscle vessel barrier function.

The notion that microvascular exchange is a dynamic process has arisen from studies in a variety of microvascular models including frog (27) and rat (46) mesentery and pig heart (29). Despite the need for understanding the mechanisms controlling the distribution of substrates and wastes in this major tissue, little is known, given the difficulty of assessing flux and permeability in a thick tissue. The majority of what is known of in vivo microvascular exchange has come from the study of thin tissues (e.g., mesentery, cheek pouch, cremaster muscle) more suitable to the limitations of light microscopy. In this study, vascular trees were isolated from a skeletal muscle present in female animals; once isolated, an individual microvessel segment was perfused with micropipettes, and solute flux was measured using fluorescent dye-labeled probes by microspectrofluorometry (26).

In earlier studies of isolated coronary microvessels from a combined group of sexually mature sedentary male and female pigs, ADO was found to reduce arteriolar permeability (P_s) to albumin via activation of ADO A_1 and/or A_{2A} receptors. While the same receptors were shown to be present in coronary venules, ADO appeared to be without effect on venular P_s (56). Expansion of the data set, facilitating analysis of the venule data by sex, demonstrated that, in fact, ADO decreased P_s of venules from males and increased P_s of venules from females (29). Recent preliminary studies in rats (28) demonstrated that P_s responses to ADO depended on vessel type, maturity, and sex. In skeletal muscle microvessels from juvenile females, the response to ADO was a robust reduction in permeability to rat serum albumin (P_s^{RSA}), leading us to use this model as a first test of our hypotheses in this study: P_s^{RSA} responses to ADO differ between abdominal skeletal muscle arterioles and venules.

Responses to ADO appear to be modulated by activation of four ADO receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3), cloned from a variety of species, linked to different second messenger systems leading subsequently to functionally distinct end points (16). ADO-induced changes in vascular tone (magnitude, duration, and direction) differ with respect to the species, tissue, and receptor subtypes. In pig heart, ADO causes vasodilation primarily via activation of A_{2A} receptors (2, 4, 20), although A_1 , A_{2A} , and A_{2B} receptors are expressed in coronary vasculature (21, 41). In mice, ADO-induced coronary vasodilation is mediated by a combination of A_1 and/or A_3 receptors (53, 54). In rat skeletal muscle,

vasodilatation responses to ADO are mediated primarily via A_{2A} and/or A₁ receptors (6, 10, 38). In the present study, therefore, a variety of available pharmacological tools and molecular approaches were used in addition to assessment of barrier function to test our second hypothesis: that acute changes in P_s^{RSA} responses to ADO are the consequence of the combined involvement of the ADO receptor subtypes, which differ between skeletal muscle arterioles and venules of juvenile female rats.

Materials and Methods

Experimental animals and microvessel preparation

All animal care and research was conducted in accordance with the National Research Council's "Guide for the Care and Human Use of Laboratory Animals" under protocols approved by the Office of Laboratory Medicine at the University of Missouri. Studies were carried out on 77 sexually immature female (40) Sprague-Dawley rats (40 days of age, 100–150 g; Hilltop Lab Animals, Scottsdale, PA). Rats were anesthetized with an intraperitoneal injection of 130 mg/kg thiobutabarbital (Inactin; Sigma, St. Louis, MO). Following removal of fur and skin from the anterior abdomen, the abdominal wall muscle was excised carefully and placed in cold (4°C) mammalian Krebs solution containing 0.15 mM dialyzed bovine serum albumin (BSA; Sigma).

Dissection of the microvessels from the rat abdominal skeletal muscle was modified from that for porcine coronary ventricle (30). A dissecting microscope (Zeiss, Thornwood, NY) aided in the isolation procedure from the excised abdominal wall muscle (40–50 × 30–40 mm). An arteriolar plexus, dissected from the internal surface of the abdominal muscle (transversus abdominis muscle), contained arterioles <100 µm in internal diameter (ID) that branched from larger feed arteries arising from the cranial or caudal epigastric artery. Given that arterioles and venules in skeletal muscle run in parallel, isolation of the arteriolar plexus resulted in isolation of a venular plexus. The venules were distinguished from the arterioles by the absence of a muscular wall and larger resting diameter. These plexuses were mounted at approximately their in vivo resting length gently on a Sylgard (Dow Corning, Midland, MI) pad over the surface of an inverted organ culture dish and kept submersed in Krebs-albumin.

Measurement of skeletal muscle microvessel permeability

The plexus was transilluminated and viewed at ×10 with a fixed-stage inverted microscope (Diavert, Leica, or Olympus IX70). The light path of the microscope was split 50/50 and projected simultaneously to a video system and an analog microscope photometer (PTI, Brunswick, NJ). Vessels were imaged using a black and white charge-coupled device (CCD) camera (Dage-MTI 72, Michigan City, IN) or a low-light camera (PTI) and displayed on a video monitor (projecting a field of view of 0.65 × 0.78 to 1.30 × 1.56 mm; Sony). A pseudocolor picture was generated using NIH Image software (National Institutes of Health, Bethesda, MD).

Single microvessels in the plexus were cannulated with a drawn and beveled (tip diameter 20 ± 5 µm) glass theta micropipette (WPI, Sarasota, FL) that contained an unlabeled

washout solution [rat serum albumin (RSA) in Krebs, 10 mg/ml] in one-half of the pipette and fluorescent dye-labeled RSA (see below) in the lumen of the other half.

Fluorescence intensity (I_f) from the microvessel lumen and surrounding vessel wall was defined by an adjustable rectangular window in the light path ($3D \times 2D$, width $\times$ length). The photometer output (voltage) was recorded as a function of time on the strip chart recorder (Cole Parmer). Solute flux (J_s , mmol/s) per unit surface area (S , cm^2) and constant concentration gradient (C , mmol/ cm^3) ($J_s/S \cdot C$, cm/s) was determined from the relationship

$$J_s/S\Delta C = (1/\Delta I_o)(dI_f/dt)_i(D/4)$$

where I_o is the fluorescence intensity of the test solute in the microvessel lumen, $(dI_f/dt)_i$ is the initial change in fluorescence intensity as solute moves across the vessel wall, and D is microvessel diameter (cm). For each measurement of P_s , the vessel was first perfused with unlabeled RSA for at least 1 min; the perfusate was then switched rapidly to labeled RSA, J_s was measured during 2 min of perfusion with the labeled RSA, and the perfusate was returned back to unlabeled RSA. This perfusion sequence was repeated at least three times so that recording was continued for at least 9 min for each condition. All measurements were carried out at 15°C and a low hydrostatic pressure of 15 cmH_2O in arterioles and 12 cmH_2O in venules to maintain constant microvessel tone (thereby minimizing changes in diameter) and to minimize the contribution of convective coupling (solvent drag) to the measure of net flux (30), respectively. Earlier studies demonstrated that working at the lower temperature was without significant effect on the magnitude of P_s and, more importantly, the direction of the P_s response to ADO or other reagents studied to date (33). Calculations of P_s took into account steady-state changes in diameter.

Experimental protocols

All of the tested solutions were applied to the vessel topically. Basal permeability to RSA (P_s^{con}) was measured during perfusion with dye-labeled RSA (10 mg/ml) and superfused with Krebs-BSA (1 mg/ml). P_s was then measured again in the presence of ADO or ADO analogs-Krebs-BSA suffusion following pretreatment with ADO or ADO analogs suffusion for 10 min. To assess P_s dose responses, increasing concentrations of ADO or ADO analogs were applied continuously in the suffusate. If the analogs were dissolved in DMSO, the suffusate under control conditions contained DMSO at the same concentration. In experiments where the impact of the ADO receptor antagonist on ADO or ADO analog-induced P_s^{RSA} responses was determined, P_s was measured with Krebs-BSA suffusate containing both agonist and antagonist after P_s^{RSA} had been measured during Krebs-BSA suffusion without and with antagonist.

Mammalian Krebs solution

The components of this solution included the following (in mM): 141.4 NaCl, 4.7 KCl, 2 $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.2 MgSO_4 , 1.2 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 5 glucose, 3 NaHCO_3 , and 1.5 HEPES. The pH

and osmolality of the solution were 7.40 ± 0.05 and 294 (292–298) mosM, respectively, at room temperature.

Krebs-serum albumin solutions

BSA was prepared for the suffusion solution, while RSA was prepared for perfusion solution. For stock solutions of BSA or RSA (Sigma), the protein was dissolved in double-distilled water (ddH₂O) at a concentration of 100 mg/ml. Diafiltration was performed in an Amicon Stirred Cell (Millipore) through a 30,000 Nominal Molecular Weight Limit (NMWL; Millipore) filter with a volume of glucose-free Krebs equal to three times the volume of the albumin solution. The concentration of the dialyzed protein was determined by absorbance spectroscopy at 280 nm and then adjusted to a 100 mg/ml stock solution, aliquoted, and stored at -20°C . All solutions containing albumin at a 10 mg/ml final concentration were prepared and used daily.

Fluorescent dye-labeled albumin

Skeletal muscle microvessel protein flux was detected using RSA labeled with a red fluorescent dye, Alexa Fluor-546 (Molecular Probes, Eugene, OR). Alexa Fluor-546 and RSA (3:1, molar/molar; starting concentration 0.125 M albumin) were mixed and reacted for 30 min at room temperature, followed by removal of free fluorescent dye with D-Salt Polyacrylamide Desalting Columns (Pierce, IL). The perfusate consisted of 1 part conjugated RSA and 9 parts unlabeled, dialyzed RSA (wt/wt) in Krebs at a final concentration of RSA of 10 mg/ml.

Reagents

All ADO agonists and antagonists (Sigma) used are provided in Table 1.

RT-PCR

Extraction of mRNA from single isolated arterioles and venules (ID $<100\ \mu\text{M}$, 1–2 mm in length) using paramagnetic oligo(dT) polystyrene beads [Dynabeads Oligo(dT)₂₅; Dynal, Carlsbad, CA] was described previously (57). In brief, individual isolated microvessels were homogenized in LiCl lysis buffer. The crude lysate and magnetic beads were incubated for 15 min at room temperature. The beads were washed two to three times with 100 μl of solutions A [10 mM Tris-HCl, pH 7.5, 150 mM LiCl, 1 mM EDTA, 0.1% CH₃(CH₂)₁₁OSO₃ Li (wt/vol)] and B (10 mM Tris-HCl, pH 7.5, 150 mM LiCl, 1 mM EDTA), respectively. The mRNA was eluted from the beads by addition of 10 μl of RNase-free H₂O. Rat brain homogenate was used as a positive control for ADO receptors (16). Total RNA from rat brain was extracted using RNeasy Total RNA Isolation System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following digestion of DNA to oligonucleotides with DNase I (Promega, Madison, WI), first-strand cDNA synthesis from microvessel mRNA and rat brain total RNA was conducted using reverse transcriptase Sensiscript RT kit (Qiagen, Valencia, CA). Primers for specific ADO receptors are shown in Table 2. Complementary DNA amplification was performed using HotStarTaq DNA polymerase in accordance with the manufacturer's instructions (Qiagen). PCR for ADO A₁, A_{2A}, A_{2B}, and A₃ receptors was conducted for 40–43 cycles at 94°C for 45 s (denature),

60°C for 1 min (anneal), and 72°C for 1 min (extension) following an initial denaturation step of 95°C for 15 min. A final extension of 72°C for 10 min was added after the 43rd amplification cycle. PCR products were separated by electrophoresis through 2% (wt/vol) agarose gel and visualized under UV light with ethidium bromide.

Immunoblot analysis

The expression of ADO receptor protein in arterioles and venules isolated from the juvenile female rat abdominal skeletal muscle was assessed by immunoblot analysis as described previously (57). In brief, each sample consisted of one to two arterioles or venules (ID <100 μ m, 1 mm in length) homogenized in solubilization buffer [50 mM Tris-HCl (pH = 7.4), 6 M urea, and 2% SDS (vol/vol) (47)]; the cell lysates were subjected to SDS-PAGE [10% (wt/vol) for A_{2A} and A_{2B}, 12% for A₁ and A₃], and protein was transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed with specific polyclonal antibodies for ADO receptors (Table 3) and incubated with secondary antibody (horseradish peroxidase-conjugated IgG; 1:1,000 dilution for A₁ and A₃, 1:1,500 dilution for A_{2B}, and 1:4,000 dilution for A_{2A} receptor detection; Pierce, Rockford, IL). Protein was detected by enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Pierce). Specificity of antibodies for ADO A₁, A_{2A}, and A_{2B} receptors was confirmed by control antigenic peptide competition. Antigenic peptides for A₁, A_{2A}, and A_{2B} were preabsorbed with their respective primary antibodies overnight at 4°C [antibody-peptide (vol/vol), according to the manufacturer's instructions; A₁ = 1:1, A_{2A} = 1:10, and A_{2B} = 1:10] and used as control for primary antibodies to show blockade of ADO receptor immunoreactivity. A₃ receptor antibody specificity was tested by using rabbit γ -globulin (1:200 dilution; Jackson ImmunoResearch Laboratories) instead of A₃ receptor antiserum as the primary antibody to perform immunoblot (Fig. 1, *top*). To monitor protein loading, monoclonal antibodies for β -tubulin (1:10,000; Chemicon, Temecula, CA) and β -actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with PVDF membrane in the presence of ADO receptor antibody.

Immunofluorescence assay

The cellular distribution of ADO A₁, A_{2A}, A_{2B}, and A₃ receptors in arterioles and venules in situ was determined using dual-color immunofluorescence and confocal laser-scanning microscopy. Cryostat sections (7- μ m thick) of abdominal skeletal muscle were fixed in methanol-acetone (1:1, -20°C for 10 min). Sections were washed with 0.1 M PBS (pH 7.4) and blocked with 5% (vol/vol) normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBS for 1 h. Sections were then incubated with dual primary antibodies, anti-ADO receptor polyclonal antibodies (used for immunoblot; Table 3) and anti-CD31 (platelet endothelial cell adhesion molecule; PECAM) monoclonal antibody (1:50 dilution; Serotec Immunological Excellence, Raleigh, NC), at room temperature for 1 h followed by three washes with PBS. Secondary antibodies used were Alexa Fluor-488-labeled goat anti-rabbit IgG (10 μ g/ml; Molecular Probes) for ADO receptor antibodies and Alexa Fluor-568-labeled goat anti-mouse IgG (6 μ g/ml; Molecular Probes) for CD31 antibody. Secondary antibodies were applied for 1 h, and sections were washed with PBS ($\times$ 3), mounted with anti-fading medium (MOWIOL 488; Calbiochem, San Diego, CA), and viewed with confocal (Radiance 2000 Confocal Microscopy System; Zeiss, Thornwood,

NY) laser (krypton-argon)-scanning microscopy in the Cytology and Molecular Core of University of Missouri-Columbia. Immunofluorescence negative controls were performed via incubation of cross sections in the absence of primary antibodies.

Statistical analysis

Permeability responses ($P_s^{\text{test}}/P_s^{\text{con}}$) to ADO or ADO receptor agonist or antagonist are presented as the ratios of the paired measures of flux under stimulating conditions relative to that basal level. Dose-response curves were fitted to the data points by nonlinear regression analysis by use of GraphPad Prism version 4.0b (GraphPad Software, San Diego, CA). The concentration of agonist causing 50% of the maximal response was designed IC_{50} . All values are expressed as means $\pm$ SE. Unpaired t -test was used to test for mean differences between two groups. One-sample t -test was utilized to determine whether P_s^{test} differed from P_s^{con} , testing the null hypothesis $P_s^{\text{test}}/P_s^{\text{con}}=1$. Statistical significance was defined as $P < 0.05$.

Results

Basal skeletal muscle microvascular P_s^{RSA}

For all experiments, basal arteriole P_s^{RSA} was $9.9 \pm 0.4 \times 10^{-7}$ cm/s (range $3.8\text{--}19.8 \times 10^{-7}$ cm/s, ID = 39 ± 1 μm , $n = 77$), and basal venule P_s^{RSA} was $10.7 \pm 0.5 \times 10^{-7}$ cm/s (range $3.4\text{--}19.3 \times 10^{-7}$ cm/s, ID = 46 ± 2 μm , $n = 66$). Arterioles and venules chosen for the study ran in parallel, and while the diameters of the arterioles were less than those of the venules, basal P_s^{RSA} did not differ between the two vessel types, and for neither vessel type was basal P_s^{RSA} correlated with microvessel size.

Concentration-dependent P_s^{RSA} response to ADO

ADO suffusion produced a concentration-dependent decrease in P_s^{RSA} of both arterioles and venules (Fig. 2). The maximal reduction of P_s^{RSA} elicited by ADO did not differ between arterioles ($44.4 \pm 2.9\%$ of baseline) and venules ($54.0 \pm 5.4\%$, Table 4). The arteriolar P_s^{RSA} response was more sensitive to ADO suffusion [$\log(IC_{50}) = -9.8 \pm 0.2$ M] than the venular P_s^{RSA} response [$\log(IC_{50}) = -8.4 \pm 0.2$ M; $P < 0.01$]. Compared with basal diameter, ADO suffusion caused a slight (but significant) increase in arteriolar diameter and was without effect on venule diameter (Table 5). Therefore, the maintenance of temperature at 15°C had the desired effect of minimizing diameter changes without influencing the effect of ADO on permeability.

Detection of ADO A_1 , A_{2A} , A_{2B} , and A_3 receptors

RT-PCR and Western blot analyses of the expression of gene transcripts (messenger RNA) and protein for ADO receptors were conducted on rat skeletal muscle microvessels. Rat brain was employed as a positive control. Figure 3 shows representative RT-PCR analysis of whole individual arterioles and venules isolated from abdominal skeletal muscles. Messenger RNA for A_1 (Fig. 3A), A_{2A} (Fig. 3B), A_{2B} (Fig. 3C), and A_3 (Fig. 3D) was

detected in all arterioles and venules. Coexpression of the four ADO receptor subtypes in both arterioles and venules was confirmed by immunoblot analysis. The representative immunoblot analysis for the presence of A_1 (molecular mass = 36 kDa, $n = 4$), A_{2A} (molecular mass = 53 kDa for rat brain, molecular mass = 67 kDa for microvessels, $n = 6$), A_{2B} (molecular mass = 52 and 40 kDa, $n = 6$), and A_3 receptor protein (molecular mass = 36 and 30 kDa, $n = 3$) is shown in Fig. 4.

Immunofluorescent analysis of abdominal skeletal muscle cross sections and confocal microscopic imaging revealed cellular distribution of ADO A_1 , A_{2A} , A_{2B} , or A_3 receptors (antibodies for receptors labeled with Alexa-488, green) with highest density in the endothelium and smooth muscle of the arteriolar and venular wall. Figure 5 shows antibody for the ADO A_{2A} receptor [green-stained vascular wall of arteriole (Fig. 5B) and venule (Fig. 5C, respectively)]. CD31 (PECAM) antibody tagged with Alexa-568 (red) labeled the inner luminal region of vascular wall in the vicinity of the endothelial cells. Antibodies for A_{2A} receptors (green) radiated to endothelial cell sites of vascular wall in the cross (arteriole, Fig. 5B, and venule, Fig. 5C, indicated by arrow) and longitudinal (venule, Fig. 5C, indicated by arrowhead) segments. Distribution of ADO A_1 , A_{2B} , and A_3 receptors on the vascular walls of both arterioles and venules did not differ from that for the A_{2A} receptors (data not shown).

Contributions of A_1 and A_2 to ADO response

A pharmacological approach was employed to determine which ADO receptor subtypes modulate microvessel P_s^{RSA} responses to ADO. At least 10 min of exposure of the microvessel to sufusate containing the nonselective A_1 and A_2 receptor antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT) at 10^{-5} ($n = 6$) and 10^{-4} ($n = 6$) M blocked the reduction in arteriole and venule P_s^{RSA} evoked by 10^{-5} M ADO suffusion. The antagonist 8-SPT alone, at these two doses, was without effect on basal P_s^{RSA} in both microvessel subtypes shown in Fig. 6 (10^{-5} M 8-SPT, $P_s^{test}/P_s^{con}=1.09 \pm 0.09$ and 1.01 ± 0.08 ; 10^{-4} M 8-SPT, $P_s^{test}/P_s^{con}=0.91 \pm 0.14$ and 0.92 ± 0.15 for arterioles and venules, respectively.)

Microvascular P_s^{RSA} responses to selective receptor activation using ADO analogs

P_s^{RSA} responses were measured after 10 min of exposure to selective A_1 and A_{2A} receptor agonists and the most-selective A_{2B} receptor agonist, cyclopentyladenosine (CPA), CGS21680, and 5'-(*N*-ethylcarboxamido)adenosine (NECA), respectively. Neither CPA (10^{-9} to 10^{-6} M) nor NECA (10^{-10} to 10^{-5} M) had a significant effect on arteriole (Fig. 7A: CPA, $n = 6$; NECA, $n = 6$) or venule (Fig. 7B: CPA, $n = 5$; NECA, $n = 7-8$; $P > 0.05$) P_s^{RSA} . CGS21680 caused a dose-dependent reduction of P_s^{RSA} in both vessel types (arteriole, $n = 3-10$; venule, $n = 3-7$). The values of $\log(IC_{50})$ for P_s^{RSA} responses to CGS21680 were -7.5 ± 1.1 M and -8.5 ± 0.4 M for arterioles and venules, respectively. The maximal reduction in P_s^{RSA} induced by CGS21680 was $45.9 \pm 4.8\%$ and $41.0 \pm 7.7\%$ of baseline for arterioles and venules, respectively. Both the sensitivity and magnitude of P_s^{RSA} responses to CGS21680 between arterioles and venules were indistinguishable (Table 4).

Effect of the A_{2A} receptor antagonist ZM241385

To further determine whether A_{2A} receptors mediated the CGS21680-induced responses, microvessel P_s^{RSA} responses to CGS21680 were measured in the presence of a selective A_{2A} receptor antagonist, ZM241385 (20, 42). At 10^{-7} M, ZM241385 abolished the fall in arteriolar and venular P_s^{RSA} induced by CGS21680 (Fig. 8, A and B). The vehicle (DMSO; 0.0001%, 1.4×10^{-5} M) or 10^{-7} M ZM241385 alone was without effect on basal P_s^{RSA} [$P_s^{test}/P_s^{con}=1.02$ ($n=5$) and 1.06 ($n=10$) for DMSO and ZM241385, respectively]. In addition, ZM241385 blocked the reduction of arteriolar ($n=5$) and venular ($n=6$) P_s^{RSA} induced by 10^{-8} M and 10^{-7} M ADO, respectively (Fig. 8C).

Reduction of P_s in response to ADO mediated by adenylyl cyclase

It is generally accepted that the ADO A_{2A} receptor is coupled to the G_{q/s} protein and is able to activate adenylyl cyclase (16). We therefore used the adenylyl cyclase inhibitor SQ22536 to test whether the cell-signaling mechanism mediating the P_s response to ADO was adenylyl cyclase dependent. While basal P_s in both vessel types was not influenced by 10^{-5} M SQ22536 (arteriole, $P_s^{test}/P_s^{con}=1.16 \pm 0.13$, $n=4$; venule, $P_s^{test}/P_s^{con}=1.15 \pm 0.13$, $n=4$), it abolished the reduction of P_s induced by 10^{-8} M and 10^{-7} M ADO for arterioles ($n=4$) and venules ($n=4$), respectively (Fig. 9).

Discussion

The present study initiated a test of the hypothesis that P_s^{RSA} responses to ADO involve signaling mechanisms regulated by more than one of the ADO receptor subtypes and that differences in responses to ADO between vessel types and tissues and following adaptation to exercise reflect differences in the receptor subtype-signaling pathways. The studies were conducted on skeletal muscle microvessels from juvenile female rats, where we observed a robust reduction in permeability from basal levels during exposure to ADO (28). The novel findings of the present study with respect to juvenile females are as follows: 1) basal permeability of skeletal muscle arterioles and venules do not differ; 2) ADO suffusion (10^{-5} M) causes a concentration-dependent reduction in P_s^{RSA} of both arterioles and venules; 3) both microvessel subtypes express mRNA and protein for the four identified ADO receptor subtypes distributed on the endothelium and vascular smooth muscle; and 4) before reproductive maturity in female rats, the reduction in P_s^{RSA} by ADO appears to be mediated primarily through ADO A_{2A} receptors, which involve adenylyl cyclase-mediated mechanisms.

Basal albumin exchange in abdominal skeletal muscle microvessels of juvenile female rats

Although skeletal muscle comprises the largest percentage of total body mass, surprisingly little information is available regarding the permeability characteristics of the microvasculature (55). The present study is the first to measure P_s to albumin in individual perfused microvessels isolated from rat abdominal skeletal muscles. The first result, contrary to expectation (43), was that basal permeability of venules was not higher than that of the arterioles. Indeed, this outcome is in contrast with that observed in other vascular beds

(venular $P_s >$ arteriolar P_s) including adult (29, 57) and juvenile porcine heart (24, 59) and skeletal muscles of adult male rat (48), mouse (48), guinea pig (43), and rabbit (50). The primary difference between the current study and those published previously is that the venous values are significantly lower than reported for other rodent skeletal muscle models (adult male) (57).

Volume balance results from the interplay of vascular permeability and interstitial resistance to solute flux as well as lymphatic function. Vessel architecture (surface area, number of vessels, vessel length, distance between vessels) and interstitial composition contribute to the interstitial resistance to solute flux. Obviously, the juvenile females are, in solute and volume balance, like the adult male animals, although their venular microvascular resistance ($R = 1/P_s$) is higher than for the males. Volume balance, however, could be achieved if one or more of the other components were changed, e.g., interstitial resistance was lower and/or lymphatic function was reduced. It remains to be determined whether the lack of an arteriole-venule permeability gradient, reflecting apparently tighter venules, is particular to juvenile and/or female rodents.

In isolated rat hindquarter preparation, the combined permeability-surface area product for albumin was reported to be $0.025 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$ (adult males) (58). With the assumption of a surface area for exchange of $70 \text{ cm}^2/\text{g}$ from cat hindlimb data (58), this product translates to a P_s of $0.6 \times 10^{-7} \text{ cm/s}$, a value significantly lower than our findings for apparent P_s^{RSA} (9.9 ± 0.4 and $10.7 \pm 0.5 \times 10^{-7} \text{ cm/s}$ for arterioles and venules, respectively). While it is reassuring that these values are within an order of magnitude of each other, several factors likely contribute to the differences in the absolute values. These include errors in the determination of the perfused surface area and differences in experimental preparation (isolated microvessels vs. isolated organ), tissue (abdominal vs. hindquarter), sex (female vs. male), and age (juvenile vs. adult) and the possibility that P_s values of vessels packed in an intact tissue are reduced by the presence of the myocytes.

P_s values of venules to RSA and to α -lactalbumin in rat mesentery have been reported as $5.6 \times 10^{-7} \text{ cm/s}$ by Rumbaut and Huxley (45) and $50 \times 10^{-7} \text{ cm/s}$ by He and colleagues (60), respectively. The value of venular P_s^{RSA} ($10.7 \pm 0.5 \times 10^{-7} \text{ cm/s}$) reported in the current study is almost twice that of venular mesentery P_s^{RSA} (45). Again, it is not known at this time whether this difference (Ref. 45 vs. the current study) reflects differences between tissues (mesentery vs. skeletal muscle), sex (male vs. female), or maturity (adult vs. juvenile). It is interesting to note that the use of the same technique for assessing P_s reduces the difference from 10- to 2-fold. Preliminary data from this laboratory (56) indicate that sex and reproductive maturity are both important variables influencing basal permeability and are features requiring additional study.

Regulation of skeletal muscle microvessel P_s^{RSA} by ADO

In skeletal muscle, interstitial ADO concentration increases significantly during hypoxia (5), ischemia, and muscle contraction (8, 22, 23, 34). For instance, in response to extensor exercise, the interstitial ADO in human vastus lateralis muscle increased fivefold from resting levels of $2.2 \pm 0.1 \times 10^{-7} \text{ M}$ at a work rate of 10 W (23). It is well known that, in

skeletal muscle, ADO is an important local metabolic vasodilator (6, 14, 39). In addition to ADO having the capacity to regulate blood flow delivery, and hence exchange, by dilating feed arterioles, ADO has been shown recently to regulate the exchange of protein by mediating permeability changes in coronary microvessels (25, 29, 30, 57).

In the present study, we found that P_s^{RSA} of two vessel types, arterioles and venules, isolated from juvenile female abdominal skeletal muscles was reduced by ADO in a concentration-dependent manner (Fig. 2). These findings are inconsistent with our global hypothesis that ADO-induced P_s^{RSA} responses would differ between arterioles and venules. What did differ with respect to vessel type was that the arteriolar P_s^{RSA} response was more sensitive to ADO suffusion than the venular response. The results from the juvenile females are most consistent with ADO acting in the two vessel types via the same signaling pathway with vessel-specific differences in ADO binding affinity, receptor number, and/or efficiency of the coupling between receptor and G protein or the signaling response. Because the pathways and associated mechanisms underlying the ADO regulation of P_s^{RSA} are unclear, we sought by molecular and pharmacological means to determine which receptor subtypes participated in P_s^{RSA} responses to ADO, providing fundamental knowledge for the future study of which signaling pathways result in a tighter barrier structure.

P_s^{RSA} responses to ADO are mediated mainly by A_{2A} receptors. To define which ADO receptor subtypes contribute to the alteration of P_s^{RSA} in response to ADO suffusion, the nonselective A_1 and A_2 receptor antagonist, 8-SPT, was chosen. 8-SPT is a theophylline derivative that lacks phosphodiesterase inhibition because of negligible intracellular penetration due to its charge at physiological pH (9, 17). The concentration of 10^{-4} M has been used to identify ADO receptor subtypes responsible for changes in skeletal muscle vascular tone (12, 18, 49). In the present study, inhibition of A_1 and A_2 receptor with 8-SPT blocked the decreased P_s^{RSA} in response to ADO (Fig. 6), suggesting that A_1 and/or A_2 but not A_3 receptors mediated the response in both arterioles and venules.

The selective A_1 and A_{2A} agonists, CPA and CGS21680, respectively, as well as the most-selective A_{2B} receptor agonist, NECA, were used to determine further the receptor subtypes involved in the ADO response. The pharmacological profile of the ligands revealed that the A_{2A} receptor agonist CGS21680 was more potent than other ADO analogs (Fig. 7; ADO > CGS21680 > CPA > NECA in arterioles, and ADO = CGS21680 > CPA = NECA in venules). Of interest, arterioles were more sensitive to ADO [$\log(IC_{50}) = -9.8 \pm 0.2$] than CGS21680 [$\log(IC_{50}) = -7.5 \pm 1.1$]. Two explanations may account for the difference. The first, that the ADO P_s^{RSA} response is the net effect of activating both A_1 and A_{2A} receptors, seems unlikely, as the A_1 agonist CPA alone was without significant effect on arteriolar P_s^{RSA} . Second, ADO may alter P_s^{RSA} via binding to ADO receptors and use of a non-receptor-mediated pathway such as an ADO transporter. It has been shown that ADO transporters can take ADO up from smooth muscle intracellular space with a K_m of $17.6 \pm 2.6 \times 10^{-6}$ M and a V_{max} of $5.1 \pm 0.5 \times 10^{-12}$ mol·min⁻¹·mg wet tissue⁻¹ in intact porcine coronary artery rings (44). It is possible that the transported intracellular ADO is converted

to AMP, which can activate AMP-activated protein kinase (AMPK), resulting in AMPK-increased nitric oxide (NO) synthesis by stimulation of endothelial NO synthase (eNOS) activity (37). Had this occurred, an increase in NO would have been expected to elevate, rather than reduce, P_s (46).

The most-selective A_{2A} receptor antagonist, ZM241385, inhibited P_s^{RSA} responses to CGS21680 and ADO (Fig. 8), providing additional evidence supporting the conclusion that ADO A_{2A} receptor activation most likely mediates the reduction of P_s^{RSA} in response to ADO.

Involvement of A_3 receptors in the P_s responses to ADO has been ruled down in this model, as the nonselective A_1 and A_2 receptor 8-SPT abolished the fall of P_s^{RSA} in response to ADO. It is noteworthy that the A_3 receptor agonist and antagonist, IB-MECA and MRS1191, respectively, have been used to determine the role of A_3 receptors in the ADO P_s^{RSA} response (57). Interestingly, it seems that neither IB-MECA nor MRS1191 activates A_3 receptors exclusively; instead, there was cross-reactivity with A_{2A} receptors. These findings are consistent with the conclusion of Fredholm et al. (16) that selective stimulation or blockade of A_3 receptors in the presence of other ADO receptors is often not possible.

The current study provides molecular evidence for expression of mRNA and protein for ADO A_1 , A_{2A} , A_{2B} , and A_3 receptor subtypes in both arterioles and venules isolated from abdominal skeletal muscles of juvenile female rats. Immunofluorescence analysis demonstrated cellular distribution of ADO A_1 , A_{2A} , A_{2B} , and A_3 receptors in the arteriolar and venular microvessels and that all receptor subtypes are located in the inner luminal region consisting of endothelium as well as smooth muscle.

The specific mechanisms whereby A_{2A} receptor activation results in increased resistance to protein flux from both vessel types require further study. We assume that vascular endothelial cells play a principle role in the regulation of permeability in our preparations, although the wall of our isolated microvessels includes other cell types such as smooth muscle cells, mast cells, and fibroblasts.

Given that ADO A_{2A} receptors are coupled to the G_{α_s} protein, ADO binding to A_{2A} receptors results in the activation of adenylyl cyclase and elevation of cellular cAMP (16, 41). It is, furthermore, likely that the enhancement of barrier structure is mediated downstream from activation of PKA, because both in vivo and in vitro studies have shown that elevating cAMP levels leads to a reduction in P_s (3, 19, 51). In this study, we demonstrated that inhibition of adenylyl cyclase blocked reduction of P_s in response to ADO (Fig. 9), consistent with the proposal that microvessel barrier resistance by ADO was primarily mediated by the ADO A_{2A} receptor-adenylyl cyclase pathway. We also found that ADO A_{2A} receptors exist in arteriolar smooth muscle cells (57). Others have shown that A_{2A} receptors are expressed on platelets, neutrophils, and mast cells, all of which they are involved in anti-inflammatory activity, especially under ischemic conditions (35). For instance, activation of A_{2A} receptors decreased TNF- α -stimulated neutrophil adherence, oxidative activity, and degranulation (52). Unfortunately, the contribution of smooth muscle

cells and mast cells to the regulation of intact microvessel exchange, specifically P_s , cannot be determined directly as yet.

What remains intriguing from this work is that, although all ADO receptor subtypes were found to be present, both as messenger RNA and as expressed protein, only A_{2A} appeared to be involved in the functional regulation of P_s . Preliminary data from sexually mature rats and from microvessels of other tissue beds indicate that a variety of permeability responses occur, implicating involvement of multiple mechanisms in the regulation of exchange in addition to the tightening of the barrier via cAMP-mediated pathways (28, 29). Comparison of the responses of skeletal muscle vessels from juvenile males and adult rats of both sexes with the present data should provide a useful tool by which the contribution of these other signaling pathways and associated mechanisms can be determined.

In conclusion, the current study is the first to assess rat abdominal skeletal muscle vasculature barrier function in excised, individually perfused microvessels. The findings shed a light on understanding the characteristics of albumin transport across the vascular barrier of skeletal muscle arterioles and venules. The results on P_s^{RSA} responses to ADO have physiological and pathophysiological significance, as ADO increases in skeletal muscle under conditions associated with contraction (7), inflammation, and hypoxia as well as ischemia (35) and subsequently affects vascular barrier function. Furthermore, other data from our laboratory indicate fundamental differences in the mechanisms determining fluid homeostasis and its regulation in male and female animals. The clear response to ADO in skeletal muscle microvessels from juvenile female rats provides the basis for a set of quantitative studies to determine the mechanisms underlying sex-based differences in fluid and solute exchange. In addition, the novel findings regarding ADO receptor distribution in rat skeletal muscle microvessels provide a molecular basis for elucidation of the entire role of ADO regulation of microvascular function.

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Fig. 1.

Tests of specificity of antibodies for adenosine (ADO) A₁, A_{2A}, A_{2B}, and A₃ receptors. *Top left*: immunoblot was performed using polyclonal antibodies for A₁, A_{2A}, A_{2B}, and A₃ receptors as described in MATERIALS AND METHODS. Rat brain was used as the positive control. Art, arterioles isolated from rat juvenile female abdominal skeletal muscles. *Top right*: the ability of antibodies for A₁, A_{2A}, and A_{2B} receptors to recognize antigen was blocked by preadsorption with their antigenic peptide, respectively, and A₃ receptor antibody specificity was tested by use of rabbit γ -globulin as primary antibody to perform immunoblot. Immunofluorescence negative controls (*bottom, A and B*) were carried out via staining with omission of primary antibodies as described in MATERIALS AND METHODS for immunofluorescence assay. *Bottom, C*: anti-fluorescence from the cross section of abdominal skeletal muscle.

Fig. 2.

ADO concentration-dependent reduction in permeability (to rat serum albumin; P_s^{RSA}). Data are expressed as the ratio of P_s^{RSA} during ADO suffusion (P_s^{ADO}) to basal P_s^{RSA} (P_s^{con}). *A*: responses of arterioles ($n = 4-9$). *B*: responses of venules ($n = 4-5$). *Significant response to ADO, e.g., the ratio (P_s^{ADO}/P_s^{con}) $\neq 1$ ($P < 0.05$). Values are means $\pm$ SE. The dashed line represents no change in P_s^{RSA} in response to ADO compared with basal P_s^{RSA} .

Fig. 3.

Expression of messenger RNA for ADO A₁ (A), A_{2A} (B), A_{2B} (C), and, A₃ (D) receptors in both microvessel types isolated from abdominal skeletal muscles of female juvenile rats, demonstrated by RT-PCR. Individual isolated arterioles (Art) and venules (Ven) <100 μ m in internal diameter (ID) and 1–2 mm in length were subjected to mRNA extraction, reverse transcription, and amplification with specific ADO A₁, A_{2A}, A_{2B}, and A₃ primers. Rat brain was used as a positive control.

Fig. 4.

ADO receptor subtype protein expressed in arterioles and venules isolated from abdominal skeletal muscles of juvenile female rats. Immunoblot analysis with anti-A₁, -A_{2A}, -A_{2B}, or -A₃ polyclonal antibody demonstrated expression of ADO A₁ (A), A_{2A} (B), A_{2B} (C), and A₃ (D) in both vessel types isolated from abdominal skeletal muscles. Samples subjected to immunoblot assay represent the pooling of 2–3 isolated arterioles or venules (ID <100 μm, length = 1 mm). Rat brain (25 μg membrane protein) was used as a positive control. β-Tubulin or β-actin indicated loading of the samples.

Fig. 5.

ADO A_{2A} receptor immunofluorescence assay in cross section of abdominal skeletal muscle in juvenile female rats. Confocal microscopic images of freeze-sectioned in situ microvessels in abdominal skeletal muscles. *Column I* (green color): immunofluorescent staining with anti-ADO A_{2A} receptor subtype antibody labeled with Alexa-488. *Column II* (red color): immunofluorescent staining with anti-CD31 (platelet endothelial cell adhesion molecule; PECAM) antibody; endothelial cell marker conjugated with Alexa-568. *Column III*: the merged picture of *columns I* and *II*. *A*: cross section of abdominal skeletal muscle. *B*: an arteriole enlarged from the section shown in *A* (indicated by square). *C*: cross (arrow) and longitudinal segments (arrow head) of venules in cross section of skeletal muscle.

Fig. 6.

The nonselective ADO A_1 and A_2 receptor antagonist, 8-(*p*-sulphophenyl)theophylline (8-SPT), blocked the P_s^{RSA} response of microvessels isolated from juvenile female rat skeletal muscle. Data are expressed as the ratio of P_s^{RSA} during 8-SPT with or without ADO (10^{-5} M) suffusion (P_s^{test}) relative to basal P_s^{RSA} (P_s^{con}) in arterioles (A) and venules (B) at 2 doses of 8-SPT (10^{-5} M or 10^{-4} M). Dashed line indicates no change compared with basal P_s^{RSA} . Nos. in parentheses indicate vessel nos. *Significant difference between ADO with or without 8-SPT.

Fig. 7.

Evidence for the contribution of the ADO receptor subtypes in the regulation of permeability response to ADO. Data are expressed as the ratio of P_s^{RSA} during a series of concentrations of ADO, A_1 receptor-selective agonist cyclopentyladenosine (CPA), A_{2A} receptor-selective agonist CGS21680, or the most-selective ADO analog for A_{2B} receptors, 5'-(*N*-ethylcarboxamido)adenosine (NECA), shown for arterioles (A) and venules (B), respectively. The gray line gives the data for ADO alone. * $P < 0.05$ for CGS21680 treatment vs. 1.

Fig. 8.

Selective A_{2A} receptor antagonist ZM241385 influences the P_s^{RSA} responses to both ADO and the A_{2A} receptor agonist CGS21680. Microvessels were isolated from abdominal skeletal muscle of juvenile female rats. Data are expressed as the ratio of P_s^{RSA} at doses of CGS21680 (10^{-10} to 10^{-6} M) with (solid symbols) and without (gray line) A_{2A} receptor antagonist ZM241385 (10^{-7} M) relative to basal P_s^{RSA} for arterioles (A; circles) and venules (B; triangles). C: arteriolar and venular P_s^{RSA} responses to 10^{-8} M and 10^{-7} M ADO, respectively, with (open symbol) and without (solid symbol) 10^{-7} M ZM241385 suffusion. Nos. in parentheses are vessel nos. Dashed line indicates ratio ($P_s^{\text{test}}/P_s^{\text{con}}$) = 1. *Significant difference between ADO alone and in addition to A_{2A} receptor blockade (ZM241385) ($P < 0.01$).

Fig. 9.

Enhancement of microvascular barrier function induced by ADO is adenylyl cyclase dependent. The adenylyl cyclase inhibitor SQ22536 (SQ), at a concentration of 10^{-5} M, was without effect on basal permeability (P_s) in both arterioles (solid triangle) and venules (open triangle). Decrease in P_s elicited by ADO (10^{-8} M for arterioles, solid circle; 10^{-7} M for venules, open circle) was abolished in the presence of 10^{-5} M SQ22536 (arteriole, solid square; venule, open square). Nos. in parenthesis indicate nos. of experiments.

Table 1
Pharmacological tools used for identification of ADO receptors

	Chemical Name	Solution Preparation	Binding Affinity, nM				Ref. No.
			A ₁	A _{2A}	A _{2B}	A ₃	
NECA	5'-(<i>N</i> -ethylcarboxamido)adenosine	acidified ddH ₂ O	6.3	10	330	113	(36)
CPA	cyclopentyladenosine	acidified ddH ₂ O	0.59	460		240	(15)
CGS21680	2- <i>p</i> -(2-carboxyethyl)phenethylamino-5'- <i>N</i> -ethylcarboxamidoadenosine hydrochloride	acidified ddH ₂ O	2,600	15.5	361,000	584	(32)
IB-MECA	1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]- <i>N</i> -methyl- β -D-ribofuranuronamide	DMSO 0.015%	54	56	54	1.1	(15)
8-SPT	8-(<i>p</i> -sulphophenyl)theophylline	ddH ₂ O	4.5	6.3*	6.3*		(9)
ZM241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol	DMSO 0.0001%	2,000	0.3	87	100,000	(15)
MRS 1191	3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-($\pm$)-dihydropyridine-3,5-dicarboxylate	DMSO 0.00032%	40,000	100,000		1,420	(31)

*K*_D or *K*_B value (equilibrium association constants) was obtained for rats. ADO, adenosine; ddH₂O, double-distilled water.

* Data for mixed A₂.

Table 2
PCR Primers for ADO A₁, A_{2A}, A_{2B}, and A₃ receptors

Receptor	Sequence of Primer	Product Size	Accession No.
A ₁	Forward 5'- ⁶³⁰ CTC CAT TCT GGC TCT GCTCG ⁶⁴⁹ -3'	207	M64299
	Reverse 5'- ⁸³⁶ ACA CTG CCG TTG GCT CTC C ⁸¹⁸ -3'		
A _{2A}	Forward 5'- ⁴⁰⁷ CCA TGC TGG GCT GGA ACA ⁴²³ -3'	150	L08102
	Reverse 5'- ⁵⁵⁶ GAA GGG GCA GTA ACA CGA ACG ⁵³⁶ -3'		
A _{2B}	Forward 5'- ¹³⁹ TGG CGC TGG AGC TGG TTA ¹⁵⁶ -3'	160	M91466
	Reverse 5'- ²⁹⁸ GCA AAG GGG ATG GCG AAG ²⁸¹ -3'		
A ₃	Forward 5'- ¹²⁰⁵ TGATCCTCAGAGCTTGCA ¹²²² -3'	397	NM_012896
	Reverse 5'- ¹⁶⁰¹ TCCTTCCCAGACACAAGA ¹⁵⁸³ -3'		

Table 3
Information about ADO receptor antibodies used for immunoblot and immunofluorescence

	Concentration for Immunoblot		Concentration for Immunofluorescence		Used	Source of Antibody
	Recommended	Used	Recommended	Used		
A ₁	1:1,000	1:250	1:100	1:50	1:50	Affinity BioReagents
A _{2A}	1:1,000–5,000	1:1,000	not tested	1:40	1:40	Alpha Diagnostic International
A _{2B}	1:100–1,000	1:200	1:20–100	1:20	1:20	Chemicon
A ₃	1:200–1,000	1:200	not tested	1:30	1:30	Santa Cruz Biotechnology

Table 4
Log(IC₅₀) and maximal responses for ADO- and A_{2A}-selective agonist CGS

Log(IC ₅₀)		% Decrease from Basal P_s^{RSA}	
Arteriole	Venule	Maximal concentration	
		Arteriole	Venule
ADO	-9.8±0.2*†	-8.4±0.2	ADO 10 ⁻⁵ M 44.4±2.9 54.0±5.4
CGS	-7.5±1.1	-8.5±0.4	CGS 10 ⁻⁶ M 45.9±4.8 41.0±7.7

Values are means ± SE. CGS, CGS21680; P_s^{RSA} , permeability to rat serum albumin.

* $P < 0.05$ vs. CGS in arteriole.

† $P < 0.05$ vs. ADO in venule.

Table 5
Microvascular diameter (μm) before and after ADO or agonist/antagonist suffusion

	Arterioles			Venules		
	Before	After	n	Before	After	n
ADO	41 $\pm$ 2	43 $\pm$ 3 *	8	54 $\pm$ 2	55 $\pm$ 2	5
ADO + 8-SPT	36 $\pm$ 4	35 $\pm$ 6	9	36 $\pm$ 6	40 $\pm$ 4	6
NECA	42 $\pm$ 6	45 $\pm$ 6	6	48 $\pm$ 5	49 $\pm$ 6	8
CGS21680	45 $\pm$ 2	47 $\pm$ 2 *	6	50 $\pm$ 6	51 $\pm$ 7	3
CGS21680 + ZM241685	43 $\pm$ 3	43 $\pm$ 2	6	36 $\pm$ 3	38 $\pm$ 3	6
CPA	38 $\pm$ 2	41 $\pm$ 2 *	6	54 $\pm$ 6	56 $\pm$ 6 *	6

Values are mean $\pm$ SE.

* Significant difference between paired values ($P < 0.05$). For abbreviation definitions, see Table 1.