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The Highly Selective Caspase-1 Inhibitor VX-765 Provides Additive Protection Against Myocardial Infarction in Rat Hearts When Combined With a Platelet Inhibitor

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Abstract

Use of ischemic postconditioning and other related cardioprotective interventions to treat patients with acute myocardial infarction (AMI) has failed to improve outcomes in clinical trials. Because P2Y₁₂ inhibitors are themselves postconditioning mimetics, it has been postulated that the loading dose of platelet inhibitors routinely given to patients treated for AMI masks the anti-infarct effect of other intended cardioprotective interventions. To further improve outcomes of patients with AMI, an intervention must be able to provide additive protection in the presence of a P2Y₁₂ platelet inhibitor. Previous studies reported an anti-infarct effect using a peptide inhibitor of the pro-inflammatory caspase-1 in animal models of AMI. Herein we tested whether a pharmacologic caspase-1 inhibitor can further limit infarct size in open-chest, anesthetized rats treated with a P2Y₁₂ inhibitor. One hour occlusion of a coronary branch followed by 2 hours of reperfusion was used to simulate clinical AMI and reflow. One group of rats received an intravenous bolus of 16 mg/kg of the highly selective caspase-1 inhibitor VX-765 30 minutes prior to onset of ischemia. A second group received a 60 µg/kg intravenous bolus of the P2Y₁₂ inhibitor cangrelor 10 minutes prior to reperfusion followed by 6 µg/kg/min continuous infusion. A third group received treatment with both inhibitors as above. Control animals received no treatment. Infarct size was measured by tetrazolium stain and volume of muscle at risk by fluorescent microspheres. In untreated hearts, 73.7% ± 4.1% of the ischemic zone infarcted. Treatment with either cangrelor or

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Authors Contributions

Xi-Ming Yang and Nicole A. Housley performed experiments and acquired data. James M. Downey and Michael V. Cohen designed the experiments, participated in data analysis, and wrote the manuscript. Diego F. Alvarez and Jonathon P. Audia contributed to analytical discussions and writing the text.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

VX-765 alone reduced infarct size to $43.8\% \pm 2.4\%$ and $39.6\% \pm 3.6\%$ of the ischemic zone, respectively. Combining cangrelor and VX-765 was highly protective, resulting in only $14.0\% \pm 2.9\%$ infarction. The ability of VX-765 to provide protection beyond that of a platelet inhibitor alone positions it as an attractive candidate therapy to further improve outcomes in today's patients with AMI.

Keywords

ischemia/reperfusion injury; heart disease

Introduction

Death of cardiac muscle during ischemia/reperfusion (I/R) is a serious complication leading to heart muscle failure in patients treated for acute myocardial infarction (AMI). Recent clinical trials of interventional therapies such as ischemic postconditioning^{1,2} and cyclosporine³ aimed at preserving cardiomyocyte viability in these hearts have been disappointing. It is critical to note that the standard of care for patients with AMI includes treatment with a P2Y₁₂ platelet inhibitor prior to reperfusion. We postulate that because P2Y₁₂ inhibitors are themselves postconditioning agents, they render any additional interventions that work through convergent mechanisms redundant.^{2,4} Thus, it is imperative that efficacy testing of novel cardioprotective interventions in animal models of AMI must be performed in the presence of a P2Y₁₂ inhibitor to validate potential utility for augmenting outcomes in today's patients. Only interventions that can provide additional protection beyond that from a platelet inhibitor will have any chance of clinical efficacy.

To obtain added protection, new interventional therapies must protect independently of classical postconditioning mechanisms. Intriguingly, there is emerging evidence to suggest I/R injury and cardiomyocyte death leads to the release of damage-associated molecular patterns (DAMPs)—danger signals that activate innate inflammatory pathways. One such DAMP is mitochondrial DNA,^{5,6} which is released from damaged mitochondria and is proposed to contribute to cell killing by triggering inflammasome formation.⁷ Inflammasomes are multiprotein complexes that sense a panoply of danger signals through the family of nucleotide oligomerization domain-like receptors and ultimately recruit and activate the pro-inflammatory cysteine protease, caspase-1. In turn, active caspase-1 elicits inflammation by activation of interleukins IL-1 β and IL-18. A previous report indicates that inhibition of caspase-1 protects the reperfused heart against infarction. Treatment with a caspase-1-selective, inhibitory substrate-mimetic Ac-Tyr-Val-Ala-Asp-chloromethylketone (YVAD) preserved contractile function and prevented enzyme washout from human atrial trabeculae subjected to simulated I/R.⁸ Because mitigating IL-18 or IL-1 β release and circulation offered similar protection in that study, it was concluded that interleukins were causing the injury.⁸ Treatment with YVAD also reduced infarct size in an open-chest rabbit model,⁹ and infarct size was increased by 50% in mice overexpressing caspase-1.¹⁰ In addition, treatment with a pan-caspase inhibitor, carbobenzoxy-Val-Ala-Asp-[O-methyl]-fluoromethylketone (z-VAD), also reduced infarct size in isolated heart models of I/R injury.^{11,12} Although inhibition in the z-VAD studies was assumed to target apoptosis, which

involves caspases-3 and -9, caspase-1 would have been blocked as well and could have accounted for the protection.

In the present study, we aimed to determine whether inhibition of caspase-1 could provide additive protection against infarction to that from the P2Y₁₂ inhibitor cangrelor. We tested the highly selective caspase-1 inhibitor VX-765¹³ in our open-chest rat myocardial infarct size model both by itself and in conjunction with the platelet P2Y₁₂ receptor antagonist cangrelor.

Methods

Surgical Preparation

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health¹⁴ and all efforts were made to minimize suffering. Experimental protocols were approved by the University of South Alabama Institutional Animal Care and Use Committee. Sprague-Dawley adult male rats (~500 g) were anesthetized with 100 mg/kg intraperitoneal sodium pentobarbital, and anesthetic plane confirmed by absence of toe-pinch reflex and the breathing pattern. Additional intravenous boluses of 5 mg/kg were administered approximately every 30 minutes to maintain a surgical plane of anesthesia. Animals were mechanically ventilated with 100% O₂ at a rate of 65 breaths/min. The ventral neck was dissected and catheters were inserted into the right jugular vein and carotid artery. After a left thoracotomy, the heart was exposed and a snare was placed around the left anterior descending branch of the left coronary artery. Arterial blood pressure was continuously recorded. The snare was tightened to create regional ischemia. All hearts were subjected to 60 minutes of regional ischemia and 2 hours of reperfusion. Animals were humanely euthanized by exsanguination during deep anesthesia at protocol terminus and hearts were harvested.

Infarct Area and Risk Zone

After removal, hearts were mounted on a Langendorff apparatus and perfused at 100 mm Hg with normal saline solution through the aortic root. After 2 minutes of perfusion, the coronary artery branch was reoccluded, and 2 to 9 µm green fluorescent microspheres (Microgenics Corp, Fremont, California) in normal saline were infused into the heart. The ischemic area was visualized as myocardium without fluorescent microspheres. The heart was flash frozen and cut into 2-mm slices perpendicular to its long axis. Slices were incubated for 8 to 10 minutes in 1% triphenyltetrazolium chloride (GFS Chemicals, Powell, Ohio) at 37°C and then put into 10% formalin for tissue preservation and enhancement of color contrast between living (stained) and infarcted (unstained) tissue. The ischemic zones were identified under ultraviolet light and were traced on a plastic overlay. A photograph was made of the slices with the overlay under white light. Areas on the image were measured by planimetry and volumes were calculated by multiplying areas by slice thickness. Infarct size is presented as the percentage of ischemic zone volume for each heart.

Protocol

Untreated control hearts received no treatment and underwent the I/R sequence as described above. Five minutes prior to occlusion, the vehicle control group received intravenous bolus injection of dimethyl sulfoxide (DMSO) (VX-765's solvent) prepared by dissolving 0.5 mL of 100% DMSO/kg body weight in normal saline to a final volume of 0.9 mL. VX-765-treated animals received a 16 mg/kg intravenous bolus (dissolved in DMSO and diluted in saline to a volume of 0.9 mL) of VX-765 30 minutes prior to occluding the coronary artery. Cangrelor-treated animals received a 60 µg/kg intravenous bolus of cangrelor in saline 10 minutes prior to reperfusion followed by a continuous infusion of 6 µg/kg/min until the heart was removed for infarct size measurement. In the combination test group, VX-765 and cangrelor were both administered as indicated above. VX-765 is a prodrug and must be enzymatically converted to VRT-043198, which inhibits caspase-1.¹³ We, therefore, reasoned that administration of the drug 30 minutes prior to the onset of ischemia would allow sufficient time for it to be converted to its active form prior to I/R.

Statistical Analysis

Data are expressed as mean ± standard error of mean from at least 5 independent experiments. Data were analyzed by 1-way analysis of variance followed by Newman-Keuls multiple comparison post hoc test. Differences with $P < 0.05$ were considered significant. All analyses were performed using SigmaStat 2.03 software.

Results and Discussion

In a majority of experimental infarct size studies in rats injury has been induced using a 30-minute period of ischemia. In the present study, we extended the ischemic time to 60 minutes to increase infarction size in the untreated hearts. This more stringent protocol allowed us to better appreciate additive protective effects since assay sensitivity is lost as the interventions reduce infarct size toward zero.

At baseline, all groups had similar blood pressures and heart rates (data not shown). There was no significant change during drug infusion. There was a mild decrease of blood pressure in all groups during coronary occlusion with partial recovery following reperfusion. Figure 1 shows infarct size expressed as a percent of the ischemic (risk) zone in these hearts. Compared to untreated or vehicle control-treated hearts, either VX-765 or cangrelor alone furnished similar and statistically significant reductions in infarct size (ie, protection). Strikingly, combination therapy using VX-765 and cangrelor elicited a clearly additive protective effect. Together, these data reveal a potential utility for VX-765-mediated inhibition of caspase-1 as a therapeutically relevant strategy to treat patients with AMI undergoing reperfusion therapy who have already been loaded with a P2Y₁₂ platelet inhibitor.

Risk zone is known to be an important variable influencing infarct size. As the volume of the risk zone decreases, the size of the infarct does not always decrease proportionately. Therefore, if groups that are to be compared have different risk zone sizes, any conclusion about differences in infarct size could be spurious. In Figure 2, risk zone volume is plotted

against infarct volume for all experimental points. It is readily apparent that average risk zone size and range are similar for all groups. The risk zone/infarct volume relationship for rats treated with either cangrelor or VX-765 alone is shifted downward relative to that in control rats. Additionally, the combination of cangrelor and VX-765 shifted the relationship further down confirming the additive protection achieved by combination therapy.

The additive protective effect of combining the caspase-1 inhibitor VX-765 with the P2Y₁₂ receptor inhibitor cangrelor observed in the present study is strongly suggestive that the 2 agents protect by different mechanisms. In previous studies, we combined ischemic preconditioning¹⁵ or ischemic postconditioning⁴ with cangrelor and in neither case was a smaller infarct size seen when compared to cangrelor alone. We interpreted those observations to suggest that cangrelor's anti-infarct effect used the same mechanism as conditioning. To further test that hypothesis, we examined the effect of 7 different blockers of signal transduction components known to block both pre- and postconditioning's protection.⁴ All 7 blockers eliminated cangrelor's anti-infarct effect, but none restored platelet reactivity in the animal's blood. We concluded that cangrelor had conditioned the hearts and the protection against infarction was independent of any effect on coagulation. Similar results were seen with ticagrelor¹⁵⁻¹⁷ and clopidogrel⁴ suggesting that this is a class effect. Thus, P2Y₁₂ blockers appear to be conditioning mimetics that, when given to patients as a loading dose prior to recanalization of the thrombosed artery, induce a postconditioned state in the heart and render any additional postconditioning intervention redundant. Importantly, caspase-1 inhibition by VX-765 appears to target an additional mechanism of cell killing beyond that targeted by cangrelor revealing a novel, conditioning-independent mechanism of cardiomyocyte protection.

We chose VX-765 for the present study as it is more selective for caspase-1 than YVAD, and thus our findings further support the hypothesis that protection is the result of targeting of caspase-1 since 3 different molecules (YVAD,^{8,9} z-VAD,^{11,12} and VX-765) known to inhibit caspase-1 have the same anti-infarct effect. However, as with any pharmacological treatment, there is always the chance that some off-target action was responsible for the observed effect. But that would require all 3 molecules to have the same nonspecific property.

Recent reports suggest that ticagrelor may provide additional protection over that from clopidogrel because of its ability to increase tissue adenosine levels,^{16,17} and ticagrelor is now widely used before recanalization interventions. We used cangrelor in the present study because an intravenous drug simplifies the protocol. When we compared salvage with intravenous cangrelor to that from oral ticagrelor, we found their anti-infarct effects to be essentially identical in a rat model (infarct size was 55.0% and 56.5% of that in untreated rats, respectively).¹⁵ Also, like ticagrelor, cangrelor's protection is adenosine-dependent as the adenosine receptor blocker 8-sulfophenyltheophylline completely blocks it.⁴ Thus, we think that a cangrelor-treated animal represents a clinically relevant P2Y₁₂ model.

The present study confirms previous studies concluding that inhibition of caspase-1 is cardioprotective. Because VX-765 is more selective than YVAD for caspase-1, it further supports the hypothesis that caspase-1 is the target. Most importantly, it also reveals that a

caspase-1 inhibitor can provide protection that is additive to that from a P2Y₁₂ inhibitor indicating that it has the potential to provide additive protection against infarction in patients undergoing reperfusion therapy for AMI. It must be noted that these experiments with VX-765 were all conducted with exposure of the animals to the compound prior to induction of myocardial ischemia. This mode of administration is obviously not suitable for treatment of patients with AMI who present only after the onset of myocardial ischemia. Whether a rapidly acting caspase-1 inhibitor administered at the time of reperfusion would still be protective is unknown.

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Figure 1.

Open circles depict individual hearts, whereas filled circles show group means and standard error of mean (SEM). Cang indicates cangrelor (started 10 minutes prior to reperfusion and maintained during the entire course of reperfusion); VX, VX-765 (intravenous bolus 30 minutes before ischemia); $*P < .001$ Cang, VX versus untreated, vehicle. $**P < .001$ Cang + VX versus untreated, vehicle, Cang, VX.

Figure 2.

Plot of risk zone volume against infarct volume for all experimental points in the 5 groups studied. Regression lines are also plotted for each group. A downward shift of the regression line indicates protection compared to the control group since for any risk zone volume infarct size is smaller.