The effect of C1 inhibitor on myocardial ischemia and reperfusion injury

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Abstract

Background—Activation of the complement system has been demonstrated to be an important mechanism in the mediation of myocardial ischemia and reperfusion (MIR) injury. C1 inhibitor (C1INH) has been shown to be beneficial in experimental MIR models. The underlying mechanism of this effect has been assumed to result primarily from inhibition of complement system activation. We recently demonstrated that C1INH plays a direct role in suppression of leukocyte transmigration in the mouse intestinal ischemia and reperfusion model. The purpose of this study was to investigate the mechanism of the beneficial effect of C1INH in mouse MIR model.

Methods—C57BL/6, C1INH-deficient (C1INH$^{-/-}$) and C3-deficient mice (C3$^{-/-}$) were subjected to 30 min (C57BL/6 and C1INH$^{-/-}$) or 60 min (C3$^{-/-}$) occlusion of the left anterior descending branch (LAD) of the coronary artery followed by 4h reperfusion. C1INH or reactive center cleaved inactive C1INH (iC1INH) was injected intravenously 5min before reperfusion.

Results—Myocardial infarct size relative to the area at risk or relative to left ventricular area was significantly reduced in C1INH treated WT, C1INH$^{-/-}$ and C3$^{-/-}$ mice compared with vehicle-treated mice. MIR induced an increase in myocardial polymorphonuclear neutrophil accumulation and plasma cardiac specific troponin I levels in vehicle-treated MIR mice while C1INH treatment significantly attenuated these effects. iC1INH had a similar protective effect.

Conclusions—These results suggested that C1INH prevented MIR injury in mice and that this cardioprotective effect may not solely result from complement inhibition, but might be also contributed by inhibiting leukocyte recruitment into ischemic tissue, an effect that is not mediated via protease inhibition.

Keywords

myocardial ischemia and reperfusion; complement inhibition; leukocyte transmigration

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1. Introduction

Myocardial infarction (MI) is one of the largest killers of Americans. Approximately every 34 seconds, an American will have a MI. In 2006, mortality from MI in the United States was 141462 [1]. Reperfusion of ischemic myocardium is an absolute necessity to salvage tissue from eventual death and for ultimate limitation of infarct size. However, reperfusion after ischemia is associated with pathologic changes that cause additional myocardial damage and may significantly contribute to myocardial infarction size. This so-called 'reperfusion injury' results from the activation of a series of inflammatory mediators.

Complement activation and neutrophil stimulation are believed to be two major components involved in ischemia reperfusion injury (IRI) [2, 3]. As early as 1971 Hill and Ward demonstrated in a rat model that complement was activated locally in ischemic myocardium and contributed to the local inflammatory response [4]. More recent studies suggested that following myocardial ischemia reperfusion (MIR) complement may be activated through the classical and alternative pathways as well as the lectin pathway [5-8]. Therefore, blockade of the complement system became a major therapeutic target in ischemia and reperfusion (IR) injury. Several agents that inhibit all or part of the complement system, such as sCR1, C1 inhibitor (C1INH), and antibodies against C5a have been shown to reduce MIR injury [9-14]. Neutrophils have been implicated as a primary mechanism underlying IR injury. Polymorphonuclear neutrophils (PMNs) infiltration of the affected tissue plays a pivotal role in IR-induced cardiac dysfunction, which include oxygen free radical generation, degranulation and release of proteases, and release of other inflammatory products that amplify the recruitment and activation of greater numbers of neutrophils into the affected myocardium [15-17]. Thus, inhibition of neutrophil infiltration could be another therapeutic option for reperfusion injury.

C1INH, a member of the serpin family of protease inhibitors, inhibits all three pathways of complement activation [18-20]. Like most other serpins, C1INH reacts with target proteases (such as C1s) to form high molecular weight complexes. However, reactive center-cleaved C1INH (iC1INH) loses the ability to complex with target proteases [21, 22]. Cardioprotective effects of C1INH were observed in animal models of MIR [9, 11]. Several studies were performed in humans to explore the potential use of C1INH against MIR injury [23-25]. These protective effects have been assumed to result from its ability to inhibit complement activation. However, the precise mechanism of action behind the positive effects seen with C1INH in MIR injury has not been established. We have recently analyzed the mechanism of C1INH-mediated alleviation of intestinal IR injury [26]. It appears that the protective effect of C1INH in this model is mediated both by inhibition of complement activation and of leukocyte infiltration. In earlier studies, we demonstrated that C1INH suppresses leukocyte rolling and infiltration, which is at least partly by virtue of its ability to interact with E- and P-selectins on the endothelial surface [26, 27]. In the present study, we used C3 deficient and C1INH deficient mice to investigate the cardioprotective role and mechanism of the beneficial effect of treatment with C1INH in a mouse MIR model.

2. Materials and methods

2.1. Animals

Male C57BL/6 (wild type (WT)) mice were purchased from Jackson Laboratories (Bar Harbor, ME). C3 deficient ($C3^{-/-}$) mice were obtained from Michael Carroll, PhD, Immune Disease Institute, Boston, MA. C1INH deficient ($C1INH^{-/-}$) mice were maintained by our laboratory [28]. All experiments were performed with mice at 8 to 12 weeks of age, weighing 20-25g. Both genders of $C3^{-/-}$ and $C1INH^{-/-}$ mice were used in the experiment. All

experimental procedures and protocols used in this investigation were reviewed and approved by the animal Care and Use Committee of the Immune Disease Institute.

2.2. Reagents

C1INH (Berinert) was generously provided by CSL Behring (King of Prussia, PA). iC1INH was prepared by incubating native C1INH with trypsin agarose (Sigma, St. Louis, MO) [29]. Briefly, C1INH was incubated with trypsin agarose (1IU/8μl) at room temperature for 60 min and then subjected to centrifugation (5,000 g) at 4°C. The supernatants were collected and used immediately. The function of C1INH and iC1INH was characterized using its ability to complex with C1s. C1INH and iC1INH were incubated separately with C1s (1:1 molar ratio) at 37°C for 60 min, subjected to SDS-PAGE, and stained with Coomassie blue. Native C1INH reacts with C1s to form a 200-kDa complex. However, iC1INH loses the ability to complex with C1s and is therefore regarded as inactive. In addition, iC1INH migrates on SDS-PAGE with a slightly smaller apparent molecular weight than intact active C1INH (105 kDa)

2.3. Surgical procedure

Mice were premedicated with pentobarbital (60mg/kg, i.p.), then the trachea intubated and the animals ventilated on a SAR small animal ventilator (D-79232, Harvard Apparatus, Germany) and maintained under anesthesia with isofluane. Depth of anesthesia was initially verified by loss of righting reflex and by recurrent testing of hind paws withdrawal and corneal reflex throughout the experimental protocol. The ECG was recorded throughout the experiments. A left thoracotomy was performed and direct visualization of the left anterior descending (LAD) coronary artery obtained. The LAD was occluded 1-2 mm below the tip of the left atrium using 8-0 black polyamide monofilament suture (AROSurgical, CA) tied over a 2-mm piece of polyethylene (PE)-10 tubing. Successful coronary occlusion was verified by the development of a pale color in the distal myocardium and by ST segment elevation on ECG. After a 30 min (C57BL/6 and C1INH$^{-/-}$) or 60 min (C3$^{-/-}$ mice) ischemia period, the PE-tubing was removed to allow for 4h reperfusion. Reperfusion of the previously occluded coronary bed was confirmed by visual inspection, and ST segment almost went back to the baseline level on ECG. The chest was closed and the animal was removed from the respirator after the automatic respiration returned. Sham animals underwent the same procedure without tightening up the suture around the LAD artery. Either C1INH (iC1INH) 0.4 IU/g or normal saline was administrated intravenously 5 min before coronary reperfusion. At lease 6 animals were used for each group.

2.4. Infarct size (IS) and area at risk (AAR)

Four hours following reperfusion, blood samples were collected for further analysis. Mice were then euthanatized with a lethal dose of pentobarbital, and their hearts were rapidly excised. After removal of the left atrium, the aorta was cannulated with a blunt 22-gauge needle and rinsed with PBS. The LAD was ligated at the site of prior occlusion, 0.5 ml of 1% Evans blue solution was then slowly injected into the aorta in order to delineate the AAR. This caused dye to enter the nonischemic region of the left ventricle and leaves the ischemic area (area at risk, AAR) unstained. The heart was then cooled at −20 °C for 30 min and the left ventricle was cut transversely into four slices from base to apex, the first cutter blade being positioned at the site of the coronary occlusion. All slices were incubated at 37 °C for 20 min with 1% TTC (2, 3, 5-triphenyl-tetrazolium chloride). After TTC staining, viable myocardium (ischemic non-necrotic tissue) is brick red and the infarct (ischemic necrotic tissue) appears pale white. Evan's blue stained area is defined as the area not at risk. Slices were fixed in 3.5 % formaldehyde at 4 °C overnight. Slices were then weighed, and color digital images of both sides of each slice were obtained and then manually measured by using Adobe Photoshop software. The left ventricular (LV) area, the area at risk (AAR),

and the infarct size (IS) were outlined on each color image and quantified by a blind observer. The size of the infarction was determined by the following equation: $IS = I_1 \times Wt_1 + I_2 \times Wt_2 + I_3 \times Wt_3 + I_4 \times Wt_4$, where I is percent infarcted areas by planimetry from subscripted numbers 1-4 slices, and Wt is the weight of the same numbered slice. The results are expressed as the infarcted area relative to the total weight of the left ventricle (IS/LV), the ischemic area at risk relative to the total weight of the left ventricle (AAR/LV), and the infarcted area relative to the area at risk (IS/AAR).

2.5. Histology & quantitation of neutrophil infiltration

For histological analysis, the heart slices from additional MIR groups (n=4 in each group) were fixed with 10% formalin and then embedded in paraffin and sectioned into 5μm-thick sections. To selectively stain PMNs in the myocardium, the slices were stained with Naphtol AS-D Chloroacetate Esterase (Sigma-Aldrich, USA) counterstained with Hematoxylin. The Naphthol AS-D chloracetate is hydrolyzed by an esterase specific for PMN leukocyte and reacted with a diazonium salt to result in a stable red compound. Thus, PMNs appear red. After staining, sections were reviewed microscopically at a power of 400×. PMNs were then counted by a blinded observer and presented as per 400× power field of section. Four fields from 4 independent slices for a total of 16 fields from each animal were determined. The average was used as the extent of PMN infiltration within the ischemic and reperfused zone.

2.6. Cardiac enzyme analysis

Blood samples were centrifuged for 10 min at 4°C after collection; the plasma was then stored at −80 °C until further analysis. A commercially available cardiac-specific troponin (TnI) kit was used for these determinations.

2.7. Statistical analyses

All results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, LaJolla, CA), with significance defined as $p < 0.05$. A one-way ANOVA analysis of variance with Bonferroni's multiple comparison tests was used for all analyzed data.

3. Results

3.1. C1INH reduced myocardial infarction

Infarct analysis was performed on 4 heart sections from ligation level to the apex. Two sides of each section were measured and the average was taken for analysis. There was no significant difference in AAR (as a percentage of the LV, AAR/LV) between experimental and control groups. Thus, the amount of LV undergoing ischemia between groups was not significantly different. A marked cardioprotective effect of C1INH or iC1INH was observed in the treated WT group. IS/LV and IS/AAR fell from 23.55±3.62 and 49.10±6.35 to 10.10±2.19 and 22.58±4.25 with C1INH treatment, and to 11.38±2.27 and 26.75±4.78, respectively, with iC1INH treatment (p<0.05, n=6). Although $C3^{-/-}$ mice are more resistant to ischemia, prolongation of ischemic time to 60 min resulted in an infarction size that was comparable with that of untreated WT mice. Treatment with C1INH reduced the infarct size from 24.28±2.58 to 10.73±0.97 (IS/LV) and 55.55±7.92 to 25.70±1.95 (IS/AAR) (p<0.05, n=6). The size of the infarction following MIR in $C1INH^{-/-}$ mice was not significantly different from that of WT mice but was reversed by C1INH treatment, with IS/LV and IS/AAR decreased from 27.43±4.79 and 52.90±4.26 to 9.60±1.24 and 28.08±2.19, respectively (p<0.05, n=6) (Fig. 1).

3.2. C1INH reduced neutrophil accumulation in the myocardium

HE stained microscopic images of myocardium and infiltration of neutrophils into the myocardium exposed to 30 min (60 min for C3$^{-/-}$ mice) LAD occlusion and 4h reperfusion are presented in Fig. 2 and Fig. 3A, respectively. The number of neutrophils was significantly increased in MIR groups as compared with the sham control group. Neutrophil numbers were not significantly different between C3$^{-/-}$, C1INH$^{-/-}$ and WT mice after MIR. In all three groups, neutrophil counts in the C1INH and iC1INH treated myocardium were significantly lower than those in vehicle-treated mice (Fig. 3B, $P < 0.05$, n=4).

3.3. C1INH treatment resulted in reduced plasma Troponin I (TnI) levels

Baseline TnI plasma levels were similar (average 5±1 ng/ml) in all three sham control groups (Fig. 4). Levels were significantly increased (approximately 7-fold) in all groups following reperfusion. Myocardial protection was accompanied by significantly reduced plasma concentrations of TnI in both the C1INH and iC1INH treated groups ($P < 0.05$, n= 10) (Fig. 4).

4. Discussion

Our data demonstrate that treatment with C1INH significantly prevented myocardial injury secondary to MIR. The C1INH-mediated cardioprotection was characterized by a reduction of myocardial infarct size, decreased accumulation of neutrophils in the myocardium and reduction of plasma levels of TnI compared to mice given the vehicle alone without C1INH.

A role for complement activation in ischemia and reperfusion injury has been clear for several years. During reperfusion, complement may be activated through the classical, alternative and lectin pathways, leading to generation of biologically active products, such as C5a and the terminal membrane attack complex (MAC), and ultimately contributes to endothelial cell disruption [5-8]. Hill and Ward reported the first evidence that complement might be involved in IRI about four decades ago [4]. More recent studies have shown in several organ systems that complement system is a key mediator of IRI [30-32]. Therefore, blockade of the complement system became a therapeutic target for experimental IR. Studies using cobra venom factor suggested that depletion of complement protected the ischemic myocardium from reperfusion injury [33, 34]. The effect of C1INH has been studied in several experimental IR models and human studies, especially in MIR models [9, 11, 35, 36]. The protective mechanism of C1INH has been assumed to result from its ability to inhibit complement activation which would result in the reduction of PMN infiltration into the ischemic myocardium. In the present study, C1INH was shown to attenuate the severity of myocardial infarction from IR, which confirmed previous studies. Parallel to these morphological criteria, plasma TnI levels, the biochemical marker for myocardial necrosis, was measured. Again, clear attenuation of the increases of this plasma marker was found in the C1INH treated group.

In order to determine whether the effect of C1INH was mediated solely via inhibition of complement activation, two approaches were taken. First, the effect of C1INH in C3 deficient mice was analyzed and secondly, the effect of inactivated, reactive center-cleaved C1INH was tested. In pre-experiment, C3$^{-/-}$ mice were subjected to 30min ischemia followed by 4h reperfusion and couldn't show significant myocardial infarction (data are not shown here). But by prolonging the period of ischemia to 60 min, the C3$^{-/-}$ mice showed infarct sizes comparable with WT mice, together with other evidence of tissue damage (elevated TnI levels) and neutrophil infiltration. Moreover, we have shown that even though C3$^{-/-}$ mice are relatively resistant to MIR injury, C1INH did reverse the myocardial injury in 60 minutes of ischemia model. At the same time, we have shown that iC1INH also has the

effect of protecting the ischemic tissue from reperfusion injury. C1 inhibitor is a member of the serpin family of serine protease inhibitors. Protease inhibition occurs as a result of SDS-resistant covalent complex formation between the inhibitor and the protease. iC1INH, which lost the ability of complex formation due to cleavage at its reactive center, is absolutely incapable of protease inhibition. This has been demonstrated in many other studies [37, 38]. Taken together, these data indicate that the protection was not only due to complement inhibition but also required the participation of another mechanism. Interestingly, the amount of myocardial damage in C1INH$^{-/-}$ mice was essentially the same as in wild type mice, which suggests that the concentration of C1INH in wild-type mice is insufficient to provide protection from IR injury. With the dose of C1INH in the present study, blood levels of C1INH very likely are increased by three- to fivefold [39].

Polymorphonuclear neutrophils (PMNs) have been implicated as a primary mediator of damage in IRI [17]. The neutrophil accumulation is thought to be the prime cellular mediator of microvascular plugging and local tissue destruction in IRI. Once sequestered, neutrophils generate oxidants and proteases that result in tissue damage, and release inflammatory products that amplify the recruitment and activation of greater numbers of neutrophils into the effected myocardium, thereby extending the severity of tissue damage [3, 40, 41]. It has been shown that inhibition of PMN activation or depletion of PMNs may reduce myocardial injury after IR in animal models [42, 43]. We observed significantly increased neutrophil accumulation in the vehicle-treated ischemic myocardial tissue, which is in agreement with previous findings [36, 44]. In addition, C1INH treatment resulted in reduced neutrophil counts in the reperfused myocardium. These data demonstrated that C1INH reduced reperfusion injury, possibly as a consequence of the reduction in the number of neutrophils accumulating in tissues. Furthermore, iC1INH, as with its effect in reducing overall myocardial damage, also resulted in diminished neutrophil infiltration. Therefore, the decrease in accumulation of neutrophils was not a result of decreased complement activation, as might have been expected.

Endothelial dysfunction plays a critical role in the pathogenesis of reperfusion injury in the myocardium [45-47]. Neutrophils start to interact with endothelial cells by rolling, followed by subsequent firm adherence, which is then followed by transendothelial migration and direct interaction with myocytes. The rolling process along the endothelial surface is mediated by selectins on the endothelium and sialylated glycoprotein on the neutrophil, most likely sialyl Lewis^x or the sialomucin P-selectin glycoprotein ligand-1 (PSGL-1) [48, 49]. Inhibition of neutrophil-endothelial adhesion has been shown to protect from MIR injury [50]. Studies in mice deficient in P-selectin, ICAM-1, and CD18 have shown significant reduction in PMN accumulation and necrosis following MIR [51, 52]. We have demonstrated that C1INH suppresses the migration of neutrophils across endothelial cells in other inflammatory models [27]. This effect very likely depends on the presence of the tetrasaccharide sialyl Lewis^x attached to one or more of the six N-linked carbohydrates on C1INH, which suppresses the interaction of selectin ligands with both E- and P-selectins. The mechanism by which C1INH exerts its cardioprotective effect could, at least in part, be explained by its ability to suppress the interaction of selectins with their ligands on neutrophils.

In conclusion, we have demonstrated that C1INH is effective in protection from MIR injury in mice, and that this effect was observed with a single dose application previously proven safe for use in humans. These protective effects are, at least in part, attributable to direct inhibition of neutrophil accumulation.

Acknowledgments

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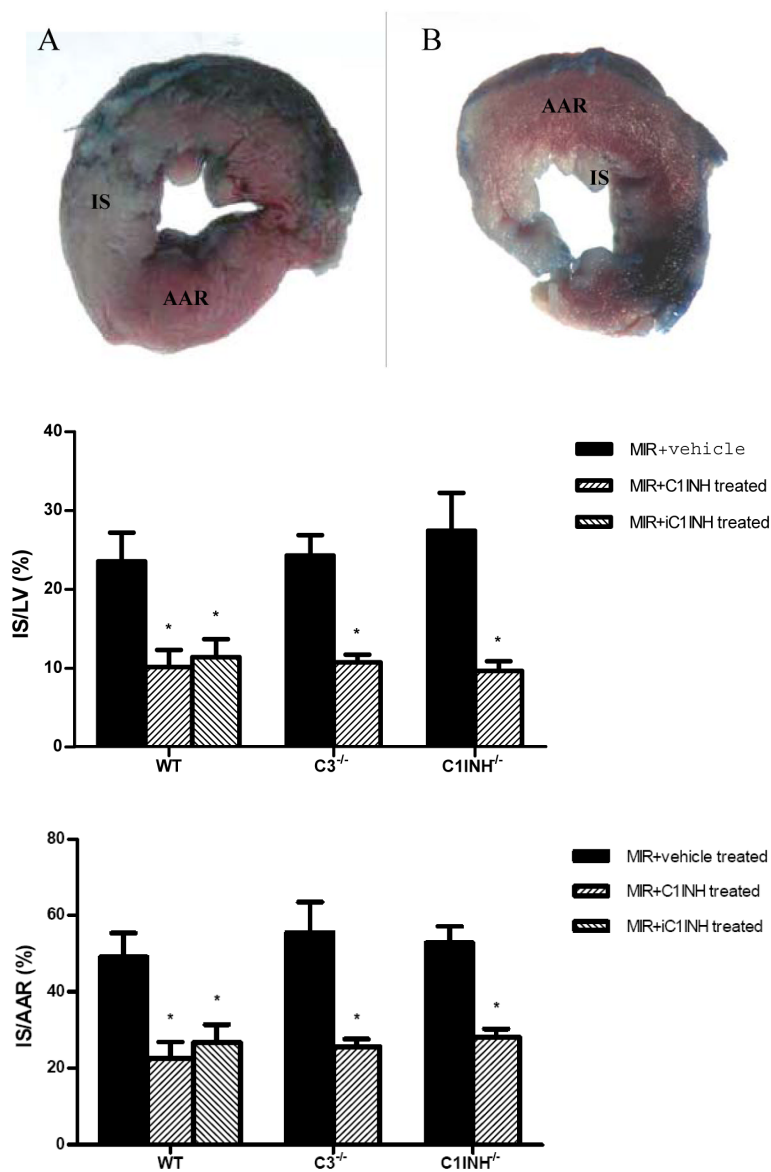
Fig. 1.
Myocardial infarct analysis. A, B, Selective photographs of left ventricle slices from mice subjected to 30 minutes ischemia and 4 hours reperfusion and then infused with Evans blue and stained with triphenyltetrazolium chloride, treated with vehicle (A) and treated with C1 inhibitor (B). Blue: non-ischemic region; Red: ischemic non-necrotic tissue (AAR); Pale white: ischemic necrotic tissue (IS). C: The infarct size following IR was expressed as a percentage of the total weight of the left ventricle (IS/LV). D: The infarct size following IR was expressed as a percentage of the total weight of the area at risk (IS/AAR). Values are means ± SEM, n=6. * P<.05 C1INH or iC1INH treated mice vs. vehicle–treated mice. IS: the infarct, ischemic necrotic area. AAR: area at risk, the ischemic area. LV: the left ventricle. IR: ischemia and reperfusion.
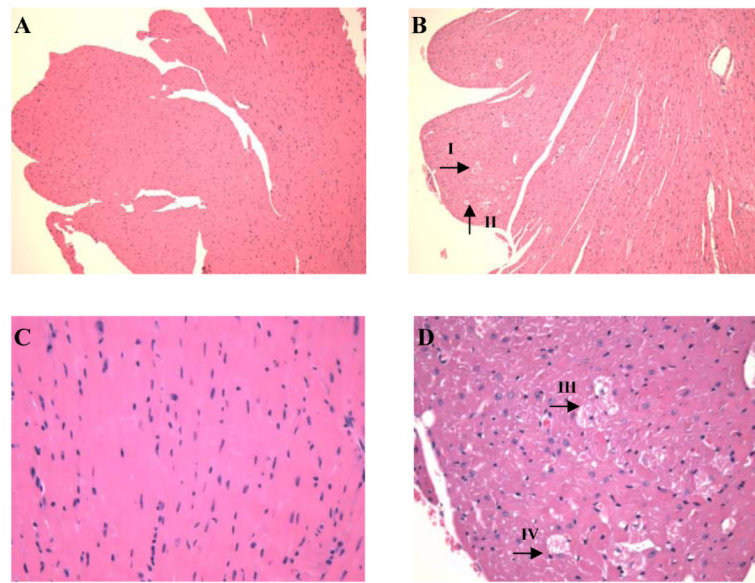
Fig. 2.
Representative H&E staining myocardium images. (A) Normal myocardium: all cardiomyocytes are of uniform color and there are no vacuoles (magnification, ×10); (B) Myocardium following 30 minutes ischemia and 4 hours reperfusion: there is uneven staining of cardiomyocytes. Scattered cardiomyocytes have vacuolated cytoplasm and pyknotic nuclei (arrow I and arrow II) (magnification, ×10); (C) High power view of normal myocardium (magnification, ×40); (D) High power view of injured myocardium after IR (arrow III and arrow IV) (magnification, ×40). H&E: hematoxylin and eosin. IR: ischemia and reperfusion.
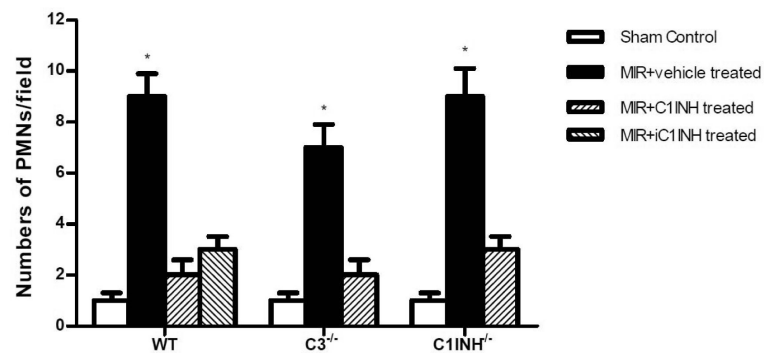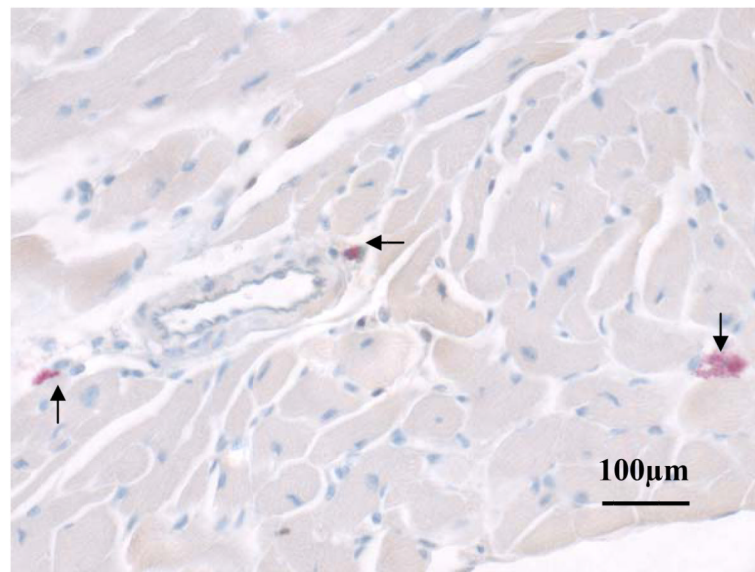
Fig. 3.
Neutrophils infiltration analysis. **A:** Red appeared neutrophils after Naphtol AS-D Chloroacetate Esterase staining and counterstained with Hematoxylin. B: myocardial PMN accumulation within the ischemic areas of the heart after IR injury. Values are means ± SEM, n=4. * P<.05 vehicle–treated mice vs. C1INH or iC1INH treated and sham control mice.
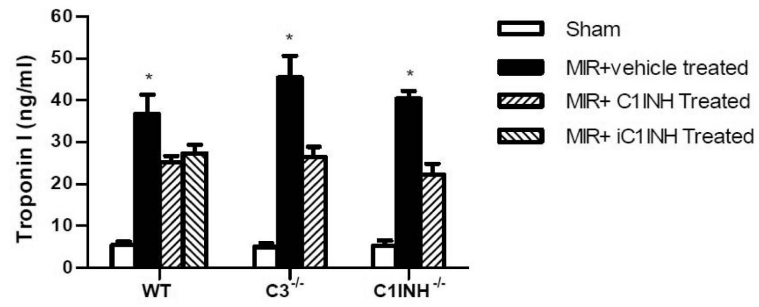
Fig. 4.
The effect of C1INH on plasma cardiac Troponin I levels after myocardial IR injury. Values are means ± SEM, n=10. * P<.05 vehicle–treated mice vs. C1INH or iC1INH treated and sham control mice.