GAP-134 ([2S,4R]-1-[2-Aminoacetyl]4-Benzamidopyrrolidine-2-Carboxylic Acid) Prevents Spontaneous Ventricular Arrhythmias and Reduces Infarct Size During Myocardial Ischemia/Reperfusion Injury in Open-Chest Dogs
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Experimental Study

GAP-134 ([2S,4R]-1-[2-Aminoacetyl]-4-Benzamidopyrrolidine-2-Carboxylic Acid) Prevents Spontaneous Ventricular Arrhythmias and Reduces Infarct Size During Myocardial Ischemia/Reperfusion Injury in Open-Chest Dogs

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The antiarrhythmic dipeptide, GAP-134, ([2S,4R]-1-[2-aminoacetyl]-4-benzamido-pyrrolidine-2-carboxylic acid) was evaluated in canine ischemia/reperfusion model. In dogs subjected to 60-minute ischemia and 4-hour reperfusion, GAP-134 was administered 10 minutes before reperfusion as a bolus + intravenous (IV) infusion. The doses administered were 0.25 µg/kg bolus + 0.19 µg/kg per hour infusion; 2.5 µg/kg + 1.9 µg/kg per hour; 25 µg/kg + 19 µg/kg per hour; 75 µg/kg + 57 µg/kg per hour. Ventricular ectopy was quantified during reperfusion, including premature ventricular contractions (PVC) and ventricular tachycardia (VT). Total incidence of VT was reduced significantly with the 2 highest doses of GAP-134 (1.7 ± 0.8; 2.2 ± 1.4 events; P < .05) compared to controls (23.0 ± 6.1). Total PVCs were reduced significantly from 11.1 ± 1.6% in control animals to 2.0% ± 0.7% and 1.8% ± 0.8% after the 2 highest doses of GAP-134. Infarct size, expressed as percentage of left ventricle, was reduced significantly from 19.0% ± 3.5% in controls to 7.9% ± 1.5% and 7.1% ± 0.8% (P < .05) at the 2 highest doses of GAP-134. GAP-134 is an effective antiarrhythmic agent with potential to reduce ischemia/reperfusion injury.

Keywords: gap junction; arrhythmia; ischemia; infarct size; ventricular tachycardia

Introduction

Spontaneous ventricular arrhythmias that occur during and after ischemia/reperfusion injury are associated with conduction abnormalities and increased nonuniform anisotropy. Reentrant circuits originate in myocardial cells of the infarcted epicardial border zone due to decreased gap junctional conductance, reduced side-to-side coupling, and transverse conduction slowing. Therapeutic strategies aimed at maintaining or increasing gap junction intercellular communication in the border zone may reduce conduction abnormalities and provide antiarrhythmic benefit. The gap junction modifier, rotigaptide, has demonstrated efficacy in 2 canine models of VT/VF and has cardioprotective...
effects in the setting of ischemia/reperfusion injury. However, this hexapeptide requires intravenous (IV) dosing and thus its use is restricted to in-a-hospital setting. GAP-134 is a dipeptide analog of rotigaptide with oral bioavailability and demonstrated efficacy in a canine atrial fibrillation (AF) model of tachypacing-induced heart failure and a canine model of postoperative AF. Similar to the parent compound rotigaptide, GAP-134 reduced dye uptake in cells subjected to simulated ischemia and prevented conduction velocity slowing in rat atrial strips subjected to metabolic stress. In this study, we investigate the antiarrhythmic and cardioprotective effects of GAP-134 in a dog model of ischemia/reperfusion injury.

Methods

Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO). GAP-134 ([2S,4R]-1-[[2-aminoacetyl]-4-benzamido-pyrrolidine-2-carboxylic acid) was manufactured by Wyeth Research.

Canine Surgical Preparation

Purpose-bred beagle dogs, weighing 8 to 10 kg, were anesthetized with sodium pentobarbital (30 mg/kg, IV). The animals were intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus, Inc., Holliston, MA), adjusted to deliver a tidal volume of 30 mL/kg at a frequency of 12 breaths/min. Blood pressure was recorded from the right femoral artery using a Millar Mikro-tip catheter (Millar Instruments, Inc, Houston, TX) interfaced with Po-Neh-Mah data acquisition software. A standard limb lead II electrocardiogram was recorded continuously to monitor heart rate and rhythm. The heart was exposed through a left thoracotomy at the sixth intercostal space and suspended in a pericardial cradle. The left circumflex (LCX) coronary artery was exposed proximal to the first obtuse marginal branch and instrumented with a Transonic ultrasonic flow probe (Model 1.5RB, Transonic Systems Inc, Ithaca, NY) for continuous monitoring of phasic coronary artery blood flow. A ligature stenosis was placed around the LCX coronary artery such that the hyperemic response to a brief 10-second occlusion was reduced by 30%. A Silastic umbilical tape was placed around the LCX coronary artery and through a polyethylene sleeve to create a snare occluder. Complete LCX coronary artery occlusion was initiated by tightening the snare occluder around the vessel. All dogs were subjected to 60 minutes of LCX coronary artery occlusion followed by 4 hours of reperfusion. Restoration of blood flow was performed by slowly releasing the Silastic ligature. Blood flow was restored to preischemic levels over a 10-minute period. The ligature stenosis reduces hyperemia during reperfusion. Infarct size was assessed after 4 hours of reperfusion.

GAP-134 or vehicle (saline) was administered 10 minutes prior to reperfusion in a randomized blinded fashion. A bolus injection (5 mL volume over 5 minutes) of drug was followed by a continuous infusion for the duration of the 4-hour reperfusion period (10 mL total volume, 42 µL/min, Harvard Pump, Holliston, MA). Four doses of GAP-134 were established based on an IV pharmacokinetic study in dogs administered a 1 mg/kg bolus of GAP-134 (data not shown). These data were used to simulate a bolus + infusion protocol that would produce steady state plasma concentrations of 1 nmol/L, 10 nmol/L, 100 nmol/L, and 300 nmol/L GAP-134. The doses administered were 0.25 µg/kg bolus + 0.19 µg/kg per hour infusion (n = 6); 2.5 µg/kg bolus + 1.9 µg/kg per hour infusion (n = 7); 25 µg/kg bolus + 19 µg/kg per hour infusion (n = 6); 75 µg/kg bolus + 57 µg/kg per hour infusion (n = 5); vehicle control (n = 7).

Administration of Radiolabeled Microspheres for Determination of Regional Myocardial Blood Flow

Regional myocardial blood flow (RMBF) was determined using radiolabeled microspheres (103Ru, 100 µCi, 15 µm in diameter, New England Nuclear, Boston, MA) by the reference withdrawal method. During the 60-minute ischemic period, each dog received an injection of microspheres 45 minutes after occlusion of the LCX coronary artery. Microspheres were injected into the left atrial appendage via an inserted catheter and reference blood samples were drawn from the femoral artery and carotid artery.

Determination of Myocardial Infarct Size and Area at Risk

At completion of the study period, hearts were excised immediately after the electrical induction of ventricular fibrillation (VF). Histochemical
determinations of the anatomic area at risk and zone of infarction were accomplished with a dual perfusion technique. The aorta was perfused in a retrograde fashion with 0.25% Evans blue dye, and the LCX coronary artery was perfused with 1.5% triphenyltetrazolium chloride (TTC) in 20 mmol/L potassium phosphate buffer (pH 7.4, 37°C). The heart was cut into 1-cm thick transverse sections and fixed in 10% phosphate buffered formalin. Both surfaces of each ventricular section were traced onto clear plastic overlays and digitized using a flatbed scanner. The PC-Draft software program (Innovative Data Design, Concord, CA) was used to calculate the area of the infarct zone and the area at risk from the digitized heart sections.

**Determination of RMBF**

Myocardial tissue samples weighing 0.1 to 0.5 g (wet weight) were dissected from the subepicardial, midmyocardial, and subendocardial sections of the heart in the nonischemic and ischemic zone, which included the posterior papillary muscle. Four transverse sections from each heart were used so that blood flow to each region represents the average of 4 samples for each experiment. The level of radiolabeled microsphere incorporation into each myocardial tissue sample was measured in a Cobra Quantum Series gamma counter (Packard Instrument Co, Meriden, CT). The mean RMBF from the inner two thirds of the myocardium was used to determine whether an excess of collateral blood supply (>0.18 mL/min per gram tissue) was present during LCX occlusion. RMBF greater than 0.18 mL/min per gram tissue in the inner two thirds of the ventricular wall or refractory ventricular fibrillation requiring more than 3 attempts at cardioversion using low energy pulses (10 Joules) were exclusion criteria.

**Arrhythmia Analysis**

Arrhythmias induced by ischemia/reperfusion injury were analyzed according to the Lambeth Convention. Premature ventricular complexes, defined as discrete and identifiable premature QRS complexes (premature in relation to the previous ventricular sinus beat) were counted for 2-minute periods every 5 minutes during the first 120 minutes of reperfusion. The number of PVCs recorded in a 2-minute period was divided by the total number of heart beats for that same 2-minute period to provide a measure of PVC incidence, expressed as percentage of total beats. Arrhythmia incidence declined significantly in the last 2 hours of reperfusion, thus arrhythmia analysis and antiarrhythmic efficacy measures were focused on the first 2 hours of reperfusion. A run of 4 or more consecutive PVCs was defined as ventricular tachycardia (VT). Ventricular fibrillation was defined as a signal from which individual QRS deflections could not be distinguished from one another and where heart rates were no longer measurable.

**Statistical Analysis**

Incidences of spontaneous ventricular arrhythmias (PVC and VT) were compared using a repeated measures analysis of variance (ANOVA). Total numbers of PVCs or VT during the first 120 minutes of reperfusion were compared using a one-way ANOVA followed by Dunnett post hoc test. Infarct size and RMBF comparisons between vehicle-control and GAP-134-treated dogs were performed using a one-way ANOVA followed by Dunnett post hoc test.

**Results**

**Canine Ischemia/Reperfusion Exclusions and Hemodynamic Data**

A total of 46 dogs were used in the ischemia/reperfusion injury study. In all, 37 dogs successfully completed the study; 7 dogs were excluded because RMBF was greater than 0.18 mL/min per gram tissue. The calculations and cutoffs for exclusion of dogs for high RMBF have been described previously. Average RMBF for the omitted dogs was 0.29 ± 0.04 mL/min per gram tissue and infarct area was 4.6% ± 0.5% of the left ventricle or 13.1% ± 2.2% of the area at risk. Two additional dogs were omitted due to intractable ventricular fibrillation during ischemia or reperfusion. Incidence of ventricular fibrillation was not specific to any 1 group of animals.

**Effect of GAP-134 on Spontaneous Ventricular Arrhythmias During Reperfusion**

Spontaneous ventricular arrhythmias were induced by ischemia/reperfusion injury. These arrhythmias
had varying origins and largely occurred during the first 120 minutes of reperfusion after the ischemic episode. During treatment with the 2 highest dose levels of GAP-134 (25 µg/kg bolus + 19 µg/kg per hour infusion; 75 µg/kg bolus + 57 µg/kg per hour infusion), PVC incidence over time was significantly reduced compared to saline-treated controls in the same period of reperfusion (repeated measures ANOVA, \( P < .05 \); Figure 1A). The total number of PVCs recorded during the first 120 minutes of reperfusion was reduced by 82% in the 25 µg/kg bolus + 19 µg/kg per hour infusion group and by 83% in the 75 µg/kg bolus + 57 µg/kg per hour infusion group compared to saline-treated controls (Figure 1B).

As shown in Figure 2A, the trend in VT incidence over time was also significantly different from control animals at the 2 highest doses of GAP-134 (repeated measures ANOVA, \( P < .05 \)). The total incidence of VT dropped by 92.5% and 90.4% at the 2 highest doses of GAP-134 (\( P < .05 \); Figure 2B).

**Effect of GAP-134 on Infarct Size After 4 Hours Reperfusion**

The effects of GAP-134 on infarct size after 60 minutes of ligature occlusion and 4 hours of reperfusion are illustrated in Figure 3A. Area of the left ventricle at risk, infarct size as a percentage of the area at risk, and infarct size as a percentage of left ventricle are shown. The area at risk was similar in all 5 groups, indicating that an anatomically similar region of the left ventricle was at risk of ischemia and reperfusion injury. In control vehicle–treated dogs, infarcts expanded to 40.9 ± 7.1% of the area at risk and 19.0% ± 3.5% of the left ventricle at the end of 4 hours of reperfusion. After treatment with the 2 highest doses of GAP-134 (25 µg/kg bolus + 19 µg/kg/hour infusion; 75 µg/kg bolus + 57 µg/kg/hour infusion), infarct size expressed as percentage of area at risk was reduced significantly to 18.8% ± 3.1% and 19.5% ± 4.1% compared to control (\( P < .05 \)). Expressed as a percentage of the left ventricle, infarct size was also reduced significantly to 7.9% ± 1.5% and 7.1% ± 0.8% at the 2 highest doses of GAP-134, respectively.

The RMBF of control and GAP-134-treated dogs is shown in Figure 3B. The data expressed represent the regional blood flow in the inner two thirds of the myocardium, reflecting myocardial blood flow in the subendocardial and midmyocardial layers of the left ventricular wall. In each of the treatment groups, RMBF in the region of the left ventricle supplied by the LCX coronary artery (infarct zone) was reduced significantly compared to the region of the left ventricle supplied by the left anterior descending coronary artery (normal zone). The data presented in Figure 3B indicate that each group of animals was
subjected to an equivalent degree of ischemia during the 60-minute ligature occlusion.

Discussion

Studies investigating pathological alterations in gap junction electrophysiology during ischemia/reperfusion injury have been limited by a lack of pharmacological tools that preserve cardiac gap junction integrity and function. GAP-134 is a well-characterized gap junction modifier with demonstrated efficacy in both in vitro and in vivo assays of gap junction communication and cardiac conduction velocity.7-9 In the current study, we demonstrate...
that similar to the parent gap junction modifier, rotigaptide, GAP-134 is an effective antiarrhythmic compound in the setting of canine ischemia/reperfusion injury and arrhythmogenesis. The activity of GAP-134 against ventricular arrhythmias is accompanied by a robust cardioprotective effect that limits infarct expansion over 4 hours of reperfusion. The growing body of preclinical literature for GAP-134 and rotigaptide strongly supports the use of gap junction modifiers for the treatment and prevention of cardiac arrhythmias.

The role of gap junctions in ischemia/reperfusion arrhythmias and cardioprotection continues to evolve. Electrical uncoupling of cardiac myocytes, increased tissue resistance, and conduction abnormalities coincide with the timing and frequency of ventricular arrhythmias. In canine myocardial infarction, reentrant circuits are built on lines of functional block in regions of high anisotropy. Increased transverse conduction due to redistribution of connexin 43 (Cx43) carries impulses away from common pathways. These gap junction–related changes combined with the environmental changes occurring during necrosis and infarction promote arrhythmogenesis and reinforce the need to restore gap junction integrity and anisotropy as an antiarrhythmic strategy. Further studies investigating the action of GAP-134 in the border zone of infarcts are ongoing to better understand the antiarrhythmic actions of this novel class of compounds. Ischemic preconditioning, a well-described antiarrhythmic and cardioprotective mechanism, delays gap junction uncoupling, suggesting that gap junction modification with GAP-134 may act to mimic aspects of ischemic preconditioning. Carbenoxolone, a well-known gap junction uncoupler, ameliorates the protection afforded by preconditioning; this finding further suggests that agents capable of increasing or maintaining gap junction integrity and function may be therapeutically useful.

Gap junction uncoupling encompasses several events within the cell including alterations in gap junction turnover, translocation, and phosphorylation status of the connexin. Dephosphorylation of Cx43 during ischemia reduces gap junction intercellular communication; however, the mechanism by which dephosphorylation occurs remains unknown. Multiple kinases are involved in the regulation of Cx43 through phosphorylation including PKA, PKG, PKC, p38 MAP kinase, AMP-dependent kinase, and CaMK-II. The control of dephosphorylation likely involves numerous changes in kinase activity and phosphatase activation. Further studies are needed to correlate changes in intercellular gap junction resistance with kinase-mediated phosphorylation to better understand the key signaling pathways responsible for decreased gap junction function during ischemia/reperfusion injury. During ischemic preconditioning and rotigaptide treatment, preservation of phosphorylation of specific Cx43 residues has been demonstrated and these signaling events are well correlated with function, cardioprotection, and antiarrhythmic benefit. Any intervention that prevents deterioration of gap junction function or delays this process has the potential to deter the onset of arrhythmias. It is difficult to separate the cardioprotective actions of GAP-134 from its antiarrhythmic actions. Given the magnitude of reduction in ventricular ectopy during reperfusion observed in this study, it is possible that the cardioprotective actions of GAP-134 are due to increased perfusion of the tissue and maintenance of normal sinus rhythm rather than a direct cardioprotective action. Further studies are needed to better understand the cardioprotective mechanisms activated by GAP-134 and the role of gap junction uncoupling during ischemia/reperfusion injury. Gap junction uncoupling has also been linked to preservation of myocardium during ischemia/reperfusion injury. Studies performed with gap junction uncouplers such as heptanol, 18β-glycyrrhetinic acid, halothane, and palmitoleic acid in rats, rabbits, and dogs have demonstrated a reduction in infarct size. This cardioprotective effect resulting from gap junction uncoupling has been attributed to limiting the spread of hypercontracture and necrotic signaling during infarct expansion. However, the tested agents lack selectivity for gap junctions and have multiple effects on targets such as ion channels that may result in a reduction in the spread of infarction. The results of this study with GAP-134 and previous studies using rotigaptide and ischemic preconditioning provide support for preservation of gap junction integrity in preventing the spread of infarction during ischemia/reperfusion injury.

The barbiturate-anesthetized, open-chest beagle was chosen as the experimental model for this study given the large body of literature already published using the same model. Infarct size determinations are subject to variability from study to study and it is well described that infarct expansion is affected by gender, age, and species. Burmeister and Reynolds demonstrated that mongrel dogs have
approximately 50% larger infarcts compared to beagles in a well-controlled ischemia/reperfusion study. To control for this variability, beagles were age-matched and weight-restricted within this study. Furthermore, GAP-134-treated groups were compared to a similarly matched control group that was treated with vehicle during the ischemic period. It is expected that the cardioprotection observed in this model will translate into significant and measurable cardioprotection in other animal models of ischemia/reperfusion injury. Barbiturate anesthesia is associated with increased infarction compared to conscious canine ischemia/reperfusion injury experiments. However, the mechanism for this effect has not been elucidated and the robust cardioprotection and antiarrhythmic effect observed with GAP-134 was compared directly to barbiturate anesthetized controls within this study.

Although the exact mechanism of action of the antiarrhythmic peptides remains elusive, we can conclude that GAP-134 is an effective antiarrhythmic and cardioprotective agent in the setting of canine ischemia/reperfusion injury. Further studies are needed to understand the direct actions of this small peptide on gap junction function, connexin phosphorylation, connexin redistribution, and cardiac conduction.

References


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