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A comparison between ranolazine and CVT-4325, a novel inhibitor of fatty acid oxidation, on cardiac metabolism and left ventricular function in rat isolated perfused heart during ischemia and reperfusion

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Abstract

Inhibition of fatty acid oxidation has been reported to be cardioprotective against myocardial ischemic injury; however, recent studies have questioned whether the cardioprotection associated with putative fatty acid oxidation inhibitors such as ranolazine and trimetazidine are due to changes in substrate oxidation. Therefore, the goals of this study were to compare the effects of ranolazine with a new fatty acid oxidation inhibitor, CVT-4325, on carbohydrate and fatty acid oxidation and on left ventricular (LV) function in the response to ischemia/reperfusion in rat isolated perfused hearts. Metabolic fluxes were determined in hearts perfused in an isovolumic Langendorff mode using ^{13}C-NMR isotopomer analysis or in isolated working hearts using ^{14}C-glucose and ^{3}H-palmitate, with and without 10 μM ranolazine or 3 μM CVT-4325. Isovolumic perfused hearts were also subjected to 30 min of low flow ischemia (0.3 ml/min) and 60 min of reperfusion, and working hearts to 15 min zero flow ischemia and 60 min reperfusion. Regardless of the experimental protocol ranolazine had no effect on carbohydrate or fatty acid oxidation whereas CVT-4325 significantly reduced fatty acid oxidation up to ~7 fold with a concomitant increase in carbohydrate oxidation. At these same concentrations, while ranolazine significantly improved LV functional recovery following ischemia/reperfusion, CVT-4325 had no significant protective effect. These results demonstrate that at pharmacologically relevant concentrations, ischemic protection, by ranolazine was not mediated by inhibition of fatty acid oxidation and conversely that inhibition of fatty acid oxidation with CVT-4325 was not associated with improved LV functional recovery.

Introduction

Despite significant advances in treatment (Theroux et al., 2000), coronary heart disease still accounts for 1 in 5 deaths in the United States. During the past 30 years numerous experimental interventions have been reported to limit ischemic injury in experimental animals; however, with the exception of timely reperfusion, none have translated into routine clinical practice (Bolli et al., 2004). Glucose-insulin-potassium (GIK) therapy has yielded encouraging results (Fath-Ordoubadi and Beatt, 1997; Diaz et al., 1998; van der Horst et al., 2003; Bolli et al., 2004); however, despite its potential GIK therapy has not been widely accepted. This is due, at least in part, to the complexity of the treatment regimen and the limitations associated with infusion of large volumes of fluid into patients with compromised cardiac function (van der Horst et al., 2003). An alternative approach that potentially avoids these complications is direct pharmacological modulation of cardiac fatty acid and carbohydrate metabolism (Stanley et al., 1997b). This has been the focus of many studies of putative fatty acid oxidation inhibitors such as trimetazidine and ranolazine for the treatment of ischemic disease (Fragasso et al., 2002; Chaitman et al., 2004a).

However, despite the considerable interest in metabolic modulation as a therapy for myocardial ischemia, there is little consensus regarding the mechanism(s) underlying its possible cardioprotective effects. Recently, we reported that while increasing glucose and insulin levels improved left ventricular (LV) functional recovery following ischemia in the isolated perfused heart, this was not associated with a significant effect on glucose oxidation (Wang et al., 2005). Conversely, in the same study, stimulation of glucose oxidation with dichloroacetate did not improve LV functional recovery following ischemia. Furthermore, while there is substantial evidence that trimetazidine and ranolazine are protective against the deleterious effects of myocardial ischemia in experimental and clinical settings (Gralinski et al., 1994; Zacharowski et al., 2001; MacInnes et al., 2003; Belardinelli et al., 2006), the data supporting

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their effect to inhibit fatty acid oxidation is less clear. For example, Saeedi et al. (Saeedi et al., 2005) demonstrated that while trimetazidine significantly improved LV functional recovery in hypertrophied hearts, it had no effect on either glucose or palmitate oxidation. Similarly, MacInnes et al. (MacInnes et al., 2003) reported that trimetazidine did not inhibit ß-oxidation in cardiomyocytes and while ranolazine did inhibit fatty acid oxidation, this was only by 12% at a concentration of 100µM, which exceeds the human therapeutic range by 10-fold. More recent reports suggest that the protective effect of ranolazine could be mediated via alterations in Ca^{2+}-homeostasis (Fraser et al., 2005; Belardinelli et al., 2006; Fraser et al., 2006), thus providing an alternative mechanism of action.

The controversy regarding both the mechanism of action of ranolazine as well as the putative beneficial effects of inhibiting of fatty acid oxidation may be attributed to several factors including a) the use of different perfused heart models, such isovolumic Langendorff perfused heart and ejecting “working” heart preparations; b) different types of ischemia, such as zero versus low flow ischemia; c) different exogenous fatty acid concentrations and d) different methods for measuring substrate utilization. Furthermore, the effects of putative “partial fatty acid oxidation inhibitors” such as ranolazine have not been directly compared to the effects more potent fatty acid oxidation inhibitors. Recently we have developed a new potent fatty acid oxidation inhibitor, CVT-4325, which has an IC50 of 0.9 µM for inhibiting fatty acid oxidation in the presence of 1.2 mM palmitate (Fraser et al., 2003a). Therefore, the goal of this study was to directly compare the metabolic effects of ranolazine with CVT-4325 in two different heart perfusion models and measured substrate utilization using both ^{13}C-glutamate NMR isotopomer analysis and radioisotope techniques.

We found that regardless of the perfusion technique, experimental conditions and methods used to measure rates of substrate oxidation, ranolazine (10 µM) had no effect on glucose or fatty acid oxidation or glycolysis, whereas CVT-4325 (3 µM) reduced fatty acid oxidation by ~7 fold with a concomitant increase in glucose oxidation. Furthermore, while

ranolazine significantly improved recovery of LV function following ischemia/reperfusion, CVT-4325 had no significant protective effect. These results demonstrate that at pharmacologically relevant concentrations, ischemic protection by ranolazine cannot be attributed to inhibition of fatty acid oxidation. Moreover these data also suggest that direct inhibition of fatty acid oxidation may not be an effective approach for improving functional recovery following ischemia/reperfusion.

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Methods

Materials: Unless otherwise noted chemicals were obtained from Fisher Scientific (Santa Clara, CA) or Sigma/Aldrich (St. Louis, MO). Essentially fatty acid free bovine serum albumin was obtained from either Sigma or Serologicals Proteins Inc. (Kankakee, IL). Radioisotopes were obtained from NEN (Boston, MA) and ^{13}C-labeled substrates were from Cambridge Isotope Laboratories (Andover, MA). Ranolazine and CVT-4325 were obtained from the bio-organic chemistry group at CV Therapeutics; the structures of these drugs have been previously reported (McCormack et al., 1998; Elzein et al., 2004). Both drugs were prepared as stock solutions of 10 mM and 3 mM respectively in DMSO. The resulting DMSO concentrations in the perfusate was <0.1% and was the same for both drugs. In a series of normoxic perfusion experiments, this concentration of DMSO was found to have no adverse effects on function or metabolism.

Animals: Animal experiments were approved either by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham or Institutional Animal Care and Use Committee of CV Therapeutics and followed the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996). Male Sprague-Dawley rats (Charles Rivers Laboratories) weighing 300-350 g were used throughout.

Isovolumic Langendorff Heart Perfusion Experiments: Hearts were perfused at 37°C as previously described in isovolumic Langendorff mode (Wang et al., 2005) with a Krebs-Henseleit solution containing 5mM glucose, 1.0 mM sodium lactate, 0.1 mM sodium pyruvate, 1.0 mM sodium palmitate, 3 % BSA and 100 μU/ml insulin. Cardiac function was continuously recorded via a fluid filled balloon placed into the left ventricle (LV), connected to a pressure transducer and LV end diastolic pressure (EDP) was set to 5 mmHg by adjusting balloon

volume at the beginning of the experiments and the balloon volume was subsequently left unchanged for the remainder of the experiment. To ensure consistency with previous studies using this model, hearts were paced at a constant rate of 320 beats/min throughout the experiment.

In all experiments hearts were initially perfused in the absence of drug. After 30 min hearts were randomly assigned to an untreated control group, a ranolazine (10 μM) group, or a CVT-4325 (3 μM) group and drugs were present for the remainder of the experiments. Hearts were either perfused under aerobic conditions for 60 min or were subjected to low flow ischemia (LFI, 0.3ml/min) for 30 min followed by 60 min of reperfusion where flow was restored to achieve a perfusion pressure of 75 mmHg as described previously (Wang et al., 2005).

In both the aerobic and LFI experiments, hearts were perfused with [U-^{13}C]palmitate, [3-^{13}C]lactate and [2-^{13}C]pyruvate for the final 30-45 mins of the protocol at which time hearts were freeze-clamped, acid extracted and ^{13}C-NMR spectra collected as previously described (Lloyd et al., 2004; Wang et al., 2005). ^{13}C-NMR isotopomer analyses of heart extracts as previously described in detail elsewhere (Lloyd et al., 2003; Wang et al., 2005) were performed to determine the relative contribution of substrates to total acetyl-CoA entering the tricarboxylic acid cycle (TCA) cycle.

The concentration of ranolazine used here (i.e., 10 μM) reflects the upper end of the proposed therapeutic concentration range (Belardinelli et al., 2006). The concentration of CVT-4325 was chosen based on the results of preliminary experiments that showed that the maximal inhibition of palmitate oxidation under the conditions of these experiments was achieved at a concentration of ~3 μM (Fraser et al., 2003b). Additional studies showed that at ~3 μM the predominant pharmacological activity of CVT-4325 in the presence of 1.2 mM palmitate was the inhibition of fatty acid oxidation (IC50=0.9 μM) and stimulation of glucose oxidation (IC50=5.8 μM) (Data not shown). The concentrations of ranolazine and CVT-4325 in the perfusate were

assayed at the end of the experiments and the mean concentrations determined to be 12.6±1.3 μM and 2.6±0.2 μM respectively.

Working Heart Perfusion Experiments: Hearts were perfused as previously described in an ejecting (i.e., "working heart") mode (Neely et al., 1967) at 37 °C with a Krebs-Henseleit solution containing 5.5 mM glucose, 1.2 mM palmitate, 3 % BSA and 100 μU/ml insulin and continuously equilibrated with a 95 % CO_2, 5 % O_2 gas mixture. After 10 min of perfusion in Langendorff mode, hearts were switched to working mode, with a constant left atrial preload of 11.5 mmHg and aortic afterload of 80 mmHg, and paced at a constant rate of 300 beats/min to ensure consistency with previous studies using this model (Fraser et al., 1999). Aortic systolic and diastolic pressures were measured via a pressure transducer attached to the aortic outflow line and cardiac output and aortic flow were measured using in-line ultrasonic flow probes. Left ventricular minute work (LV work), calculated as (cardiac output) x (left ventricular developed pressure, LVDP) with LVDP = aortic systolic pressure - preload pressure, was used as an index of mechanical function. LV work was measured continuously. Glucose and fatty acid oxidation rates were measured simultaneously using dual labeled substrates (^{14}C-glucose and ^{3}H-palmitate) as previously described (Lopaschuk and Barr, 1997). Rates of palmitate and glucose oxidation are expressed as μmol substrate metabolized/min/g dry wt.

For the aerobic experiments, ranolazine (10 μM, n=3) and CVT-4325 (3 μM, n=5) were added to the perfusate after 5 min of aerobic perfusion and recirculated for 60 min. Hearts were paced at a constant rate of 300 beats/min throughout the experiment.

In the ischemia/reperfusion experiments hearts were perfused aerobically for 30 min followed by 15 min global, zero-flow ischemia induced by clamping off both the preload and afterload lines. After 15 min of global, zero flow ischemia, coronary flow was restored by removing the clamps and reperfusion continued for 60 min. It has already been shown using a

very similar protocol that 10 μM ranolazine improved functional recovery on reperfusion (MacInnes et al., 2003), therefore in these experiments, hearts were either untreated (n=9) or treated with CVT-4325 (3 μM, n=6) added after the first 5 min of preischemic aerobic perfusion. Pacing can alter the response to ischemia/reperfusion by potentially exacerbating ischemic injury as well as obscuring the incidence of arrhythmias on reperfusion; therefore in these ischemia/reperfusion experiments hearts were not paced.

Statistics: All data are presented as means±SEM, with 5 to 6 replicates in each group unless stated otherwise. Unpaired T-tests, one-way and repeated measure ANOVA were used where appropriate followed by a Dunnett's Multiple Comparison Test using Prism 4.0c (GraphPad Software Inc., San Diego CA). Statistically significant differences between groups were defined as $p < 0.05$.

Results

Experiments using isovolumic Langendorff heart preparations: LV functional data from both the aerobic and LFI experiments are summarized in Table 1. Prior to the addition of drugs there was no difference in baseline contractile function between any of the three groups; however, MVO_2 was significantly lower in the groups subsequently assigned to ranolazine and CVT-4325 compared to the control group and RPP/MVO_2 was increased in the group subsequently assigned to CVT-4325. To compare the effects of the two drugs on cardiac function under normoxic perfusion conditions, functional parameters 30 min following addition of drugs were compared to function in the same hearts at baseline, prior to addition of drugs. Ranolazine resulted in a small (<10%), but significant increase in +dP/dt but had no effect on any of the other functional parameters including MVO_2. In contrast, CVT-4325 had a significant positive inotropic effect increasing LV developed pressure (LVDP) and rate-pressure product (RPP) by ~30 % and ±dP/dt by more that 40 %, which was accompanied by a significant increase in coronary flow relative to baseline. However, despite the increase in RPP with CVT-4325 there was no increase in oxygen consumption (MVO_2), consequently, there was a significant increase in efficiency as defined by the ratio of RPP/MVO_2. Thirty minutes after perfusion with ranolazine there were no differences in function between control and ranolazine groups; however, there was a significant increase in all functional parameters in the CVT-4325 group compared to controls.

The relative contributions of glucose, palmitate, lactate and pyruvate to total TCA cycle flux from ^{13}C-NMR glutamate isotopomer analysis during aerobic perfusion are summarized in Figure 1. Consistent with previous reports with similar substrate mixtures (Chatham et al., 1999), palmitate contributed ~85 % of acetyl-CoA entry into the TCA cycle, lactate ~10 %, and the remainder was from glucose and pyruvate. Ranolazine had no effect on the relative contributions of any of the substrates to the TCA cycle; however, CVT-4325 inhibited palmitate

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oxidation by ~80 %, which was accompanied by a more than 4-fold increase in lactate and pyruvate oxidation and approximately 9-fold increase in glucose oxidation.

The effects of ranolazine and CVT-4325 on LV EDP during LFI are summarized in Figure 2. Ranolazine treatment appeared to shift the EDP curve to right (Fig 2A); however, due to the large variance in the data, there was no difference between groups when analyzed via repeated measures ANOVA. Nevertheless, the time to onset of contracture during LFI, defined as the time EDP reached ≥10 mmHg was significantly delayed in the ranolazine group (Fig 2B) and the EDP averaged over the duration of LFI was significantly lower with ranolazine treatment (Fig 2C).

The time course of recovery of RPP during reperfusion is summarized in Figure 3. ANOVA indicated a significant drug effect and post-hoc test indicates significant differences between ranolazine and other two groups at individual time points between 2 and 10 minutes. Thus, upon restoration of flow ranolazine treatment resulted in significantly faster recovery of contractile function, whereas CVT-4325 had no beneficial effect.

LV function at the end of reperfusion is summarized in Figure 4 and Table 1. After 60 min reperfusion ranolazine treatment improved functional recovery compared to the control group; in contrast, despite the marked reduction in fatty acid oxidation, CVT-4325 did not improve recovery of LV function post ischemia; indeed, relative to pre-ischemic values, recovery of LV function was significantly lower than that observed in the control group. During reperfusion oxygen consumption in the CVT-4325 group was significantly lower; however, in contrast to normoxic perfusion efficiency (RPP/MVO_2) was not significantly different between groups. At the end of reperfusion, LV EDP was significantly lower in the ranolazine group compared to the control group (20±4 vs. 35±4 mmHg; $p<0.05$); CVT-4325 had no effect on end reperfusion EDP (33±5 mmHg; $p>0.05$ Vs control).

Substrate oxidation in the three groups during reperfusion is summarized in Figure 5. Similar to the results of experiments in normoxic conditions, ranolazine had no effect on the

relative utilization of palmitate, lactate, glucose or pyruvate; whereas, CVT-4325 significantly decreased palmitate utilization with a concomitant increase in lactate, glucose and pyruvate oxidation. However, compared to normoxic perfusion the inhibition of fatty acid oxidation by was significantly attenuated.

Working heart experiments: To ensure that the results above were not specific to the isovolumic perfused heart preparation, fatty acid and carbohydrate oxidation rates were also determined in isolated perfused working hearts (Figure 6). Similar to the results of experiments using the isovolumic Langendorff heart preparation, ranolazine had no effect on the rates of either glucose or fatty acid oxidation; however, CVT-4325 decreased fatty acid oxidation by ~4-5 fold with a concomitant increase in glucose oxidation.

Previously 10 μM ranolazine has been shown to significantly improve functional recovery following zero flow ischemia and reperfusion in a working perfused heart model (MacInnes et al., 2003); however, the effect of CVT-4325 on the response of LV function to ischemia in this model has not been previously reported. Therefore, to determine whether the lack of protection seen with CVT-4325 in response to LFI in the isovolumic perfused heart, was specific to that model, we examined the response of the working heart to zero-flow ischemia and reperfusion with and without CVT-4325. Similar to the isovolumic perfused heart experiments pre-ischemic cardiac work was increased in the CVT-4325 group compared to controls (9.2±0.2 vs. 7.9±0.4 mmHg L/min; $p < 0.025$). Following ischemia/reperfusion, cardiac work was ~40 % of pre-ischemic levels in both control and CVT-4325 groups; however, there were no differences in cardiac function between these two groups (Figure 7).

DISCUSSION

In light of the controversies regarding the effect of ranolazine on cardiac metabolism and the proposed cardioprotective effects of inhibiting fatty acid oxidation we compared, for the first time, the effects of ranolazine with a new potent fatty acid oxidation inhibitor, CVT-4325, on fatty acid and carbohydrate oxidation in the heart. The results demonstrate that at pharmacologically relevant concentrations, ranolazine had no effect on fatty acid or carbohydrate oxidation and that this was independent of a) the perfusion technique, b) the method used to measure substrate oxidation, or c) the perfusion protocol. The lack of effect of ranolazine was in contrast to CVT-4325, which markedly reduced fatty acid oxidation with a concomitant increase in carbohydrate oxidation in both the isovolumic and working heart preparations. Moreover, despite having no effect on substrate utilization, ranolazine significantly improved the recovery of LV function following ischemia, whereas CVT-4325 did not improve functional recovery despite its effect on fatty acid oxidation. This side-by-side comparison of ranolazine with CVT-4325 provides compelling evidence that the mechanism underlying the protection seen with ranolazine cannot be attributed to alterations in substrate utilization either before ischemia or during reperfusion. Furthermore, the fact that CVT-4325 did not improve functional recovery following either LFI or zero flow ischemia suggests that direct inhibition of fatty acid oxidation may not be an effective approach for improving functional recovery following ischemia/reperfusion.

Ranolazine has been shown to be protective against myocardial ischemic injury in a number of experimental settings (Clarke et al., 1996; McCormack et al., 1996; MacInnes et al., 2003) and has also found to be efficacious as an anti-anginal agent in patients with coronary artery disease (Chaitman et al., 2004b). Early studies with ranolazine suggested that its protection was mediated by stimulation of glucose oxidation, secondary to partial inhibition of fatty acid oxidation (Clarke et al., 1996; McCormack et al., 1996). However, more recent

findings that have shown that ranolazine improved cardiac function in response to various stressors, including ischemia/reperfusion and heart failure, in the absence of any changes in fatty acid or glucose metabolism (Gralinski et al., 1994; Matsumura et al., 1998; Maruyama et al., 2000; Chandler et al., 2002; MacInnes et al., 2003). Our observations that ranolazine had no effect on fatty acid or glucose oxidation is entirely consistent with these recent studies. The reason for the discrepancies between the earlier reports and our study regarding the metabolic effect of ranolazine is unclear; however, it may be related to the concentration of the drug used since high concentrations of ranolazine, outside the therapeutic range have been reported to inhibit fatty acid oxidation (MacInnes et al., 2003).

Importantly, the lack of effect of ranolazine on fatty acid and carbohydrate oxidation seen here was observed using two different techniques for evaluating substrate utilization and in two different working heart models. We cannot rule out the possibility that in vivo administration of ranolazine could lead to systemic metabolic effects, which could contribute to its cardioprotection; however, Chandler *et al.,* (Chandler et al., 2002) showed *in vivo* in a canine model of heart failure that while ranolazine improved cardiac function, it had no effect on the myocardial uptake of fatty acids or carbohydrates. Thus, there is consistent evidence demonstrating that ranolazine is cardioprotective in a number of experimental and clinical settings; however, on balance recent evidence suggests that the mechanism of action is unlikely to be a consequence of alterations in carbohydrate and fatty acid oxidation.

Recent studies have suggested that the mechanism underlying the cardioprotection seen with ranolazine may be due at least in part to reduced intracellular sodium dependent calcium overload secondary to inhibition of the late sodium current (Fraser et al., 2005; Belardinelli et al., 2006; Fraser et al., 2006). Consistent with this hypothesis, the present study showed that ranolazine treatment delayed the onset of ischemic-contracture, reduced the average LVEDP during ischemia and lowered LVEDP at the end of reperfusion. The recovery of coronary flow at the end of reperfusion was also was greater in the ranolazine group

compared to either the control- or CVT-4325-treated hearts, which could also be a consequence of reduced contracture (i.e., lower LVEDP).

The fact that CVT-4325 did not improve function recovery despite its marked inhibition of fatty acid oxidation and concomitant increase in glucose oxidation is consistent with our earlier study where we showed that increasing glucose oxidation with dichloroacetate also did not improve functional recovery following ischemia/reperfusion despite a 5-fold reduction in palmitate oxidation (Wang et al., 2005). One potential criticism of our earlier study was that we used 0.32 mM palmitate while, many studies demonstrating protection associated with increasing glucose oxidation used fatty acid concentrations in the 1-1.2 mM range (Lopaschuk et al., 1993). We also used low-flow ischemia in an isovolumic Langendorff preparation rather than zero-flow ischemia in an ejecting heart preparation, which are also more common in such studies. Here we show that inhibition of fatty acid oxidation in hearts perfused with 1-1.2 mM palmitate afforded no protection regardless of whether the hearts were perfused in an isovolumic or working mode preparation. Furthermore, the lack of myocardial protection accompanying marked inhibition of fatty acid oxidation (4- to 7-fold) with CVT-4325 was also independent of whether low flow ischemia or zero flow ischemia was used. However, it is important to note that here and in our earlier study (Wang et al., 2005), metabolic interventions were initiated prior to ischemia; therefore, we cannot completely rule out the possibility that inhibition of fatty acid oxidation during reperfusion might afford some benefit as previously suggested (Finegan et al., 1996).

One frequently cited explanation for the beneficial effect of increasing glucose oxidation relative to fatty acid oxidation is that it improves efficiency because the amount of ATP produced per unit oxygen consumed is ~12 % greater when glucose is oxidized compared to palmitate (Stanley et al., 1997a). Interestingly, we found that CVT-4325 did improve efficiency (Table 1); however, the magnitude of this change was greater than can be attributed to the decrease in palmitate oxidation. Furthermore, despite this increase in efficiency functional recovery was not

improved. It should be noted however that this increase in efficiency was associated with a significant increase in RPP, which could adversely affect the response to ischemia. The mechanisms underlying the increase in contractility and efficiency associated with CVT-4325 are unknown at this time and warrant further study.

A potential limitation associated the use of any pharmacological inhibitors is their specificity. Therefore, CVT-4325 was tested for potential pharmacological activity (using the MDS-Pharma, Spectrum Screen) at 169 targets that included G-protein coupled receptors (e.g. ß-adrenergic receptors), nuclear hormone receptors (e.g. estrogen receptor-α), and transporters (e.g. serotonin). CVT-4325 at 10 μM inhibited ($\geq$70 %) 5HTA2 (serotonin), HT1 (histamine), L-type calcium channel and serotonin transporter (unpublished data on file at CVT). Thus, at the concentration used in the present study (3 μM) the predominant pharmacological activity of CVT-4325 in the presence of 1.2 mM palmitate was the inhibition of fatty acid oxidation (IC50=0.9 μM) and stimulation of glucose oxidation (IC50=5.8 μM). Nevertheless, it is conceivable that the lack of myocardial protection associated with CVT-4325 may be due to potentially adverse effects associated with targets independent of fatty acid oxidation inhibition.

It should also be noted that we compared only single concentrations of ranolazine and CVT-4325 rather than using multiple overlapping concentrations, which would have provided a more comprehensive comparison of these agents. However, the primary purpose of this study was to examine the effects of ranolazine at a therapeutically relevant concentration on cardiac metabolism and the response to ischemia, and to compare this directly with an inhibitor of fatty acid oxidation. At 300 μM, MacInnes et al. (MacInnes et al., 2003) reported that ranolazine inhibited ß-oxidation in cardiomyocytes by ~30%; demonstrating the potential for ranolazine to inhibit fatty acid oxidation at high concentrations. However, since the 10 μM reflects the upper end of the proposed therapeutic concentration range for ranolazine (Belardinelli et al., 2006) it is highly unlikely that its effect on fatty acid oxidation is therapeutically relevant. CVT-4325 is

clearly a potent inhibitor of fatty acid oxidation, reducing palmitate oxidation by 80% at a concentration of only 3 μM; consequently, we cannot entirely rule out the possibility that at lower concentrations with more modest inhibition of fatty acid oxidation CVT-4325 may have demonstrated some beneficial effects.

There were some differences in protocols between the isovolumic Langendorff and working heart experiments. For example, in the isovolumic studies physiologically relevant concentrations of lactate and pyruvate were used in addition to glucose; whereas, in the working heart studies glucose was the only carbohydrate source. Furthermore, in the isovolumic studies hearts were paced at 320 bpm in both normoxic and ischemia/reperfusion experiments whereas in the working heart experiments hearts were paced at 300 bpm in the normoxic experiments and unpaced in the ischemia/reperfusion experiments. Some of these differences such as the pacing rate would not be expected to affect the metabolic measurements or the response to ischemia; however, substrate availability and pacing during ischemia/reperfusion could potentially impact both. Nevertheless despite these differences in perfusion conditions the effects of ranolazine and CVT-4325 were remarkably consistent in these two different experimental models.

In conclusion we have shown that regardless of the perfusion conditions ranolazine had no effect on glucose or fatty acid oxidation; nevertheless, in the isovolumic perfused heart model it attenuated the development of ischemic contracture and improved recovery of function following ischemia/reperfusion. In contrast, in both the isovolumic and working heart models CVT-4325 markedly reduced fatty acid oxidation with a concomitant increase in glucose oxidation, but did not improve functional recovery. These results demonstrate that at pharmacologically relevant concentrations, ischemic protection associated with ranolazine is mediated by mechanisms other than inhibition of fatty acid oxidation and conversely that significant inhibition of fatty acid oxidation with CVT-4325 is not associated with cardioprotection.

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Figure Legends

Figure 1: Effect of ranolazine (RAN, 10μM) and CVT-4325 (3μM) on relative contributions of palmitate, lactate, glucose and pyruvate to total tricarboxylic acid (TCA) cycle flux under aerobic perfusion conditions in isovolumic Langendorff perfused heart. Values are the mean±SEM of 6 experiments in each group; *p < 0.05 vs control.

Figure 2: A) Time-course of end diastolic pressure (EDP) during LFI; B) time to onset of contracture, defined as time at which EDP ≥10mmHg; C) average EDP over duration of LFI in control, ranolazine (RAN, 10μM) and CVT-4325 (3μM) groups. Values are the mean±SEM of 5-6 experiments in each group; * p < 0.05 vs control.

Figure 3: Time-course of rate-pressure product (RPP) during reperfusion in control, Ranolazine (RAN, 10μM) and CVT-4325 (3μM) groups. There were significant differences between ranolazine and other two groups at individual time points between 2 and 10 minutes (repeated measures ANOVA, p<0.05). Values are the mean±SEM of 5-6 experiments in each group

Figure 4: A) RPP, B) +dP/dt, C) -dP/dt, D) Coronary Flow, E) MVO_2 and F) RPP/MVO_2 after 30 min LFI and 60 min reperfusion in control, ranolazine (RAN, 10μM) and CVT-4325 (3μM) groups. Data presented as % of pre-ischemic levels. Values are the mean±SEM of 5-6 experiments in each group; * p < 0.05 vs control. Absolute values for the parameters shown here are included in Table 1.

Figure 5: Relative contributions of palmitate, lactate, glucose and pyruvate to total TCA cycle flux during reperfusion in control, ranolazine (RAN, 10μM) and CVT-4325 (3μM) groups. Values are the mean±SEM of 5-6 experiments in each group; *p < 0.05 vs control; # p<0.05 Vs. normoxic values in Fig. 1.

Figure 6: Effect of ranolazine (RAN, 10μM) and CVT-4325 (3μM) on glucose and palmitate oxidation in under aerobic conditions in working heart perfusions. Values are the mean±SEM of 5-6 experiments in each group; * p < 0.05 vs control.

Figure 7: Cardiac function after 15 min no-flow ischemia and 60 min reperfusion in control and CVT-4325 (3μM) groups.

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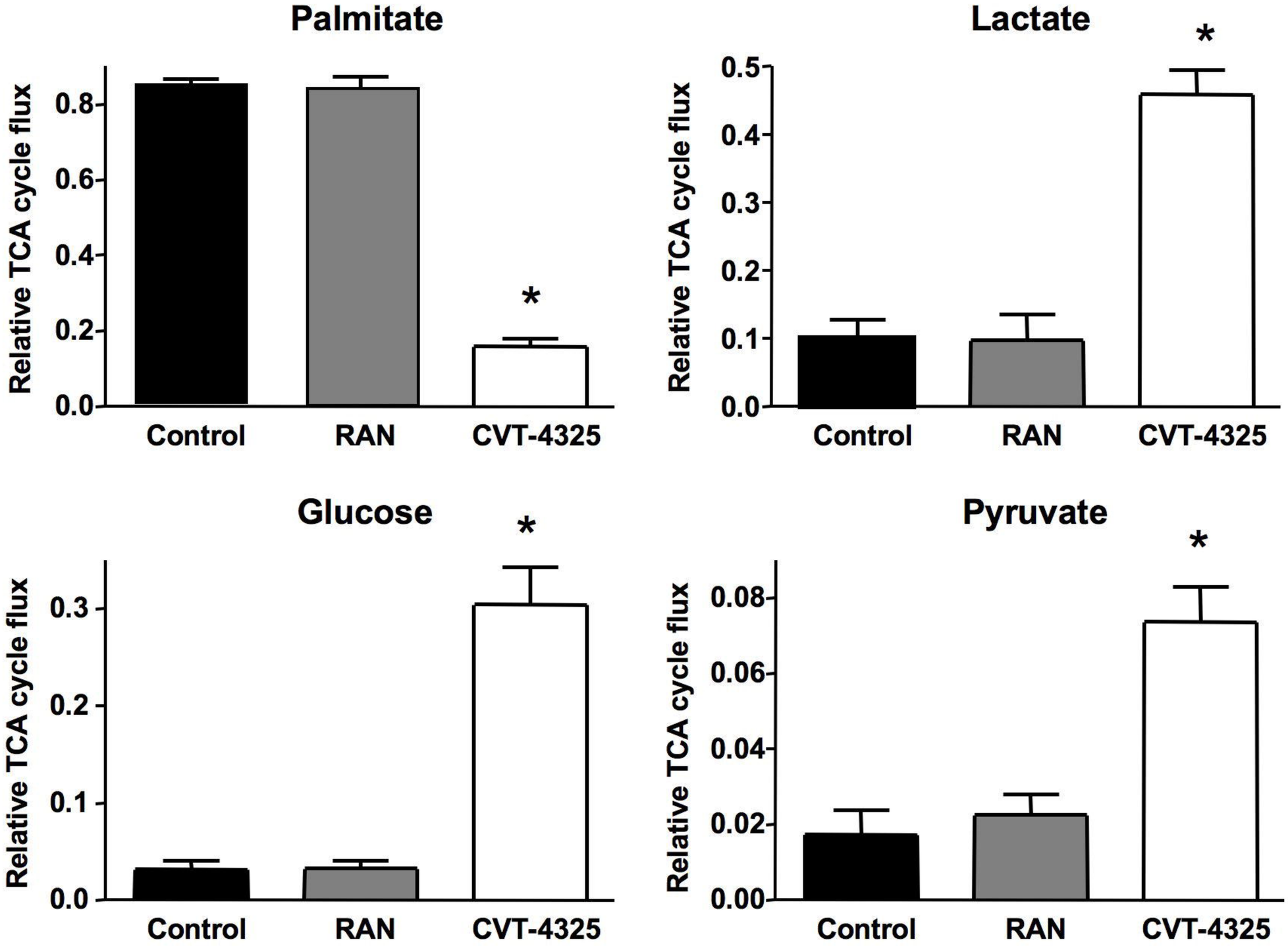
Table 1: Cardiac function data at baseline prior to addition of drug, 30 min following drug, and at the end of reperfusion in control, ranolazine (10μM) and CVT-4325 (3μM) groups. Data presented as Mean±SEM.

	LVDP	RPP	+dP/dt	-dP/dt	CF	MVO_2	RPP/MVO_2
Baseline							
Control (n=12)	108±3	34.4±0.9	3.9±0.3	2.2±0.1	9.8±0.3	4.8±0.1	7.4±0.2
Ranolazine (n=12)	107±5	34.5±1.5	4.0±0.2	2.4±0.1	8.8±0.6	4.1±0.2*	8.5±0.3
CVT-4325 (n=11)	110±4	35.3±1.3	4.1±0.3	2.5±0.2	8.6±0.5	4.2±0.2*	8.7±0.5*
Post-Drug							
Control (n=12)	110±3	35.1±0.9	4.0±0.3	2.3±0.1	9.7±0.5	4.7±0.3	7.6±0.4
Ranolazine (n=12)	110±4	34.4±1.2	4.3±0.2$^{\#}$	2.5±0.1	8.6±0.5	4.1±0.2	8.7±0.4
CVT-4325 (n=11)	148±4$^{*\#}$	47.9±1.4$^{*\#}$	5.9±0.2$^{*\#}$	3.9±0.2$^{*\#}$	9.6±0.4$^{\#}$	4.4±0.1	9.8±0.6$^{*\#}$
End Reperfusion							
Control (n=6)	61±6	19.7±1.8	2.3±0.3	1.3±0.1	5.2±0.4	2.8±0.2	7.2±0.6
Ranolazine (n=6)	80±4*	25.8±1.2*	3.3±0.2*	2.0±0.1*	5.3±0.3	2.9±0.2	9.1±0.7
CVT-4325 (n=5)	59±6	18.9±1.8	2.2±0.2	1.5±0.3	3.8±0.5*	2.0±0.2*	9.8±1.1

LVDP = left ventricular developed pressure, mmHg; RPP = rate pressure product, mmHg/min X 10^{-3}; ±dP/dt = rate of pressure development and relaxation, mmHg/sec X 10^{-3}; CF = Coronary flow, ml/min/g wet weight; MVO_2 = Oxygen consumption, μmoles/min/g wet weight; RPP/MVO_2 = Cardiac efficiency, mmHg/μmol/g wet weight X 10^{-3}. * $p < 0.05$ vs control; # $p < 0.05$ vs baseline.



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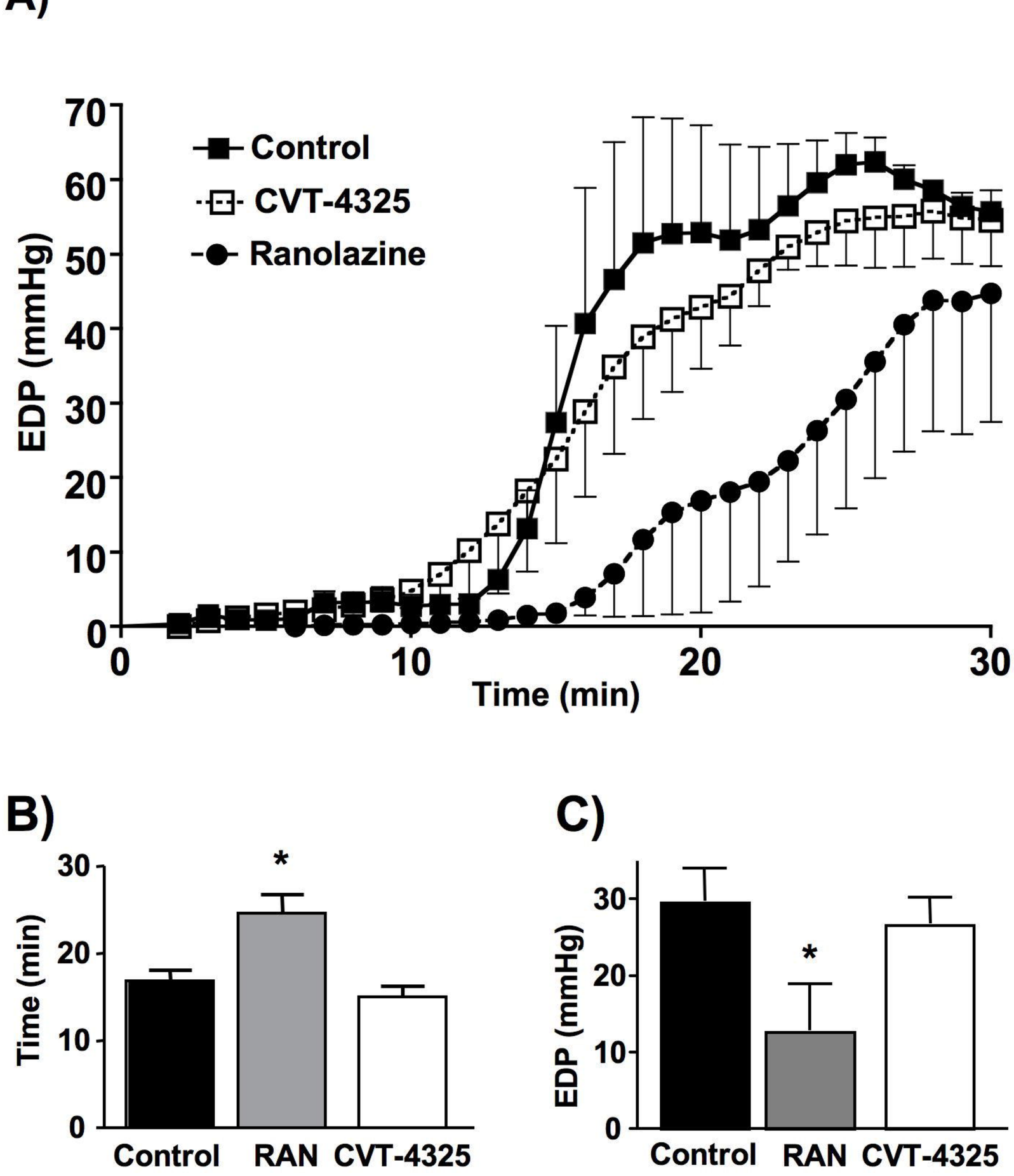

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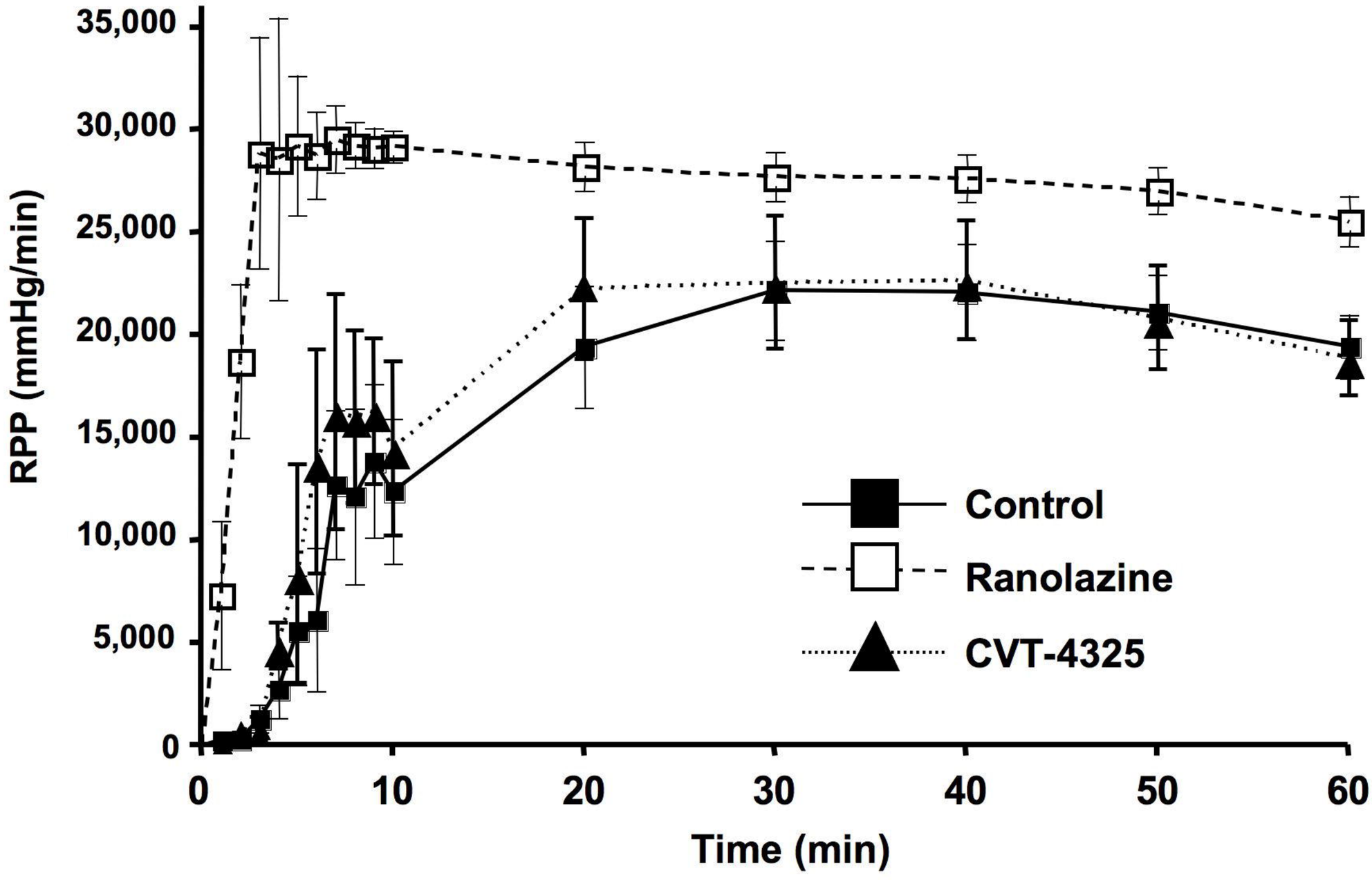
Figure 3



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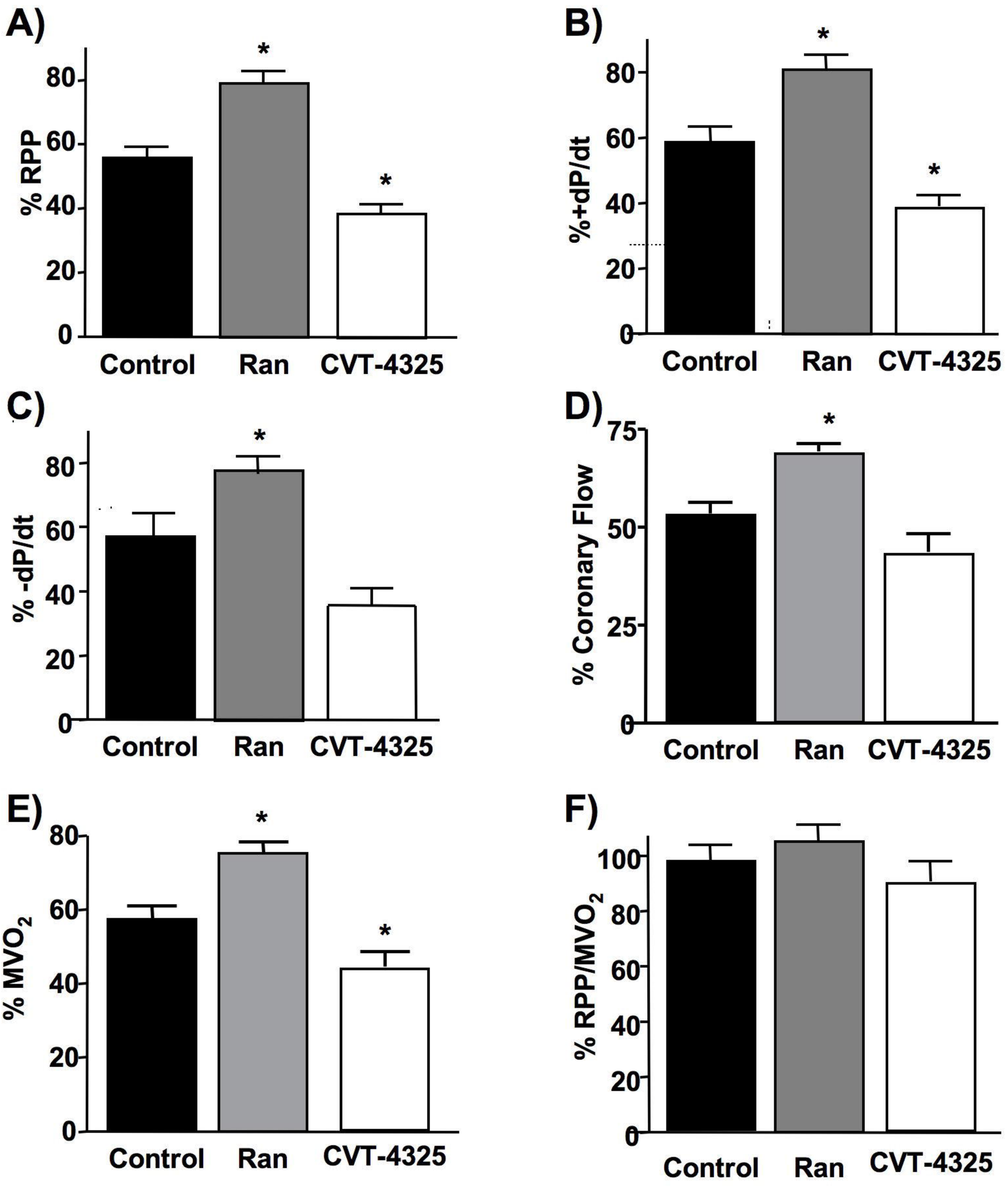

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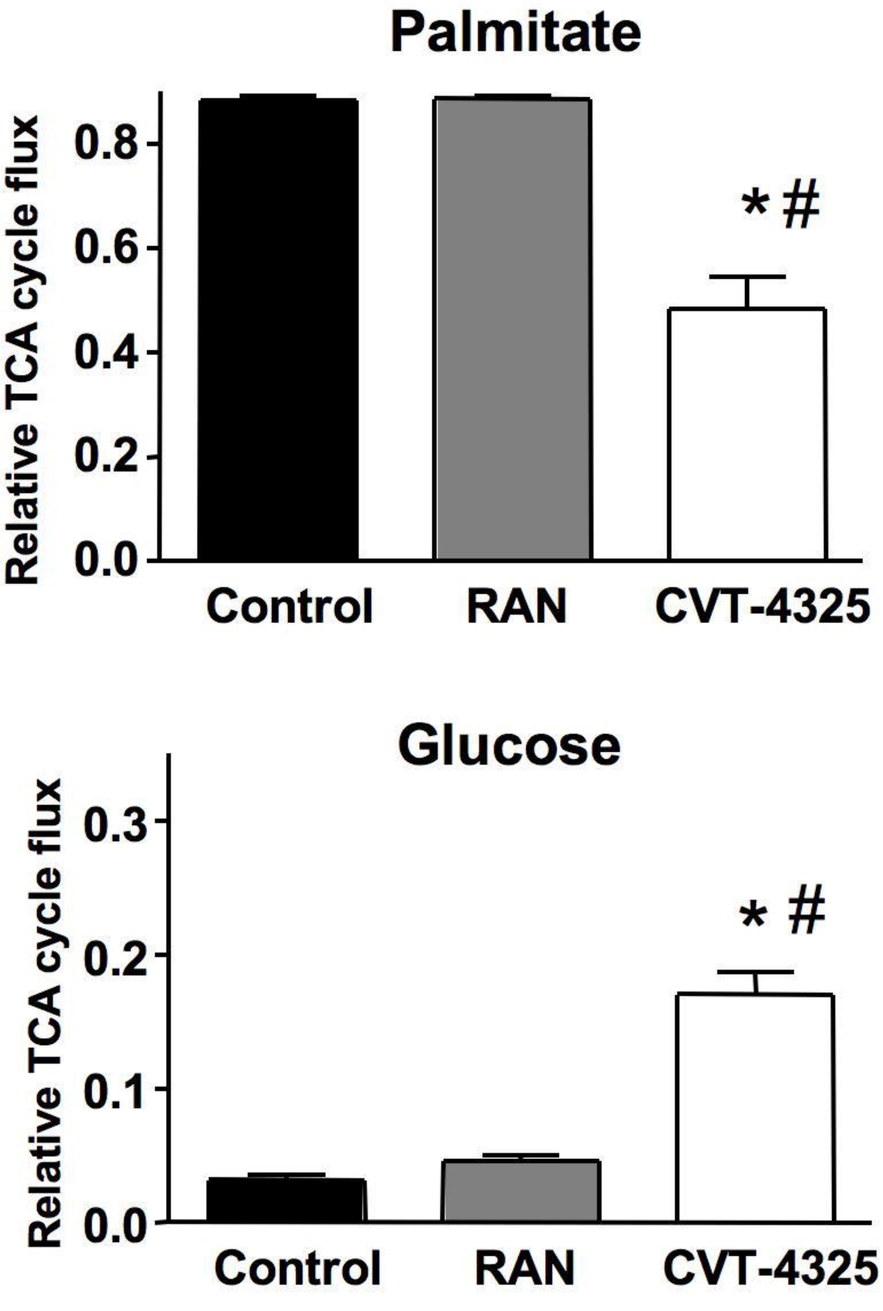

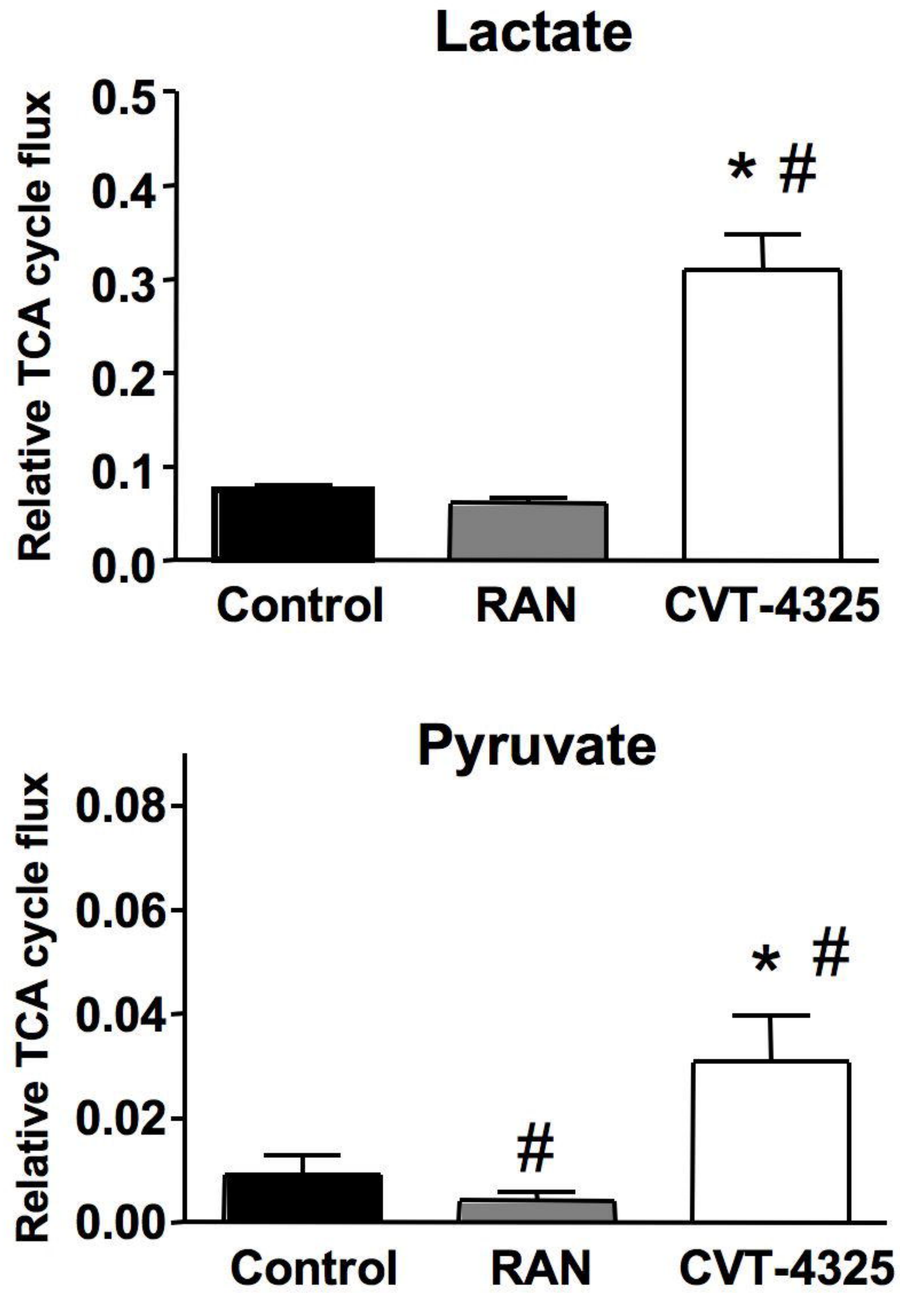

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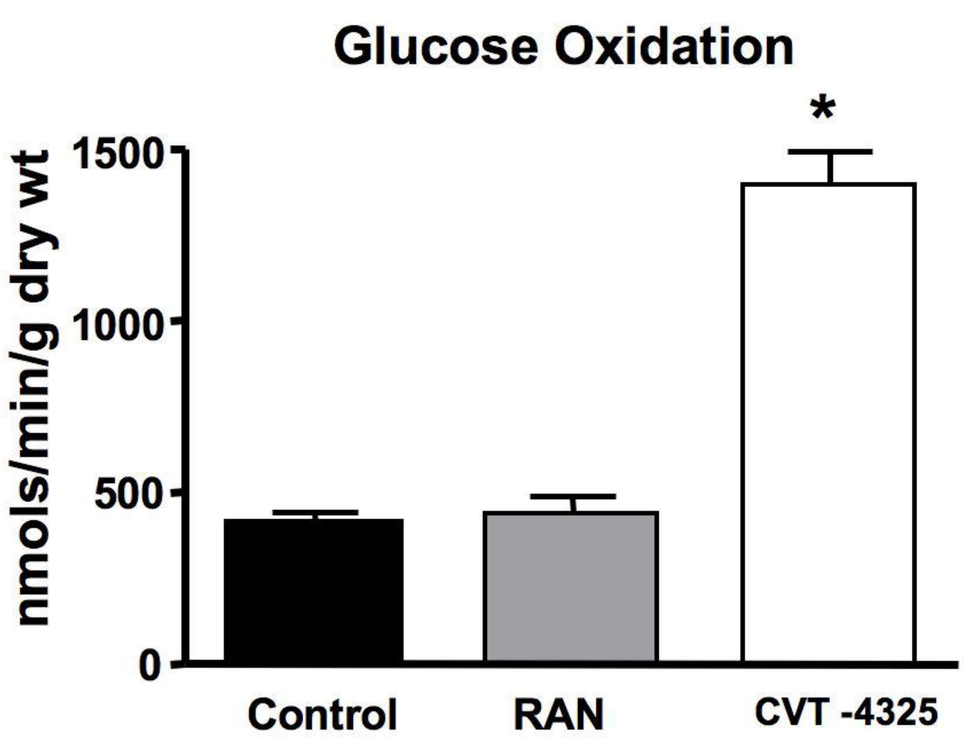

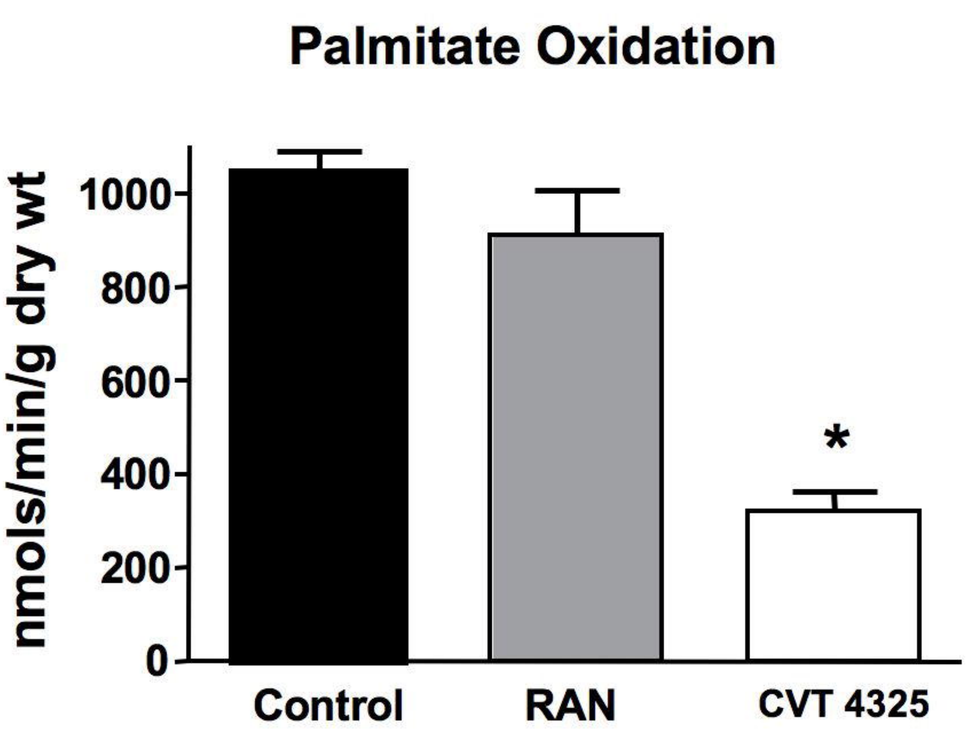

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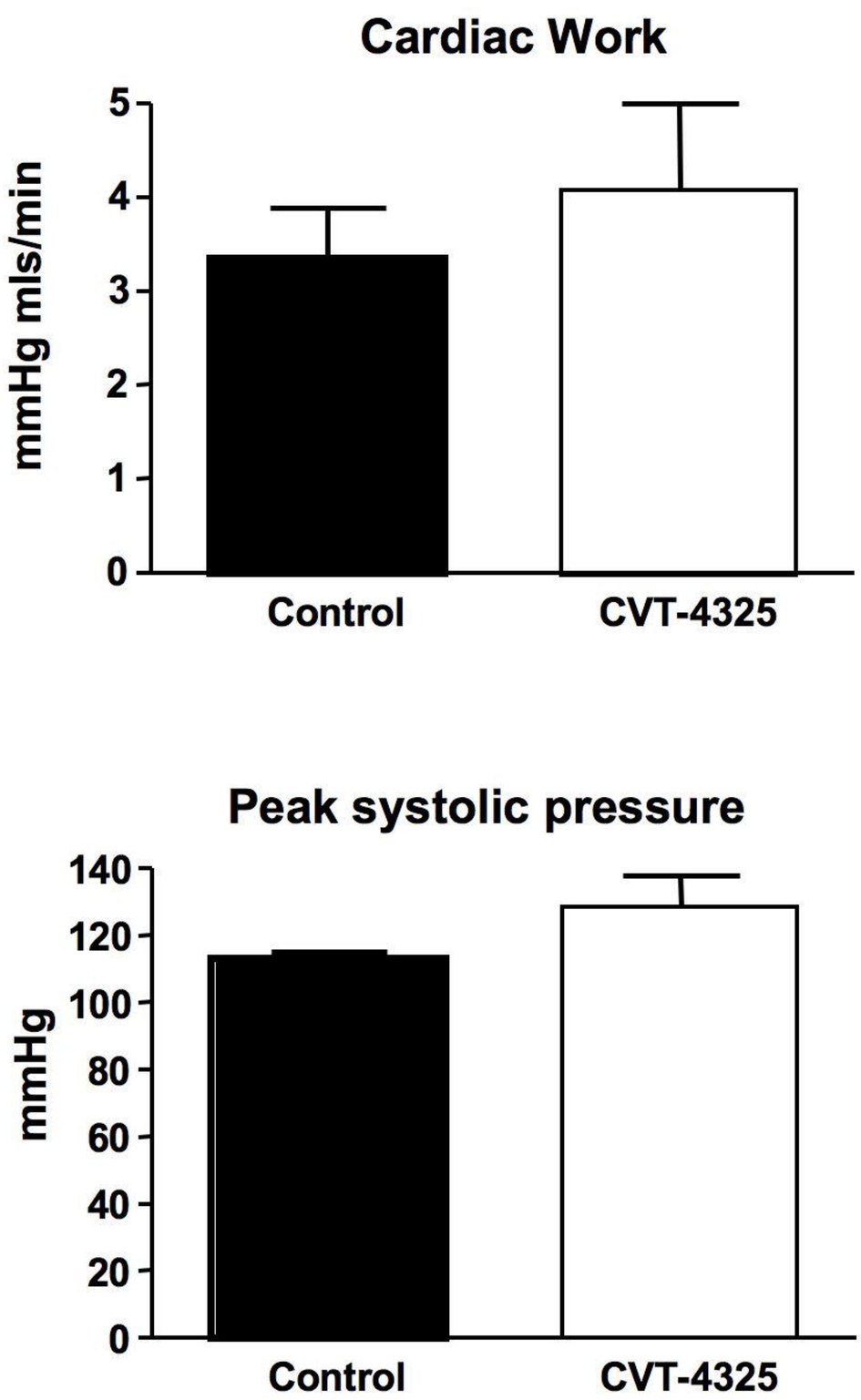

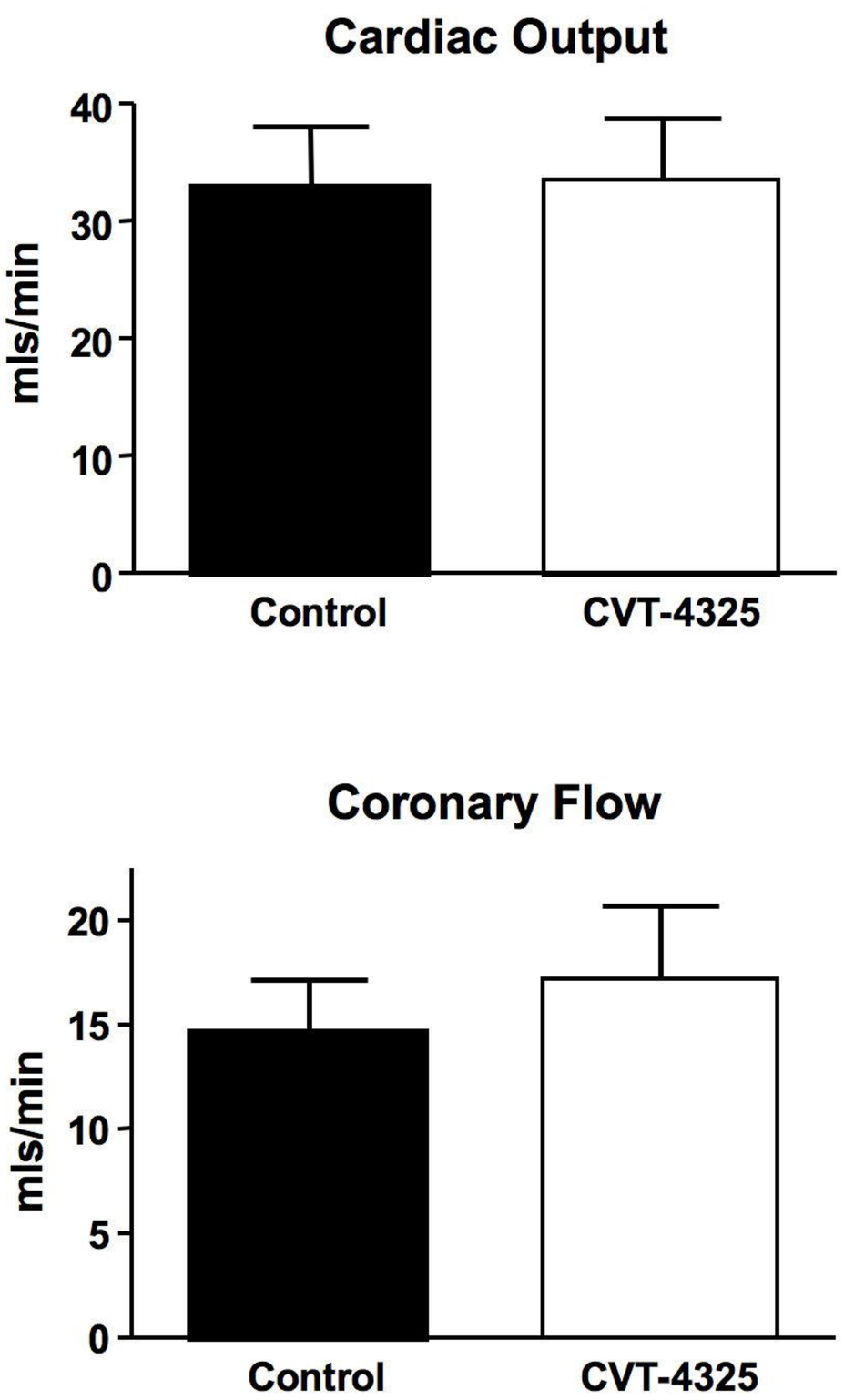

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