Adenosine inhibition of neutrophil damage during reperfusion does not involve K$_{ATP}$-channel activation

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Zhao, Zhi-Qing, James C. Todd, Hiroki Sato, Xin-Liang Ma, and J. Vinten-Johansen. Adenosine inhibition of neutrophil damage during reperfusion does not involve K$_{ATP}$-channel activation. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1677–H1687, 1997.—This study tests the hypothesis that cardioprotection exerted by adenosine A$_2$-receptor activation and neutrophil-related events involves stimulation of ATP-sensitive potassium (K$_{ATP}$) channels on neutrophils during reperfusion. The adenosine A$_2$ agonist CGS-21680 (CGS) inhibited superoxide radical generation from isolated rabbit polymorphonuclear neutrophils (PMNs) in a dose-dependent manner from 17.7 ± 2.1 to 7.4 ± 1.3 nmol/5 × 10$^6$ PMNs (P < 0.05). Pinacidil, a K$_{ATP}$-channel opener, partially inhibited superoxide radical production, which was completely reversed by glibenclamide (Glib). Incremental doses of Glib in combination with CGS (1 µM) did not alter CGS-induced inhibition of superoxide radical generation. CGS significantly reduced PMN adherence to the endothelial surface of aortic segments in a dose-dependent manner from 189 ± 8 to 50 ± 6 PMNs/mm$^2$ (P < 0.05), which was also not altered by incremental doses of Glib. Infusion of CGS (0.025 mg/kg) before reperfusion reduced infarct size from 29 ± 2% in the Vehicle group to 15 ± 1% in rabbits undergoing 30 min of ischemia and 120 min of reperfusion (P < 0.05). Glib (0.3 mg/kg) did not change the infarct size (28 ± 2%) vs. the Vehicle group and did not attenuate infarct size reduction by CGS (16 ± 1%). Glib did not change blood glucose levels. Cardiac myeloperoxidase activity was decreased in the ischemic tissue of the CGS group (0.15 ± 0.03 U/100 mg tissue) compared with the Vehicle group (0.37 ± 0.05 U/100 mg tissue; P < 0.05). We conclude that adenosine A$_2$-receptor activation before reperfusion partially reduces infarct size by inhibiting neutrophil activation and that this effect does not involve K$_{ATP}$-channel stimulation.

Adenosine 5'-triphosphate-sensitive potassium channel; infarct size; neutrophil; reperfusion injury

STUDIES (15, 27) HAVE demonstrated that exogenous administration of adenosine and adenosine receptor-specific analogs attenuates myocardial injury from ischemia and reperfusion. A$_2$-receptor activation exerts its predominant modulation of myocardial injury during the ischemic phase, whereas A$_2$-mediated cardioprotection is primarily exerted during reperfusion (29, 45). Although mechanisms by which cardioprotection mediated by adenosine during ischemia and reperfusion have not been fully elucidated, recent evidence strongly implicates that activation of ATP-sensitive potassium (K$_{ATP}$) channels plays an important role in adenosine A$_2$-receptor-mediated cardioprotection (6, 7, 22, 35, 37). It has been suggested that the opening of K$_{ATP}$ channels on myocytes during ischemia results in potassium efflux and cell hyperpolarization, which would abbreviate the duration of the action potential and reduce Ca$^{2+}$ influx through voltage-gated calcium channels. Opening of the K$_{ATP}$ channels before ischemia results in significant cardioprotection (7, 18, 40). Several laboratories have shown that pretreatment with a selective adenosine A$_1$-receptor agonist attenuates myocardial injury from ischemia-reperfusion, and the protective effect can be abolished by the K$_{ATP}$-channel antagonist glibenclamide (7, 22, 40). This may explain how activation of A$_1$ receptors can protect ischemic myocardium. Recently, however, several studies (1, 13, 24, 42) have shown that opening of the K$_{ATP}$ channels in vascular smooth muscle and endothelial cells is involved in adenosine and adenosine A$_2$-receptor-mediated vasodilation in addition to an adenosine 3',5'-cyclic monophosphate-dependent mechanism. Furthermore, with the use of whole cell patch-clamp techniques, it has been reported that there are potassium currents in neutrophils (23). Accordingly, selective K$_{ATP}$-channel openers inhibit superoxide radical generation by zymosan-activated neutrophils (31). Neutrophil activation and accumulation in the myocardium are suggested to be major pathological events in reperfusion injury (12). In vitro studies show that inhibition of neutrophil function and prevention of neutrophil-induced endothelial cell damage are primarily mediated by adenosine A$_2$ receptors (8, 9). In addition, in vivo studies (29, 32) also show that treatment by a selective adenosine A$_2$-receptor analog before reperfusion protects the myocardium from reperfusion injury. These data suggest that there is a link between K$_{ATP}$-channel activity and neutrophil function. Therefore, activation of A$_2$ receptors by exogenous and endogenous adenosine may have a protective effect on neutrophil-mediated events in reperfusion injury by opening K$_{ATP}$ channels. However, it is not known whether cardioprotection by A$_2$-receptor activation during reperfusion could be exerted by opening K$_{ATP}$ channels on the neutrophil and thereby inhibiting neutrophil activation. Accordingly, the present study was designed to test the hypotheses that 1) adenosine A$_2$-mediated inhibition of neutrophil activation such as superoxide generation and adherence to endothelium is linked to K$_{ATP}$-channel activation, 2) an adenosine A$_2$-receptor analog and endogenous adenosine stimulates cardioprotection during reperfusion by opening K$_{ATP}$ channels on neutrophils, and 3) adenosine A$_2$-receptor activation attenuates myocardial injury during the reperfusion phase by inhibiting neutrophil accumulation via a link with the K$_{ATP}$ channel.
METHODS

Surgical preparation. The experimental procedures complied with the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society as well as with state and federal regulations. The experimental protocol was approved by the institutional Animal Care and Use Committee. New Zealand White rabbits of either sex weighing 4–5 kg were initially anesthetized with an intramuscular injection of ketamine HCl (35 mg/kg) and xylazine (6 mg/kg). Subsequent anesthesia (100 mg/ml of ketamine plus 2 ml/g of xylazine) was continuously administered intravenously at a rate of 1.3 ml/h during the experiment. The neck was opened with a central midline incision, and a tracheotomy was performed. The rabbits were spontaneously ventilated with oxygen-enriched room air. Acidosis was counteracted with intravenous sodium bicarbonate as necessary. Catheters were placed in the right femoral artery for the measurement of blood pressure and blood sampling and in the femoral vein for the infusion of drugs and saline. The chest was opened by a median sternotomy, and the pericardium was incised anteriorly to expose the heart. A high-fidelity transducer-tipped catheter (model MPC-500, Millar Instruments, Houston, TX) was secured in the left ventricular (LV) cavity via a small puncture wound in the apex and a purse-string suture. A 4-0 silk thread with a tape needle was passed around a branch of the left coronary artery, and the ends of the tie were threaded through a small plastic (PE-90) tube to form a snare. All rabbits were then systemically heparinized with 300 U/kg of heparin sodium before the experiment was started.

Preparation of drugs. The A1-selective adenosine-receptor agonist CGS-21680 (CGS; 1 mg) was first dissolved in 200 µl of dimethyl sulfoxide and then diluted with 9.8 ml of 0.9% saline to achieve a final concentration of 0.1 mg/ml. Glibenclamide (Glib; 4.5 mg), a KATP-channel antagonist, was dissolved in 0.5 ml of ethanol, 0.5 ml of 1 N NaOH, and 0.5 ml of polyethylene glycol 400 to achieve a final concentration of 3 mg/ml as a stock solution (37). The dose for each rabbit was then diluted with 0.9% saline in a 1:3 ratio before the experiment. The treatment doses selected were 0.025 mg/kg of CGS and 0.3 mg/kg of Glib. Saline in the Vehicle group contained appropriate amounts of the dissolving agents.

Experimental protocol. After a 20- to 30-min postsurgical stabilization period, steady-state baseline hemodynamic measurements were acquired in duplicate, and a blood sample was drawn. A branch of the left coronary artery was reversibly occluded by pulling up on the snare to produce a zone of ischemia in the left ventricular cavity via a small puncture wound in the apex and a purse-string suture. A 4-0 silk thread with a tape needle was passed around a branch of the left coronary artery, and the ends of the tie were threaded through a small plastic (PE-90) tube to form a snare. All rabbits were then systemically heparinized with 300 U/kg of heparin sodium before the experiment was started.

In all four groups, hemodynamic data were acquired in duplicate, and blood samples were collected before ischemia, at the end of 30 min of coronary occlusion, and after 15, 60, and 120 min of reperfusion. Lidocaine was not given in any experiment to avoid any potentiation of cardioprotection by the antiarrhythmic agent (5).

Blood sampling. Blood samples (3 ml) were drawn from the femoral artery 10 min before coronary occlusion, at the end of 30 min of ischemia, and 15, 60, and 120 min after reperfusion for analysis of creatine kinase (CK) activity and measurement of pH and gases. An equal volume of 0.9% saline was replaced after each blood sampling. The CK activity was analyzed by a Hewlett-Packard model 8542A U-V is spectrophotometer.

Hemodynamic measurements. LV and aortic pressures were digitized at 250 Hz with a 12-bit analog-to-digital converter (Data Translation model DT 2821, Marlborough, MA) and stored on a hard disk. With the use of a videographics program developed in our laboratory, the instantaneous first derivative of the LV pressure (dP/dt) was calculated by computer algorithm. The data were divided into cardiac cycles, with the end-diastolic point initially identified when the positive dP/dt exceeded 200 mmHg/s during isovolumic systole and end systole identified 20 ms before the peak negative dP/dt. Pressure waveforms were visually displayed, end systole and end diastole were manually adjusted if necessary, and dysrhythmic beats were excluded. Heart rate, peak LV pressure, end-diastolic LV pressure, and aortic pressure and diastolic pressures were averaged from no less than 15 beats. The pressure-rate product, used as an index of myocardial oxygen demand, was calculated as the product of heart rate and mean arterial pressure.

Isolated coronary artery study. Vasodilator responses to CGS and pinacidil in the presence and absence of Glib were used to confirm physiologically active concentrations of the three drugs. Twenty-two coronary artery segments were isolated from anesthetized rabbits (n = 5), carefully cut into 1.5- to 2-mm-long rings, and placed in Radnoti tissue baths containing 7 ml of oxygenated Krebs-Henseleit solution as described previously (46). The vascular rings were then mounted on stainless steel hooks and connected to isometric force transducers. Changes in isometric force were digitized at 3 Hz with an analog-to-digital converter and an IBM personal computer and were analyzed with a videographics program (SPECTRUM, Winston-Salem, NC) developed in our laboratory for vascular ring responses (34). The rings were equilibrated for 1 h at an optimal tension averaging 2 g (determined from pilot studies) and precontracted with the thromboxane A2 mimetic U-46619 (Upjohn Pharmaceutical). Once a stable contraction was observed, cumulative concentration responses to CGS and pinacidil were obtained in the absence and presence of Glib (5 µM). Drug concentrations refer to final organ chamber concentrations. Responses to CGS were calculated as cumulative relaxation from the preconstriction force and are presented in grams.

Isolation of polymorphonuclear neutrophils (PMNs). Rabbits of either sex weighing between 5 and 6 kg were anesthetized with ketamine HCl and xylazine as described in Surgical preparation. Catheters were placed in the right femoral vein and carotid artery for the infusion of fluid and collection of blood. Thirty milliliters of Hespan (6% hetastarch in 0.9% saline, DuPont Pharmaceutical, Wilmington, DE) were injected into the femoral vein before sample withdrawal. Twenty milliliters of blood from the carotid artery were then mixed with 20 ml of diluting solution that contained 17 ml of Hespan and 3 ml of antiaggregating solution (composed of 16 g of citric acid and 25 g of sodium citrate at pH 5.4 in 1,000 ml of sterile water). All tubes for blood collection were kept at room temperature and sedimented for ~20–30 min. Any contaminating red blood cells in the leukocyte pellet were removed by hypotonic lysis, and leukocytes were isolated with Ficoll-
Pacque (46). With this procedure, final suspensions contained >95% neutrophils, and cell viability was >94% as determined by trypan blue exclusion.

Superoxide production by PMNs. Superoxide radical production by PMNs was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c to ferrocytochrome c as described previously (46). Briefly, PMNs (5 × 10⁶ cells/ml) obtained from 10 rabbits were prewarmed at 37°C in 160 μM cytochrome c and incubated in the presence of cytochalasin B (5 μg/ml) and test agents at designated concentrations for 5 min. The PMNs were then stimulated with platelet-activating factor (PAF; 100 nM) in a final reaction volume of 0.5 ml. All assays were run in duplicate. SOD (100 μg/ml) was added to the sample at the beginning of the assay to verify that cytochrome c reduction was due to superoxide radical generation (SOD inhibitable). Cytochrome c reduction was monitored spectrophotometrically by determining the optical density of the supernatant at 500 nm with a Vmax Kinetic Microtiter Plate Reader (Molecular Devices, Palo Alto, CA). Superoxide radical production was calculated with an extinction coefficient of 21 mM/cm for cytochrome c. Results are reported as nanomoles of SOD-inhibitable superoxide radical production per 5 × 10⁶ PMNs for 5 min.

PMN adherence assay. Reduction in the adherence component of PMN activity by CGS was observed in the control rabbits (n = 5) that were not subjected to ischemia-reperfusion and was assessed with neutrophils labeled with Zynaxis PKH26 vital fluorescent dye (Zynaxis Cell Science, Malvern, PA) as described previously (46). The labeling procedure yields cells possessing normal morphology and function (43). Segments from the rabbit aorta were removed and cut into 3- to 4-mm lengths. All rings were carefully opened and placed endothelial side up in round plastic dishes containing 3 ml of Krebs-Henseleit buffer at 37°C. Labeled neutrophils (4 × 10⁶ cells/ml) were added to the baths alone or in combination with different concentrations of test agents. PAF (100 nM) was added to the dishes 5 min after incubation with the test agents and allowed to incubate for an additional 15 min. Adherence was determined by counting the number of neutrophils adhering to the endothelial surface in six separate microscopic fields under epifluorescent microscopy (490-nm excitation, 504-nm emission) and are expressed per square millimeter of endothelium.

Determination of area at risk and infarct size. At the end of reperfusion, the snare around the coronary artery was re-tightened, and 4–5 ml of Gentian Violet blue dye (Humco, Texarkana, Texas) were injected into the left atrium to stain the normally perfused region. The rabbits were killed with a lethal dose of 50 mg/ml of pentobarbital sodium, and the heart was removed and placed in a 10% solution of cold buffered Formalin overnight for fixation. The AAR was then subdivided into the normal perfused region. The AAR and AN were determined gravimetrically as described previously (44). The AAR as a percentage of LV mass (AAR/LV) was calculated as [(weight of nonnecrotic and necrotic tissues in the AAR)/(total weight of LV)] × 100. AN as a percentage of LV mass (AN/LV) was calculated as [(weight of AN in the AAR)/(weight of LV)] × 100. AN as a percentage of the AAR (AN/AAR) was calculated as [(weight of AN in the AAR)/(total AAR)] × 100.

Measurement of blood glucose. The effect of Glib on blood glucose levels was examined in six rabbits. The concentration of blood glucose was determined by a blood glucose monitoring kit (Lifescan, Milpitas, CA) and expressed in milligrams per deciliter.

Determination of tissue myeloperoxidase activity. At the end of the experiment, tissue samples weighing 0.3 g were taken from the nonischemic and ischemic zones for analysis of myeloperoxidase (MPO) activity. MPO is specific for neutrophil accumulation in myocardium. The samples were frozen and stored at −70°C until assayed. The samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide and dissolved in 50 mM potassium phosphate buffer (pH 6) with a polyanion (PCU-2) homogenizer. The homogenates were centrifuged at 12,500 g and 4°C for 30 min. The supernatants were then collected and reacted with 0.167 mg/ml of o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide in 50 mM phosphate buffer (pH 6). The activity of MPO was measured spectrophotometrically at 460 nm and expressed as units per gram of tissue. One unit of MPO activity was defined as the quantity of enzyme degrading 1 µM peroxide/min at 25°C.

Chemicals and reagents. The following chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO): SOD, cytochalasin B, ferricytochrome c, indomethacin, sodium bicarbonate, polyethylene glycol 400, pinacidil, and Glib. The thromboxane A₂ mimetic U-46619 was donated by Upjohn (Kalamazoo, MI). PAF was purchased from Biomol (Plymouth Meeting, PA). Hanks’ buffered saline solution without Mg²⁺ and Ca²⁺ was purchased from Cellgro. CGS was a gift from Ciba-Geigy Pharmaceuticals (Summit, NJ).

Criteria for exclusion. In the in vivo study, animals were excluded from final data analysis due to 1) unclear demarcation of the AAR after coronary occlusion by Gentian Violet blue staining, 2) ventricular fibrillation that did not spontaneously convert after 1 min, and 3) failure to complete the entire protocol. Data from 40 rabbits are included in the final analysis of the in vivo study.

Statistical analysis. Dose-response curves of vascular relaxation are calculated as a percentage of the decrease of U-46619-induced isometric force. Graphic determination of the concentration of drug required to elicit 50% of maximal relaxation was obtained from stepwise dose-response curves by using a videographics program (SPECTRUM) developed in our laboratory (34). One-way analysis of variance (ANOVA) was used to analyze group differences in superoxide radical production, PMN adherence, and infarct size data. ANOVA for repeated measures was used to determine time- and group-related differences in hemodynamic, pH, and CK activity data. If a significant group-time interaction was found, Duncan’s multiple range test adjusting for baseline levels was applied to locate the sources of differences. Results are reported as means ± SE.

RESULTS

Study group. Eighty-five rabbits were initially entered into the whole study (in vivo and in vitro). Twenty-six rabbits were used in the in vitro study. Fifteen rabbits were used to test the effect of the drugs on neutrophil function, of which 10 were used to determine the production of PMN superoxide radicals and 5 were used in PMN adherence studies. For the in vivo study, 10 rabbits were used to select the doses of CGS and Glib (n = 4) and to determine the effects on blood glucose levels (n = 6). In infarct size experiments,
three rabbits developed ventricular fibrillation during the period of coronary occlusion, two of which were rapidly resuscitated. During reperfusion, two rabbits were excluded: one in the GlibR group due to failure of the snare to release at reperfusion and one in the CGSR group due to ventricular fibrillation. Data were analyzed on the remaining 40 rabbits, 10 in each group.

Dose selection of CGS and Glib. To confirm the appropriate in vivo dose of CGS with hemodynamic criteria, we tested the blood pressure and heart rate responses to an intravenous infusion (10 min for each dose) of incremental doses of CGS in four rabbits under bilateral vagotomy and β-blockade (1.5 mg/kg of metoprolol). CGS at 0.025 mg/kg was the lowest dose at which moderate hypotension and compensatory tachycardia were observed. CGS caused a concentration-dependent relaxation in rabbit coronary arteries (Fig. 1A), which was partially blocked by Glib. Relaxation caused by endothelium-independent vasodilator acidified NaNO₂ was not blocked by Glib (B). K$_{ATP}$-channel opener pinacidil relaxed coronary artery in a dose-dependent manner, which was blocked by Glib (C). Maximum relaxation by pinacidil at highest dose was 30%. *P < 0.05 vs. Glib blockade.

addition, 5 μM Glib was sufficient to completely inhibit the vasodilation induced by the K$_{ATP}$-channel opener pinacidil (Fig. 1C). The in vivo dose of Glib was estimated from the in vitro responses in PAF-stimulated PMNs. Pinacidil decreased superoxide radical generation from PAF-stimulated PMNs at a concentration of 10 μM. This response was inhibited by Glib between 5 and 10 μM (Fig. 3B). On the basis of a blood volume between 60 and 80 ml/kg body weight, a blood concentration of 7.5 μM would be achieved with an in vivo dose of 0.3 mg/kg of Glib. Therefore, 0.3 mg/kg was used in vivo.

Effect of CGS on superoxide radical generation. The coinubcation of PAF-stimulated PMNs with CGS at different concentrations from 10 to 5 μM significantly decreased the production of superoxide radicals in a dose-dependent fashion compared with PAF exposure alone (Fig. 2A). Maximal inhibition (58 ± 2% of PAF stimulated) was observed at 1 μM CGS. Inhibition by 1 μM CGS on superoxide radicals was significantly blocked by the A₂-receptor agonist CGS-15943A at concentrations of 100 nM and 1 μM (37 ± 9 and 46 ± 9 nM O$_2$/5 × 10⁶ PMNs, respectively, compared with 13 ± 0.2 nM O$_2$/5 × 10⁶ PMNs with 1 μM CGS; P < 0.01), with no further inhibition at higher doses. To determine whether PMN K$_{ATP}$ channels were involved in the inhibitory effect of CGS on superoxide radical production, we studied the effect of Glib in the presence of a fixed CGS concentration (1 μM) on superoxide radical production. Although superoxide radical generation
tended to be higher when PMNs were incubated with Glib at different concentrations from 10 nM to 5 µM; this did not reach statistical significance for CGS vs. Glib+CGS (Fig. 2B). To confirm the presence of K<sub>ATP</sub> channels on PMNs, pinacidil, a K<sub>ATP</sub>-channel opener, partially inhibited superoxide radical production at 10 and 50 µM concentrations (Fig. 3A), which was completely blocked by 5 and 10 µM Glib (Fig. 3B).

Effect of CGS on PMN adherence. As shown in Fig. 4A, when unactivated PMNs were added to the bath, very few PMNs adhered to the aortic endothelium. However, activation of PMNs with 100 nM PAF significantly increased adherence. Preincubation with CGS at concentrations from 10 nM to 5 µM resulted in a dose-dependent attenuation of PAF-stimulated PMN adherence. A maximal inhibition to basal levels was observed at concentrations of 100 nM and greater of CGS (66 ± 8%), with little increase in inhibition at higher doses. The attenuation of PMN adherence with a fixed concentration of Glib (1 µM) was unaltered by Glib at increasing concentrations from 10 nM to 5 µM (Fig. 4B), indicating that inhibition of PMN adherence by CGS is not mediated by activation of K<sub>ATP</sub> channels. Furthermore, compared with PAF-stimulated PMNs, Glib alone at concentrations of 100 nM (174.6 ± 6.4 PMNs/mm<sup>2</sup> endothelium) to 10 µM (185 ± 3.7 PMNs/mm<sup>2</sup> endothelium) did not alter PAF-stimulated PMN adherence (180 ± 3.2 PMNs/mm<sup>2</sup> endothelium).

**Fig. 3.** Effects of K<sub>ATP</sub>-channel agonist pinacidil (Pina) and K<sub>ATP</sub>-channel antagonist Glib on superoxide radical production by PAF (100 nM)-stimulated PMNs (5 × 10<sup>6</sup> cells/ml). A: Pina dose responses. B: Pina (10 µM) + Glib dose responses. Values are means ± SE in nmoles O<sub>2</sub> (SOD inhibitable) produced by PAF-activated PMNs from at least 4 separate experiments with duplicate determinations per experiment. * P < 0.05 vs. PAF-stimulated PMNs.

**Fig. 4.** Effects of adenosine A<sub>2</sub> receptor agonist CGS and K<sub>ATP</sub>-channel antagonist Glib on PAF (100 nM)-stimulated PMN (4 × 10<sup>6</sup> cells/ml) adherence to endothelium of thoracic aorta. A: CGS dose responses. B: CGS (1 µM) + Glib dose responses. Values are means ± SE expressed as adhered PMN number/mm<sup>2</sup> of thoracic aorta endothelium from at least 10 aortic segments from 5 to 6 rabbits. * P < 0.05 compared with PAF-activated PMN group.

pH, blood gases, and glucose. No significant differences were found in pH or blood gases during both the control period and ischemia among the four groups. Similarly, intravenous administration of CGS, Glib, or both in combination before reperfusion did not show any change in pH and blood gases until the end of the experiment. Furthermore, there were no differences between the two groups during the control period in blood glucose (Vehicle, 157 ± 9 vs. Glib, 161 ± 2 mg/dl). Although blood glucose levels in the Vehicle group were significantly increased over time (225 ± 10 mg/dl at 120 min of reperfusion), similar increases were observed in the Glib group (229 ± 13 mg/dl). There were no significant group differences in blood glucose concentration during the experiment.

**Hemodynamics.** Heart rate, aortic systolic and diastolic pressures, LV systolic pressure, positive dP/dt, and pressure-rate product during the course of the experiments for the four groups are shown in Fig. 5. There were no significant differences among the groups in any variable during the control period. Coronary occlusion resulted in a significant increase in heart rate and a reduction in aortic systolic and diastolic and LV systolic pressures in all groups. Heart rates at 15 min of reperfusion in the GlibR group and 120 min in the CGSRR group were significantly different relative to the other groups. The pressure-rate product, an index of myocardial oxygen demand, tended to decrease during the course of the experiment but showed no group differences at any time.

**AAR and infarct size.** The masses of the LV, AAR, and AN in grams are summarized in Table 1. The mass of the LV was similar among all four groups. The mass
of the AAR was also comparable among the four groups. However, CGS treatment before reperfusion significantly decreased the absolute mass of the AN relative to both the Vehicle and GlibR groups (P < 0.05). Glib + CGS infusion before reperfusion did not reverse the protective effect of CGS on AN mass. The AAR/LV, AN/LV, and AN/AR are shown in Fig. 6. The AAR/LV was comparable among the four groups and ranged between 27 ± 1% in the Vehicle group and 29 ± 1% in the CGS group. The AN/LV in the CGSR group was significantly less than that in the Vehicle group. As a percentage of the AAR, the AN was significantly reduced in the CGSR group by ~48 ± 1% compared with the Vehicle group (P < 0.05). Glib + CGS infusion before reperfusion did not eliminate the cardioprotective effect observed in the CGS group. In addition, when Glib alone was given before reperfusion, there was no change in infarct size relative to the Vehicle group.

To determine whether the mass of the AAR influenced the mass of the AN, the AN was correlated with the respective AAR in each experiment and is shown in Fig. 7. There was a positive relationship between the mass of the AN and the mass of the AAR in each group (P < 0.05). Treatment with CGS before reperfusion shifted this relationship downward compared with the Vehicle group, indicating that the cardioprotective effect of CGS was not related to a change in the AAR but rather by the intervention. Administration of Glib + CGS before the reperfusion period had no effect on infarct size compared with the CGSR group; the slope in this group was comparable to that of the CGSR group. These data suggest that for any given AAR, a smaller infarct size is achieved by a protective effect rather than by a change in AAR.

Table 1. Mass of myocardial tissue in left ventricle, total area at risk, and necrotic area

<table>
<thead>
<tr>
<th>Group</th>
<th>Left Ventricle, g</th>
<th>At Risk Tissue, g</th>
<th>Necrotic Tissue, g</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>3.94 ± 0.18</td>
<td>1.08 ± 0.07</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>CGSR</td>
<td>4.11 ± 0.29</td>
<td>1.15 ± 0.08</td>
<td>0.18 ± 0.03*</td>
</tr>
<tr>
<td>GlibR</td>
<td>4.08 ± 0.32</td>
<td>1.24 ± 0.08</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Glib + CGSR</td>
<td>4.09 ± 0.25</td>
<td>1.15 ± 0.10</td>
<td>0.13 ± 0.01*</td>
</tr>
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Values are means ± SE. CGSR, single bolus injection of CGS-216820 2 min before reperfusion; GlibR, intravenous infusion of glibenclamide 2 min before reperfusion; Glib + CGSR, intravenous infusion of Glib 4 min before reperfusion followed by bolus injection of CGS 2 min before reperfusion. *P < 0.05 compared with Vehicle and GlibR groups.
effect of CGS on CK activity, consistent with the infarct size data.

MPO activity. MPO activity in tissue from the nonischemic and ischemic zones in the four groups is shown in Table 2. MPO activity in the ischemic zone increased significantly above the values within the nonischemic zone in all four groups. However, administration of CGS before reperfusion exhibited significantly less (by 60%) MPO activity than in the Vehicle group. The combined treatment with Glib + CGS before reperfusion showed no effect on MPO activity relative to that in the CGS group. Furthermore, there was also no significant difference between the GlibR and Vehicle groups. These data suggest that CGS treatment reduced neutrophil accumulation in the ischemic zone after reperfusion that is not related to $K_{ATP}$-channel therapy. The lack of alteration of MPO data with Glib alone suggests that the $K_{ATP}$-channel is not involved in the protective actions of endogenous adenosine during reperfusion.

DISCUSSION

The cardioprotective role of adenosine during ischemia and reperfusion has been intensively investigated in the past several years. Studies from our and other laboratories (20, 44) strongly suggest that both exogenous adenosine and endogenous adenosine released during ischemia and reperfusion exerted a receptor-mediated cardioprotection. Recent data suggesting a link between the adenosine A$_2$ receptor and the $K_{ATP}$ channel (1, 42) and the presence of $K_{ATP}$ channels on neutrophils (31) imply that A$_2$-mediated inhibition of neutrophil activity may involve $K_{ATP}$-channel activation. Therefore, in the present study, we hypothesized that the antineutrophil and infarct reduction effects exerted by a selective A$_2$-receptor agonist CGS could be
In addition, K<sub>ATP</sub>-channel activation in the coronary vasodilation involves, in part, opening of the K<sub>ATP</sub>-channel. Glib, a K<sub>ATP</sub>-channel antagonist, inhibits neutrophil function but that this antineutrophil action was blockade with Glib at the highest dose (Fig. 1). Glib dose-dependently inhibited PAF-stimulated neutrophils and K<sub>ATP</sub>-channel opener pinacidil relaxed coronary artery segments in a dose-dependent manner that reached the same extent as blockade with Glib in the present study (4). Furthermore, pinacidil-induced vasodilation and found that the coronary vasodilator effect of CGS was partially reversed in a dose-dependent manner by the K<sub>ATP</sub>-channel antagonist Glib, suggesting that adenosine A<sub>2</sub>-receptor-mediated vasodilation involves, in part, opening of the K<sub>ATP</sub>-channels. Although the K<sub>ATP</sub>-channel opener pinacidil attenuated superoxide radical production by neutrophils, which was reversed by Glib, inhibition of superoxide radical production and neutrophil adherence to endothelium by CGS was not blocked by Glib, suggesting that the inhibitory effects of CGS on neutrophil function was not mediated mainly by the K<sub>ATP</sub>-channel. In the in vivo study, we showed that intravenous CGS administration before reperfusion attenuated infarct size and CK activity and inhibited neutrophil accumulation. The CGS protection was not reversed by Glib at a dose that has been shown to alter infarct size previously when administrated before ischemia with or without preconditioning (36, 38). These data suggest that the protective effect of adenosine A<sub>2</sub>-receptor activation during reperfusion is largely mediated by inhibiting neutrophil function but that this antineutrophil effect does not involve stimulating K<sub>ATP</sub>-channels.

Recent evidence has indicated that activation of K<sub>ATP</sub>-channels in vascular endothelial cells and smooth muscle may be involved in coronary vasodilation by adenosine and an adenosine A<sub>2</sub>-analogue (4, 24). To confirm the appropriate dose of Glib in the in vitro studies, we observed the effect of Glib on CGS- and pinacidil-induced vasodilation and found that the coronary vasodilator effect of CGS was partially attenuated (33 ± 4%) by the Glib at 5 µM, not at 1 µM. Furthermore, pinacidil-relaxed coronary artery segments in a dose-dependent manner that reached the same extent as blockade with Glib at the highest dose (Fig. 1B), suggesting that 5 µM Glib in the present study is sufficient to block smooth muscle K<sub>ATP</sub>-channel activity. In addition, K<sub>ATP</sub>-channel activation in the coronary artery is not the only mechanism of vasodilation, but rather CGS-induced dilation in the coronary artery may be only partially mediated by K<sub>ATP</sub>-channel activation. This is consistent with the observation by Kuo and Chancellor (24) that both intraluminal and extraluminal administration of Glib only partially blocked vasodilation by adenosine.

Table 2. Activity of myeloperoxidase in myocardium after ischemia and reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonischemic Zone</th>
<th>Ischemic Zone</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>0.02 ± 0.005</td>
<td>0.37 ± 0.052</td>
</tr>
<tr>
<td>CGSR</td>
<td>0.03 ± 0.003</td>
<td>0.15 ± 0.031*</td>
</tr>
<tr>
<td>GlibR</td>
<td>0.03 ± 0.010</td>
<td>0.31 ± 0.057</td>
</tr>
<tr>
<td>Glib + CGSR</td>
<td>0.02 ± 0.006</td>
<td>0.14 ± 0.026*</td>
</tr>
</tbody>
</table>

Values are means ± SE in U/100 mg tissue. *P < 0.05 compared with Vehicle and GlibR groups.

In the present study, we found that the K<sub>ATP</sub>-channel opener pinacidil attenuated superoxide radical production by PAF-stimulated neutrophils in a dose-dependent manner. However, data showing that 5 µM Glib did not alter the inhibition by CGS on superoxide radical production and neutrophil adherence suggest that A<sub>2</sub>-mediated neutrophil inhibition is independent of K<sub>ATP</sub>-channel activation.

In the present study, 0.3 mg/kg of Glib did not have an effect on blood glucose levels at 2 h of reperfusion because Glib is known to increase insulin secretion from the pancreas by blocking K<sub>ATP</sub>-channels in β-cells, suggesting that the Glib dose was not high enough to block K<sub>ATP</sub>-channels. It has been reported (38) that the change in blood glucose is related to anesthetia. Using the same dose of Glib, Thornton et al. (36) found that blood glucose levels were significantly decreased when rabbits were anesthetized with pentobarbital sodium, whereas Toombs et al. (38) found that Glib did not show an inhibition in blood glucose when ketamine and xylazine were used in combination to produce anesthetia in rabbits. Thornton et al. (36) did find that a 0.3 mg/kg Glib infusion before ischemia increases infarct size and blocks protection by preconditioning. Xylazine has been shown to inhibit insulin release and increase blood glucose by an α<sub>2</sub>-receptor mechanism (16), so this may explain why Glib did not produce any effect on blood glucose in our rabbit model.

It is generally accepted that the involvement of K<sub>ATP</sub>-channel activation in cardioprotection occurs primarily during the ischemic period. In support of the role of K<sub>ATP</sub>-channel activation in cardioprotection during ischemia, several studies (37, 38, 41) have shown that the protective effects of preconditioning and pretreatment with adenosine before ischemia can be blocked by Glib. In addition, K<sub>ATP</sub>-channel opener were in themselves cardioprotective when given before or during ischemia. Furthermore, pretreatment with an adenosine A<sub>1</sub>-receptor agonist before coronary occlusion was also blocked by Glib, indicating that A<sub>1</sub>-receptor activation is involved in K<sub>ATP</sub>-channel activation during ischemia (37, 38). However, whether K<sub>ATP</sub>-channel activation is cardioprotective during reperfusion still remains controversial. The results of Grover et al. (17) showed that an intracoronary infusion of the K<sub>ATP</sub>-channel opener cromakalim 2 min before reperfusion did not reduce infarct size in dogs subjected to 90 min of ischemia and 5 h of reperfusion, similar to the results of the present study. Similarly, Auchampach and Gross (3) showed a lack of reduction in infarct size and protection from postischemic dysfunction when the K<sub>ATP</sub>-channel openers cromakalim and aprikalim were given only during reperfusion. In contrast, several studies reported that the pharmacological opening of K<sub>ATP</sub> channels with K<sub>ATP</sub>-channel openers did reduce postischemic injury. The recent study by Iwamoto et al.
suggesting that KATP-channel activation is not involved in infarct size, CK, and tissue MPO activity, before reperfusion did not alter the inhibitory effect of neutrophils. Infusion of Glib in combination with CGS exerted specifically during reperfusion but not in blockade of the endogenous opening of channels.

Although the cardioprotective effects exerted by KATP-channel activation on myocytes during reperfusion has not been fully elucidated, studies from several laboratories addressed neutrophil inhibition as a mechanism by which KATP-channel openers may reduce reperfusion injury. Pieper and Gross (31) showed that the selective KATP-channel opener bimakalim inhibited both superoxide radical generation and luminal-enhanced chemiluminescence in opsonized zymosan-activated canine neutrophils. It has also been shown that intravenous bimakalim before reperfusion reduced transmural MPO activity, a maker of neutrophil accumulation in myocardium, in the ischemic area (26). These data, therefore, support an important role for KATP-channel openers in neutrophil activation and accumulation and neutrophil-induced myocardial damage during reperfusion, particularly in infarct size studies. In the present study, we hypothesized that the cardioprotective effect of CGS would be mediated by opening of the KATP channels on neutrophils. Infusion of Glib in combination with CGS before reperfusion did not alter the inhibitory effect of CGS on infarct size, CK, and tissue MPO activity, suggesting that KATP-channel activation is not involved in exogenous adenosine A2-receptor-mediated cardioprotection during reperfusion. Recently, it has been shown that endogenous adenosine, released during acute global hypoxia in the guinea pig in vivo, mediated atrioventricular nodal conduction delay and shortening of atrial action potential duration and that these effects were not blocked by Glib, indicating that endogenous adenosine did not activate KATP channels on myocardial conduction tissue (39). In agreement with this study, we found that infusion of Glib alone before reperfusion did not exacerbate infarct size, CK activity, and MPO activity. These were consistent with the in vitro data that coinubcation of aortic segments and PMNs with Glib alone did not change PMN adherence. These results suggest that KATP channels do not play a major role in the cardioprotective mechanisms of the adenosine A2-receptor agonist and that A2-mediated inhibition of neutrophil actions does not involve KATP-channel activation.

Recently, cardioprotection by an adenosine A2 receptor during reperfusion has received considerable attention. Studies have shown that exogenous administration of an adenosine A2-receptor analog before reperfusion resulted in significant reduction in infarct size and improved myocardial wall function (21, 29, 32). In agreement with these data are some reports indicating that adenosine A2 activation before ischemia had no protection on infarct size (35) and stunned myocardium in dogs (40), implying that A2 receptors are predominantly involved in the myoprotective mechanisms by treatment with an adenosine A2 agonist before reperfusion. Although the exact protective mechanisms of A2-receptor activation on the myocardium from a reperfusion injury are still unknown, a number of studies have been performed to determine the cellular effects of A2-receptor activation. Cronstein and co-workers (8, 9) reported that adenosine and an adenosine A2-receptor analog inhibited superoxide radical production by human PMNs and attenuated adherence to cultured endothelial cells by N-formyl-methionyl-leucyl-phenylalanine-stimulated human PMNs. Findings by Nolte et al. (28) indicated that adenosine reduces postischemic neutrophil-endothelium interaction via A2 receptors in a hamster dorsal skinfold model. A previous report from our laboratory (46) has also demonstrated that adenosine markedly attenuated the adherence of PAF-stimulated canine PMNs to coronary endothelium and protected activated PMN-induced vasoconstriction and endothelial dysfunction. These protective effects were not blocked by the adenosine A1-receptor antagonist, indicating that A2 receptors are involved in the inhibition of neutrophil-endothelial interaction by adenosine. The injurious effects of PMNs on reperfused myocardium relate to their adherence and generation of reactive oxygen species and proteolytic enzymes (2, 10, 11, 25, 33). Activation and accumulation of neutrophils at sites of reperfused tissue not only aggravate myocardial injury by mechanical plugging of microvessels that cause an increase in capillary flow resistance, and thereby participate in the "no-reflow" phenomenon (12), but also may affect the endothelial function by releasing neutrophil-derived vasoactive substances, which decrease vascular responses to prostacyclin and endothelium-derived relaxing factor. In vitro data obtained in the present study showing that CGS significantly reduces superoxide radical generation and neutrophil adherence are consistent with the inhibition of MPO activity in the AAR in vivo, suggesting that inhibition of neutrophil activity is possibly the main mechanism of cardioprotection by CGS during reperfusion.

Activation of KATP channels during ischemia has recently been shown to be an important protective mechanism by the adenosine A1 receptor. The results of the present study demonstrate that although A2 activation stimulates KATP channels and the KATP-channel opener pinacidil inhibits superoxide radical production by neutrophils, blockade of KATP channel during reperfusion does not eliminate the exogenous and endogenous adenosine A2-receptor-mediated cardioprotection, suggesting that the antineutrophil effects of adenosine A2-receptor activation are not mediated by...
the stimulation of $K_{\text{ATP}}$ channels as has been shown in other studies for myocyte $K_{\text{ATP}}$ channels (14, 26).

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REFERENCES


29. Tan, P., H. S. Klopfenstein, and J. Vinten-Johansen. Interactive software for analysis of global or segmental cardiac systolic


