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Cardioprotection with a novel adenosine regulating agent mediated by intravascular adenosine

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Abstract

Adenosine is cardioprotective in models of myocardial stunning and infarction, but the precise compartment within the heart in which adenosine elicits its cardioprotective effects has not been determined. The goals of the present study were to (i) investigate the effects of a novel adenosine regulating agent, GP531 (5-amino-1-β-D-(5-benzylamino-5-deoxyribofuranosyl)imidazole-4-carboxamide), on post-ischemic myocardial function, and (ii) examine the contribution of endogenous adenosine in the intravascular and interstitial compartments in mediating the beneficial effects. Pigs were instrumented for measurement of myocardial segment shortening, and for sampling of coronary venous blood and myocardial interstitial fluid for determination of adenosine concentration. Myocardial dysfunction was induced by 4×8 min coronary occlusions, and recovery of regional function was monitored for 2 h. In control pigs, function recovered to $24 \pm 2\%$ of baseline after 2 h. Treatment with GP531 improved functional recovery to $55 \pm 3\%$. GP531-mediated cardioprotection was prevented by adenosine receptor blockade with 8-sulfophenyltheophylline ($23 \pm 2\%$). GP531 did not affect basal adenosine levels, but caused a 2-fold greater increase in vascular adenosine concentration with ischemia (54.6 ± 10.6 vs. 28.1 ± 8.0 μM in controls, $P < 0.05$). In contrast, the interstitial adenosine concentration was not significantly different in treated vs. untreated control pigs (9.4 ± 3.9 vs. 15.0 ± 1.3 μM in controls). These data indicate that (1) GP531 improves recovery of myocardial function following ischemia-reperfusion injury via an adenosine receptor-dependent mechanism, and (2) the cardioprotection is associated with increased intravascular, but not interstitial, adenosine concentration during ischemia. Therefore, we conclude that cardioprotection elicited by GP531-enhanced endogenous adenosine is dependent on an intravascular site of action.

Keywords: Adenosine; Myocardial ischemia, myocardial stunning; Myocardial ischemia, reperfusion injury; Coronary vessel

1. Introduction

The cardioprotective effects of adenosine are the subject of extensive investigation. Recent studies have shown that exogenously administered adenosine decreases myocardial infarct size (Toombs et al., 1992; Yao and Gross, 1994) and attenuates myocardial stunning (Norton et al., 1991, 1992; Sekili et al., 1995). Moreover, the importance of endogenously produced adenosine is underscored by demonstration of cardioprotection using agents which inhibit the uptake (Abd-Elfattah et al., 1993; Kirkebøen et al., 1994) and/or metabolism (Dhasmana et al., 1983; Dorheim et al., 1991) of adenosine. Convincing evidence of the cardioprotective effects of endogenous adenosine is also provided by studies of ischemic preconditioning, which is augmented by agents which enhance local adenosine concentrations (Itoya et al., 1994; Tsuchida et al., 1994), and is prevented by adenosine receptor antagonists (Liu et al., 1991, 1994; Yao and Gross, 1994; Hoshida et al., 1994). Despite this extensive evidence, however, neither the source nor the compartmental site of action of endogenously produced adenosine has been conclusively determined. The conventional viewpoint suggests that adenosine is derived primarily from cardiomyocytes during ischemia, and that elevated interstitial adenosine elicits cardioprotection via activation of adenosine receptors on the myocytes. This is supported by evidence that the cardioprotective effects of adenosine or preconditioning are prevented by selective antagonism of adenosine A_1 receptors (Auchampach and Gross, 1993; Zhao et al., 1994) (which are located primarily on myocytes) and the observation that cardioprotection elicited by adenosine transport in-

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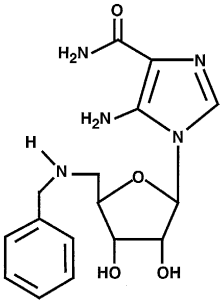

Fig. 1. Chemical structure of GP531 (5-amino-1-β-D-(5-benzylamino-5-deoxyribofuranosyl)imidazole-4-carboxamide).

hibitors (Galiñanes et al., 1993) or by adenosine deaminase inhibitors (Dorheim et al., 1991) is associated with elevated interstitial adenosine concentrations.

An alternative mechanism is suggested by several independent lines of evidence that enhanced intravascular, rather than interstitial, adenosine may contribute significantly to adenosine's cardioprotective effects. For example, preconditioning-induced cardioprotection has been associated with increased adenosine in coronary venous effluent (Kitakaze et al., 1993, 1994), but with decreased interstitial adenosine concentration during ischemia (Van Wylen, 1994). In agreement with this concept, Todd et al. (1995) have reported adenosine-mediated cardioprotection using a stable polyadenylated analog of adenosine which was confined to the vascular space. Taken together, these studies suggest that the critical site of action for adenosine during ischemia might be within the intravascular, rather than the myocardial interstitial, compartment. To date, no studies have been reported in which adenosine was measured simultaneously in the intravascular and interstitial compartments during ischemia.

Therefore, the present studies were designed to investigate the effects of the adenosine regulating agent, GP531 (5-amino-1-β-D-(5-benzylamino-5-deoxyribofuranosyl)imidazole-4-carboxamide, Fig. 1), on recovery of post-ischemic myocardial function in a porcine model of cardiac stunning. In addition, the compartmental release of adenosine was assessed by simultaneous measurement of adenosine in the myocardial interstitial fluid by microdialysis, and in the coronary vasculature by sampling coronary venous blood. The results indicate that GP531 attenuates myocardial stunning via an adenosine receptor-dependent mechanism, and that this cardioprotection is associated with elevated intravascular, but not interstitial, adenosine concentrations.

2. Materials and methods

2.1. General surgical preparation

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23), and were approved by the Institutional Animal Care and Use Committee. Yucatan minipigs (5–6 months of age, 19–31 kg) of either sex were premedicated with xylazine (2 mg/kg, i.m.) and atropine (0.1 mg/kg). Surgical anesthesia was induced with isoflurane, and maintained with pentobarbital (8–10 mg/kg per h). The animals were positioned in a dorsal recumbent position, a tracheotomy was performed via an incision in the mid-line of the neck, and the lungs were ventilated with room air supplemented with oxygen to maintain $P_aO_2 \geq 100$ mmHg. The right internal jugular vein and carotid artery were cannulated for administration of drugs and fluids, and monitoring of arterial pressure and blood gases, respectively. The heart was exposed via a mid-line sternotomy, and a catheter-tip pressure transducer (Millar Instruments, Houston, TX, USA) was inserted into the ventricle through an incision near the apex. A silk ligature was passed around the left anterior descending coronary artery below the first marginal branch for use as a snare. The vessel was occluded briefly (< 15 s) and the ischemic zone was identified by the appearance of cyanosis in the perfused region. Regional myocardial segment shortening was assessed as an index of myocardial function using a sonomicrometer (Triton Technology, San Diego, CA, USA). Piezo-electric sonomicrometry crystals were placed in the sub-endocardium at the center of the ischemic zone, and in the region perfused by the left circumflex coronary artery to measure ischemic and non-ischemic zone segment shortening, respectively. Proper placement of the crystals was verified post-mortem. Hemodynamic variables monitored throughout the study were: arterial pressure, left ventricular pressure and its first derivative (dP/dt), and heart rate. A temperature probe was placed inside the pericardium to monitor core temperature, which was maintained at 38°C throughout the experiment with a heating blanket.

A separate group of animals was used for experiments measuring myocardial and coronary venous adenosine concentrations. Microdialysis probes were placed in the ischemic zone for measurement of myocardial interstitial fluid adenosine concentration, and a 40 mm long cannula was placed in the great cardiac vein at the level of the arterial snare for sampling coronary venous blood. A snare was placed around the coronary vein downstream of the cannula to prevent back-flow of blood during sampling.

Microdialysis probes were constructed and implanted as originally described by Van Wylen et al. (1990). Two probes were placed in the subendocardial layer of the myocardium near the center of the ischemic zone for each experiment, and the effluent from both probes was collected into a single vial. After placement, probe integrity was evaluated by verifying that outflow from each probe was equal to the perfusion rate (5 μl/min). A minimum recovery of 1 h was allowed after probe implantation before collection of baseline samples, a time which is sufficient to allow for stabilization of interstitial metabolite concentrations (Van Wylen et al., 1990). Proper placement

of the probes was verified post-mortem. Dialysate samples were collected for 2 min to provide sufficient sample volume for analysis. Samples were immediately placed in ice, and then stored at −20°C. Coronary venous blood samples were obtained by quickly withdrawing 0.5 ml of blood into a syringe containing 0.5 ml of iced stopping solution composed of: erythro-9(2-hydroxy-3-nonyl)-adenine (EHNA, 5 μM), α,β-methylene-adenosine 5′-diphosphate (AOPCP, 62 μM), dipyridamole (0.2 mM), EDTA (4.2 mM), and heparin (25 000 U/l) (Feldman et al., 1992). To facilitate mixing of the blood and stopping solution, glass beads were added to the syringes, and the syringes were agitated during withdrawal. Samples were immediately transferred to microcentrifuge tubes, chilled to 4°C, centrifuged ($500 \times g$ for 3 min, pellet discarded), and stored at −20°C.

2.2. Protocol

The protocol for inducing myocardial stunning is illustrated graphically in Fig. 2. The animals were allowed to stabilize for 1 h after instrumentation. Regional ischemia was induced by tightening the snare on the coronary artery for 8 min, followed by 8 min of reperfusion. A total of four occlusion/reperfusion cycles were used to induce myocardial stunning. Regional segmental function and systemic hemodynamics were then monitored for 2 h following the final coronary artery occlusion. The effect of GP531 on recovery of post-ischemic function was studied in five groups of animals: (1) vehicle (saline, $n = 6$); (2) low-dose GP531 (50 μg/kg per min × 5 min loading dose, 10 μg/kg per min continuous infusion, $n = 6$); (3) high-dose GP531 (500 μg/kg per min × 5 min loading dose, 100 μg/kg/min continuous infusion, $n = 5$); (4) saline vehicle + 8-SPT (5 μg/kg per h, i.c., $n = 4$); (5) high-dose GP531 + 8-SPT, 5 μg/kg per h, i.c., $n = 4$).

Intravenous infusions of GP531 were begun 30 min prior to the first occlusion. In the 8-SPT groups, 8-SPT was infused into the coronary artery via an indwelling 30 gauge needle beginning 35 min prior to the first occlusion and continuing until 30 min after the fourth occlusion. This dose of 8-SPT has previously been shown to abolish adenosine-mediated coronary vasodilatation (Bullough et al., 1994). In all studies, plasma samples were obtained prior to the first occlusion and after the fourth occlusion for determination of GP531 concentration (by high-performance liquid chromatography, HPLC).

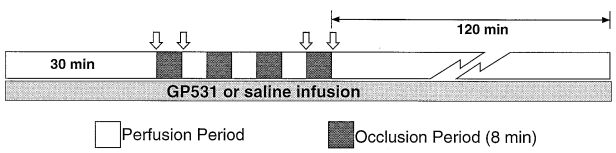

Fig. 2. Protocol used to induce myocardial stunning in minipigs. In the 8-SPT-treatment groups, intracoronary 8-SPT infusions were begun 5 min prior to beginning intravenous infusions of GP531 or saline vehicle. Coronary venous and interstitial fluid sampling timepoints are indicated by the large arrows.

The effects of GP531 on interstitial and coronary venous adenosine during stunning were studied in two additional groups of pigs: GP531 100 μg/kg per min ($n = 6$) and vehicle ($n = 6$). In these two groups, blood and interstitial fluid dialysate samples were collected before and after the first occlusion (immediately upon reperfusion), and before and after the fourth occlusion (immediately upon reperfusion, Fig. 2). Blood samples were not obtained during occlusion periods due to inadequate coronary venous blood flow. In all experiments, venous plasma samples were obtained prior to the first occlusion and after the fourth occlusion for determination of GP531 concentration.

2.3. Analytical methods for determination of adenosine content

Analyses for adenosine concentrations in dialysate and plasma were performed in a blinded manner. Analysis of plasma samples for adenosine content was performed using a liquid chromatography/mass spectrometry system (LC/MS/MS) because of the high degree of specificity and sensitivity available with this technique. Samples (0.2 ml) were spiked with an internal standard (8-bromoadenosine; Sigma, St. Louis, MO, USA) and treated with acetonitrile to precipitate proteins. Samples were injected onto a strong cation exchange column (SCX cartridge, YMC, Wilmington, NC, USA). The effluent from the HPLC column passed via a heated nebulizer to a PE SCIEX API III-Plus triple quadrupole mass spectrometer, operated in positive ion mode. The protonated molecular ions of adenosine and the internal standard at m/z 267.7 and 345.9, respectively, were selected to pass the first quadrupole mass filter and react with the argon collision gas in the second quadrupole. The resulting daughter ions of m/z 135.8 and 213.7, respectively, were then selected to pass the third quadrupole (identical to the first) and activate the electron multiplier target. The mass chromatograms were processed automatically by the PE SCIEX software program MACSPEC. Peak area ratios of adenosine to the internal standard and the concentrations of the calibration standards were fitted by weighted linear least squares regression. Peak area ratios of unknown samples were converted to concentrations of adenosine using these computer-generated parameters. Calibration curves were generated each day which were linear (correlation coefficient > 0.9987) and reproducible over the calibrated range of 18.71 nM–1.497 μM. Samples of pig plasma containing adenosine at concentrations above the upper limit of the calibration curve were appropriately diluted with control (blank) plasma and reanalyzed.

Measurement of adenosine in interstitial fluid dialysate was accomplished by injecting samples directly onto a microbore HPLC column (Bioanalytical Systems C-18,

100 × 1 mm; 3 μm particle size). Adenosine was eluted after 17.5 min using KH_2PO_4 buffer (10 mM, pH 3.0) at a flow rate of 50 μl/min. Eluents were monitored at 254 nm, and adenosine was quantified against a standard curve generated each day.

2.4. Criteria for acceptable experiment

Of 33 animals introduced into the protocol, eight were excluded from the study for the following reasons: (1) failure to survive for the entire protocol – 5 control; (2) sonomicrometry crystals not properly located in the ischemic zone – 1 control; (3) microdialysis probes not properly located in the ischemic zone – 1 control, 1 treated.

2.5. Data analysis

Myocardial interstitial fluid adenosine concentrations were calculated from dialysate adenosine concentrations by multiplying the dialysate adenosine concentration by the inverse of the recovery rate for the microdialysis probe ([adenosine] × 1/recovery rate). The recovery rate was determined by submerging the probe in Krebs buffer containing known concentrations of adenosine (1, 10 and 100 μM), perfusing the probes with buffer at 5 μl/min, and measuring the adenosine concentrations in both as described above. Recovery rate was calculated as:

$$[\text{adenosine}]_{\text{dialysate}}/[\text{adenosine}]_{\text{buffer}}.$$

The recovery rate was determined to be 19.0 ± 0.5% for all concentrations of adenosine tested. Concentrations of adenosine in plasma were corrected for dilution with stopping solution.

Comparison of data between groups was made using repeated-measures analysis of variance. The Student's paired *t*-test with Bonferroni correction was used to compare values within a group to their corresponding baseline values. Differences were considered significant at $P \leq 0.05$. All data are expressed as mean ± S.E.M.

3. Results

3.1. Hemodynamic effects

Systemic hemodynamic effects of GP531 are summarized in Table 1. Baseline values for systemic arterial pressure, left ventricular developed pressure and left ventricular dP/dt were similar in all groups and were unaffected by infusion of GP531 in either protocol. In all groups, global cardiac function, assessed as left ventricular dP/dt, decreased during the first occlusion, and remained depressed throughout the protocol. Except for higher left ventricular and arterial pressures in the low-dose GP531 group, there were no differences in any hemodynamic values between treated and untreated groups. There was an overall tendency for arterial and left ventricular developed pressure to decline over the course of the protocol, but these trends were not significant, and were unaffected by GP531 infusion (Table 1).

Plasma concentrations of GP531 were at steady state prior to the first occlusion, averaging 2.0 ± 0.1 and 13.1 ± 2.0 μM at 10 and 100 μg/kg per min, respectively.

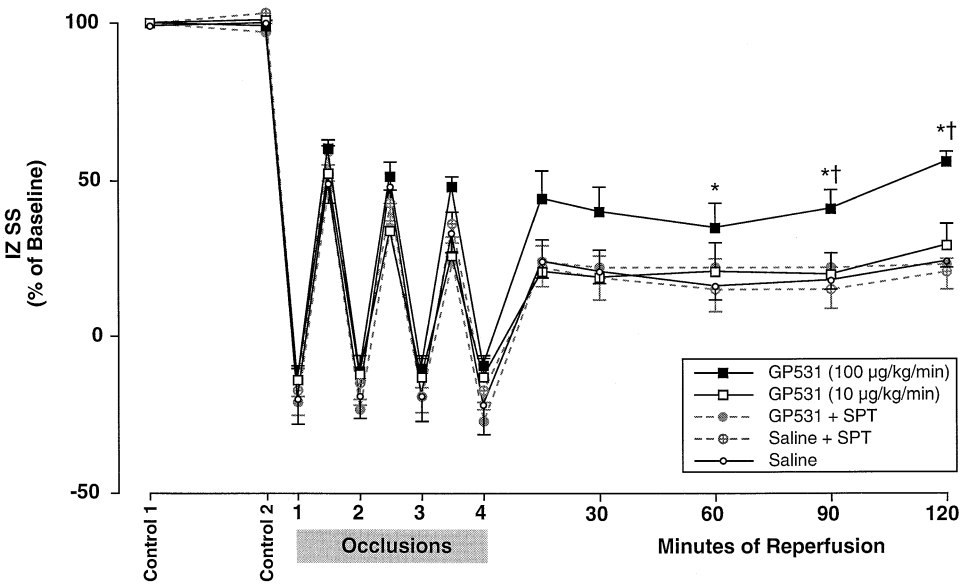

Fig. 3. Effects of treatment with GP531 (10 or 100 μg/kg per min, i.v.) and 8-sulfophenyltheophylline (8-SPT, 5 mg/kg per h, i.c.) on recovery of post-ischemic myocardial segment shortening. Results are expressed as percent of baseline value. IZ SS = ischemic zone myocardial segment shortening; Control 1 = pre-GP531 (or pre-8-SPT) baseline; Control 2 = pre-occlusion 1 baseline; * $P < 0.05$ vs. saline; † $P < 0.05$ vs. GP531 + 8-SPT.

3.2. Effects of GP531 on post-ischemic function

The effects of treatment with GP531 on post-ischemic segmental function are summarized in Fig. 3. Marked ischemic zone dyskinesis was elicited during each of the four 8-min coronary artery occlusions, with partial recovery during the intervening reperfusion periods. The degree of dyskinesis and recovery during the occlusion-reperfusion cycle was not affected by either dose of GP531. However, ultimate recovery of segment shortening was significantly improved with the high-dose infusion of GP531. In saline-treated pigs, segment shortening recovered to $24 \pm 2\%$ of baseline following 2 h of reperfusion. This recovery was unchanged by the 10 μg/kg per min infusion of GP531 at any timepoint, averaging $25 \pm 6\%$ after 2 h of reperfusion. In contrast, recovery of segment shortening was significantly ($P < 0.05$) improved in animals treated with 100 μg/kg per min GP531, averaging $33 \pm 6\%$, $38 \pm 6\%$, and $55 \pm 3\%$ at 60, 90, and 120 min of reperfusion, respectively (Fig. 3).

Fig. 3 also illustrates that adenosine receptor blockade with 8-SPT prevented the improvement in functional recovery elicited by GP531. Combined GP531 (100 μg/kg per min) and 8-SPT (5 mg/kg per h, i.c.) resulted in a recovery of function to $23 \pm 2\%$ of baseline after 2 h of reperfusion (P = NS vs. saline). 8-SPT alone had no effect on recovery of segment shortening at any timepoint, with recovery averaging $21 \pm 6\%$ after 2 h.

3.3. Effects of GP531 on adenosine concentration

The effects of GP531 (100 μg/kg per min, i.v.) on coronary venous adenosine concentration are summarized in Fig. 4A. Adenosine concentration in coronary venous blood from saline-treated control pigs averaged 0.26 ± 0.06

Table 1
Summary of hemodynamic variables in pigs treated with GP531

	Baseline	GP531	Occl 1	Occl 4	15 min	60 min	120 min
HR (beats / min)							
Saline	99 ± 9	97 ± 10	95 ± 9	96 ± 9	98 ± 9	102 ± 9	101 ± 8
10 μg/kg per min	94 ± 10	96 ± 11	102 ± 9	106 ± 8	109 ± 8	111 ± 7	103 ± 6
100 μg/kg per min	95 ± 8	96 ± 7	96 ± 8	93 ± 8	93 ± 8	91 ± 9	85 ± 7
SPT	85 ± 9	104 ± 13	91 ± 10	112 ± 6	115 ± 5	116 ± 7	97 ± 1
SPT + GP531	84 ± 14	91 ± 12	95 ± 10	95 ± 6	99 ± 4 [a]	106 ± 3	101 ± 3
MAP (mmHg)							
Saline	110 ± 1	111 ± 1	106 ± 2	97 ± 6	99 ± 9	99 ± 11	94 ± 11
10 μg/kg per min	130 ± 4 [a]	129 ± 4 [a]	127 ± 5 [a]	123 ± 5 [a]	125 ± 7 [a]	121 ± 7	113 ± 5
100 μg/kg per min	97 ± 8	96 ± 7	94 ± 7	89 ± 6	92 ± 6	90 ± 5	83 ± 5
SPT	120 ± 10	118 ± 10	114 ± 12	107 ± 13	111 ± 12	106 ± 14	98 ± 11
SPT + GP531	120 ± 5	118 ± 6	115 ± 3	111 ± 8	115 ± 9	112 ± 11	102 ± 10
LVDP (mmHg)							
Saline	107 ± 2	106 ± 3	102 ± 3 [b]	94 ± 6 [b]	99 ± 8	99 ± 10	95 ± 10
10 μg/kg per min	137 ± 4	136 ± 5 [a]	127 ± 5 [a,b]	121 ± 5 [a,b]	130 ± 7 [a]	126 ± 8	119 ± 5
100 μg/kg per min	99 ± 13	98 ± 12	92 ± 11 [b]	86 ± 11 [b]	90 ± 10	90 ± 8	89 ± 8
SPT	127 ± 9	125 ± 9	117 ± 11	106 ± 10	116 ± 11	110 ± 13	103 ± 12
SPT + GP531	126 ± 8	123 ± 8	118 ± 3	116 ± 10	120 ± 11	115 ± 12	105 ± 10
$LV\mathrm{d}P/\mathrm{d}t$							
Saline	1914 ± 101	1914 ± 105	1623 ± 89 [b]	1421 ± 112 [b]	1371 ± 132 [b]	1281 ± 142 [b]	1268 ± 161 [b]
10 μg/kg per min	2114 ± 48	2071 ± 41	1723 ± 71 [b]	1631 ± 79 [b]	1666 ± 105 [b]	1587 ± 89 [b]	1468 ± 89 [b]
100 μg/kg per min	1884 ± 303	1786 ± 250	1488 ± 229 [b]	1384 ± 205 [b]	1359 ± 213 [b]	1291 ± 186 [b]	1359 ± 237 [b]
SPT	2093 ± 321	2325 ± 214	1867 ± 82	1829 ± 135	1619 ± 88	1483 ± 88	1257 ± 122
SPT + GP531	2393 ± 203	2374 ± 306	2057 ± 203	1758 ± 197	1844 ± 154	1628 ± 103	1623 ± 114
NIZ SS							
Saline	24 ± 2	24 ± 2	24 ± 2	22 ± 1	22 ± 2	20 ± 1	19 ± 2
10 μg/kg per min	21 ± 2	22 ± 3	21 ± 2	21 ± 2	20 ± 2	19 ± 2	20 ± 2
100 μg/kg per min	22 ± 2	22 ± 2	23 ± 2	22 ± 2	21 ± 2	20 ± 3	21 ± 2
SPT	24 ± 2	24 ± 3	23 ± 2	22 ± 2	22 ± 2	21 ± 2	21 ± 2
SPT + GP531	23 ± 2	22 ± 2	23 ± 2	23 ± 3	22 ± 2	20 ± 3	20 ± 3

Saline = vehicle control, $n = 6$; 10 μg/kg per min = low-dose GP531, $n = 6$; 100 μg/kg per min = high-dose GP531, $n = 5$; SPT = 8-sulfophenyltheophylline, 5 mg/kg per h, i.c., $n = 4$; SPT + GP531 = SPT + GP531 (100 μg/kg per min, i.v.), $n = 4$; HR = heart rate; MAP = mean aortic pressure; LVDP = left ventricular developed pressure; NIZ SS = non-ischemic zone segment shortening (% of end-diastolic length). Data expressed as mean ± S.E.M.

[a] Different from saline control, $P < 0.05$.

[b] Different from baseline, $P < 0.05$.

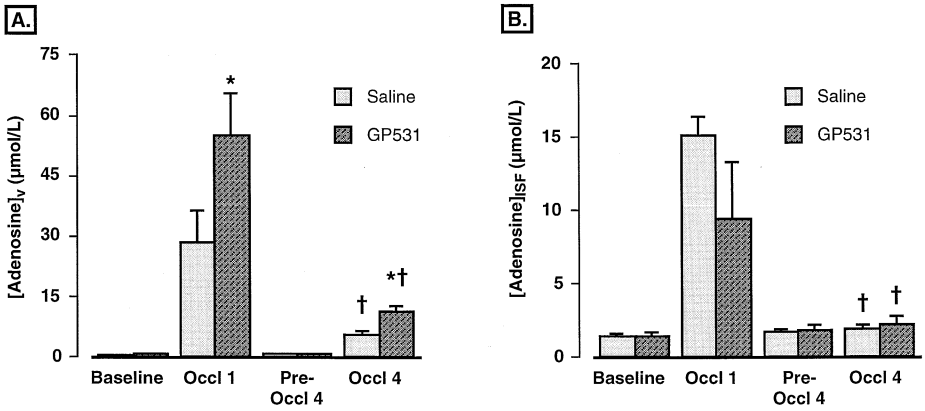

Fig. 4. Effects of GP531 (100 μg/kg per min, i.v.) and coronary artery occlusion on adenosine concentrations in coronary venous blood (A) and myocardial interstitial fluid (B). Samples were obtained prior to and at the end of occlusion 1 (Occl 1) and occlusion 4 (Occl 4). * $P < 0.05$ vs. saline; † $P < 0.05$ vs. occlusion 1.

μM at baseline, and increased to 28.09 ± 7.99 μM following the first occlusion ($P < 0.05$). Adenosine concentration returned to baseline by the end of the third reperfusion period (pre-occlusion 4, 0.45 ± 0.12 μM), and increased again during the fourth occlusion (5.27 ± 0.96 μM, $P < 0.05$), although the magnitude of this increase was significantly smaller than that observed in the first occlusion. Treatment with GP531 (100 μg/kg per min) did not significantly affect baseline adenosine (0.48 ± 0.23 μM, P = NS), but significantly enhanced the increase in adenosine concentration elicited during the first occlusion period (54.60 ± 10.61 μM, $P < 0.05$ vs. saline control). As with saline-treated control animals, coronary venous adenosine returned to baseline prior to the fourth occlusion (0.33 ± 0.05 μM). During the fourth occlusion, ischemia in the presence of GP531 elicited a 2-fold greater increase in adenosine compared to that observed in control pigs (11.14 ± 1.30 μM, $P < 0.05$).

Fig. 4B shows the effects of GP531 on interstitial fluid adenosine concentration. Baseline myocardial interstitial adenosine averaged 1.30 ± 0.27 μM in control pigs, and increased to 14.99 ± 1.34 μM at the end of occlusion 1. Adenosine concentration returned to baseline prior to occlusion 4 (1.59 ± 0.20 μM, P = NS vs. baseline), and was not increased significantly at the end of occlusion 4 (1.88 ± 0.22 μM). Treatment with GP531 did not change baseline adenosine (1.37 ± 0.30 μM), and adenosine concentration in GP531-treated animals was not significantly different from saline-treated animals during occlusion 1 (9.35 ± 3.86 μM), pre-occlusion 4 (1.69 ± 0.41 μM), or occlusion 4 (2.09 ± 0.67 μM) (all P = NS vs. control).

4. Discussion

The present study was designed to evaluate the cardioprotective effects of GP531 in a model of reversible ischemic injury, and to examine its effects on adenosine concentrations in the intravascular and interstitial compartments during ischemia. The results indicate that (1) GP531 significantly improved recovery of post-ischemic myocardial function and (2) GP531 caused a 2-fold increase in coronary venous adenosine during ischemia, with no effect on the interstitial concentration of adenosine. In addition, the cardioprotective effect of GP531 was prevented by the non-selective adenosine-receptor antagonist 8-SPT. These data suggest that the cardioprotective effect of GP531 is mediated by selective enhancement of intravascular adenosine during ischemia.

GP531 is a structural analog of the prototype adenosine regulating agent, acadesine (Fig. 1). The cardioprotective (Galiñanes et al., 1992a,b; Young and Mullane, 1991; Hori et al., 1994; Zhao et al., 1995) and antithrombotic (Bullough et al., 1994) effects of acadesine are well documented in animal models, and an adenosine-dependent mechanism is implicated by the prevention of acadesine's effects by adenosine receptor blockade (Hori et al., 1994; Zhao et al., 1995; Gruver et al., 1994). Moreover, acadesine has been shown to increase adenosine concentration in coronary venous effluent (Hori et al., 1994; Gruber et al., 1989) and in myocardial tissue biopsies (Hori et al., 1994) in animal models of myocardial ischemia. In contrast, studies measuring interstitial concentrations of adenosine using the microdialysis technique report no enhancement of interstitial adenosine by acadesine (Zhao et al., 1995). Although these prior studies indirectly support a role for elevated intravascular adenosine in cardioprotection, no prior study has assessed the effects of acadesine or other adenosine regulating agent on interstitial and vascular adenosine concentrations in the same hearts. The present findings with GP531 support the results from previous studies documenting cardioprotection with acadesine, and extend those observations by directly demonstrating differential regulation of adenosine in the intravascular vs. interstitial compartments.

The preservation of post-ischemic segment shortening in pigs treated with GP531 is not surprising, considering

numerous prior studies with acadesine in other species (for review, see Mullane and Young, 1993), and with other agents modulating production and/or metabolism of adenosine (Abd-Elfattah et al., 1993; Dhasmana et al., 1983; Itoya et al., 1994; Zughaib et al., 1993). Most analogous to the current study is a recent report by Kirkebøen et al. (1994), who demonstrated attenuation of myocardial stunning following five repeated coronary occlusions in pigs administered the adenosine transport inhibitor, R-75231. Interestingly, the magnitude of improvement in regional contractile function is nearly identical in the two studies (saline = approx. 30%; treated = approx. 60%), suggesting a similar mechanism of cardioprotection by the two agents. The demonstration of cardioprotection in the absence of hemodynamic changes is also similar in the two studies, indicating that the beneficial effect on cardiac function is not due to indirect effects on cardiac loading conditions. In the present study, however, GP531 was administered intravenously, in contrast to the intracoronary infusion of R-75231. Moreover, the adenosinergic mechanism of cardioprotection in the present study was demonstrated directly by measurement of adenosine and by the use of the adenosine receptor antagonist, 8-SPT, whereas the involvement of adenosine in the study of Kirkebøen et al. (1994) was only inferred from prior biochemical analyses reporting inhibition of adenosine transport by R75231 (Van Belle and Janssen, 1991; Van Belle et al., 1992). Nevertheless, these two studies, utilizing a similar model of myocardial stunning, suggest that pharmacological enhancement of adenosine in the heart provides cardioprotection beyond that observed with the concentrations of adenosine normally produced during ischemia.

4.1. Role of intravascular vs. interstitial adenosine

The current study is the first documentation of a cardioprotective effect associated with enhanced intravascular adenosine concentration in the absence of enhanced interstitial adenosine. The mechanism by which GP531 enhances intravascular adenosine but not interstitial adenosine in the present studies is not clear. One possible interpretation of the present findings is that the intravascular and interstitial spaces represent separate, independently regulated adenosine compartments. This concept is plausible because of the tremendous capacity of the endothelium to metabolize adenosine (Nees et al., 1985). Thus, changes in adenosine concentration within the vascular lumen might not be reflected in the interstitial compartment (Van Wylen, 1994; Randhawa et al., 1995). Because high-energy phosphate and purine metabolism are regulated differently in myocytes and endothelial cells (Borst and Schrader, 1991; Headrick et al., 1992), it is interesting to speculate that GP531 has a greater affect on net adenosine production in intravascular cells, and that this effect is not detectable in the interstitium due to the endothelial adenosine barrier.

The observation that enhanced intravascular adenosine without a concomitant increase in interstitial adenosine can confer cardioprotection is surprising, initially, in view of the widely accepted concept that adenosine's cardioprotective properties in the intact heart arise from activation of A_1 or A_3 receptors on the cardiomyocyte (Liu et al., 1991; Auchampach and Gross, 1993; Zhao et al., 1994; Tsuchida et al., 1993). However, several reports exist which may be interpreted to question the pivotal role of elevated interstitial adenosine in cardioprotection, and which suggest a potential role of intravascular adenosine. For example, studies by Forman and colleagues (Norton et al., 1991; Pitarys et al., 1991) and by Toombs et al. (1992) demonstrated adenosine-mediated cardioprotection with intravenous infusions of adenosine of between 100 and 150 μg/kg per min. A similar cardioprotective effect was demonstrated by Sekili et al. (1995), with intracoronary infusions of 2 mg/min. Based on previous work by Nees et al. (1985), and recent work by Van Wylen (1994) and by Randhawa et al. (1995), these doses of exogenously infused adenosine might not be expected to traverse the endothelial barrier, thus providing little or no increase in interstitial adenosine beyond that already produced by ischemia itself. Thus increased interstitial adenosine might not be responsible for cardioprotection observed in those studies (Toombs et al., 1992; Norton et al., 1991; Sekili et al., 1995; Pitarys et al., 1991). This view is supported by a recent study (Silva et al., 1995) in which adenosine deaminase inhibition with pentostatin enhanced interstitial adenosine concentration 150-fold above that in untreated hearts, but failed to decrease myocardial infarct size. Moreover, Zhao et al. (1995) recently reported the converse scenario, demonstrating a reduction in infarct size in rabbits treated with acadesine without an enhancement of interstitial adenosine concentration. Taken together, these studies (Zhao et al., 1995; Silva et al., 1995) are consistent with the concept that elevated interstitial adenosine concentration is neither necessary nor sufficient for eliciting cardioprotection. Alternatively, in support of the present findings, several studies suggest that elevated intravascular adenosine might have a role in cardioprotection. Kitakaze et al. (1993, 1994) reported that the ischemia-induced elevation in coronary vascular adenosine concentration is enhanced in preconditioned hearts, while Van Wylen has reported that ischemia-induced increases in interstitial adenosine concentrations are attenuated in preconditioned hearts (Van Wylen, 1994). Recently, Todd et al. (1995) reported that polyadenylic acid, a stable adenosine receptor agonist confined to the vascular space, reduced myocardial infarct size in rabbits. These studies support the concept, originally proposed by Rubio and colleagues (Balcells et al., 1992), that intravascular adenosine can significantly affect myocardial function, and further suggest that elevated intravascular adenosine levels might be more relevant for cardioprotection than interstitial adenosine concentration. Evidence supporting this hypothesis has been

critically reviewed recently (Engler and Gruber, 1992; Mullane and Bullough, 1995), and rests primarily on reports of (i) differential changes in vascular and interstitial adenosine concentrations with ischemia (Kitakaze et al., 1993; Van Wylen, 1994); (ii) the high capacity for endothelial cells to produce adenosine (Borst and Schrader, 1991; Smolenski et al., 1994); and (iii) observations of cardioprotection with low doses of adenosine (Sekili et al., 1995) or high-molecular weight adenosine analogs (Todd et al., 1995) that are likely confined to the vascular space. A corollary to this hypothesis is that the adenosine released from cardiomyocytes may subserve some cardioprotective role, but that the magnitude of the rise in interstitial adenosine probably reflects the magnitude of ischemia and damage to the cardiomyocyte, rather than serving as a predictor of eventual recovery of function. Thus, the observation that interstitial adenosine concentrations were attenuated slightly by ischemic preconditioning (Van Wylen, 1994), or treatment with GP531 in the present study, might reflect the cardioprotection and less severe ischemic insult in treated hearts.

4.2. Mechanism of action of GP531

The mechanism and site of action of GP531 are incompletely defined. Neither GP531 nor the prototype compound, acadesine, is a ligand for A_1, A_2 or A_3 receptors or the NBTI-sensitive adenosine transporter, and thus it is unlikely that this class of compound has a direct, receptor-mediated effect. This is supported by the lack of hemodynamic effects with GP531 in the present study (Table 1), and the failure of GP531 to affect collateral or reperfusion blood flow (measured with fluorescent microspheres) in an earlier canine infarct study (unpublished observation). Furthermore, GP531 does not inhibit adenosine deaminase, adenosine kinase, or AMP deaminase (unpublished observations). Importantly, in the present study, adenosine concentrations were enhanced by GP531 only during the ischemic periods and not when the myocardium was normally perfused, suggesting that regulation of adenosine is affected only in metabolically compromised cells (Bullough et al., 1994; Gruber et al., 1989; Mullane and Young, 1993; Mullane and Bullough, 1995). This requirement for ischemia to unmask the effects of GP531 on adenosine release undoubtedly contributes to the localization of adenosine release and the lack of systemic hemodynamic effects which are commonly observed with other pharmacologic strategies that modulate adenosine levels.

It is possible that the improved post-ischemic function observed in GP531-treated animals could have been caused by a direct positive inotropic effect in the post-ischemic myocardium, although GP531 did not alter contractile function prior to induction of ischemia. However, a study utilizing a rabbit model repetitive ischemic injury has been performed in which the GP531 infusion was terminated 30 min after the last ischemic episode. In those experiments, the improvement in post-ischemic myocardial function in GP531-treated animals was comparable to that observed in the present study (data not published), suggesting that the improved functional recovery was related to an effect during the ischemia/reperfusion period, and not to a direct effect on the myocardium during the post-ischemic period.

A second possibility is that the GP531-enhanced vascular adenosine improved functional recovery by increasing coronary blood flow. Because coronary artery blood flow was not measured in the present studies, this possibility cannot be directly excluded. However, intravascular adenosine concentrations returned to baseline levels within minutes of reperfusion in the present study, so it is difficult to ascribe the long-term (2 h) improvement in post-ischemic function to a direct effect of adenosine on coronary flow. Conversely, it is possible that increased intravascular adenosine during the critical early reperfusion period could have improved long-term vascular function by inhibiting leukocyte and/or platelet activation via stimulation of adenosine A_2 receptors on those cells. Neutrophils have long been cited as important mediators of reperfusion injury (Mullane et al., 1983; Engler et al., 1983; Romson et al., 1983), and adenosine has been shown to inhibit neutrophil-mediated vascular dysfunction by inhibiting neutrophil adhesion (Cronstein et al., 1990), and by inhibiting the release of neutrophil-derived pro-inflammatory mediators (Cronstein et al., 1983, 1986). However, it is important to note that similar effects of GP531 on both adenosine levels and on post-ischemic function have previously been demonstrated in the isolated buffer-perfused rat heart (Bullough et al., 1995). Thus, while inhibition of neutrophil (and/or platelet) activation may be an important contributor in mediating the cardioprotective effect of GP531 in vivo, this mechanism is not essential for GP531 to exhibit a cardioprotective effect.

In summary, the present studies demonstrate the cardioprotective effects of GP531 in a pig model of ischemia-reperfusion injury. These cardioprotective effects are observed in the absence of significant effects on baseline myocardial function or peripheral hemodynamics. The cardioprotective effects of GP531 are associated with augmentation of ischemia-induced increases in adenosine concentration in coronary venous blood but not in the interstitial space, and are prevented by co-administration of the adenosine-receptor antagonist 8-SPT. We conclude that the cardioprotective effect of GP531 is mediated by selective enhancement of intravascular adenosine during ischemia, that increased interstitial adenosine is not critical for adenosine-mediated cardioprotection, and that the interstitial space and intravascular space represent separate, independently regulated compartments for adenosine.

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