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Rotigaptide (ZP123) Prevents Spontaneous Ventricular Arrhythmias And Reduces Infarct Size During Myocardial Ischemia/Reperfusion Injury In Open-Chest Dogs

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Non-Standard Abbreviations: PVC, premature ventricular complex; LVEDP, left ventricular end diastolic pressure; GJIC, gap junction intercellular communication; RMBF, regional myocardial blood flow.

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Abstract

The antiarrhythmic and cardioprotective effect of increasing gap junction intercellular communication during ischemia/reperfusion injury has not been studied. The antiarrhythmic peptide, rotigaptide (previously ZP123), which maintains gap junction intercellular communication was tested in dogs subjected to a 60-min coronary artery occlusion and 4 hr reperfusion. Rotigaptide was administered IV 10-min before reperfusion as a bolus + IV infusion at doses of: 1ng/kg bolus + 10ng/kg/hr infusion (n=6); 10ng/kg bolus + 100ng/kg/hr infusion (n=5); 100ng/kg bolus + 1000ng/kg/hr infusion (n=8); 1000ng/kg bolus + 10μg/kg/hr infusion (n=6); vehicle control (n=5). Premature ventricular complexes (PVC's) were quantified during reperfusion. Four or more consecutive PVC's was defined as ventricular tachycardia (VT). Total incidence of VT was reduced significantly with the two highest doses of rotigaptide (20.3±10.9; 4.3±4.1 events; $p<0.05$) compared to controls (48.7±6.0). Total PVC's were reduced significantly from 25.1±4.2% in control animals to 11.0±4.4% and 1.7±1.3% after the two highest doses of rotigaptide. Infarct size, expressed as percent of left ventricle, was reduced significantly from 13.2±1.9 in controls to 7.1±1.0 ($p<0.05$) at the highest dose of rotigaptide. Ultrastructural evaluation revealed no differences in myocardial injury in the infarct area, area at risk, border zone or normal zone in vehicle and rotigaptide-treated animals. However, rotigaptide did increase the presence of gap junctions in the area at risk ($p=0.022$, Fisher's exact test). Rotigaptide had no effect on heart rate, blood pressure, QTc, or LVEDP. In conclusion these results demonstrate that rotigaptide is a potent antiarrhythmic compound with cardioprotective effects and desirable safety.

Introduction

Myocardial gap junctions are transmembrane channels that transmit electrical impulses via direct transfer of cytosolic ions, metabolites and small intracellular messengers between neighboring cells. During electrical excitation and depolarization of cardiomyocytes, gap junctions play a significant role in action potential propagation and are thus a major determinant of conduction velocity (Lin et al., 2003). Acute alterations in electrical coupling during myocardial ischemia and reperfusion are associated with conduction slowing and heterogeneities of repolarization that are important substrates for reentrant arrhythmias (DeGRoot et al., 2004). Ischemia/ reperfusion induced gap junction uncoupling is determined by several factors including acidosis (Kleber, 1992), calcium overload (Dekker et al., 1996) and accumulation of lipid metabolites (Wu et al., 1993). Based on these facts, therapeutics aimed at preventing uncoupling or re-establishing gap junction intercellular communication may be effective antiarrhythmic agents.

The effect of modulating gap junction intercellular communication (GJIC) on myocardial infarct size after ischemia/reperfusion injury is somewhat controversial. Several studies have demonstrated that reduced GJIC in the presence of the gap junction uncoupler heptanol (0.5-6 mM) may reduce cell-to-cell propagation of hypercontracture and cell death (Garcia-Dorado et al., 1997; Saltman et al., 2002). However, heptanol has also been shown to relax arteries, activate large conductance calcium-activated potassium channels and inhibit nifedipine-sensitive calcium current (Matchov et al., 2004). Thus, it is difficult to determine whether the cardioprotective effects of heptanol observed at millimolar concentrations are due to gap junction uncoupling or some other mechanism. Furthermore, heptanol failed to induce cardioprotection in a rabbit model of ischemia/reperfusion injury (Gysembergh et al., 2001). Reduced GJIC has

also been correlated with cardioprotection in studies from connexin43 heterozygous null mice, which develop significantly smaller infarcts compared to wild-type mice (Kanno et al., 2003). In contrast to the evidence that reduced coupling may be cardioprotective, several studies have demonstrated a cardioprotective role for GJIC preservation via dilution of 'death signals' or passage of 'survival factors' in ischemia/reperfusion injury (Blanc et al., 1998, Yasui et al., 2000; Li et al., 2002). However, investigation of the cardioprotective effect of increased GJIC has been limited due to an absence of stable compounds that specifically increase GJIC. The novel gap junction modifier, rotigaptide, represents a unique opportunity to resolve this issue.

Gap junction modifiying peptides were first described in the early 1980's (Aonuma et al., 1980) and later shown to increase gap junction intercellular communication in the absence of changes in membrane conductance or basal current (Muller et al., 1997b). The clinical development of the original antiarrhythmic peptide (AAP) (Aonuma et al., 1980) and later synthetic derivatives (HP-5 and AAP10) (Kohama et al., 1987; Dhein et al., 1994) has been limited by their instability and very short half life. Rotigaptide is a rotation-inversion of AAP10 that incorporates the unnatural D-configuration of the amino-acids to provide much improved proteolytic stability. Rotigaptide increases gap junction intercellular communication in paired guinea pig cardiomyocytes measured using dual-cell patch clamp electrophysiology (Xing et al., 2003). Rotigaptide-mediated increases in GJIC occur without alteration of basal or membrane current suggesting a direct effect of the compound on gap junctions. Rotigaptide has a low affinity for hERG and exhibits no binding to a large panel of receptors including numerous ion channels (Haugan et al., 2005). Rotigaptide also prevents metabolic stress-induced conduction velocity slowing in rat atrial strips (Haugan et al., 2005) and improves ventricular conduction velocity in

whole guinea pig hearts without effecting cellular repolarization (Eloff et al., 2003). In ischemic canine hearts, rotigaptide reduces the incidence of inducible re-entrant ventricular tachycardia demonstrating the importance of gap junction uncoupling as an arrhythmogenic substrate (Xing et al., 2003). In this study, we investigated the antiarrhythmic and cardioprotective effects of rotigaptide in the dog to elucidate the role of gap junctional intercellular communication on ischemia/reperfusion-induced arrhythmias and myocardial infarction. We present evidence of increased gap junctions in the area at risk after treatment with rotigaptide. We also present cardiovascular safety pharmacology data and a pharmacokinetic profile in dogs administered rotigaptide.

Methods

Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical (St. Louis, MO). Rotigaptide (proposed INN name for the drug formerly known as ZP123) was manufactured by Bachem AG (Bubendorf, Switzerland) for Wyeth Research. The chemical structure of rotigaptide (Ac-d-Tyr-d-Pro-d-Hyp-Gly-d-Ala-Gly-NH_2) is presented in **Figure 1**.

Methods

Surgical Preparation

Purpose-bred Beagle dogs, weighing 8-10 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.). 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 a Grass Model 7-polygraph recorder (Grass Instruments Division, Astro-Med Inc., West Warwick, RI). 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-s 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. All hemodynamic parameters were recorded throughout the experiment on a Grass Instruments multi-channel recorder interfaced with a PC computer running PO-NEH-MAH data acquisition software. Complete LCX coronary artery occlusion was initiated by tightening the snare occluder around the vessel. All dogs were subjected to 60 min of LCX coronary artery occlusion and reperfusion. Restoration of blood flow was performed by slowly releasing the Silastic ligature. Blood flow was restored to pre-ischemic levels over a ten-minute period. The ligature stenosis reduces hyperemia during reperfusion. Infarct size was assessed after 4 hours of reperfusion.

Rotigaptide or vehicle (saline) was administered 10 minutes prior to reperfusion in a randomized blinded fashion. A bolus injection (5 ml volume over 5 min) of drug was followed by a continuous infusion for the duration of the 4 hr reperfusion period (10 ml total volume, 42 µl/min, Harvard Pump, Holliston, MA). Four doses of rotigaptide were tested including 1 ng/kg bolus + 10 ng/kg/hr infusion (n=6); 10 ng/kg bolus + 100 ng/kg/hr infusion (n=5); 100 ng/kg bolus + 1000 ng/kg/hr infusion (n=8); 1000 ng/kg bolus + 10 µg/kg/hr infusion (n=6); vehicle control (n=5).

Administration of Radiolabeled Microspheres for Determination of RMBF

Regional myocardial blood flow (RMBF) was determined using radiolabeled microspheres (^{103}Ru, 100 µCi, 15 µm in diameter, New England Nuclear, Boston, MA, USA) by the reference withdrawal method (Black et al., 1998). During the 60-minute ischemic period each dog received an injection of microspheres 45-min after occlusion of the LCX coronary 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. 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 mM potassium phosphate buffer (pH 7.4, 37^{o} 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 - 0.5 g (wet weight) were dissected from the subepicardial, midmyocardial and subendocardial sections of the heart in the non-ischemic 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 four 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/g tissue) was present during LCX occlusion. RMBF greater than 0.18 ml/min/g tissue in the inner two-thirds of the

ventricular wall or refractory ventricular fibrillation requiring more than three 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 (Walker et al., 1988). Premature ventricular complexes, defined as discrete and identifiable premature QRS complexes (premature in relation to the previous ventricular sinus beat) were counted for 2 min periods every 5 min during the first 60 min of reperfusion. A run of 4 or more consecutive PVC's was defined as ventricular tachycardia (VT). VF was defined as a signal from which individual QRS deflections could not be distinguished from one another and where heart rate were no longer measurable.

Electron Microscopy and Ultrastructural Analysis

Small pieces of canine heart from vehicle-treated controls (n=3) or rotigaptide-treated dogs (100 ng/kg bolus + 1000 ng/kg/hr infusion, n=3) were fixed in Modified McDowell-Trump's fixative and processed for transmission electron microscopic examination as described previously (Keith, 1986). Samples were taken from the infarct area, infarct border zone, area at risk, and normal myocardium. Each sample was post-fixed with 1% osmium tetroxide, routinely dehydrated in a graded series of ethanols, immersed in propylene oxide and embedded in Araldite resin. Thin sections were cut on a Leica EM UC6 microtome using a diamond knife. Sections on grids were post-stained with uranyl acetate and lead citrate and viewed on a Zeiss 10C transmission electron microscope operating at 80kV. Micrographs were recorded on film and printed on photographic paper. Three hundred and six electron photomicrographs were produced and randomly coded

prior to evaluation by two investigators (JCK and RGS). The degree of myocardial injury was scored based on four parameters described previously (Trump et al., 1976; Keith, 1986). The swelling or dilation of the tubules and sacroplasmic reticulum were each scored, ranging from 0-normal to 2 for severe dilation. The structural integrity of the mitochondria were assessed for swelling and for disruption of the cristae using a scale ranging from 0-normal to 3-massive swelling and complete disruption of the cristae. The structure of the myofibrils was graded, ranging from 0-normal relaxed myofibrils, through 1-hypercontraction, to 2-disruption of myofibrils. Each photomicrograph was examined for the presence of intercellular gap junction profiles clearly associated with zona adherens junctions as described previously (Petrich et al. 2002). If intercellular profiles of gap junctions were seen, they were called present. If zona adherens junctions were present but no gap junction profiles were seen, they were called absent. If no zona adherens junctions and intercellular regions were present in the photomicrograph, the specimen was called non-evaluable for gap junctions. A representative photomicrograph containing gap junctions is seen in **Figure 2**. After the analysis was completed, the codes were revealed and the results of myocardial injury score and presence of gap junctions tabulated by myocardial region and treatment group.

Pharmacokinetics and Determination of Rotigaptide Concentrations in Dog Plasma

Rotigaptide was formulated in saline with no buffer or pH adjustment and administered via single intravenous bolus (20 µg/kg) via a cephalic vein in a separate group of 8 dogs. Blood (~3 ml) was collected from the jugular vein in tri-potassium EDTA at 5 min, 15 min, 30 min, 1, 1.5, 2, 4, 6, 8 and 12 hours after dosing. Rotigaptide concentration was determined in plasma samples by an LC/MS/MS procedure (Kjolbye et al., 2003) with a lower limit of quantitation of

1 ng/mL. The pharmacokinetic parameters for individual animals were determined using IV bolus administration, non-compartmental analysis module (Model 201) of the PK software package WinNonlin, ver. 3.2 (Pharsight, CA).

Cardiovascular Safety Profile of Rotigaptide

To further understand the cardiovascular safety of rotigaptide, 8 additional dogs (4 male, 4 female) were administered suprapharmacological doses (1, 3, and 10 mg/kg) by single intravenous bolus injection according to a Latin square crossover dosing paradigm. Effects on heart rate, systolic and diastolic blood pressure and Lead II ECG (QT, QTc, PR and QRS interval) were evaluated in all animals using radiotelemetry units implanted 14 days prior to dosing. Prior to dosing, data were collected for 30-second periods every 5 min for 24 hours. After dosing, telemetry data were collected for 30 second periods every minute for 2 hours followed by measurements every 5 minutes up to 24 hours post dose. Heart-rate corrected QT interval (QTc) was calculated using the regression relationship estimated from the predose data. The relationship between postdose QT interval and heart rate was examined by fitting a mixed model analysis of variance to the postdose data for the vehicle-control and 10 mg/kg dosages.

Statistical Analysis

Incidences of spontaneous ventricular arrhythmias (PVC and VT) were compared using a repeated measures ANOVA. Total number of PVC's or VT during the first 60 min of reperfusion were compared using a one-way ANOVA followed by Dunnet's post-hoc test. Infarct size and RMBF comparison's between vehicle-control and rotigaptide-treated dogs were

performed using a one-way ANOVA followed by Dunnet's post-hoc test. In the safety pharmacology studies, repeated measures ANOVA was used to compare mean response after administration of rotigaptide to mean response after vehicle administration. The ANOVA model used for heart rate data analysis included a random effect to account for animal to animal differences. For heart rate corrected QT comparisons, a regression model was fit to the combined predose data for vehicle-control and compound dosages. The presence of gap junctions in the ultrastructural analysis was evaluated for treatment effect by Fisher's Exact test. For all statistical analysis, $P<0.05$ was considered significant.

Results

Exclusions and Hemodynamic Data

A total of 45 dogs were used in the ischemia/reperfusion injury study. 36 dogs successfully completed the study. Four dogs were excluded because RMBF was greater than 0.18 ml/min/g tissue and five dogs were omitted due to intractable ventricular fibrillation during ischemia or reperfusion. Incidence of ventricular fibrillation was not specific to any one group of animals.

Effect of Rotigaptide on Spontaneous Ventricular Arrhythmias During Ischemia/Reperfusion Injury

Spontaneous ventricular arrhythmias were induced by ischemia/reperfusion injury. These arrhythmias had varying origins and were concentrated to the first 60 min of reperfusion after the ischemic episode. During treatment with the two highest dose levels of rotigaptide (100 ng/kg bolus + 1000 ng/kg/hr infusion or 1000 ng/kg + 10 µg/kg/hr) PVC incidence over time was significantly reduced compared to saline-treated controls in the same period of reperfusion (repeated measures ANOVA, $p<0.05$) (**Figure 3a**). At the highest dose, statistically significant reductions in PVC's were observed at the 25, 30, 35, 40 and 45 min time points after reperfusion ($p<0.05$). The total number of PVC's recorded during the first 60 min of reperfusion was reduced by 56% in the 100 ng/kg + 1000 ng/kg/hr infusion dose group and by 93% in the 1000 ng/kg + 10 µg/kg/hr infusion group compared to saline treated controls (**Figure 3b**).

As shown in **Figure 4a**, runs of VT were reduced significantly at the 25, 30, 35 and 40 min time points of reperfusion ($p<0.05$) after treatment with the highest dose of rotigaptide. The trend in VT incidence over time was significantly different from control animals at the two highest doses

of rotigaptide (repeated measures ANOVA, $p<0.05$). The total incidence of VT in the first 60 minutes of reperfusion was reduced significantly during treatment with two highest doses of rotigaptide ($p<0.05$) (**Figure 4b**). These data suggest that gap junction uncoupling is one of the primary substrates for arrhythmia formation in the ischemic/reperfused dog heart and maintenance of GJIC by rotigaptide is an effective antiarrhythmic strategy.

Effect of Rotigaptide on Infarct Size After 4 Hours Reperfusion

The effects of rotigaptide on infarct size after 60 min of ligature occlusion and 4 hours of reperfusion are illustrated in **Figure 5**. Area of the left ventricle at risk, infarct size as a percent of the area at risk and infarct size as a percentage of left ventricle are shown. The area at risk was similar in all five groups, indicating than an anatomically similar region of the left ventricle was at risk of ischemia and reperfusion injury. Infarct size, expressed as a percentage of the area at risk or as a percentage of the left ventricle was significantly reduced after treatment with the highest dose of rotigaptide ($p<0.05$).

The RMBF of control and rotigaptide-treated dogs is shown in **Figure 6**. 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 left circumflex 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 6** indicate that each group of animals was subjected to an equivalent degree of ischemia during the 60-min ligature occlusion.

Ultrastructural Analysis of Infarcted Hearts

306 transmission electron photomicrographs were evaluated by two investigators without knowledge of the treatment group. Seventeen micrographs could not be evaluated for the parameters of injury or the presence of gap junctions due to advanced necrosis. Myocardial injury scores were determined for 289 evaluable micrographs, and the results are seen in **Table 1**. The degree of damage was comparable between the vehicle and the rotigaptide treated animals. Intercellular gap junction profiles were scored present or absent in the 146 micrographs that contained intercellular regions adjacent to zona adherens junctions, and the results are displayed by myocardial region in **Table 2**. In the infarct, border zone and non-ischemic myocardial regions the frequency of gap junctions was similar between treatment groups. In the area at risk region, rotigaptide administration appeared to increase the presence of gap junctions (P = 0.022, Fisher's Exact Test).

Pharmacokinetics and Estimated Plasma Concentrations of Rotigaptide

Due to the injection of radiolabeled microspheres in the infarct study plasma concentrations of rotigaptide were not measured. However, a detailed pharmacokinetic analysis was done in the dog to estimate steady state plasma concentrations of rotigaptide in the ischemia/reperfusion study. The pharmacokinetic profile of rotigaptide following a single bolus intravenous dose of 20 µg/kg showed a linear pharmacokinetic behavior. The mean C_{max} and exposure (AUC) values were 97.3 ng/mL and 88.8 ng.hr/mL, respectively. The mean clearance (CLT) and volume of distribution (Vdss) values were 0.226 L/hr/kg and 0.237 L/kg, respectively. Based on the data from the 20 µg/kg IV bolus dose in dogs, and assuming linear pharmacokinetics, the extrapolated

PK parameters of rotigaptide 60 min after bolus and start of the infusion at the highest dose in the arrhythmia study were estimated to be a C_{max} of 199 ng/mL and exposure (AUC) of 182 ng.hr/mL, respectively. Rotigaptide has previously demonstrated canine antiarrhythmic efficacy at plasma concentrations ranging from 1-50 ng/ml (Xing et al., 2003).

Hemodynamic Effects of Rotigaptide

In the present study heart rate (HR) was unchanged from the respective baseline in control and rotigaptide treated animals (**Table 3**). Comparisons among groups did not reveal differences in HR. Mean arterial blood pressure (MABP) was also not different from baseline in control and rotigaptide-treated animals and no difference in blood pressure was observed in a comparison among groups.

In a separate study from the ischemia/reperfusion experiments above, the cardiovascular safety profile for rotigaptide was determined in male and female dogs administered an intravenous bolus injection of 1, 3, or 10 mg/kg. Rotigaptide did not produce any compound-related changes in heart rate or arterial blood pressure. There was no evidence of abnormal atrial or ventricular arrhythmia, QTc prolongation or morphologic changes in any of the ECG's examined after administration of vehicle-control (saline) or rotigaptide. PR, QRS and QTc values for animals treated with 10 mg/kg rotigaptide were comparable to those values observed after treatment with vehicle-control. The exposure ratio at the highest dose in the safety pharmacology study was ~300-times the efficacious exposure of the highest dose shown in **Figure 3**.

Discussion

The present study demonstrates a beneficial antiarrhythmic effect of improving gap junction communication with rotigaptide during reperfusion injury. Furthermore, it provides the first evidence that increasing gap junction intercellular communication during ischemia reperfusion injury reduces infarct size. The antiarrhythmic and cardioprotective effects of rotigaptide were observed at concentrations shown to increase gap junctional conductance in paired cardiomyocytes (Xing et al., 2003), improve conduction velocity in acidotic guinea pig ventricle (Eloff et al., 2003) and inhibit metabolic stress-induced conduction velocity slowing in rat atrial strips (Haugan et al., 2005). Despite a large body of data describing the pharmacology and antiarrhythmic effects of rotigaptide, its exact molecular target remains unknown. In very recent studies, rotigaptide has been shown to alter the phosphorylation status of Cx43 in cardiomyocytes during ischemia. Treatment with rotigaptide prevented dephosphorylation of Ser297 and Ser368 on Cx43 and prolonged significantly the time until asystole in a rat Langendorff model (Axelson et al., 2005). Changes in gap junction function during ischemia likely involve both phosphorylation and dephosphorylation of specific sites in Cx43. These data suggests that the antiarrhythmic effect of rotigaptide may be mediated through modulation of Cx43 phosphorylation during ischemia. Rotigaptide also increases Cx43 expression in cultured cardiomyocytes (Stahlhut et al., unpublished 2005). These new studies provide further evidence that rotigaptide has downstream effects on connexins and gap junctions.

The extent of gap junction uncoupling during ischemia is directly related to the duration of ischemia and well correlated with the onset of ischemic rigor and calcium overload (Delmar et al., 1987). Upon reperfusion of the ischemic myocardium rapid normalization of pH and

cytosolic calcium elicit further hypercontracture and the genesis of reperfusion arrhythmias (Yamazaki et al., 1986). These arrhythmias occur most often through re-entrant circuits that develop in the injured myocardium due to slowed conduction and conduction block (Yamazaki et al., 1986; Dillon et al., 1988). Confocal microscopy and thin section electron microscopy have shown that gap junctions are severely disturbed in the infarct and border zone leading to heterogeneous conduction and increased susceptibility to arrhythmias (Smith et al., 1991; Peters et al., 1995). The dramatic reduction in reperfusion arrhythmias observed with rotigaptide suggests that under these pathological conditions the gap junction may be the predominant factor implicated in arrhythmogenesis and thus agents that improve gap junction intercellular communication may be effective for the prevention of VT post-myocardial infarction. These results are in agreement with previously published data on rotigaptide demonstrating a reduction in re-entry VT in ischemic canine hearts subjected to programmed extra-stimulation to induce VT and three dimensional activation mapping to confirm re-entry (Xing et al., 2003). Interestingly, rotigaptide does not appear to be efficacious against focal VT in canine hearts subjected to programmed extra-stimulation (Xing et al., 2005). Thus, the spontaneous arrhythmias observed in this study likely originate from re-entry circuits in the ischemic zone. Rotigaptide had no effect on heart rate or mean arterial blood pressure confirming the previously reported lack of effect of rotigaptide on cardiac contractility or systemic hemodynamics (Xing et al., 2003; Kjolbye et al., 2003; Haugan et al., 2005).

Myocardial reperfusion of the ischemic heart remains the best strategy so far to limit infarct size. Reperfusion interrupts the progression of necrosis and salvages tissues in the border zone of the infarct. Despite its proven utility, reperfusion is also associated with detrimental cardiac effects

including extension of myocyte hypercontracture and a deleterious inflammatory response against host tissue, mediated by free radicals, neutrophils and complement activation. The process of gap junction uncoupling during ischemia/reperfusion injury is well documented but the role of gap junctions as a determinant of final infarct size is controversial (Garcia-Dorado et al., 1997; Blanc et al., 1998; Yasui et al., 2000; Gysembergh et al., 2001; Saltman et al., 2002; Li et al., 2002; Kanno et al., 2003; Matchov et al., 2004). It has been suggested that gap junction intercellular communication may act to dilute potentially lethal signals during reperfusion injury thereby limiting the spread of cell death. In rat neonatal cardiomyocytes, preservation of cell-to-cell coupling is associated with a reduction in apoptosis (Yasui et al., 2000). Low cell density in culture or inhibition of gap junction formation through Cx43 antisense treatment leads to increased cell death (Yasui et al., 2000). It has been suggested that one or more humoral factors may transfer through gap junctions acting as "survival signals" to reduce cell death. Gap junctions also permit transfer of antioxidants from non-ischemic cells to ischemic cells, which protect the cell against further oxidative stress and prevent cardiomyocytes from induction of apoptosis (Inserte et al., 2000). Further studies are required to specifically identify those apoptosis-affecting humoral factors that are transferred through gap junctions to prevent spread of cell death.

The observed preservation of gap junctions in the rotigaptide treated hearts is a possible indication of improved conduction in the ischemic heart. However, we did not assess functional coupling or conduction velocity in this model so it remains to be determined if the gap junctions are functional. Previous rotigaptide studies have demonstrated increased conduction velocity (Eloff et al., 2003, Haugan et al., 2005) and reduced dispersion of the action potential (Kjolbye et

al., 2003) in intact cardiac muscle. Whether these effects result from improved gap junction preservation or decreased degradation of gap junctions requires further study. Several studies have demonstrated reduced conduction velocity after ischemia induced gap junction uncoupling or after administration of gap junction uncouplers (see Rohr, 2004 for review), further suggesting that preservation of gap junctions should result in sustained or increased conduction velocity. Aside from cardiac tissue the potential benefit of increasing gap junction communication in bone osteoblast membrane has been linked to improved bone density in rat osteoporosis (Petersen et al., 2005).

The effect of improving gap junction coupling or reducing uncoupling in the setting of ischemia/reperfusion injury has not been studied extensively in vivo due to a lack of compounds that selectively increase gap junction coupling. The reduction in infarct size with rotigaptide demonstrated in this study, provides the first evidence that improved gap junction communication during ischemia/reperfusion may protect the myocardium. These data are supported by previous studies demonstrating an important role for gap junctional intercellular communication in the transfer of cardioprotective factors during ischemic preconditioning (Li et al., 2002). Other mechanisms of cardioprotection including altered intracellular calcium dynamics, ATP preservation or improved perfusion of the infarct and border zone due to increased gap junction communication may be responsible for the decrease in infarct size observed with rotigaptide. Further studies investigating these cardioprotective pathways are required to clearly identify the mechanism of rotigaptide-induced cardioprotection.

Despite our finding of a protective effect for rotigaptide, several investigators have studied the effect of inhibiting gap junction communication during reperfusion injury. Four gap junction uncouplers with different mechanisms of action (heptanol, 18α-glycyrrhetinic acid, halothane and palmitoleic acid) have demonstrated a protective effect against myocardial necrosis when administered during reperfusion. These agents limit cell-to-cell hypercontracture and reduce overall infarct size in rat, rabbit and dog models of ischemia/reperfusion injury (Garcia-Dorado et al., 1997; Schlack et al., 1994). The mechanism of action of the most commonly used uncoupler, the organic solvent heptanol, remains unknown. It reportedly reduces the open probability of gap junction channels by eliciting a conformational change at the interface between gap junction proteins and membrane lipids (Takens-Kwak et al., 1992; Christ et al., 1999). However, heptanol has also been shown to relax arteries, activate large conductance calcium-activated potassium channels and inhibit nifedipine-sensitive calcium current (Matchov et al., 2004). The non-selective actions of this agent were clearly apparent in the mouse heart, where 20% of hearts treated with 1 mM heptanol exhibited spontaneous arrest (Li et al., 2002). Rotigaptide is a selective gap junction modifier with no observed cardiovascular side effects at doses well above efficacy. Thus, it is possible that the role of gap junctions in the spread of infarction has been mislead by poor uncouplers and a lack of good gap junction couplers.

In conclusion, our data demonstrate that increased gap junction intercellular communication in the presence of rotigaptide prevents spontaneous reperfusion-induced ventricular arrhythmias and protects the heart against the spread of infarction. Rotigaptide represents a novel approach to acute prevention of VT/VF post-myocardial infarction.

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Legends for Figures

Figure 1. Chemical structure of rotigaptide.

Figure 2. Representative electron photomicrograph of canine myocardium displaying gap junction profiles. The slender arrows deliminate the gap junction profile in the intercellular space, while the larger arrows identify the zona adherens junctions. Original magnification 8,800x.

Figure 3. Effect of rotigaptide on the incidence of (a) and total (b) premature ventricular complexes (PVC's) in the first 60 min of reperfusion. Incidence values are expressed as the mean, whereas total PVC values are expressed as mean±SEM for n=5-8 animals. The trend in PVC incidence over the first 60 min of reperfusion in control and rotigaptide treated dogs was compared using a repeated measures one-way ANOVA. The total number of PVC's in control and rotigaptide treated dogs was compared using a one-way ANOVA followed by Dunnet's post-hoc test. Asterisks indicate a statistically significant difference in PVC's compared to control ($p<0.05$); # indicates a statistically significant difference in the overall trend of PVC's during the first 60 min of reperfusion ($p<0.05$).

Figure 4. Effect of rotigaptide on the incidence of (a) and total (b) ventricular tachycardia (VT) in the first 60 min of reperfusion. Incidence values are expressed as the mean, whereas total VT values are expressed as mean±SEM for n=5-8 animals. The trend in VT incidence over the first 60 min of reperfusion in control and rotigaptide treated dogs was compared using a repeated measures one-way ANOVA. The total number of VT episodes in control and rotigaptide treated dogs was compared using a one-way ANOVA followed by Dunnet's post-hoc test. Asterisks

indicate a statistically significant difference in VT compared to control ($p<0.05$); # indicates a statistically significant difference in the overall trend of VT during the first 60 min of reperfusion ($p<0.05$).

Figure 5. Effect of rotigaptide on infarct size after 60 min of left circumflex coronary artery occlusion and 4 hours of reperfusion. Values represent mean±SEM for n=5-8 animals per group. Infarct size as a percent of the area at risk or left ventricle in rotigaptide-treated animals were compared to vehicle-control animals using a one-way ANOVA followed by Dunnet's post-hoc test. Asterisk indicates a significant reduction in infarct size compared to control ($p<0.05$).

Figure 6. Regional myocardial blood flow (RMBF) in the infarct and normal zone during a 60 min occlusion of the left circumflex coronary artery. Values represent mean±SEM for n=5-8 experiments. RMBF in the infarct zone was significantly reduced compared to the normal zone. Four dogs exhibiting RMBF above 0.18 ml/min/g tissue were omitted from the study.

Tables

Table 1. Myocardial Ultrastructural Injury Scores* After Ischemia/Reperfusion in Dogs Treated with Vehicle or Rotigaptide.

Group	t-tubules 0-2	Sarcoplasmic Reticulum 0-2	Mitochondria 0-3	Myofibrils 0-2	Mean Injury Score 0-10
Vehicle					
Infarct	0.65+1.02	0.73+0.95	1.69+1.38	0.82+1.05	**3.89**
Border Zone	0.30+0.53	0.33+0.58	1.86+0.43	1.46+0.47	**3.95**
Area at Risk	0.61+1.06	0.61+1.05	1.05+1.55	0.81+1.05	**3.08**
Normal	0.11+0.19	0.11+0.19	0.26+0.35	0.18+0.17	**0.66**
Rotigaptide					
Infarct	0.39+0.68	0.47+0.81	1.88+0.36	1.45+0.33	**4.19**
Border Zone	0.67+1.01	0.70+1.06	1.05+0.55	1,28+0.33	**3.70**
Area at Risk	0.30+0.28	0.34+0.30	1.25+1.01	0.75+0.43	**2.64**
Normal	0.09+0.09	0.14+0.13	0.63+0.59	0.22+0.21	**1.08**

*Injury scores were determined by scoring t-tubules and sarcoplasmic reticulum-0-normal, 1-mild dilation, 2-severe dilation; mitochondria- 0-normal, 1-mild swelling, 2-moderate swelling, 3-severe swelling & disruption; and myofibril structure – 0-normal, 1-hypercontraction, 2-disruption of myofibrils.

Table 2. Ultrastructural Documentation of Gap Junctions After Ischemia /Reperfusion in Dogs Treated with Vehicle or Rotigaptide.

Group / Region	Photomicrographs With Gap Junctions Present per Photomicrographs Deemed Evaluable
Vehicle / Infarct	2 of 19
Vehicle / Border Zone	2 of 17
Vehicle / Area at Risk	1 of 13
Vehicle / Non-Ischemic	15 of 19
Rotigaptide / Infarct	5 of 15
Rotigaptide / Border Zone	5 of 16
Rotigaptide / Area at Risk	10 of 22*
Rotigaptide / Non-Ischemic	9 of 22

*Significantly different higher than Vehicle /Area at Risk (P = 0.022, Fisher's Exact Test).

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Table 3. Mean heart rate and mean arterial blood pressure at baseline and for 4 hours after administration of Rotigaptide in a canine model of ischemia/reperfusion injury.

Treatment	Baseline	1 hour	2 hour	3 hour	4 hour
Mean Heart Rate					
Saline Control	151±9	128±5	144±10	152±6	159±6
1ng/kg bolus + 10ng/kg/hr Rotigaptide	160±6	151±15	168±9	179±9	178±8
10ng/kg bolus + 100ng/kg/hr Rotigaptide	178±22	154±9	178±10	172±7	161±7
100ng/kg bolus + 1000ng/kg/hr Rotigaptide	160±9	156±12	169±12	172±9	170±7
1000ng/kg bolus + 10µg/kg/hr Rotigaptide	157±10	146±8	157±5	163±6	165±6
Mean Arterial Blood Pressure					
Saline Control	99±7	84±9	88±9	87±9	89±9
1ng/kg bolus + 10ng/kg/hr Rotigaptide	98±6	79±3	87±3	87±3	84±3
10ng/kg bolus + 100ng/kg/hr Rotigaptide	103±6	93±8	93±6	90±5	82±6
100ng/kg bolus + 1000ng/kg/hr Rotigaptide	90±5	85±7	85±4	88±4	87±4
1000ng/kg bolus + 10µg/kg/hr Rotigaptide	81±10	79±7	77±9	82±8	78±8

Values represent mean ± SEM for n=5-8 dogs per group.

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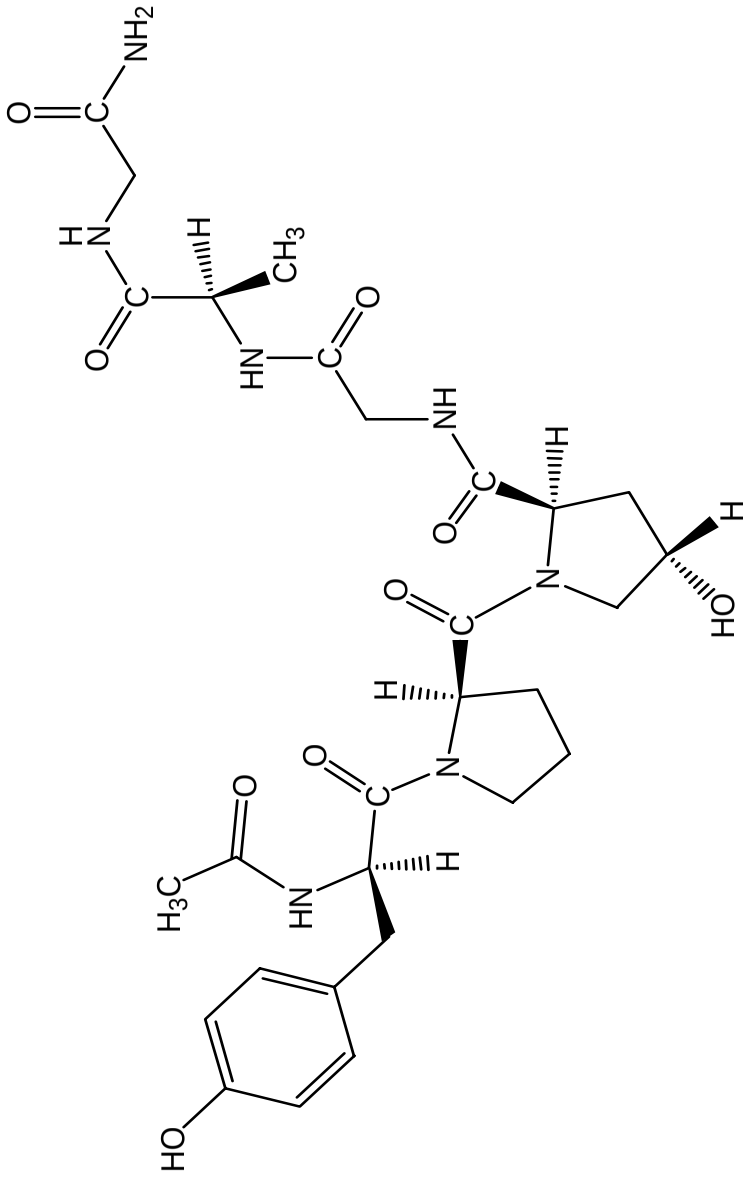

Figure 1

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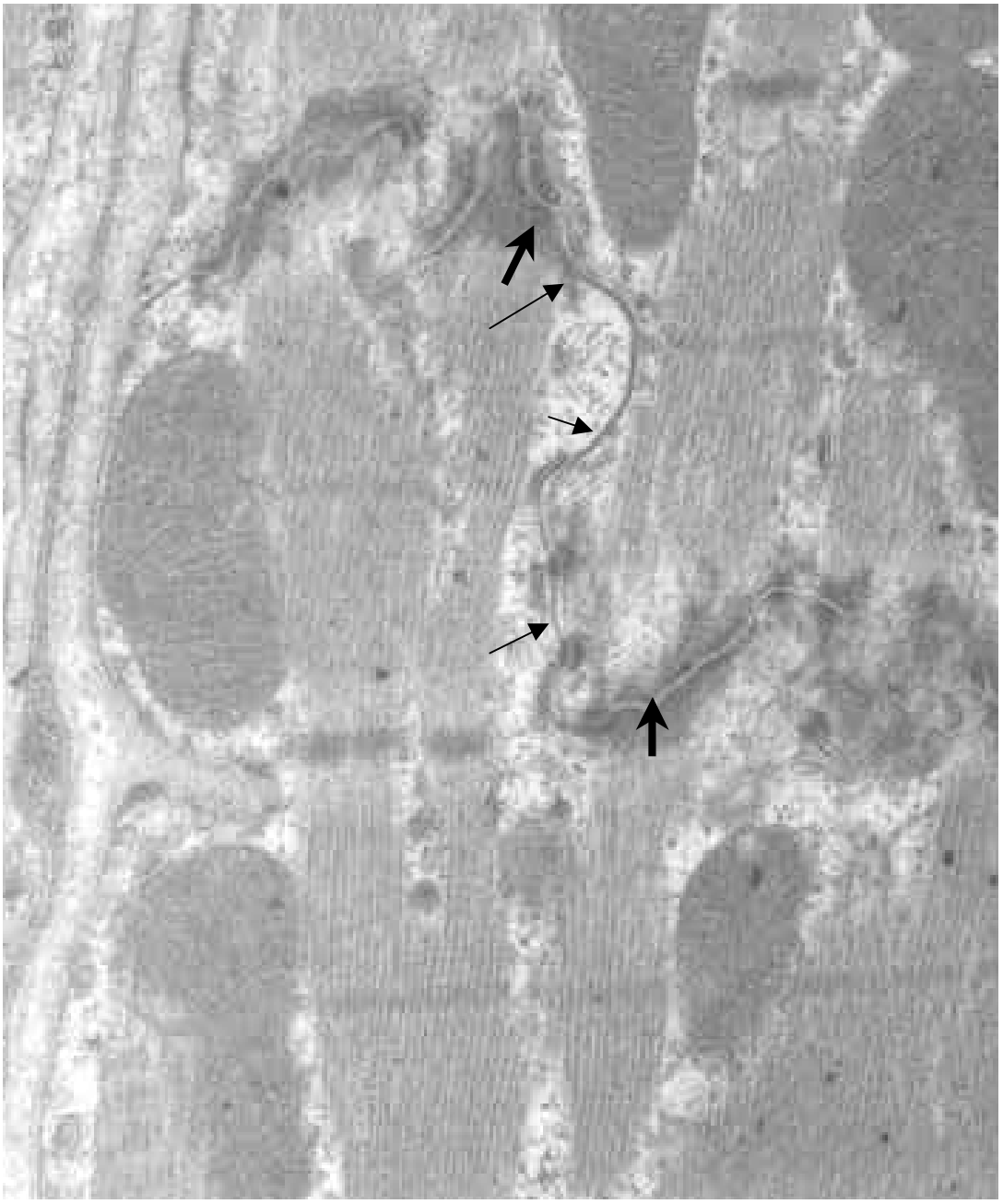

Figure 2

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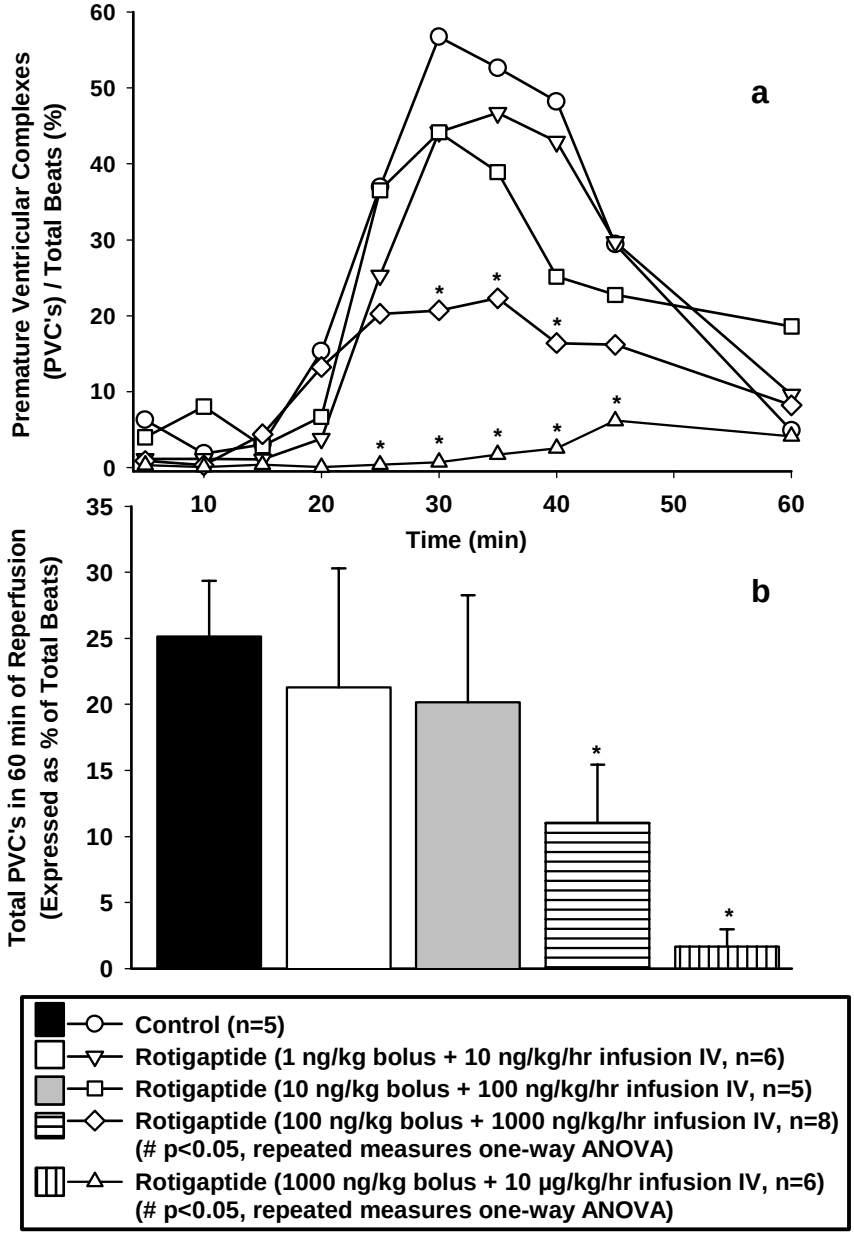

Figure 3.

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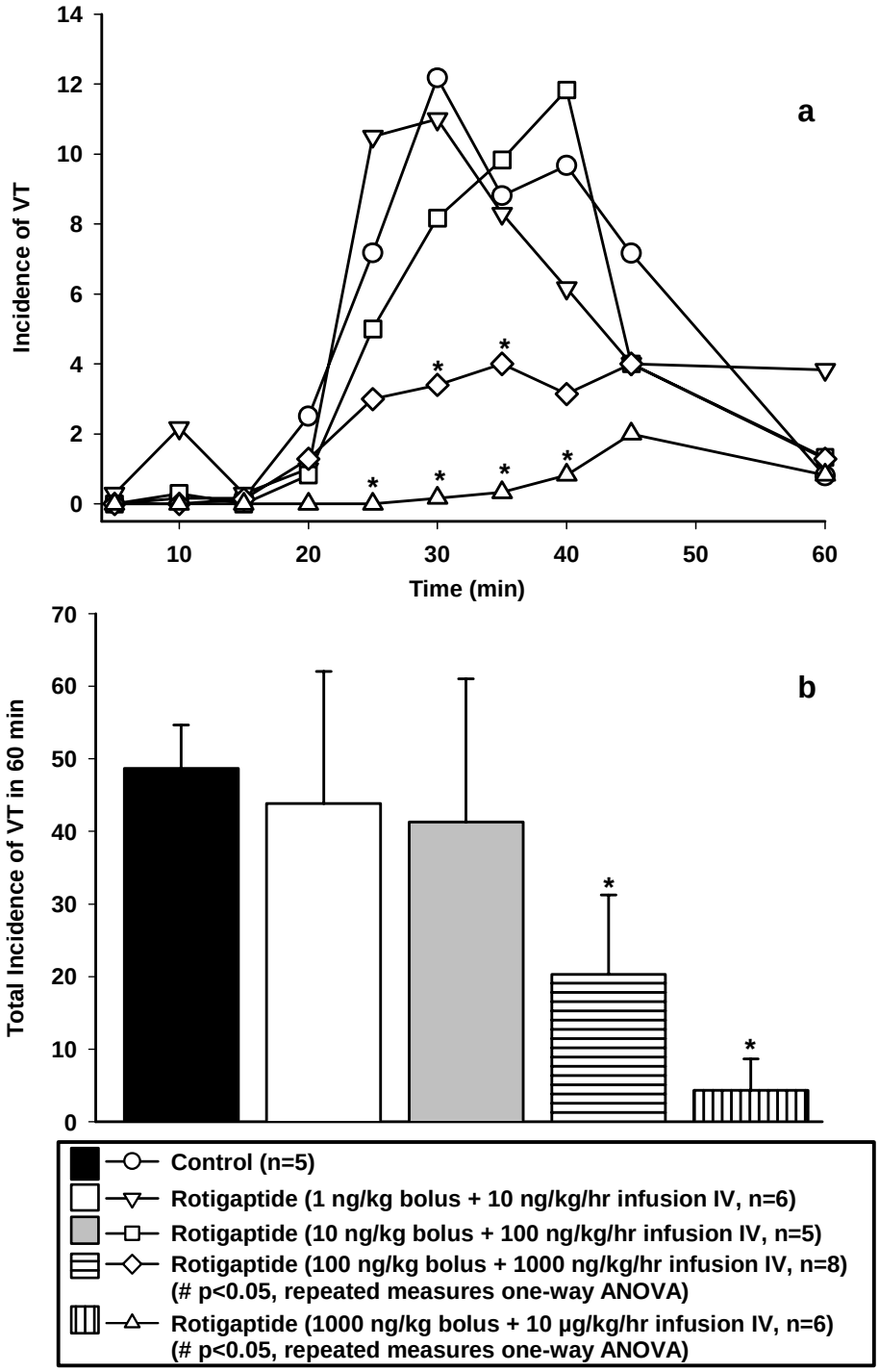

Figure 4.

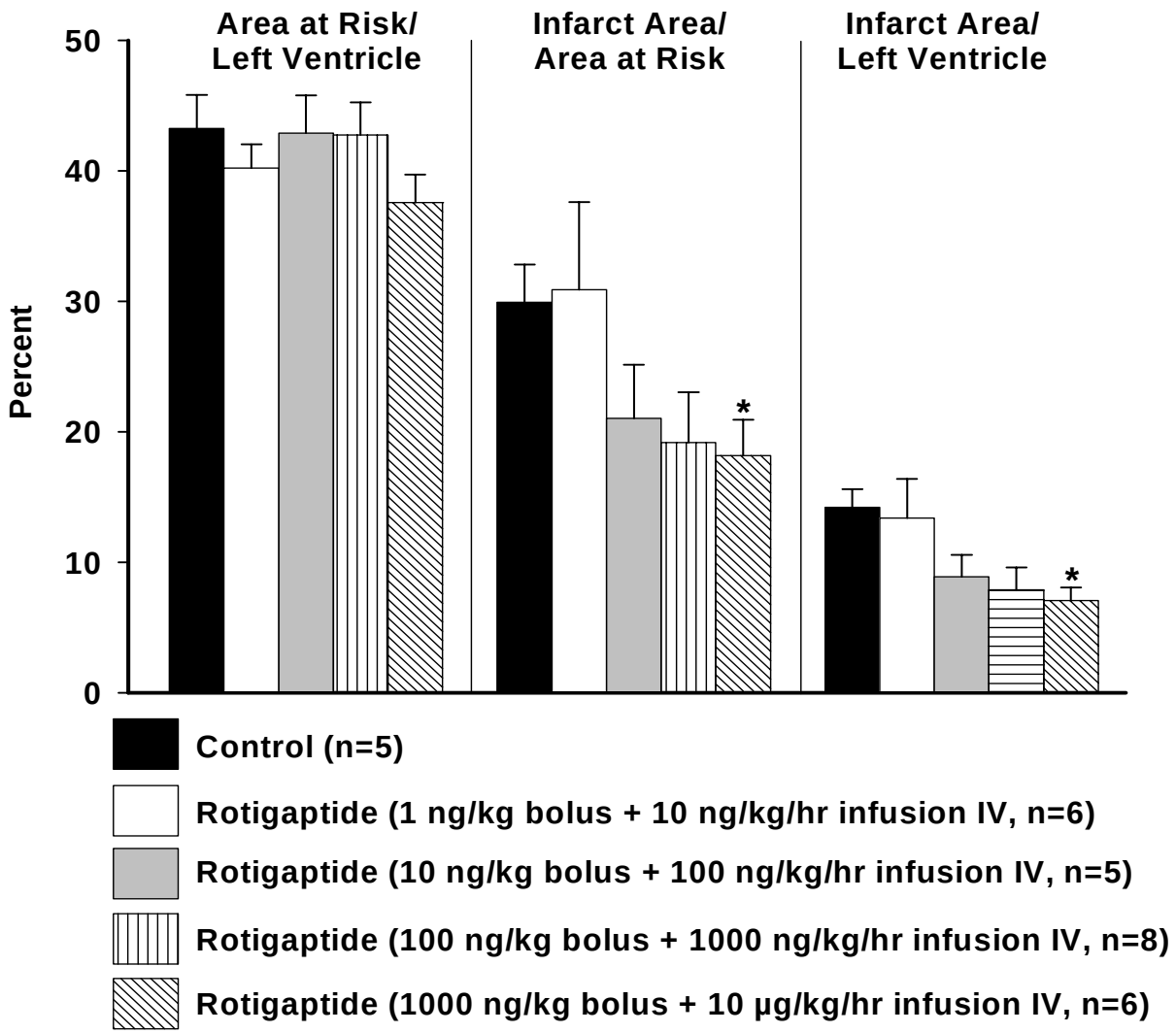

Figure 5.

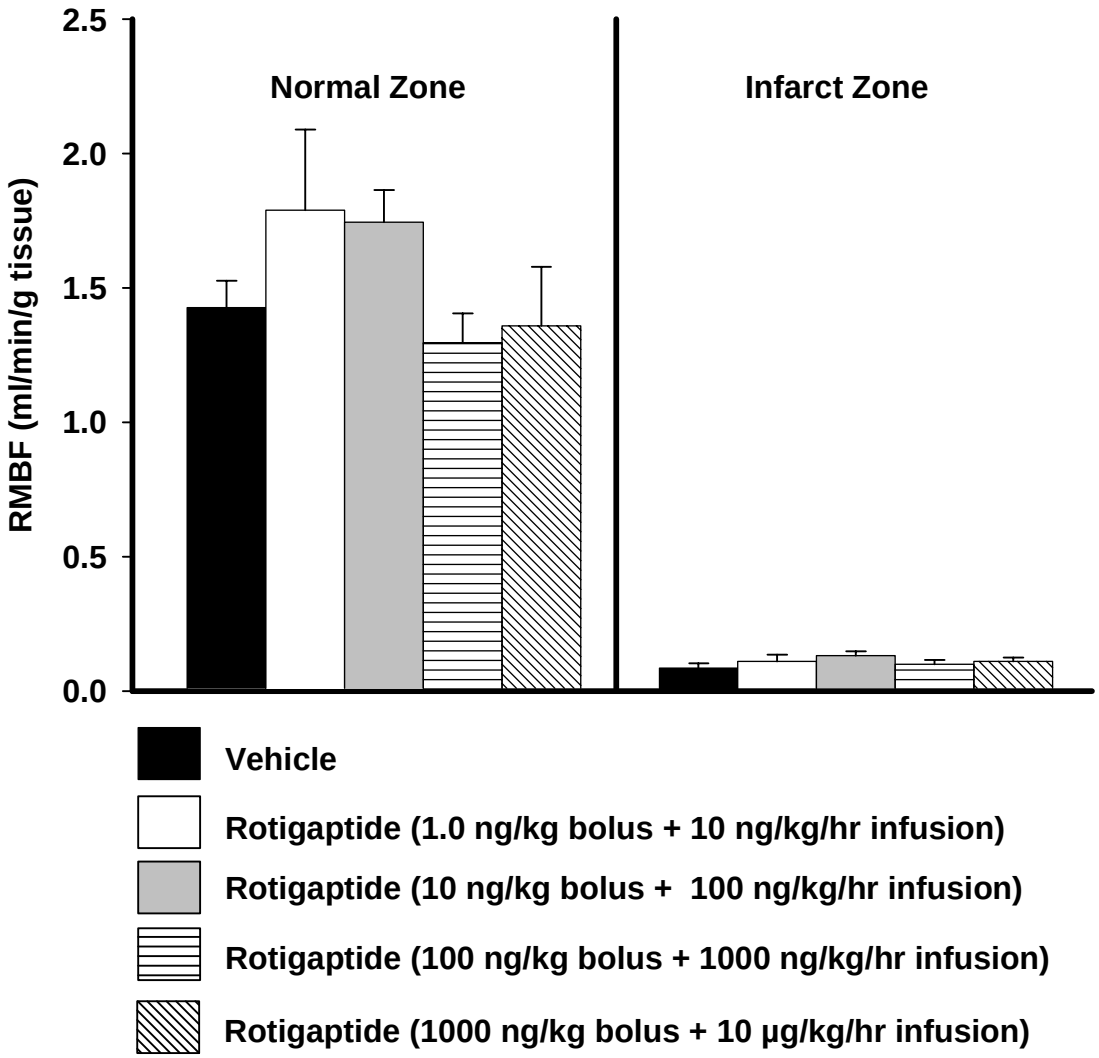

Figure 6.