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Midazolam Selectively Potentiates the A_{2A}- but not A_1-receptor–mediated Effects of Adenosine

Role of Nucleoside Transport Inhibition and Clinical Implications

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Background: **Inhibition of adenosine metabolism offers a unique approach to harness the cardioprotective properties of adenosine in a site- and event-specific manner. Benzodiazepines inhibit adenosine metabolism by blocking nucleoside transporter. Therefore, the authors studied the binding affinities of structurally different benzodiazepines to nucleoside transporter and benzodiazepine-induced potentiation of A_1-adenosine (negative dromotropy) and A_{2A}-adenosine (coronary vasodilation) receptor–mediated effects.**

Methods: **In membranes from porcine striatum and guinea pig ventricle, competition binding assays to displace [^{3}H]nitrobenzylmercaptopurine riboside ([^{3}H]NBMPR) from nucleoside transporter were performed using alprazolam, chlorodiazepoxide, diazepam, flurazepam, and midazolam. The augmentation by the most potent benzodiazepine of A_1- and A_{2A}-adenosine receptor–mediated responses, elicited by exogenous administration of adenosine or brief periods of global hypoxia, was subsequently studied in guinea pig Langendorff-perfused hearts.**

Results: **All benzodiazepines completely displaced [^{3}H]NBMPR in a concentration-dependent manner with Hill coefficients not significantly different from unity in both striatal and ventricular membranes. Midazolam was the most potent inhibitor of nucleoside transporter (ventricle: $pK_i = 5.22 \pm 0.41$, K_i = 6 μM). In isolated hearts, midazolam (5, 10, 20 μM) significantly augmented coronary flow in a concentration-dependent manner in the presence of adenosine (30 nM), an effect reversed by ZM 241385, a selective A_{2A}-receptor antagonist. In contrast, midazolam did not increase the effect of adenosine (30 nM) on atrioventricular conduction. Similarly, midazolam potentiated A_{2A}- but not A_1-receptor–mediated effects of endogenous adenosine released during hypoxia.**

Conclusions: **Structurally distinct benzodiazepines inhibit nucleoside transporter to different degrees. Midazolam selectively augments A_{2A}- but not A_1-receptor–mediated effects of adenosine by inhibiting nucleoside transporter. (Key words: Cardiac conduction, coronary flow; guinea pig; isolated heart; radioligand binding.)**

ADENOSINE is an important regulator of cardiac function. Its cardiac effects are predominately mediated by A_1 and A_{2A} receptors. Activation of A_1 receptors reduces oxygen demand by causing a negative chronotropic and dromotropic effect, and by inhibiting catecholamine-stimulated increases in ventricular contractility, whereas activation of A_{2A} receptors increases oxygen supply by causing coronary vasodilation. The physiologic properties of adenosine convey cardioprotection during myocardial ischemia.[1,2] Therefore, therapeutic modalities that exploit the cardioprotective effects of endogenous adenosine are being developed. Augmentation of the effects of adenosine *via* inhibition of adenosine metabolism offers a unique approach to selectively harness its properties in an event- and site-specific manner.[3,4] The utility of this approach was recently demonstrated in the clinical setting. Acadesine, a drug that weakly inhibits adenosine metabolism,[5] reduced the incidence of early cardiac death and myocardial infarction after coronary artery bypass grafting.[6]

Benzodiazepines reduce adenosine degradation by

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weakly inhibiting the nucleoside transporter, the major mechanism whereby the effect of adenosine is terminated through reuptake into cells and reincorporation into the intracellular nucleoside pool.[7,8] Significant differences in binding affinity to nucleoside transporter have been noted among benzodiazepines in human erythrocytes,[8] atrial membranes,[9] and neurons.[10] Similarly, in functional studies, benzodiazepines augmented the negative inotropic effect of exogenous adenosine in guinea pig atrial strips.[11,12] However, binding and functional studies have never been conducted in cardiac tissue for the most commonly used benzodiazepine in the perioperative setting, midazolam. Furthermore, it remains to be shown whether benzodiazepines can selectively augment the effects of endogenous adenosine during ischemia or global hypoxia in the whole heart.

Therefore, we studied the binding affinity for nucleoside transporter of midazolam, relative to that of four structurally distinct benzodiazepines, by performing competition radioligand binding assays on membranes derived from heart and brain. Furthermore, we studied whether the benzodiazepine with the greatest affinity for nucleoside transporter can selectively augment the effects of (1) exogenously administered adenosine and (2) endogenously released adenosine during global hypoxia in guinea pig isolated hearts.

Materials and Methods

Chemicals

The benzodiazepines alprazolam, chlorodiazepoxide, diazepam, and flurazepam were purchased from Research Biochemicals (RBI, Natick, MD) and prepared as stock solutions in ethanol. Midazolam was obtained from Roche Laboratories (Nutley, NJ). *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR, RBI), its tritiated derivative [^{3}H]NBMPR (New England Nuclear, Dupont, Boston, MA), the selective A_{2A}-adenosine receptor antagonist ZM 241385, (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol), a gift from Dr. Simon Poucher (Zeneca Pharmaceuticals, London, UK), and its tritiated derivative [^{3}H]ZM 241385 (New England Nuclear) were prepared as stock solutions in dimethylsulfoxide. Stock solutions were further diluted in normal saline. Neither dimethylsulfoxide nor ethanol had a significant nonspecific effect in binding experiments. The concentration of dimethylsulfoxide did not exceed 0.01% (vol/vol) in the perfusion medium for the isolated heart experiments and did not affect coronary flow or the stimulus-to-ventricle (S-V) interval, a measure of atrioventricular conduction time (AVCT).[13,14]

Membrane Preparations

Membranes for binding studies were prepared from porcine brain striatum and guinea pig ventricle in ice-cold Tris-HCl buffer (50 mM, *p*H 7.4). The protein content of membrane suspensions was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The membranes were stored at −80°C until used for binding assays.

Binding Assays

Equilibrium saturation binding studies were performed to determine the dissociation constant (K_d) of NBMPR. Membrane suspensions were incubated for 120 min at 25°C with varying concentrations of [^{3}H]NBMPR (0.17–10 nM). Nonspecific binding was defined as binding not displaced by an excess (10 μM) of unlabeled NBMPR and was subtracted from total binding to calculate specific binding. K_d was 1.9 and 1.0 nM for guinea pig ventricular and porcine striatal membranes, respectively. Competition assays to determine the affinities of alprazolam, chlorodiazepoxide, diazepam, flurazepam, and midazolam for the [^{3}H]NBMPR binding site were performed by incubating membrane suspensions for 90 min at 25°C with 4 nM [^{3}H]NBMPR and progressively higher concentrations of the respective benzodiazepines in a final volume of 300 μl. Similarly, competition binding assays were performed with porcine striatal membranes to determine the affinity of midazolam for A_{2A} receptors labeled with 4 nM [^{3}H]ZM 241385. Protein content, final volume, incubation time, and temperature were 10–50 μg, 300 μl, 120 min, and 25°C, respectively. Nonspecific binding was defined as binding not displaced by 30 μM 5′-*N*-ethylcarboxamidoadenosine (NECA). In all assays, separation of free from bound radioligand was conducted by vacuum filtration with glass fiber filters (type 32 glass, Schleicher & Schuell, Keene, NH) using a Brandel cell harvester (Biomedical Research & Development Laboratories, Inc., Gaithersburg, MD). The radioactivity trapped on the filter was counted using a liquid scintillation counter. All determinations were conducted in triplicate. Binding was linear over the range of protein concentrations used (protein content 10–50 μg for pig striatum and 100–300 μg for guinea pig ventricle). Assay times and temperatures were chosen to assure equilibrium conditions.

Isolated Perfused Hearts

All protocols involving animals were reviewed and approved by the Animal Use Committee of the Univer-

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sity of Florida Health Science Center. Hartley guinea pig hearts were isolated and perfused according to the Langendorff method as previously described.[15] The perfusate was supplied through a nonrecirculating system fed by a peristaltic pump at constant flow, set to exceed maximal coronary flow, with the outflow variably split between coronary flow and overflow. With this arrangement, administration of drugs at the inflow allowed delivery of a fixed drug concentration regardless of changes in coronary flow. The oxygen tension, temperature, and *p*H of the perfusate were maintained at 500–600 mmHg, 36 ± 0.5°C, and 7.3–7.4, respectively, unless stated otherwise. Because of the limited oxygen-carrying capacity of salt solutions, a partial pressure of oxygen > 500 mmHg in the perfusate represents normoxia for a nonworking heart preparation.[16] The left atrium was excised to facilitate placement of a small plastic catheter into the left ventricle to prevent increases in wall tension through fluid accumulation. Hearts were paced at a fixed cycle length of 270 ms (222 beats/min). A unipolar electrode was placed on the interatrial septum to continuously acquire an electrogram. The S-V interval was used for the analysis of AVCT because neither the stimulus-to-atrium nor the His bundle-to-ventricle intervals are affected by adenosine or adenosine-regulating agents.[14] Coronary flow was measured as total perfusate flow using an ultrasonic transit time flow probe (Model 2N, Transonic Systems, Ithaca, NY) placed in the perfusate line. Data were continuously recorded to the hard disk of an IBM-compatible PC (PII-300 MHz, Gateway 2000, North Sioux City, SD) using an analog-to-digital data board (TL-1-125, Axon Instruments, Foster City, CA).

Experimental Protocols

Effects of Midazolam on Coronary Flow and Atrioventricular Conduction in Normoxic Hearts. After equilibration and acquisition of baseline coronary flow and AVCT measurements, hearts were exposed to 5 and 10 μM midazolam for 25 min at each concentration. Subsequently, 1 μM ZM 241385 was administered in the continued presence of 10 μM midazolam for 10 min. The coronary flow and AVCT were recorded at the end of each intervention. To control for possible time-related deterioration of the heart preparation or agonist-induced desensitization, return to baseline values was confirmed after a 30-min washout period, and the coronary flow response after a 2.5-μg bolus dose of adenosine was measured. Bolus doses of adenosine were administered as 100-μl aliquots over 2 s by syringe pump into the perfusate line proximal to the flow probe. As the maximal A_{2A}-adenosine receptor-mediated response was a twofold to threefold increase in coronary flow (fig. 1) in hearts not treated with midazolam, data were retained for hearts in which at least a doubling of coronary flow was observed.[16,17]

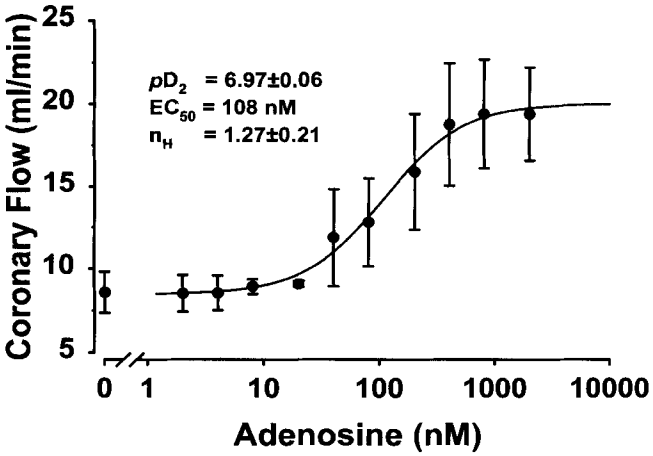

Fig. 1. Concentration–response relationship for the coronary vasodilation caused by adenosine in guinea pig isolated hearts. Points indicate the mean ± SD of single measurements in each of three hearts. EC_{50} = concentration that causes a half-maximal effect; n_H = Hill coefficient; $pD_2 = -\log_{10}(EC_{50})$.

Effects of Midazolam on Coronary Flow and Atrioventricular Conduction in the Presence of Exogenously Administered Adenosine. In a first set of experiments, the changes in coronary flow and AVCT caused by a bolus dose of 0.5 μg adenosine were measured in the absence and presence of 5 μM midazolam followed by a third bolus dose after a 30-min washout period. Coronary flow and the AVCT were recorded before each bolus dose and at peak coronary flow. To assure the validity of the experimental design, the reproducibility of the response to three consecutive bolus doses of adenosine was assessed in hearts not exposed to midazolam.

In a second set of experiments, the effects of midazolam were assessed in hearts exposed to a continuous infusion of 30 nM adenosine, a concentration just sufficient to increase coronary flow (fig. 1). In the continued presence of adenosine, midazolam was administered at progressively higher concentrations of 5, 10, and 20 μM for 15 min at each concentration followed by the administration of 1 μM ZM 241385 for 20 min concurrently with 20 μM midazolam and adenosine. The coronary flow and AVCT were recorded before each intervention. Postintervention measurements were performed after a 30-min washout, followed by testing the coronary flow

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response to a bolus dose of 2.5 μg adenosine. A doubling of coronary flow was considered indicative of a viable preparation.

Effects of Midazolam on Coronary Flow and Atrioventricular Conduction in the Presence of Endogenously Released Adenosine. Hearts were exposed to three consecutive 3-min episodes of hypoxic perfusion by changing the perfusate to Krebs-Henseleit solution gassed with 20% O_2, 5% CO_2, and 75% N_2 (temperature 36°C, *p*H 7.3–7.4, partial pressure of oxygen 200 ± 20 mmHg) to stimulate the release of endogenous adenosine. Each 3-min period of hypoxic perfusion was followed by a 20-min normoxic recovery. After control measurements, 5 μM midazolam was administered for 15 min before a second episode of hypoxia. ZM 241385 (1 μM) was then infused for 5 min in the continued presence of midazolam before the last episode of hypoxia. Coronary flow and AVCT were recorded before and after each intervention and at peak coronary flow. To validate the experimental design, the reproducibility of the response to three consecutive exposures to hypoxic perfusion was assessed in hearts not exposed to midazolam or ZM 241385.

Data Analysis

Binding parameters describing the results of competitive displacement experiments (affinity constant [K_i] and Hill coefficient) were determined using the radioligand binding analysis program GraphPad v 2.0 (GraphPad Software Inc., San Diego, CA). K_i values were calculated using the Cheng-Prusoff transformation.[18] Because values of K_i are not normally distributed, mean values were calculated from the logarithmically transformed data (pK_i) for each experiment.[19] The S-V interval and the coronary flow were measured at the times indicated. Changes in coronary flow were determined by integrating the recorded flow-time curves to generate the area under the curve using TableCurve 2D (Jandel Scientific, San Rafael, CA).

All data are presented as mean ± SD. Before parametric testing, the assumption of normality was validated using the Kolmogorov-Smirnov test with Lilliefors correction (SSPS v9.0, SPSS, Inc., Chicago, IL). Differences between mean values were analyzed by one- or two-way repeated measures analysis of variance of raw data, followed by Student-Newman-Keuls testing. A P value < 0.05 was considered to indicate statistically significant differences.

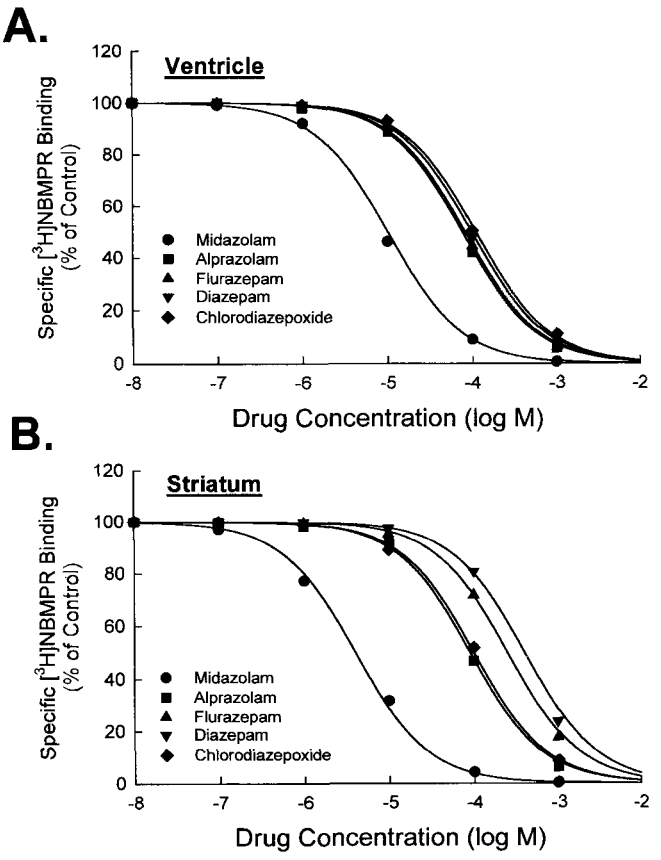

Fig. 2. Competition by benzodiazepines for binding sites labeled by [^{3}H]NBMPR in membranes prepared from guinea pig ventricle and porcine striatum. The competition curves depict results of a single representative experiment from a series of four to eight experiments. The K_i and pK_i values for benzodiazepines to compete with [^{3}H]NBMPR are given in table 1.

Results

Binding of Benzodiazepines to Nucleoside Transporter

Specific binding of [^{3}H]NBMPR to membranes prepared from guinea pig ventricles and porcine striatum was displaced in the presence benzodiazepines (alprazolam, chlorodiazepoxide, diazepam, flurazepam, and midazolam; fig. 2). Although the displacement of [^{3}H]NBMPR was concentration-dependent and complete (fig. 2), the ligand potencies were significantly different (table 1). In both tissues, midazolam was the most potent inhibitor of the nucleoside transporter. The rank orders of potency for the ligands to displace the specific binding of [^{3}H]NBMPR from guinea pig ventricle and from porcine striatum were midazolam > alprazolam = flurazepam = diazepam = chlorodiazepoxide and midazo-

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Table 1. Binding Affinities of Benzodiazepines for Nucleoside Transporter as Determined by Competition for [^{3}H]NBMPR Binding Sites in Membranes Prepared from Guinea Pig Ventricle or Porcine Striatum

	K_i, μM (pK_i ± SD)			
Benzodiazepine	Ventricle	n	Striatum	n
Midazolam	6.0 (5.22 ± 0.41)	7	2.8 (5.56 ± 0.38)	4
Alprazolam	39.8 (4.40 ± 0.16)	7	46.8 (4.33 ± 0.22)	4
Flurazepam	39.8 (4.40 ± 0.20)	6	120.2 (3.92 ± 0.13)*	4
Diazepam	46.8 (4.33 ± 0.20)	7	204.2 (3.69 ± 0.20)*	4
Chlorodiazepoxide	53.7 (4.27 ± 0.31)	7	49.0 (4.31 ± 0.29)	4

K_i indicates the equilibrium dissociation constant. Values are mean ± SD of the results of n experiments performed in triplicate. Numbers in parentheses are mean pK_i ± SD where $pK_i = -\log_{10}(K_i)$ and were determined by use of the Cheng-Prusoff equation.

$P < 0.05$: *, ventricle compared to striatum. Note: analysis of all binding studies gave Hill coefficients that were not significantly different than unity.

lam > alprazolam = chlorodiazepoxide > flurazepam = diazepam, respectively. When mean values of pK_i for all drugs were analyzed across tissues, it was found that drug affinities for nucleoside transporter were dependent on the tissue source of the nucleoside transporter to a significant degree ($P = 0.034$). Both diazepam and flurazepam had significantly higher affinities for nucleoside transporter in guinea pig ventricle than for nucleoside transporter in porcine striatum (table 1 and fig. 2).

Midazolam Augments Coronary Flow (A_{2A}-receptor-mediated) but Does Not Delay Atrioventricular Conduction (A_1-receptor-mediated) in the Presence of Adenosine

Midazolam Alone in Normoxic Heart Preparations. Exposure of normoxic hearts (n = 4) to midazolam caused a significant, concentration-dependent, reversible increase in coronary flow (table 2). ZM 241385, a selective A_{2A}-adenosine receptor antagonist, eliminated approximately 50% of this effect ($P = 0.003$). The S-V interval tended to shorten in parallel to the increase in coronary flow. In contrast to the shortening of the S-V interval during the administration of an adenosine bolus dose (see below), this change did not achieve statistical significance ($P = 0.08$). These effects were fully reversible as washout values for coronary flow, and the S-V interval did not differ from control measurements. Likewise, all hearts demonstrated at least a doubling of coronary flow after a 2.5-μg bolus dose of adenosine (data not shown).

Exogenous Administration of Adenosine to Normoxic Heart Preparations. A bolus dose of 0.5 μg adenosine caused a transient, reproducible increase in coronary flow from 5.1 ± 0.6 to 7.9 ± 1.3 ml/min ($P = 0.034$; figs. 3A and 3B) and tended to shorten the S-V interval by approximately 2 ms ($P = 0.07$; figs. 3C and 3D) in a control group of three hearts. In the presence of 5 μM midazolam, the increase in coronary flow after an adenosine bolus—expressed as the area under the flow curve—was significantly greater than at baseline or after washout of midazolam ($P = 0.002$; n = 6; figs. 4A and 4B). The presence of 5 μM midazolam caused similar increases in baseline coronary flow (from 6.5 ± 1.2 to 7.4 ± 1.0 ml/min; $P < 0.05$) and in peak coronary flow (from 11.1 ± 1.8 to 12.7 ± 1.7 ml/min; $P < 0.01$). The S-V interval shortened in parallel with the coronary flow increase for all three adenosine administrations. At the peak increase of coronary flow, AVCT decreased from 52.3 ± 2.6 ms to 50.7 ± 2.4 ms ($P = 0.006$). However, this change in AVCT was not affected by midazolam (figs. 4C and 4D).

Figure 5 demonstrates the interaction between a constant concentration of adenosine (30 nM) and successively higher concentrations of midazolam (5, 10, and 20 μM). In the absence of midazolam, adenosine caused minimal to no change in coronary flow as shown by the concentration-response relationship depicted in figure 1. In the presence of 30 nM adenosine, coronary flow increased 66 ± 14%, 123 ± 31%, and 165 ± 44% above control measurements during administration of 5, 10, and 20 μM midazolam, respectively ($P < 0.001$; n = 4). Approximately 70% of the maximal increase in coronary flow caused by 20 μM midazolam in the presence of adenosine was antagonized by ZM 241385. Adenosine

Table 2. Effect of Midazolam on Coronary Flow (A_{2A}-adenosine Receptor-mediated) and atrioventricular conduction time (A_1-adenosine Receptor-mediated) Responses in Normoxic Guinea Pig Isolated Perfused Heart

Intervention	Coronary Flow (ml/min)	S-V Interval (ms)
Control	6.0 ± 0.5	50.0 ± 3.5
Midazolam 5 μM	7.1 ± 0.8*	49.3 ± 3.1
Midazolam 10 μM	8.7 ± 1.5*,†	47.5 ± 4.4
Midazolam 10 μM + ZM 241385	7.4 ± 0.8*	50.0 ± 4.5
Washout	5.7 ± 0.4	51.5 ± 4.0

Values expressed as mean ± SD for four hearts. Atrioventricular conduction time was measured as the stimulus-to-ventricle (S-V) interval from the electrogram. $p < 0.05$: *, compared to both Control and Washout; †, compared to both midazolam 5 μM and midazolam 10 μM + ZM 241385, 1 μM, a selective A_{2A}-adenosine receptor antagonist.

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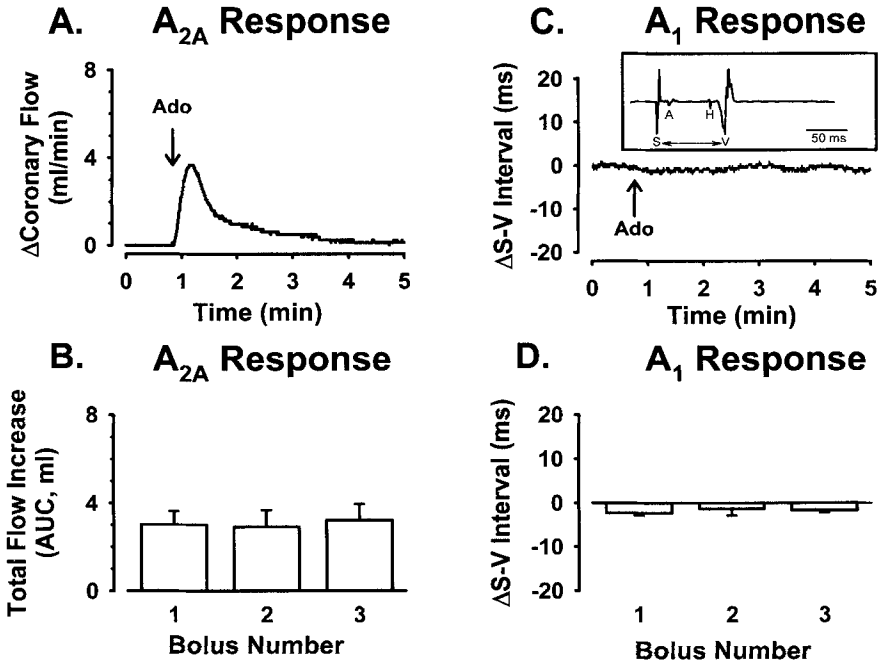

Fig. 3. Reproducible effect of an adenosine bolus dose on coronary flow and stimulus-to-ventricle (S-V) interval. Shown are representative examples and summary data of the A_{2A}-adenosine receptor–mediated vasodilator response (*A* and *B*) and of the A_1-adenosine receptor–mediated S-V interval prolongation (*C* and *D*). (*C, inset*) Representative electrogram used to measure the S-V interval (arrows). Adenosine-induced increases in coronary flow are expressed as changes in the area under the flow–time curve in three hearts. Changes in S-V interval were measured at peak increase in coronary flow. Summary data are expressed as mean ± SD. A = atrium; Ado = adenosine; H = His bundle; S = stimulus artifact; V = ventricle.

and midazolam did not significantly affect the S-V interval (values ranged from 49.0 ± 2.4 to 52.5 ± 2.6 ms), whereas the addition of ZM 241385 significantly prolonged AVCT to 56.5 ± 5.5 ms ($P = 0.007$; n = 4). The changes in coronary flow and S-V interval were reversible on washout of all drugs (data not shown).

Effects of Midazolam in Hypoxic Heart Preparations. Exposure of hearts to three consecutive episodes of endogenous adenosine release caused by hypoxic perfusion reproducibly increased coronary flow from a baseline of 5.7 ± 1.5 to a peak flow of 11.2 ± 2.2 ml/min ($P = 0.01$; n = 3). The hypoxia-induced increases in

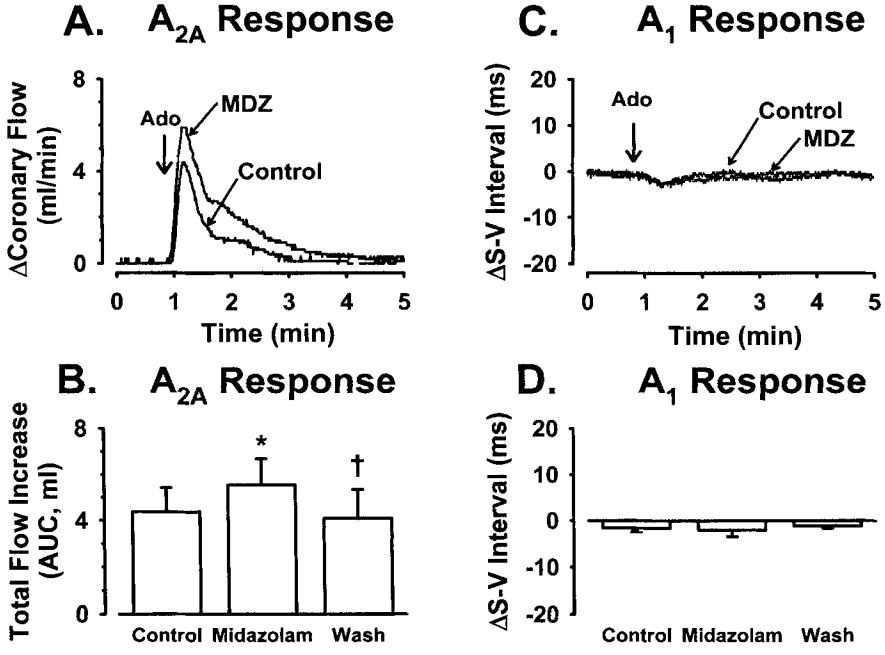

Fig. 4. Selective augmentation by midazolam of the coronary flow response after an adenosine bolus dose. Shown are representative examples and summary data of the effect of midazolam (5 μM) on A_{2A}-adenosine receptor–mediated increases in coronary flow (*A* and *B*) and on A_1-adenosine receptor–mediated changes in the S-V interval (*C* and *D*). Summary data are expressed as mean ± SD. $P < 0.05$ compared with *control and †midazolam.

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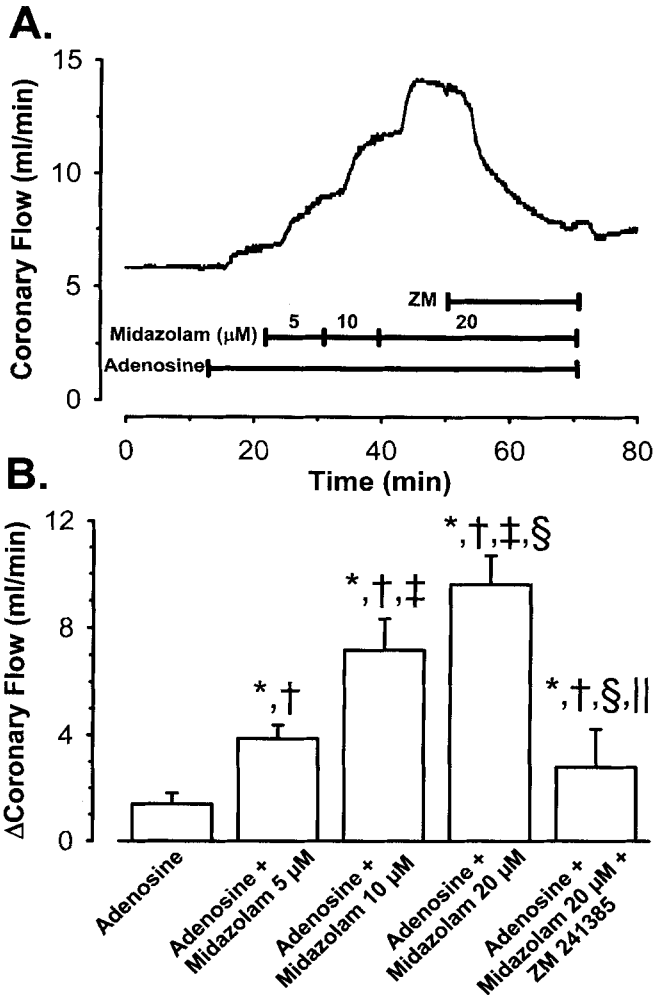

Fig. 5. Concentration-dependent potentiation by midazolam of A_{2A}-receptor–mediated increases in coronary flow caused by adenosine (30 nM). (*A*) Representative example of the effect of increasing concentrations of midazolam on coronary flow. ZM 241385, a specific A_{2A}-adenosine receptor antagonist, attenuated the increase in coronary flow. Solid bars represent the duration of drug administration. (*B*) Summary data for changes in coronary flow as depicted in (*A*). Data expressed as mean ± SD for four experiments. $P < 0.05$ compared with *baseline, †adenosine, ‡adenosine + midazolam 5 μM, §adenosine + midazolam 10 μM, and ‖adenosine + midazolam 20 μM.

coronary flow, expressed as the areas under the flow-time curve, for the three episodes were 9.8 ± 1.3, 10.1 ± 2.3, and 11.5 ± 0.5 ($P = 0.45$; n = 3). During hypoxia, coronary flow increased linearly with a stable rate of increase of 2.8 ± 0.5 ml/min^2 ($P < 0.67$ for the three consecutive episodes). The S-V interval did not shorten consistently during the period of hypoxic perfusion.

Administration of 5 μM midazolam during hypoxic perfusion increased the coronary flow response by > 50% ($P = 0.005$; n = 6; figs. 6A and 6B). Similar to previous experiments, coronary flow increased reversibly from a baseline of 5.6 ± 0.6 ml/min to a new baseline of 7.4 ± 0.9 ml/min in the presence of midazolam. This new baseline was used to determine the total flow increase in response to hypoxic perfusion in the continued presence of midazolam (figs. 6A and 6B). ZM 241385 attenuated the midazolam-induced increase in coronary flow. The presence of ZM 241385 also attenuated the rate of increase in coronary flow to 2.0 ± 0.8 ml/min^2, compared with 2.9 ± 1.0 ml/min^2 during control and 2.8 ± 0.9 ml/min^2 in the presence of 5 μM midazolam ($P = 0.04$). However, the total increase in coronary flow caused by global hypoxia was similar during control (hypoxia 1) and in the presence of both midazolam and ZM 241385 (hypoxia 3). The S-V interval did not change significantly or consistently during the consecutive hypoxic episodes (figs. 6C and 6D).

Binding of Midazolam to A_{2A}-adenosine Receptors

To determine whether the selective augmentation of adenosine-induced coronary vasodilation is caused by direct activation by midazolam of A_{2A}-adenosine receptors, in competition binding assays we assessed the affinity of midazolam to porcine striatal A_{2A}-adenosine receptors labeled with [^{3}H]ZM 241385. At concentrations used in the isolated heart experiments, midazolam did not displace specific binding of [^{3}H]ZM 241385 (fig. 7). In contrast, the adenosine receptor agonist NECA caused concentration-dependent, complete displacement with a Hill coefficient not different from unity and a value of K_i (pK_i ± SD) of 78 nM (7.13 ± 0.16), indicating the presence of A_{2A}-adenosine receptors in the membrane preparation.

Discussion

Our results demonstrate that (1) among five commonly used benzodiazepines, midazolam is the most potent inhibitor of nucleoside transporter in heart (guinea pig ventricle) and brain (porcine striatum), and (2) midazolam can be used as an "adenosine-regulating agent" to selectively enhance the cardiac actions of adenosine in an event- and site-specific manner in the guinea pig isolated heart.

Binding of Benzodiazepines to the Nucleoside Transporter

A novel finding of the current study is that among five commonly used benzodiazepines, midazolam is the most potent inhibitor of nucleoside transporter in two distinct

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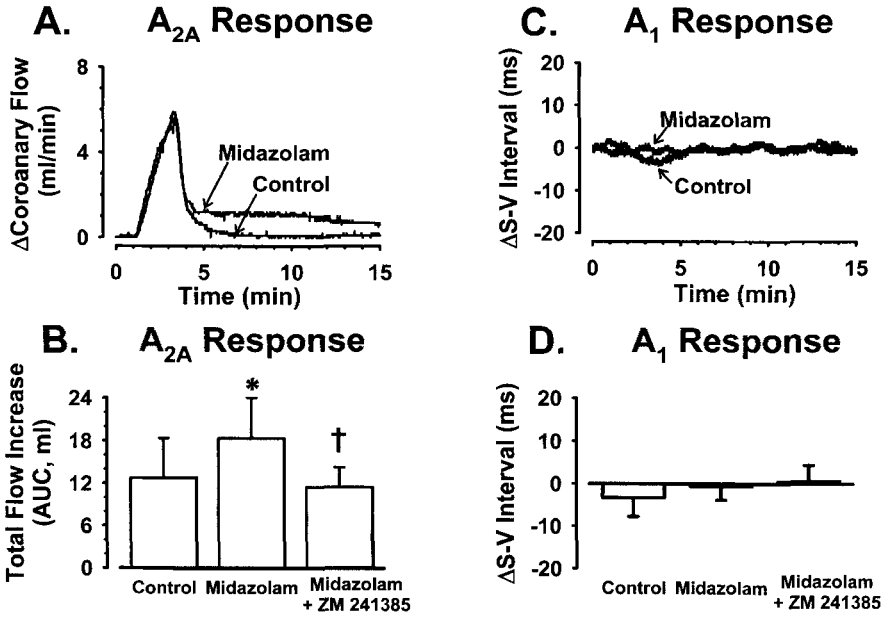

Fig. 6. Midazolam increases the coronary flow response to adenosine endogenously released during global hypoxia. (*A*) Representative example of tracings of the coronary flow response in the absence and presence of midazolam (MDZ) from a single heart. (*B*) Summarized data for six experiments as shown in (*A*). The flow increase was measured as the area under the flow–time curve. Atrioventricular conduction was not significantly affected, as shown in the example in (*C*) and the summary data in (*D*). Summary data are expressed as mean ± SD; $P < 0.05$ compared with *control and †MDZ (5 μM).

tissues from different species. In agreement with our results, other investigators also found significant differences among benzodiazepines in their ability to displace [^{3}H]NBMPR binding to the nucleoside transporter in various tissues.[7,9,20] Midazolam, the benzodiazepine most widely used in the perioperative setting, was only studied in human erythrocytes.[9] No prior study assessed midazolam binding to transporter in cardiac and neural tissue. All benzodiazepines tested completely displaced [^{3}H]NBMPR in a concentration-dependent and competitive manner from heart and brain (table 1 and fig. 2). The rank order of potency of benzodiazepines to compete with [^{3}H]NBMPR for binding to guinea pig ventricle differed slightly from the rank order of potency of benzodiazepines to compete for binding to porcine striatum. Although this difference in rank order of potency of benzodiazepines to compete with [^{3}H]NBMPR for binding sites in these two tissues may reflect the existence of distinct subtypes of nucleoside transporter, these slight differences in the rank order of ligand potency are not unusual and have been described for other binding studies.[9,20,21] In addition, species-dependent factors may contribute to this difference.

The results of competition binding studies indicate that benzodiazepines are weak inhibitors of transporter at concentrations that facilitate GABAergic transmission *in vivo*. For example, the K_i value of midazolam to displace [^{3}H]NBMPR from guinea pig ventricular membranes was approximately 5 μM (table 1). According to the law of mass action, in the absence of transporter substrate (*i.e.*, adenosine), 5 μM midazolam will only inhibit 50% of nucleoside transporter. The affinity of benzodiazepines for the nucleoside transporter is thus more than 1,000-fold lower than that of prototypical high-affinity nucleoside transporter ligands such as NBMPR (K_d = 1.6 nM in guinea pig ventricle[22,23]) or dipyridamole (K_i = 1.9 nM in human erythrocytes[9]). To achieve relatively selective inhibition by midazolam of nucleoside transporter and thus minimize nonspecific effects provided the rationale of using a concentration of

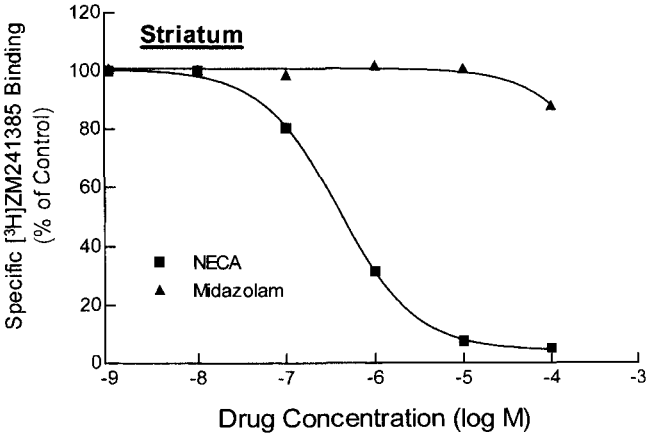

Fig. 7. Competition by midazolam and the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) for binding sites labeled by the A_{2A}-adenosine receptor antagonist [^{3}H]ZM 241385 in membranes prepared from porcine striatum. The competition curves depict results of a single representative experiment from a series of three experiments.

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midazolam at its K_i value in most of the experimental protocols. Although this concentration of midazolam is approximately an order of magnitude lower than those used in previous isolated heart studies,[7,8,12,17] it is nonetheless at the upper end of the clinically relevant dose spectrum (6 μM [2 μg/ml] during bolus administration and 1.7 μM for deep hypnosis[24,25]). Furthermore, the high degree of protein binding of benzodiazepines (> 90%, at least under physiologic conditions) further reduces the free concentration of drug *in vivo*. Therefore, it is unlikely that under physiologic conditions a significant midazolam-induced potentiation of the cardiac effects of adenosine would occur. Regardless, the major goal of this study was to demonstrate the feasibility and rationale of using the benzodiazepine with the greatest binding affinity to nucleoside transporter, namely, midazolam, as an "adenosine-regulating agent" to selectively enhance the cardiac actions of adenosine in an event- and site-specific manner.

Effects of Midazolam on Adenosine Receptor-Mediated Responses

Midazolam selectively augmented coronary vasodilation (A_{2A}-receptor-mediated) but not atrioventricular conduction delay (A_1-receptor-mediated) caused by adenosine, regardless of whether the nucleoside was exogenously administered or endogenously released during global hypoxia. Midazolam alone caused a concentration-dependent increase in coronary flow without prolonging atrioventricular conduction (table 2). This finding is in agreement with a previous study in constant pressure perfused guinea pig isolated hearts that also showed a concentration-dependent increase in coronary flow caused by midazolam, despite a concomitant decrease in myocardial oxygen consumption.[17] Based on the actions of ZM 241385, a potent adenosine receptor antagonist that is > 100-fold more selective for A_{2A} receptors than for other adenosine receptor subtypes (*i.e.*, A_1, A_{2B}, and A_3 receptors),[26] only approximately half of this coronary vasodilator response can be ascribed to increased adenosine levels. Although the etiology of the ZM 241385-insensitive component cannot be determined with the design of the current study, it may be secondary to enhanced GABA receptor activity.[27] In any case, the focus of our study was neither to compare the potency of benzodiazepines as coronary vasodilators nor to define further the underlying mechanism(s) mediating the effects of midazolam on coronary flow, but rather to demonstrate that benzodiazepines can potentiate the cardiac effects of adenosine.

In agreement with our finding that midazolam consistently augmented the effects of exogenous adenosine on coronary flow, pretreatment of rats with diazepam has been shown to enhance the A_{2A}-adenosine receptor-mediated relaxation of isolated pulmonary artery rings.[28] Likewise, diazepam enhanced coronary flow increases caused by exogenous adenosine in anesthetized dogs.[29] Our observation of an augmentation of the cumulative coronary flow during hypoxic perfusion extends these findings to pathophysiologic situations wherein adenosine release is increased. The time course of the coronary flow increase in the presence of midazolam suggests that the increase in flow is predominantly caused by a slowed return to baseline coronary flow (figs. 4A and 6A), lending further support to the hypothesis that midazolam delays the elimination of extracellular adenosine.

Although benzodiazepines were previously shown to sensitize guinea pig atria to the negative inotropic effect of exogenously administered adenosine (A_1-receptor-mediated),[11,12] we did not observe a prolongation of AVCT in the presence of midazolam and adenosine. On the contrary, exogenous adenosine, administered as a bolus dose, caused a transient but significant shortening of AVCT. This counterintuitive finding may be explained by the increase in shear stress caused by increased flow, which was shown to alter Ca^{2+} transients and cause an inverse relationship between coronary flow and AVCT.[30]

Mechanism of Differential Augmentation of A_{2A}- versus A_1-adenosine Receptor-mediated Effects by Midazolam

Three factors may explain the differential augmentation of A_{2A}- *versus* A_1-adenosine receptor-mediated effects by midazolam. First, this finding may be a result of differential receptor reserve of A_{2A}- and A_1-adenosine receptor-mediated responses. Receptor reserve is a phenomenon whereby submaximal receptor occupancy elicits a maximal response.[13] In tissues with a large receptor reserve, a small increase in receptor occupancy produces a large response. Significant differences for receptor reserve among distinct receptor subtypes mediating the effects of an endogenously released substance such as adenosine are an important determinant of organ and tissue responsiveness and form the basis of agonist selectivity *in vivo*.[13,15,31,32] In guinea pig isolated hearts, the receptor reserve for A_{2A}-receptor-mediated coronary vasodilation (70%[13]) is much greater than that for A_1-receptor-mediated slowing of atrioventricular nodal conduction (2%[32]). Therefore, the concentration of adenosine required to cause half-maximal cor-

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onary vasodilation (EC_{50}= 108 nM; fig. 1) is > 40-fold lower than that required to produce half maximal slowing of atrioventricular conduction (EC_{50}= 4,200 nM[31]). This phenomenon, at least in part, explains how concentrations of adenosine that are subthreshold for slowing atrioventricular conduction can markedly increase coronary flow.[13] Midazolam may cause small increases in the interstitial concentration of adenosine, sufficient to elicit a significant A_{2A}-receptor-mediated response but subthreshold to concurrently affect A_1-receptor-mediated effects. Consistent with this explanation, adenosine levels in normoxic isolated hearts are just below the inflection point of the steep portion of the concentration-response relationship.[1,33] However, in the setting of ischemia, where coronary flow is limited or absent, this hierarchy in adenosine-mediated responses may be overcome, and adenosine accumulation may be sufficient to convey a protective effect *via* activation of both A_1 and A_{2A} receptors.[34,35] In keeping with this interpretation, we found a small but significant increase in AVCT (A_1-receptor-mediated) in the presence of 20 μM midazolam and 30 nM adenosine only, when the concomitant increase in coronary flow was attenuated by the A_{2A}-adenosine receptor antagonist ZM 241385.

A second factor that may also contribute to an apparent differential responsiveness of A_1 and A_{2A} receptors to endogenous and exogenous adenosine is the phenomenon of "compartmentalization." The concentration of adenosine in the interstitium differs from that in the coronary perfusate (arterial compartment).[36] An analysis using a mathematical model of adenosine metabolism in guinea pig heart indicates that the concentration of adenosine in the interstitium is approximately threefold lower than that in the arteries during the exogenous administration of adenosine and is not a linear function of arterial adenosine concentration.[36] How transient mild hypoxia, which inhibits adenosine kinase[37] and thus increases adenosine release from cardiomyocytes, affects the relative adenosine concentration in these compartments is not known. However, the hypoxia-induced increase in coronary flow may augment oxygen delivery to the atrioventricular node and cause sufficient adenosine washout to keep the increase in interstitial adenosine levels subthreshold for activation of A_1-adenosine receptors. Alternatively, the atrioventricular node may have a higher density of nucleoside transporter sites, which more efficiently remove adenosine, and thus render the atrioventricular node less susceptible to the effects of the nucleoside.

Third, our finding that midazolam selectively amplified A_{2A}- but not A_1-adenosine receptor-mediated responses may simply be a result of direct activation of A_{2A} receptors by midazolam. However, this conclusion is not supported by the results of our competition binding experiments that indicate no significant affinity of midazolam to A_{2A} receptors.

Clinical Implications and Future Directions

The therapeutic benefits of adenosine in the heart are undisputed. The potential cardioprotective effects of exogenous and endogenous adenosine provide the rationale for adding adenosine to crystalloid cardioplegic solutions and explain the improvement in cardiac morbidity and mortality observed with acadesine administration during coronary artery bypass grafting.[6] Exogenously administered adenosine is the drug of choice to treat supraventricular tachyarrhythmias involving the atrioventricular node as part of the reentrant circuit. In a similar manner, the clinical utility of using endogenously released adenosine as an effective antiarrhythmic agent has been demonstrated in humans.[38] Conti *et al.* used the prototypical blocker of nucleoside transporter dipyridamole as an adenosine-regulating agent to terminate supraventricular tachyarrhythmias by a mechanism involving interstitial accumulation of endogenous adenosine acting at the A_1 receptor.[39] In the current study, we extend the results of this prior work to the A_{2A} receptor. Midazolam, by a mechanism involving inhibition of nucleoside transporter and subsequent accumulation of extracellular adenosine, selectively augmented the effects of the nucleoside on coronary flow without affecting AVCT (site-specific action) during global hypoxia (event-specific action). Therefore, benzodiazepines have the therapeutic potential to be used as "adenosine regulating agents." Our results may provide the framework whereby a new series of benzodiazepines could be developed that have even greater nucleoside transporter blocking properties while still retaining their allosteric enhancing effects on GABA receptor-mediated anxiolysis and sedation. By promoting the known cardioprotective effects of adenosine, benzodiazepines of this type may potentially mitigate hypoxia- or ischemia-induced organ injury, particularly in the perioperative setting.

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