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Cl-IB-MECA Reduces Ischemia/Reperfusion Injury in Mice by Activating the A_3 Adenosine Receptor

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Abbreviations

AR, adenosine receptor; CB-MECA, -N^6-(3-chlorobenzyl)adenosine-5'-*N*-methylcarboxamide; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide; ECG, electrocardiogram; IB-MECA, N^6-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide; [^{125}I]I-AB-MECA; N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5'-*N*-methylcarboxamide; LAD, KO, knock-out; left anterior descending; α-MHC, α-myosin heavy chain; NECA, adenosine-5'-*N*-ethylcarboxamide; PE, polyethylene; WT, wild-type

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Abstract

We used pharmacological agents and genetic methods to determine whether the potent A_3 adenosine receptor (AR) agonist 2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide (Cl-IB-MECA) protects against myocardial ischemia/reperfusion injury in mice via the A_3AR or via interactions with other AR subtypes. Pretreating wild-type (WT) mice with Cl-IB-MECA reduced myocardial infarct size induced by 30 min of coronary occlusion and 24 h of reperfusion at doses (30 and 100 μg/kg) that concomitantly reduced blood pressure and stimulated systemic histamine release. The A_3AR-selective antagonist MRS 1523, but not the A_{2A}AR antagonist ZM 241385, blocked the reduction in infarct size provided by Cl-IB-MECA, suggesting a mechanism involving the A_3AR. To further examine the selectivity of Cl-IB-MECA, we assessed its cardioprotective effectiveness in A_3AR gene 'knock-out' (A_3KO) mice. Cl-IB-MECA did not reduce myocardial infarct size in A_3KO mice *in vivo* and did not protect isolated perfused hearts obtained from A_3KO mice from injury induced by global ischemia and reperfusion. Additional studies using WT mice treated with compound 48/80 to deplete mast cell contents excluded the possibility that Cl-IB-MECA was cardioprotective by releasing mediators from mast cells. These data demonstrate that Cl-IB-MECA protects against myocardial ischemia/reperfusion injury in mice principally by activating the A_3AR.

Introduction

Several different A_3 adenosine receptor (AR) agonists including the N^6-benzyl adenosine-5'-*N*-methylcarboxamide derivatives IB-MECA, Cl-IB-MECA, and CB-MECA, have been shown to be effective at protecting against myocardial ischemia/reperfusion injury in animal models of infarction and myocardial stunning (Auchampach et al., 1997b; Tracey et al., 1997; Tracey et al., 1998; Jordan et al., 1999; Thourani et al., 1999a; Thourani et al., 1999b; Kodani et al., 2001; Takano et al., 2001; Auchampach et al., 2003; Tracey et al., 2003). However, it remains uncertain whether these agents are effective by activating the A_3AR or by non-specific interactions with other AR subtypes. This issue has been difficult to address, since useful A_3AR antagonists have only recently been developed.

The goal of this investigation was to test the cardioprotective effectiveness of Cl-IB-MECA in an *in vivo* mouse model of infarction and in an isolated mouse heart model of global ischemia and reperfusion. A second goal of this investigation was to establish definitively whether Cl-IB-MECA exerts cardioprotection by activating A_3ARs. Our experimental approach involved the use of the rodent A_3AR antagonist MRS 1523 (Li et al., 1998), the potent A_{2A}AR antagonist ZM 241385, and mice with genetic deletion of the A_3AR gene (A_3KO mice; Salvatore et al., 2000). We examined whether cardioprotection provided by Cl-IB-MECA is sensitive to blockade by ZM 241385, since the possibility has been raised that N^6-benzyladenosine-5'-*N*-methylcarboxamide A_3AR agonists may be beneficial in animal models of tissue injury via interactions with the A_{2A}AR rather than the A_3AR (Murphree et al., 2002; Lappas et al., 2005). Finally, since

A_3AR agonists induce mast cell degranulation in rodents (Linden, 1994; Hannon et al., 1995; Van Schaik et al., 1996), we also examined the cardioprotective profile of Cl-IB-MECA in mice that had been depleted of mast cell contents by chronic treatment with compound 48/80.

Materials and Methods

Materials

Cell culture reagents, G418, pcDNA3.1, and Lipofectamine were purchased from Invitrogen (Carlsbad, CA). ZM 241385 was from Tocris Cookson, Inc. (Ellisville, MO), adenosine deaminase was from Roche Applied Science (Indianapolis, IN), and all remaining drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO). N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5'-*N*-methylcarboxamide ([^{125}I]I-AB-MECA) was synthesized and purified by HPLC, as previously described (Olah et al., 1994; Auchampach et al., 1997a). cAMP and histamine radioimmunoassay kits were obtained from GE Healthcare (Piscataway, NJ) and Immunotech (Marseille, France), respectively.

Animals

All experiments were performed with 10-14 week old male mice (weighing ~25-30 g). Wild-type (WT) FVB/N and C57BL/6 mice were purchased from Taconic Farms Inc. (Germantown, NY). A_3KO mice were generated by embryonic stem cell targeting and genotyped by Southern blotting, as previously described (Salvatore et al., 2000). A_3KO mice used had been transferred to the C57BL/6 genetic background by backcrossing greater than 12 generations. Transgenic mice cardiac-specifically overexpressing the A_3AR (A_3tg.1) were generated on the FVB/N genetic background, as described previously (Black et al., 2002). All animals in the study received humane care in accordance with the guidelines established by the Medical College of Wisconsin, which

conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Radioligand Binding Assays and cAMP Accumulation Assays

Competition radioligand binding assays using [^{125}I]I-AB-MECA were conducted with membranes prepared from HEK 293 cells expressing recombinant mouse A_1 or A_3ARs, and cAMP accumulation assays were performed with HEK 293 cells transfected with mouse A_{2A} or A_{2B}ARs, as described previously (Auchampach et al., 1997a; Takano et al., 2001; Kreckler et al., 2006).

***In vivo* Mouse Model of Infarction**

Surgical Preparation. Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.) and placed on a warm heating pad to maintain body temperature at 37 ± 0.3° C. A polyethylene (PE)-60 tube was inserted into the trachea and connected to a mouse ventilator (Hugo Sachs Elektronic Model 845; Hugstetten, Germany). The mice were respirated (tidal volume = 225 μl; rate ~ 100 strokes/min) with room air supplemented with 100% oxygen to maintain blood gases within normal physiological limits. The electrocardiogram (ECG) was obtained with needle electrodes using the limb lead II configuration and recorded continuously using a Powerlab data acquisition system (ADInstruments, Colorado Springs, CO).

A left thoracotomy was performed at the fourth intercostal space to expose the heart, the pericardium was removed, and an 8.0 nylon suture was passed below the left anterior descending (LAD) coronary artery 1-3 mm from the tip of the left atrium with the aid of a dissecting microscope. Myocardial ischemia was induced by tying the suture over a piece of wetted gauze, and reperfusion was initiated by loosening the suture. Successful performance of coronary occlusion and reperfusion was verified by visual inspection (i.e., by the development of a pale color in the distal myocardium upon occlusion and the return of a bright red color due to hyperemia after release) and by observing changes in the ECG (i.e., widening of the QRS complex and ST segment changes). The chest wall was then closed with a 7-0 polypropylene suture with one layer through the chest wall and muscle, and a second layer through the skin and subcutaneous tissue. Subsequently, the mouse was removed from the ventilator and kept in a warm chamber supplied with 100% oxygen. The endotracheal tube was removed after approximately 60 min when the mice began to recover their righting reflex and respiratory rate was ~140 breaths/min.

Experimental Protocol. Following a 30-min stabilization period, all mice were subjected to 30 min of coronary occlusion and 24 h of reperfusion. Cl-IB-MECA or equivalent vehicle (0.2 ml of 50% DMSO in normal saline) was administered as i.v. boluses (tail vein injection) at the doses indicated beginning 10 min before the coronary occlusion. In studies using AR antagonists, the drugs were administered by i.v. injection 15 min before the administration of Cl-IB-MECA. Heart rate was monitored at baseline and during the 30 min occlusion period from the ECG recording.

Measurement of Ischemic Area and Infarct Size. After 24 h of reperfusion, the mice were administered heparin (1 U/g i.p.) and anesthetized with pentobarbital. Following anesthesia, a thoracotomy was performed and the ascending aorta was cannulated with PE-10 tubing. To delineate the ischemic area at risk, the LAD was re-occluded and a 5% solution of phthalo blue dye in normal saline was injected into the aortic root. The heart was excised, washed with PBS, embedded in agarose, and frozen at -20° C. To delineate infarcted tissue, the left ventricle was sliced into 5-6 transverse pieces using a microtome, incubated in a 1% solution of triphenyltetrazolium chloride for 10 min at 37° C to stain viable tissue red, and fixed in 10% formaldehyde overnight. The slices were weighed and photographed from both sides using a dissecting microscope mounted with a SPOT Insight digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The left ventricular, ischemic, and infarcted areas were measured by digital plainimetry. Infarct size is presented as a percentage of the ischemic risk region.

Measurement of Systemic Blood Pressure and Plasma Histamine Levels. Parallel experiments were performed on two separate groups of mice to determine the effect of

Cl-IB-MECA on systemic blood pressure and plasma histamine levels. For blood pressure measurements, the mice were anesthetized with pentobarbital and respirated as described above. A saline-filled cannula (stretched PE-10 tubing) was inserted into the left femoral artery and connected to a pressure transducer (ADInstruments model MLT 0699). Blood pressure was recorded continuously by a PowerLab data acquisition system (ADInstruments).

For measurement of plasma histamine levels, the mice were anesthetized, connected to the respirator, and subjected to a thoracotomy to expose the heart. Blood samples (0.15 ml) were collected by cardiac puncture into EDTA-coated syringes containing the histamine N-methyltransferase inhibitor SKF-91488 (final concentration = 20 μM) at baseline and at 15, 30, and 45 min after the administration of Cl-IB-MECA. The blood samples were centrifuged (1,000 x g for 10 min at 4° C) and the plasma was analyzed for histamine content by radioimmunoassay.

Langendorff-Perfused Mouse Heart Model of Global Ischemia and Reperfusion

Experimental Setup. Mice were anesthetized with pentobarbital. As soon as deep anesthesia was achieved, the hearts were excised quickly and arrested in ice-cold perfusion buffer. The hearts were cannulated (20 gauge stainless steel) via the aorta and perfused by the Langendorff method using Krebs-Henseleit buffer containing (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, EDTA 0.5, and glucose 11. The buffer was equilibrated with 95% O_2 / 5% CO_2 at 37° C to maintain the pH at 7.4. The left ventricle was ventilated with a PE drain, and a fluid-filled balloon

was inserted into the ventricle via the mitral valve. The balloon was connected to a pressure transducer for continuous measurement of left ventricular pressure. The hearts were immersed in perfusate maintained at 37° C, and the balloon was inflated to a diastolic pressure of ~5-10 mmHg. Coronary flow was monitored by an in-line flow probe connected to a flowmeter (Transonics Systems Inc, Model T206 Ithaca, NY). The left ventricular pressure signal was acquired by a PowerLab data acquisition system (ADInstruments) and processed to yield heart rate and left ventricular dP/dt.

Protocol. Isolated hearts were perfused for 20 min to allow for stabilization and then perfused for an additional 15 min while pacing at 420 beats/min (ventricular pacing, 2 ms square waves, 20% above threshold). Baseline measurements of function were acquired immediately before subjecting the hearts to 20 min of no-flow global ischemia and 45 min of reperfusion. To examine the effect of Cl-IB-MECA on functional recovery, hearts were perfused with buffer containing 100 nM Cl-IB-MECA for 10 min before ischemia and throughout the reperfusion period.

Statistical Analysis

K_i and EC_{50} values are reported as geometrical means with corresponding 95% confidence intervals. All other data are presented as arithmetic means ± SEM. Hemodynamic variables and plasma histamine concentrations were analyzed by two-way repeated measures ANOVA (time and treatment) to determine whether there was a main effect of time, a main effect of treatment, or a time-treatment interaction. If global tests showed a main effect or interaction, post-hoc tests were performed using unpaired

or paired analyses, as appropriate. Infarct size, ischemic area at risk size, and left ventricular functional recoveries in the isolated heart studies were compared by one-way ANOVA followed by a Student's *t*-test with the Bonferroni correction or an unpaired *t*-test, as appropriate.

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Results

Selectivity of Cl-IB-MECA for recombinant mouse ARs

Preliminary assays were conducted with transfected HEK 293 cells to determine the affinity and selectivity of Cl-IB-MECA and its parent compound IB-MECA for recombinant mouse ARs. Competition radioligand binding assays were performed to compare the binding affinity of the ligands for A_1 and A_3ARs. Since high affinity agonist binding to A_{2A}ARs can not be accurately measured in heterologous expression systems due to poor receptor coupling (Murphree et al., 2002) and since agonist radioligands are not available for the A_{2B}AR, we determined the potency of Cl-IB-MECA and IB-MECA to stimulate cAMP production in HEK 293 cells expressing mouse A_{2A} or A_{2B}ARs. On the basis of radioligand binding analysis, Cl-IB-MECA was determined to be 219-fold selective at binding to the high-affinity form of the mouse A_3AR versus the high-affinity form of the mouse A_1AR (Fig. 1). The dissociation constants were calculated to be 35 nM (95% confidence intervals = 33, 36 nM) for the mouse A_1AR and 0.18 nM (0.16, 0.19 nM) for the mouse A_3AR. In comparison, IB-MECA was 68-fold more potent at binding to the mouse A_3AR versus the mouse A_1AR (5.9 nM [4.4, 7.9 nM] versus 0.087 nM [0.078, 0.098 nM], respectively). In cAMP assays (Fig. 1), Cl-IB-MECA and IB-MECA displayed low potency at stimulating cAMP production in HEK 293 cells expressing either A_{2A} or A_{2B}ARs. In fact, accurate EC_{50} values could not be calculated for these ligands due to failure to reach maximal responses at their solubility limit of 10 μM. It is reasonable to estimate that Cl-IB-MECA was at least 1,000-fold less potent at stimulating cAMP production in A_{2A}AR-expressing cells compared to the potent A_{2A}AR agonist CGS 21680. In A_{2B}AR expressing cells, Cl-IB-MECA was considerably less

potent at stimulating cAMP production compared to adenosine-5'-*N*-ethylcarboxamide (NECA), the most potent A_{2B}AR agonist currently known.

Dose-Dependent Reduction in Infarct Size by Cl-IB-MECA

We initially conducted a dose-response study (10 – 100 μg/kg) with Cl-IB-MECA examining its ability to reduce infarct size in WT FVB/N mice in response to 30 min of LAD occlusion and 24 h of reperfusion. In separate groups of mice, we assessed the effect of Cl-IB-MECA on systemic blood pressure and plasma histamine concentrations. The data are presented in Fig. 2. In WT FVB/N mice, administration of Cl-IB-MECA produced a dose-dependent reduction in infarct size that was significantly different from vehicle at 30 μg/kg. At a dose of 100 μg/kg, the magnitude of the reduction in infarct size provided by Cl-IB-MECA was 36% compared to vehicle-treated mice (30 ± 3% of the ischemic area at risk vs. 47 ± 2%, respectively). Treatment with Cl-IB-MECA had no effect on heart rate during the 30-min ischemic period (Table 1).

In parallel experiments, we observed that Cl-IB-MECA also reduced blood pressure and significantly increased plasma histamine levels (Fig. 2). The decrease in blood pressure was transient peaking at 10 min after Cl-IB-MECA administration (15% reduction by 100 μg/kg Cl-IB-MECA) and returned to normal within 30 min. The increase in plasma histamine concentration was most prominent at a dose of 100 μg/kg (from 151 ± 16 to 1,763 ± 298 nM 15 min after Cl-IB-MECA administration), although a small increase was also detected at a dose of 30 μg/kg (from 150 ± 11 to 358 ± 132 nM).

Cl-IB-MECA Reduced Infarct Size in A_3AR Transgenic Mice

We have previously created transgenic mice that cardiac-specifically over-express the A_3AR using the α-myosin heavy chain (α-MHC) gene promoter and have shown that these mice demonstrate increased tolerance to ischemia/reperfusion injury (Black et al., 2002; Cross et al., 2002), supporting the theory that the A_3AR is capable of coupling to protective signaling pathways in cardiomyocytes. In the present study, we examined whether administration of a cardioprotective dose of Cl-IB-MECA provides additional protection in A_3AR transgenic mice. We used A_3tg.1 mice containing a single copy of the α-MHC-A_3AR transgene expressing 12.5 ± 3.2 fmol/mg of the high affinity, G protein-coupled form of the A_3AR in the heart (Black et al., 2002). As shown in Fig. 3, administration of 100 μg/kg of Cl-IB-MECA decreased infarct size in A_3tg.1 mice from 35 ± 4% to 25 ± 3 %. Administration of Cl-IB-MECA caused a 10% reduction in heart rate after 30 min of ischemia (Table 1) that is likely due to activation of exogenously expressed A_3ARs in pacemakers cells (Black et al., 2002). Although Cl-IB-MECA produced additional cardioprotection in A_3tg.1 mice, there was no difference in infarct size between A_3tg.1 and WT mice treated with Cl-IB-MECA reported in Figure 1.

Cl-IB-MECA Reduces Infarct Size *in vivo* via a Specific Interaction with the A_3AR

To provide evidence whether or not Cl-IB-MECA effectively reduces infarct size via an A_3AR-mediated mechanism, we initially assessed the effect of MRS 1523 (Fig. 4). MRS 1523 is a dihydropyridine compound developed by Li and colleagues (Li et al., 1998) as a rat A_3AR antagonist (K_i = 113 ± 12 nM for rat A_3ARs; 140-fold selectivity). In a

previous study (Kreckler et al., 2006) we determined that MRS 1523 binds to mouse A_3ARs with high affinity (K_i = 731 nM) and with moderate selectivity versus the A_1AR (11-fold), but excellent selectivity versus A_{2A} and A_{2B}ARs (at least 1,000-fold). As shown in Fig. 4, pretreatment with 2 mg/kg of MRS 1523 blocked the reduction in infarct size produced by 100 μg/kg of Cl-IB-MECA. This dose of MRS 1523 also inhibited the ability of Cl-IB-MECA to increase plasma histamine concentrations and reduce blood pressure (Fig. 4).

We subsequently examined the effect of the A_{2A}AR antagonist ZM 241385 on cardioprotection provided by Cl-IB-MECA. Mice were pretreated with 2 mg/kg of ZM 241385 15 min before the administration of 100 μg/kg of Cl-IB-MECA. This dose of ZM 241385 was based on the study of Ohta and Sitkovsky (Ohta and Sitkovsky, 2001). As shown in Fig. 5, we were surprised to observe that 2 mg/kg of ZM 241385 blocked the beneficial effect of Cl-IB-MECA on infarct size. However, we observed that this dose of ZM 241385 also antagonized the ability of Cl-IB-MECA to increase plasma histamine levels and to reduce blood pressure (Fig. 5.), suggesting that ZM 241385 at a dose of 2 mg/kg is sufficient to block A_3ARs. We subsequently tested ZM 241385 at a lower dose of 0.5 mg/kg. This dose only partially antagonized the hemodynamic and histamine-releasing actions of Cl-IB-MECA (Fig. 5), but blocked completely the hypotensive response induced by iv administration of 30 μg/kg of the A_{2A}AR agonist CGS 21680 (Supplemental Figure 1). As shown in Fig. 5, treatment with 0.5 mg/kg of ZM 241385 failed to block the infarct size-reducing effect of Cl-IB-MECA.

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Finally, we examined whether Cl-IB-MECA reduces infarct size in A_3KO mice. We used A_3KO mice made congenic on the C57BL/6 genetic background by back-crossing greater than 12 generations to the pure strain. Comparisons were made with WT C57BL/6 mice. Similar to FVB/N mice, pretreating WT C57BL/6 mice with 100 μg/kg of Cl-IB-MECA reduced infarct size 54% (vehicle = 52 ± 4% of the risk region; Cl-IB-MECA = 24 ± 2%), decreased systemic blood pressure 15% (with no effect on heart rate), and increased plasma histamine levels (Fig. 6 and Table 1). The onset of the hypotensive effect of Cl-IB-MECA in C57BL/6 mice was immediate (within 5 min), but more sustained (> 45 min) compared to that produced in FVB/N mice. Infarct size induced by 30 min of occlusion and 24 h of reperfusion was similar in magnitude in A_3KO mice (56 ± 4% in A_3KO mice) compared to WT mice (Fig. 6). However, unlike WT mice administration of Cl-IB-MECA (100 μg/kg) to A_3KO mice subjected to ischemia and reperfusion did not reduce infarct size (infarct size as a percent of the risk region was 56 ± 2%; Fig. 6); similarly it did not evoke systemic release of histamine (Fig. 6). These results concur with the data obtained with AR antagonists, and indicate that Cl-IB-MECA reduces infarct size by an A_3AR-mediated mechanism. Curiously, we observed that Cl-IB-MECA continued to reduce blood pressure in A_3KO mice, although the kinetics of the response was different. Rather than a rapid but sustained hypotensive effect, Cl-IB-MECA produced a delayed reduction in blood pressure that became apparent 15 min after drug administration (Fig. 6). Cl-IB-MECA produced a similar hypotensive profile in WT C57BL/6 mice pretreated with 2 mg/kg of the A_3AR antagonist MRS 1523 as well as in A_3KO mice pretreated with a high dose of ZM 241385 (2 mg/kg) that presumably blocks all four AR subtypes (Fig. 6).

Cl-IB-MECA Protects Isolated Mouse Hearts from Global Ischemia/Reperfusion Injury via the A_3AR

It has previously been reported that Cl-IB-MECA improves reperfusion contractile function of isolated mouse hearts subjected to global ischemia/reperfusion injury (Harrison et al., 2002; Peart et al., 2002). In this study, we examined whether the protective effect of Cl-IB-MECA in isolated hearts involves a specific interaction with the A_3AR. Langendorff-perfused hearts from WT or A_3KO mice were subjected to 20 min of normothermic no-flow global ischemia and 45 min of reperfusion; the hearts were treated throughout the experiment with either vehicle or 100 nM Cl-IB-MECA. Post-ischemic recovery of left ventricular function was assessed via a fluid-filled balloon placed in the left ventricle. As shown in Fig. 7, indices of left ventricular contractile function including developed pressure, +dP/dt, and -dP/dt returned to ~50% of baseline values at 45 min of reperfusion in both vehicle-treated WT and A_3KO hearts, indicating that 20 min of global ischemia followed by reperfusion results in significant contractile dysfunction in our isolated mouse heart model system and that ischemic tolerance is similar between WT and A_3KO mice. Treatment with 100 nM Cl-IB-MECA significantly improved reperfusion contractile function from ~50 to ~75% of baseline values in WT mouse hearts; this beneficial effect of Cl-IB-MECA was not apparent in hearts from A_3KO mice (Fig. 7). In both WT and A_3KO hearts, treatment with Cl-IB-MECA increased reperfusion coronary flow to pre-ischemic levels. Collectively, these results suggest that cardioprotection provided by Cl-IB-MECA in isolated mouse hearts is the result of A_3AR activation.

Cardioprotection Provided by Cl-IB-MECA is not Mediated by the Release of Stored Mediators from Mast Cells

Additional experiments were conducted to test the possibility that Cl-IB-MECA induces cardioprotection due to its ability to stimulate mast cells to degranulate. Theoretically, Cl-IB-MECA could provide cardioprotection by depleting mast cells of noxious mediators that contribute to injury during ischemia or induce adaptive changes that provide protection similar to that of myocardial preconditioning (Linden, 1994). To address this possibility, we conducted a series of experiments testing the cardioprotective effectiveness of Cl-IB-MECA in mice that had been treated chronically with compound 48/80 to deplete mast cells of stored mediators. Compound 48/80 was administered to WT mice intraperitoneally twice daily for five days beginning with a dose of 1 mg/kg on the first day, followed by an increment of 1 mg/kg per day, to a maximum of 5 mg/kg on the final day (Riley and West, 1955). On the following day (day 6), the effectiveness of Cl-IB-MECA was examined in both the *in vivo* mouse model of infarction and the isolated mouse heart model of global ischemia and reperfusion. As shown in Figs. 8 and 9, depletion of mast cell contents with compound 48/80 did not attenuate protection provided by Cl-IB-MECA (100 μg/kg) in intact mice or in isolated mouse hearts. In the *in vivo* model, treatment with Cl-IB-MECA produced a 30% reduction in infarct size in compound 48/80-treated mice (Fig. 8). In the isolated mouse heart model, Cl-IB-MECA increased post-ischemic recovery of left ventricular developed pressure from 54 to 71% of pre-ischemic baseline values after treatment with compound 48/80 (Fig. 9). Effective depletion of mast cell mediators was confirmed by the observation that Cl-IB-MECA did

not increase plasma histamine concentrations or reduce blood pressure in compound 48/80-treated mice (Fig. 8).

Discussion

We observed that pretreatment with the A_3AR-selective agonist Cl-IB-MECA produced a dose-dependent reduction in infarct size in mice subjected to 30 min of coronary artery occlusion and 24 h of reperfusion. This protective effect of Cl-IB-MECA was apparent at a dose of 30 μg/kg and produced a ~35% reduction in infarct size at a dose of 100 μg/kg. We subsequently observed that cardioprotection provided by Cl-IB-MECA was blocked completely in mice pretreated with the rodent A_3AR antagonist MRS 1523 (Li et al., 1998), suggesting involvement of the A_3AR. To further explore the cardioprotective profile of Cl-IB-MECA, we examined its effectiveness in an isolated mouse heart model of 20 min normothermic global ischemia and 45 min of reperfusion. In hearts from WT mice, treatment with 100 nM Cl-IB-MECA significantly improved several indices of left ventricular post-ischemic contractile function including developed pressure; this protective effect of Cl-IB-MECA was lost completely using hearts obtained from A_3KO mice. Collectively, these data are the first to provide definitive evidence that an A_3AR agonist provides protection against myocardial ischemia/reperfusion injury by activating the A_3AR. These data support the concept that the A_3AR plays a protective role in the ischemic myocardium.

Several previous studies have demonstrated that A_3AR agonists are effective at protecting against myocardial ischemia/reperfusion injury (Auchampach et al., 1997b; Tracey et al., 1997; Tracey et al., 1998; Jordan et al., 1999; Thourani et al., 1999a; Thourani et al., 1999b; Kodani et al., 2001; Takano et al., 2001; Auchampach et al., 2003; Tracey et al., 2003). For example, we have reported that pretreatment with IB-

MECA effectively reduced infarct size, attenuated myocardial stunning, and induced the late phase of ischemic preconditioning in a clinically relevant conscious rabbit model or a barbital-anesthetized dog model of regional ischemia/reperfusion injury (Auchampach et al., 1997b; Kodani et al., 2001; Takano et al., 2001; Auchampach et al., 2003). In the dog model, IB-MECA was shown to reduce infarct size even when administered immediately before release of the occlusion, demonstrating that it was also effective at reducing reperfusion-mediated injury (Auchampach et al., 2003). Similar results have been obtained by others using more selective A_3AR agonists in *in vivo* models as well as *in vitro* models of ischemia/reperfusion injury including isolated rodent hearts (Tracey et al., 1997; Tracey et al., 1998; Thourani et al., 1999a; Thourani et al., 1999b; Tracey et al., 2003). However, even though the A_1AR was excluded using highly selective antagonists (Tracey et al., 1997; Tracey et al., 1998; Kodani et al., 2001; Tracey et al., 2003), involvement of the A_3AR could not be proven definitively in these studies due to the lack of selective antagonists. Using the most recently developed rodent A_3AR antagonist MRS 1523 as well as A_3KO mice, we have now confirmed that protection against ischemia/reperfusion injury provided by Cl-IB-MECA in the mouse is mediated predominantly via the A_3AR.

We conducted additional experiments using ZM 241385 to address the possibility that Cl-IB-MECA may be producing cardiac protection by a mechanism involving the A_{2A}AR. We chose to address this issue, since administration of low doses of A_{2A}AR agonists has been reported to reduce myocardial reperfusion injury by suppressing inflammatory responses (Glover et al., 2005; Yang et al., 2005) and since it has been suggested that

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N^6-benzyladenosine-5'-*N*-methylcarboxamide A_3AR agonists may have higher affinity for the A_{2A}AR than originally appreciated (Murphree et al., 2002). Moreover, Yang and colleagues (Yang et al., 2003) have presented the hypothesis, in abstract form, that A_3AR agonists may act by increasing adenosine production, which subsequently provides tissue protection via the A_{2A}AR by an anti-inflammatory mechanism. ZM 241385 is a highly potent A_{2A}AR antagonist with low affinity for the mouse A_3AR (Palmer et al., 1995; Kreckler et al., 2006). Surprisingly, we found that pretreating mice with 2 mg/kg of ZM 241385 (Ohta and Sitkovsky, 2001) was also capable of blocking the infarct size-reducing effect of Cl-IB-MECA in our *in vivo* mouse model. However, at this dose ZM 241385 also inhibited the effect of Cl-IB-MECA to increase plasma histamine levels, a response in rodents known to be due to A_3AR-mediated degranulation of mast cells (Linden, 1994; Van Schaik et al., 1996), confirming that a lower dose of ZM 241385 was required to prevent blockade of A_3ARs. Using a dose of 0.5 mg/kg of ZM 241385 that only partially inhibited Cl-IB-MECA-induced histamine release (but effectively blocked the hypotensive actions of the A_{2A}AR agonist CGS 21680; Supplemental Figure 1), we found that Cl-IB-MECA continued to produce a significant reduction in infarct size. These results exclude the involvement of the A_{2A}AR in mediating the cardioprotective effects of Cl-IB-MECA and provide further evidence in support of an A_3AR-mediated mechanism. Perhaps more importantly, these results demonstrate that ZM 241385 exhibits relatively high *in vivo* potency in rodents such that doses lower than 2 mg/kg should be used to selectively block A_{2A}ARs.

We have previously reported in conscious rabbits and dogs that IB-MECA effectively reduced ischemia/reperfusion injury at doses that produced no changes in systemic hemodynamic parameters (Auchampach et al., 1997b; Kodani et al., 2001; Takano et al., 2001; Auchampach et al., 2003). This was an important observation, since the clinical use of adenosine as well as subtype-selective agonists for A_1 and A_{2A}ARs for treating ischemia/reperfusion injury carry the risk of hemodynamic side-effects including hypotension, bradycardia, and atrioventricular blockade due to activation of A_1ARs expressed in conducting tissue and A_{2A}ARs in vascular smooth muscle cells. In rodent species (but not in other species), however, it is well-known that activation of A_3ARs reduces blood pressure due to the actions of vasoactive mediators released from mast cells (Linden, 1994; Hannon et al., 1995; Fozard et al., 1996). Indeed, in the present investigation, we observed that administration of 100 μg/kg of Cl-IB-MECA produced an immediate (within 1-2 min) 10-15% reduction in blood pressure, which correlated with increased plasma histamine concentrations, but was absent in compound 48/80-treated mice. Interestingly, however, we observed that the kinetic profile of Cl-IB-MECA to reduce blood pressure differed between the two strains of mice that we used in our studies, in that the response was transient in FVB/N mice (~15-20 min) but more sustained (at least 45 min) in C57BL/6 mice. Since Cl-IB-MECA continued to produce a delayed reduction in blood pressure in WT or A_3KO mice pretreated with MRS 1523 or a non-selective dose of ZM 241385 (2 mg/kg), we conclude that Cl-IB-MECA reduced blood pressure in C57BL/6 mice by a mechanism that may not be receptor-dependent.

We did not address the specific mechanism by which Cl-IB-MECA exerted cardioprotection, although we did determine in that it is independent of mast cell degranulation. We have previously shown that the infarct size-reducing effects of IB-MECA are blocked by glibenclamide (Auchampach et al., 1997b; Auchampach et al., 2003), implicating involvement of ATP-sensitive potassium channels. We have also determined in preliminary studies using our *in vivo* mouse model of infarction and A_3KO bone marrow chimeric mice that Cl-IB-MECA appears to reduce injury when administered at the time of reperfusion by an effect on bone marrow-derived cells, suggesting an anti-inflammatory mechanism (Ge et al., 2004). We predict, therefore, that A_3AR agonists function by different mechanisms to protect against ischemia/reperfusion injury depending on whether they are administered prior to ischemia or during reperfusion. While it is unlikely to be a significant factor since we observed protection in the isolated heart model and in compound 48/80-treated mice, it is important to note that the hypotensive action produced by Cl-IB-MECA in the present investigation may have contributed to the reduction in infarct size in the *in vivo* studies.

One aspect of our ischemia/reperfusion studies using A_3KO mice requires particular attention. In an earlier study, we reported that infarct size was smaller in A_3KO mice subjected to 30 min of coronary artery occlusion and 24 h of reperfusion suggesting that deletion of the A_3AR gene induces an unexpected cardioprotective phenotype (Guo et al., 2001). However, this earlier study was conducted with A_3KO mice on a mixed background of three different genetic strains (C57BL/6, sv129, and D2). Furthermore, controls used in this previous study were not littermates, but were from a separate line

of mice interbred between the three different genetic strains. This line of control mice was considered to be the best available at that time. Based on the results of the present investigation using congenic (C57BL/6) A_3KO mice, we conclude that genetic deletion of the A_3AR gene itself has no effect on ischemic tolerance and that the results of our earlier study are likely explained by differences in the genetic background of the mice rather than specific deletion of the A_3AR gene (Guo et al., 2001). Harrison and colleagues (Harrison et al., 2002) reached a similar conclusion regarding A_3KO mice.

In summary, we have demonstrated that pretreatment with the A_3AR agonist Cl-IB-MECA protects against ischemia/reperfusion injury in mice, and that this occurs through an A_3AR-mediated mechanism that is independent of mast cell degranulation. These results further support the contention that therapeutic strategies targeting the A_3AR could be a novel and useful approach for protection of the ischemic myocardium.

Acknowledgements

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Footnotes

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Legends for Figures

Figure 1. **A.** and **B.** Competition for [^{125}I]I-AB-MECA binding to HEK 293 cell membranes expressing mouse A_1 or A_3ARs by Cl-IB-MECA (**A**) or IB-MECA (**B**). Binding is plotted as a fraction of specific binding in the absence of the competitors. The specific binding data was fitted to one (A_1AR) or two (A_3AR) binding sites and dissociation constants for the high affinity sites were calculated, as described previously (Auchampach et al., 1997a; Black et al., 2002). Values are the mean ± SEM of triplicate determinations. Protein, 50 μg/incubation; [^{125}I]I-AB-MECA, ~0.3 nM/incubation. **C.** and **D.** cAMP accumulation in HEK 293 cells expressing recombinant mouse A_{2A} (**C**) or A_{2B}ARs (**D**) in response to increasing concentrations of AR agonists. EC_{50} values were calculated when maximal responses were achieved using the following equation: $E = E_{max}*C/C+EC_{50}$, where E is the cAMP concentration observed at concentration C and E_{max} is the maximal cAMP concentration. Values are the mean ± SEM of triplicate determinations. K_i and EC_{50} values are presented as geometrical means with 95% confidence intervals.

Figure 2. **A.** Myocardial infarct size expressed as a percentage of the risk region induced by 30 min of LAD coronary occlusion and 24 h of reperfusion in WT FVB/N mice treated with increasing concentrations of Cl-IB-MECA. Cl-IB-MECA was administered i.v. 10 min before coronary occlusion. Filled circles indicate the mean ± SEM. Open circles indicate infarct size in individual animals. *$p < 0.05$ versus the vehicle-treated group. Risk region size ranged from 38 ± 2 to 41 ± 2% of the left

ventricle and was not significantly different among the treatment groups. **B.** and **C.** The effect of increasing doses of Cl-IB-MECA on mean arterial blood pressure expressed as a percentage of baseline and plasma histamine levels (nM) in FVB/N mice. Baseline mean arterial blood pressure was 88 ± 2 mmHg. All values are the mean ± SEM. In **B** and **C**, n = 6-8 mice/group. *$p < 0.05$ versus vehicle or time 0.

Figure 3. A. Myocardial infarct size (mean ± SEM; n = 8 mice/group) expressed as a percentage of the risk region induced by 30 min of LAD coronary occlusion and 24 h of reperfusion in transgenic mice that cardiac-specifically over-expressed the A_3AR (A_3ARtg.1) treated 10 min before coronary occlusion (i.v.) with either vehicle or 100 μg/kg Cl-IB-MECA. *$p < 0.05$ versus the vehicle-treated group. Risk region size was 38 ± 1 and 40 ± 2% of the left ventricle for the vehicle- and Cl-IB-MECA-treated groups, respectively. **B.** Representative images of heart slices stained with phthalo blue dye and TTC used to assess infarct size.

Figure 4. A. Myocardial infarct size expressed as a percentage of the risk region induced by 30 min of LAD coronary occlusion and 24 h of reperfusion in WT FVB/N mice treated 10 min before the coronary occlusion with vehicle or 100 μg/kg of Cl-IB-MECA in the presence or absence of the A_3AR antagonist MRS 1523. Mice were given 2 mg/kg of MRS 1523 (i.v.) 15 min before the administration of Cl-IB-MECA. Risk region size ranged from 36 ± 3 to 43 ± 2% of the left ventricle and was not significantly different among the treatment groups. **B.** and **C.** Effect of Cl-IB-MECA (100 μg/kg) on mean arterial blood pressure and plasma histamine levels in FVB/N mice pretreated

with 2.0 mg/kg of MRS 1523. Baseline mean arterial blood pressure was 89 ± 3 mmHg. All data are presented as the mean ± SEM. n = 9-10 (**A**) or 6-8 (**B** and **C**) mice/group. *p < 0.05 versus the vehicle-treated group or time 0.

Figure 5. A. Myocardial infarct size expressed as a percentage of the risk region induced by 30 min of LAD coronary occlusion and 24 h of reperfusion in WT FVB/N mice treated 10 min before the coronary occlusion with vehicle or 100 μg/kg of Cl-IB-MECA in the presence or absence of the A_{2A}AR antagonist ZM 241385. Mice were given 0.5 or 2.0 mg/kg of ZM 241385 (i.v.) 15 min before the administration of Cl-IB-MECA. Risk region size ranged from 36 ± 3 to 41 ± 2% of the left ventricle and was not significantly different among the treatment groups. **B.** and **C.** Effect of Cl-IB-MECA (100 μg/kg) on mean arterial blood pressure and plasma histamine levels in FVB/N mice pretreated with 0.5 or 2.0 mg/kg of ZM 241385. Baseline mean arterial blood pressure was 88 ± 3 mmHg. All data are presented as the mean ± SEM. n = 9-10 (**A**) or 6-8 (**B** and **C**) mice/group. *p < 0.05 versus the vehicle-treated group or time 0.

Figure 6. A and **B**. Myocardial infarct size expressed as a percentage of the risk region in WT C57BL/6 mice and A_3KO mice subjected to 30 min of coronary occlusion and 24 h of reperfusion. Cl-IB-MECA was administered i.v. 10 min before coronary occlusion. Risk region size ranged from 37 ± 2 to 42 ± 2% of the left ventricle and was not significantly different among the treatment groups. **C – F**. Mean arterial blood pressure and plasma histamine levels in WT (**C** and **E**) and A_3KO (**D** and **F**) mice after administration of 100 μg/kg Cl-IB-MECA. MRS 1523 (2 mg/kg) or ZM 241385 (2 mg/kg)

were administered to WT or A_3KO mice 15 min before the administration of Cl-IB-MECA in some of the hemodynamic studies. Baseline mean arterial blood pressure was 86 ± 4 mmHg in WT mice and 88 ± 2 mmHg in A_3KO mice. All data are presented as the mean ± SEM. n = 9-12 (**A** and **B**) or 6-8 (**C** and **F**) mice/group. *p < 0.05 versus the vehicle-treated group or time 0.

Figure 7. Recovery of left ventricular (LV) developed pressure (**A**), LV +dP/dt (**B**), LV -dP/dt (**C**), and coronary flow (**D**) expressed as a percentage of baseline of isolated Langendorff-perfused mouse hearts obtained from WT C57BL/6 or A_3KO mice after 20 min of no-flow global ischemia and 45 min of reperfusion. Baseline parameters following equilibration for WT C57BL/6 and A_3KO hearts, respectively, were: LV DP = 140 ± 5 and 149 ± 6 mmHg; LV +dP/dt = 6.7 ± 0.8 and 6.3 ± 0.8 mmHg/ms; LV -dP/dt = 4.3 ± 0.7 and 4.4 ± 0.9 mmHg/ms; coronary flow = 23.1 ± 1.5 and 22.3 ± 2.0 ml/min/g. All data are presented as the mean ± SEM. n = 8-12 hearts/group. *p < 0.05 versus the vehicle-treated group.

Figure 8. A. Effect of Cl-IB-MECA on myocardial infarct size expressed as a percentage of the risk region induced by 30 min of LAD coronary occlusion and 24 h of reperfusion in WT C57BL/6 mice treated chronically with compound 48/80 to deplete mast cells of stored mediators. Vehicle or Cl-IB-MECA (100 μg/kg i.v.) was administered 10 min before the coronary occlusion. Risk region size was 39 ± 1 and 39 ± 2% of the left ventricle for the vehicle- and Cl-IB-MECA-treated groups, respectively. **B.** and **C.** Effect of Cl-IB-MECA (100 μg/kg) on mean arterial blood pressure and

plasma histamine levels in compound 48/80-treated mice. Baseline mean arterial blood pressure was 68 ± 3 mmHg. All data are presented as the mean ± SEM. n = 8 (**A**) or 6-8 (**B** and **C**) mice/group. *p < 0.05 versus the vehicle-treated group.

Figure 9. Recovery of left ventricular (LV) developed pressure (**A**), LV +dP/dt (**B**), LV -dP/dt (**C**), and coronary flow (**D**) expressed as a percentage of baseline of isolated Langendorff-perfused mouse hearts obtained from WT C57BL/6 mice treated with compound 48/80 to deplete mast cell of stored mediators. Baseline parameters following equilibration were: LV DP = 168 ± 7 mmHg; LV +dP/dt = 7.7 ± 0.4 mmHg/ms; LV -dP/dt = 3.6 ± 0.2 mmHg/ms; coronary flow = 28.9 ± 3.3 ml/min/g. All data are presented as the mean ± SEM. n = 8 hearts/group. *p < 0.05 versus the vehicle-treated group.

Table 1. Heart rate during the ischemia/reperfusion experiments.

Treatment	Strain	Genotype	Pre-Occ	Ischemia (min)	
				15	30
Vehicle	FVB/N	WT	380 ± 14	412 ± 10	422 ± 10
Cl-IB-MECA 10 μg/kg	FVB/N	WT	370 ± 9	416 ± 9	415 ± 12
Cl-IB-MECA 30 μg/kg	FVB/N	WT	369 ± 13	397 ± 15	406 ± 11
Cl-IB-MECA 100 μg/kg	FVB/N	WT	385 ± 12	413 ± 16	399 ± 12
Vehicle	FVB/N	WT	382 ± 24	395 ± 25	384 ± 26
Vehicle	FVB/N	A_3tg.1	380 ± 25	383 ± 20	374 ± 16
Cl-IB-MECA 100 μg/kg	FVB/N	A_3tg.1	378 ± 17	345 ± 13*	335 ± 18*
ZM 241385 2.0 mg/kg	FVB/N	WT	376 ± 13	409 ± 12	418 ± 13
MECA 100 μg/kg + ZM 0.5 mg/kg	FVB/N	WT	389 ± 14	411 ± 15	420 ± 14
MECA 100 μg/kg + ZM 2.0 mg/kg	FVB/N	WT	383 ± 15	411 ± 14	421 ± 12
MRS 1523 2.0 mg/kg	FVB/N	WT	369 ± 15	408 ± 6	414 ± 12
MECA 100 μg/kg + MRS 2.0 mg/kg	FVB/N	WT	397 ± 13	417 ± 15	418 ± 8
Vehicle	C57BL/6	$A_3AR^{+/+}$	376 ± 21	402 ± 24	424 ± 20
Vehicle	C57BL/6	$A_3AR^{-/-}$	384 ± 24	399 ± 23	406 ± 16
Cl-IB-MECA 100 μg/kg	C57BL/6	$A_3AR^{+/+}$	378 ± 13	393 ± 10	406 ± 9
Cl-IB-MECA 100 μg/kg	C57BL/6	$A_3AR^{-/-}$	381 ± 21	397 ± 17	395 ± 15

Values are means ± SEM. MECA = Cl-IB-MECA; * P< 0.05 vs. the vehicle-treated group for each mouse strain by two-way repeated measures ANOVA (time and drug treatment) and Student's *t*-test with the Bonferroni correction. n=7 to 10/group.

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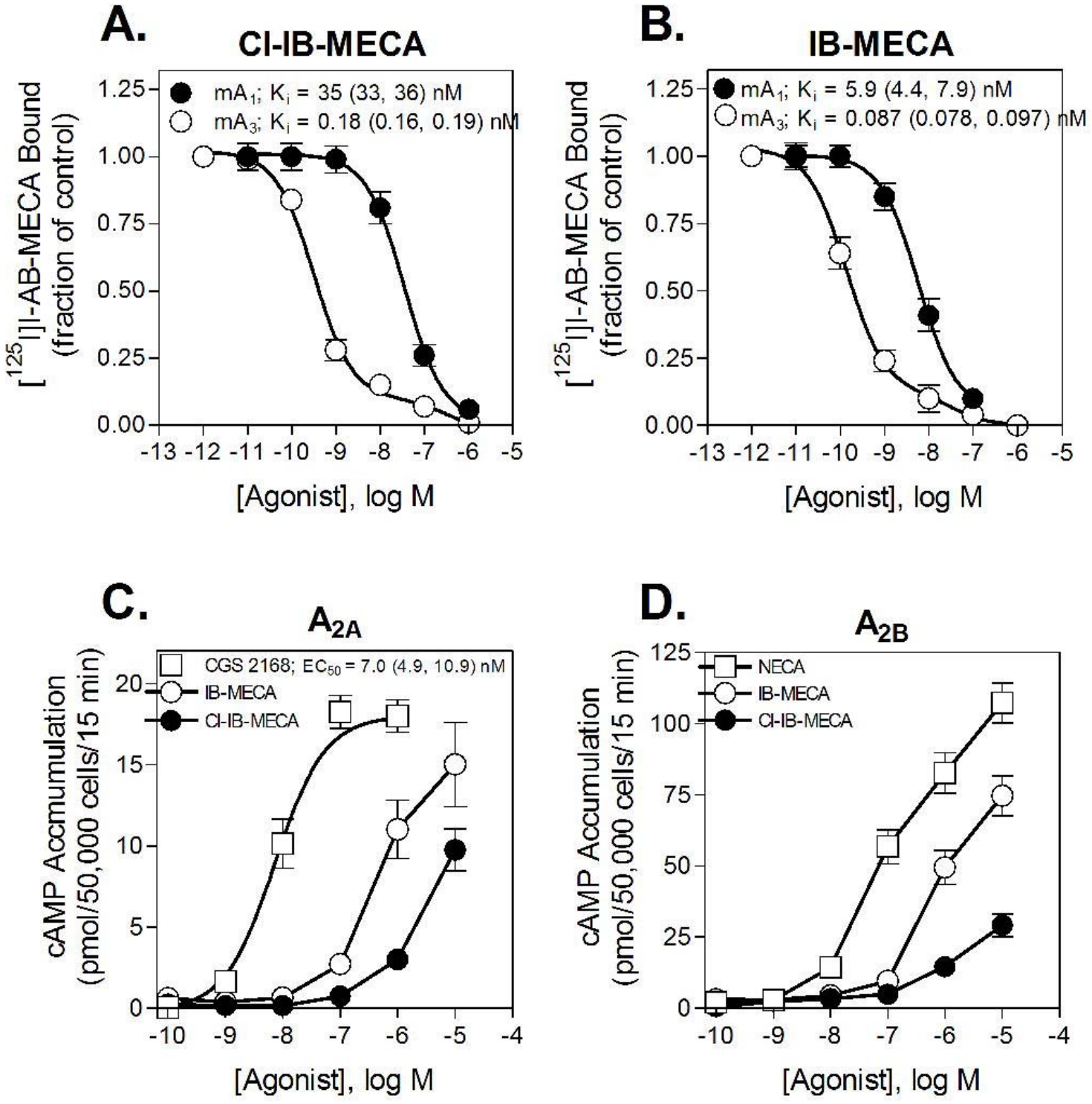

Figure 1

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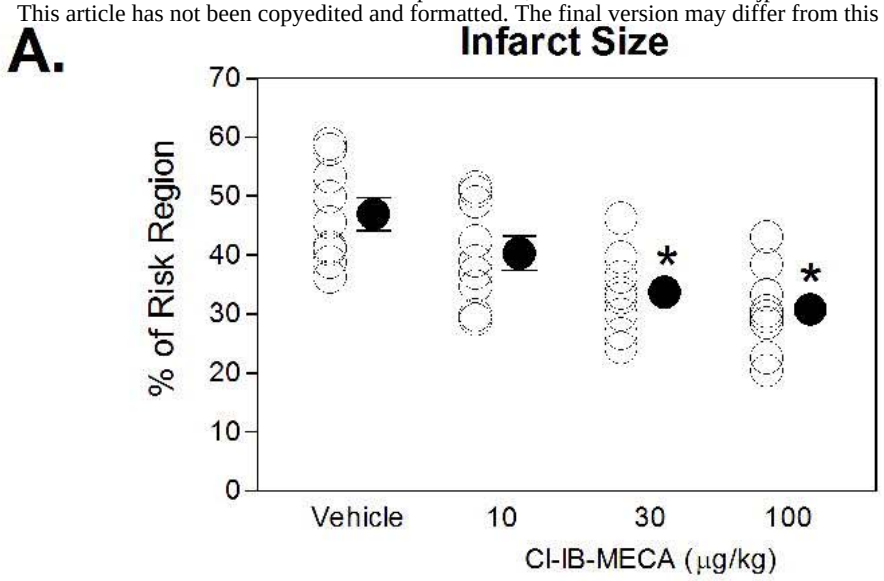

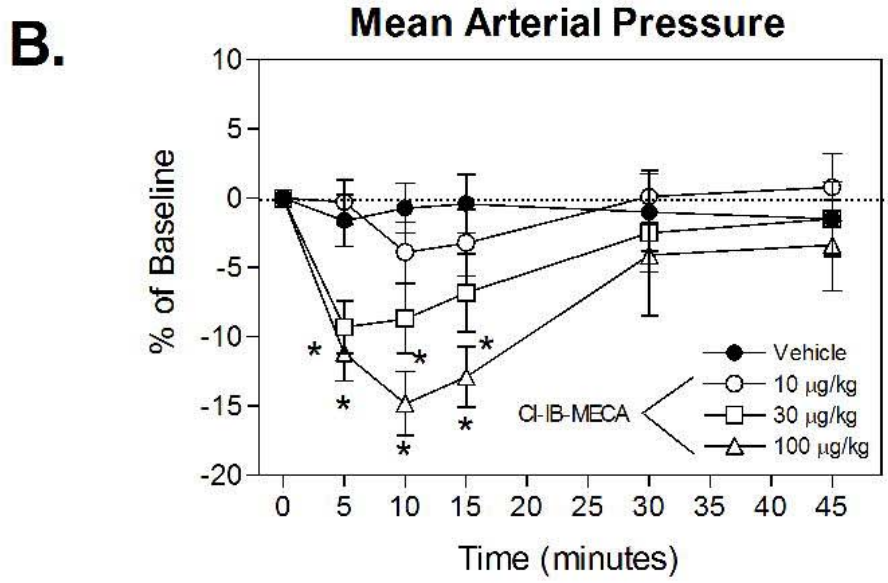

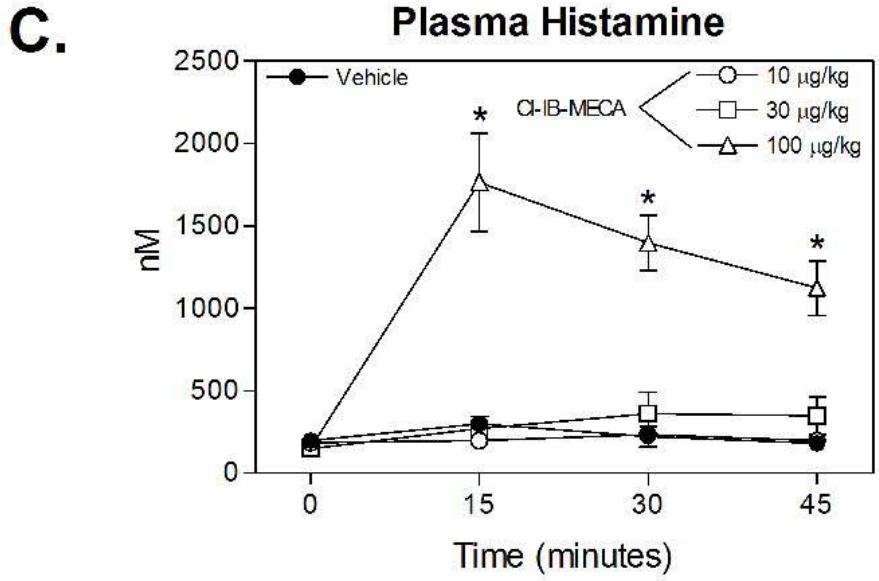

Figure 2

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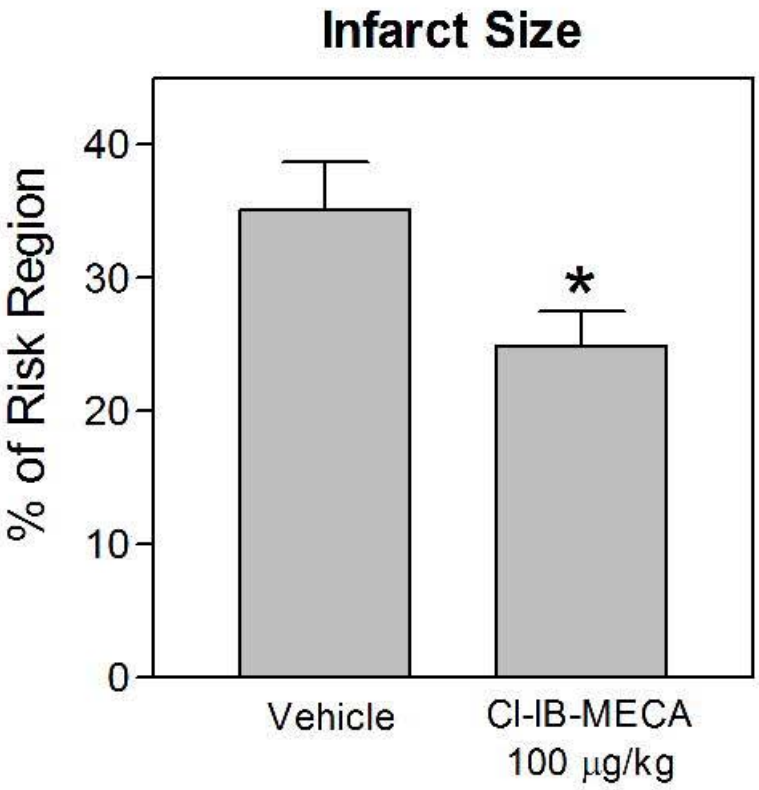

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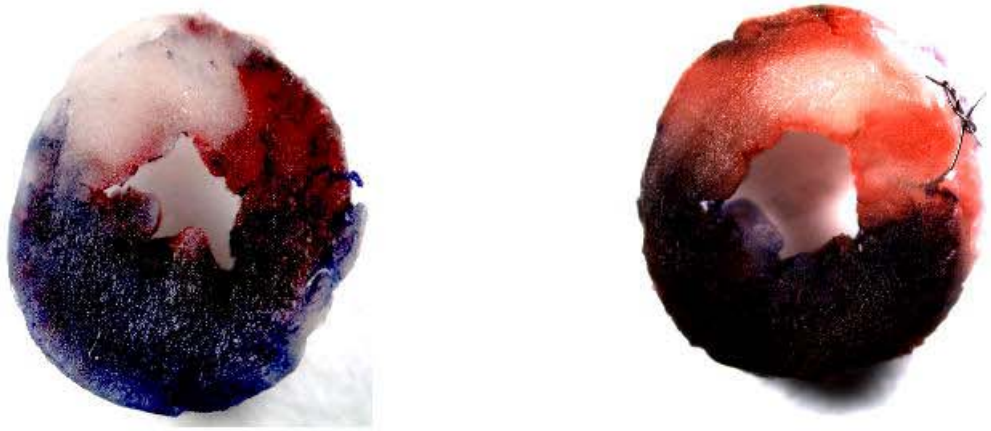

Vehicle **Cl-IB-MECA**

Figure 3

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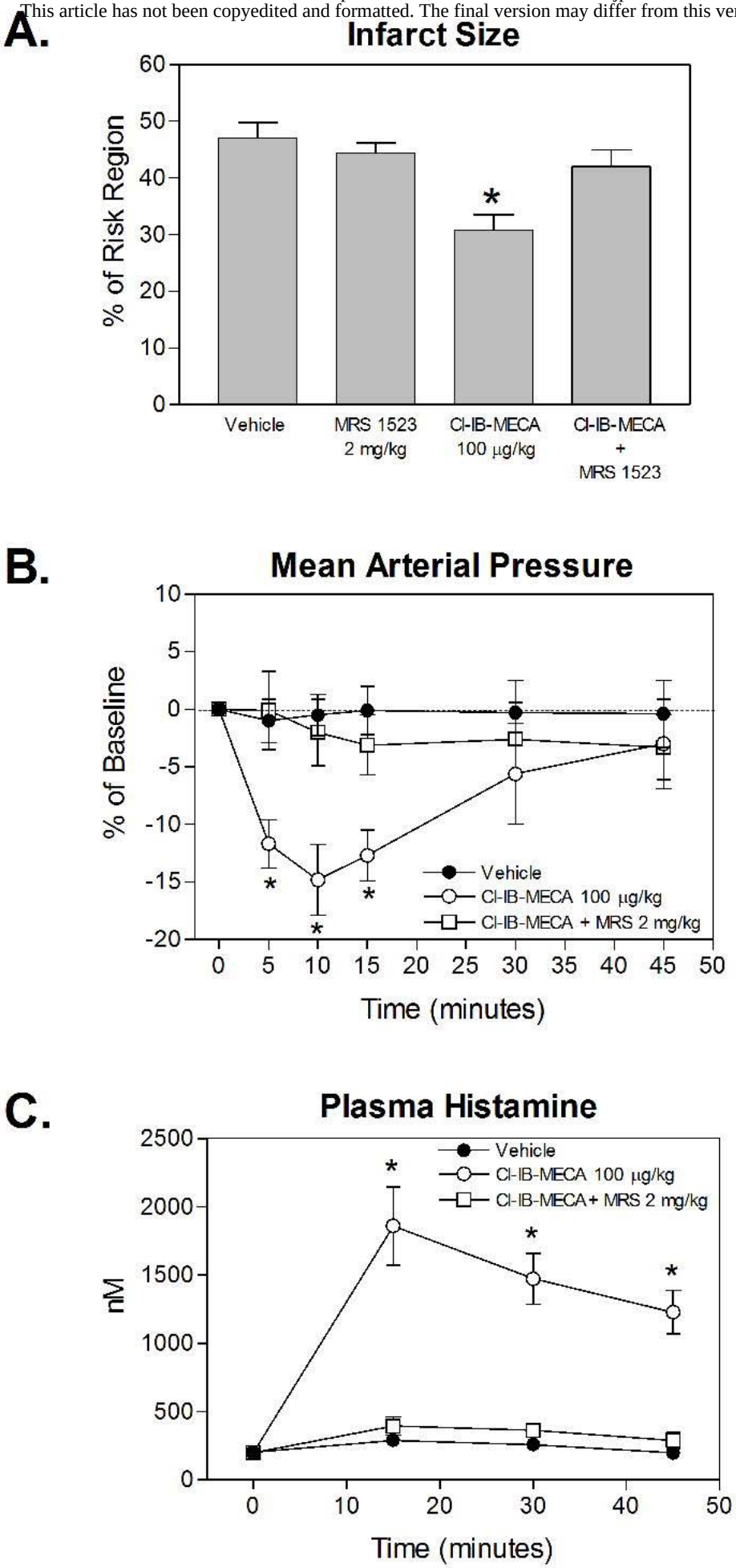

Figure 4

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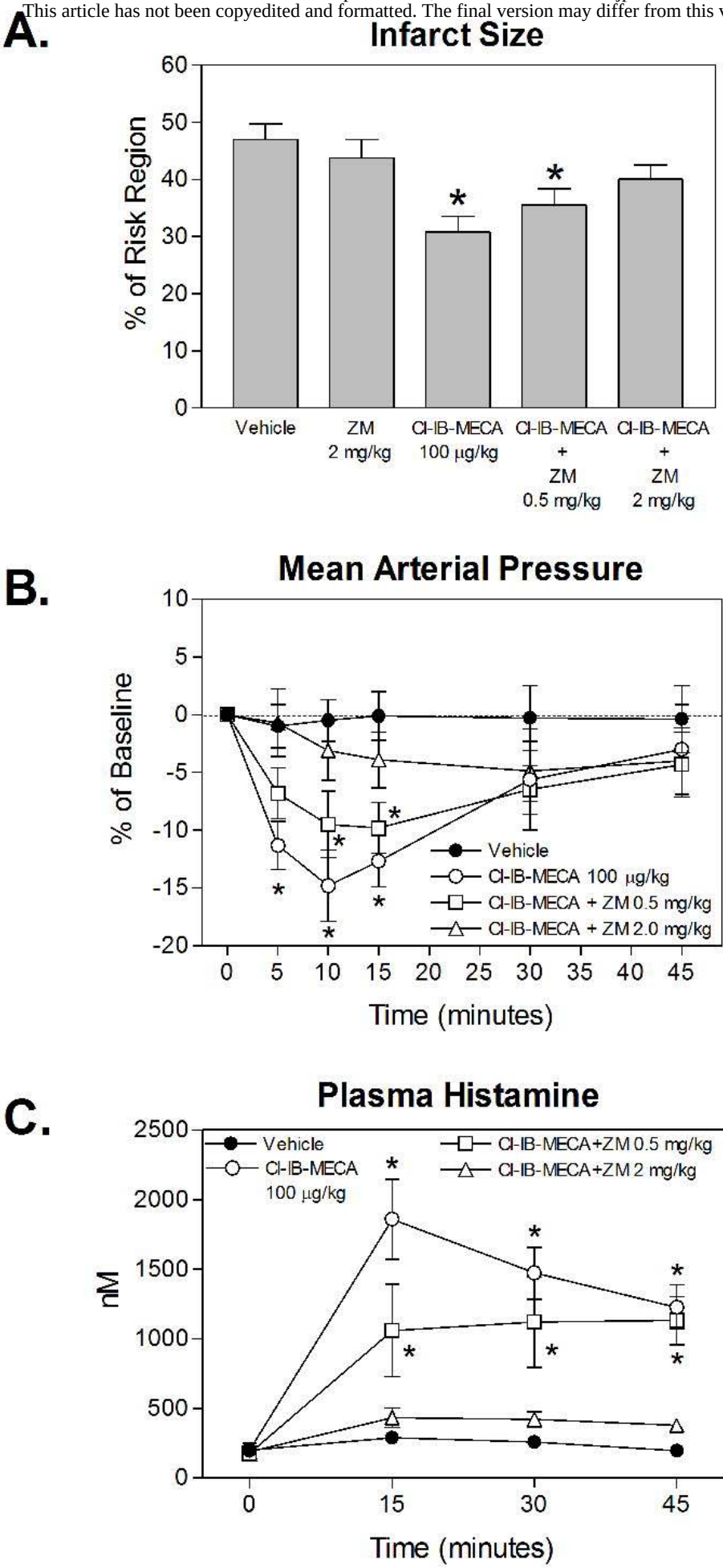

Figure 5

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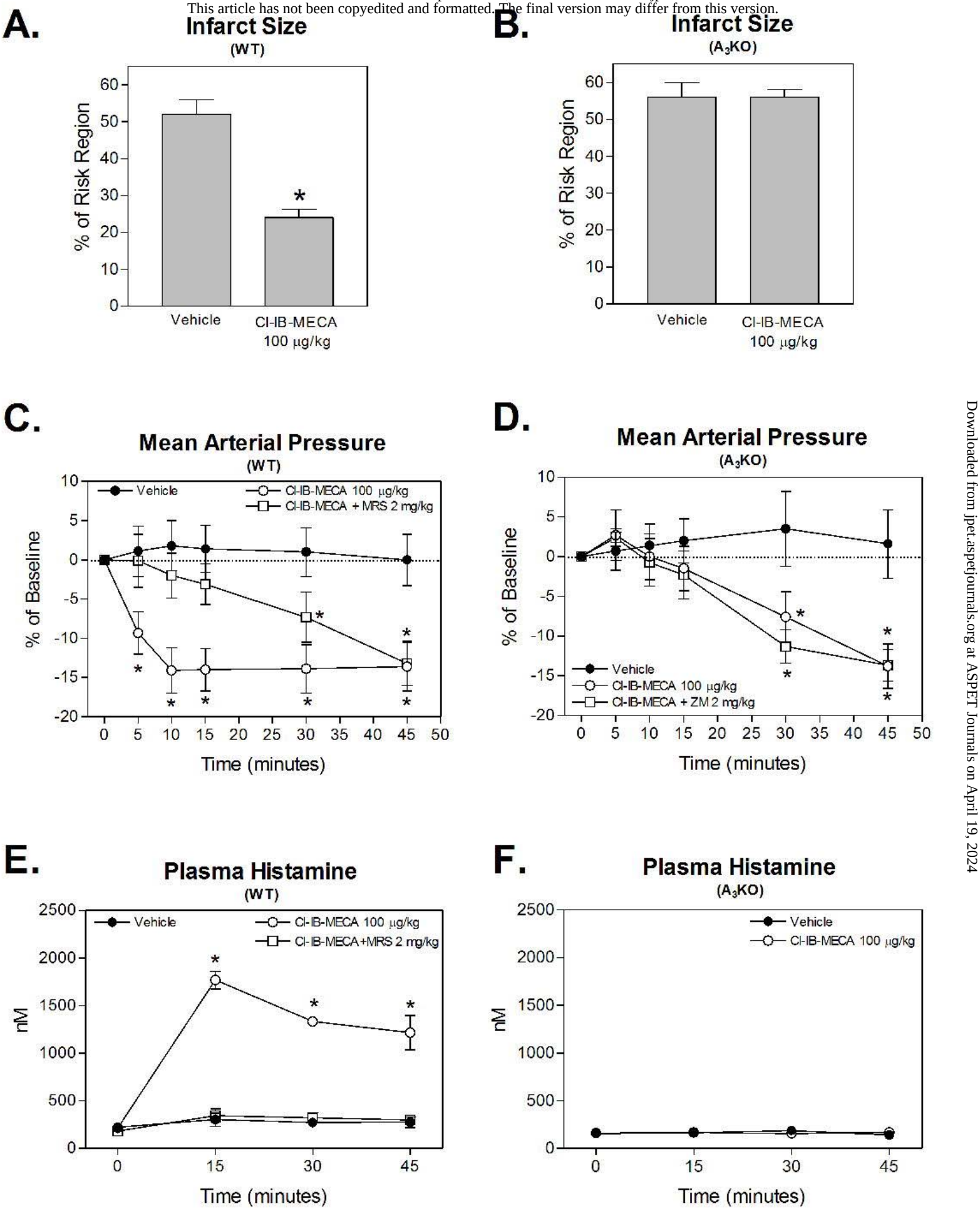

Figure 6

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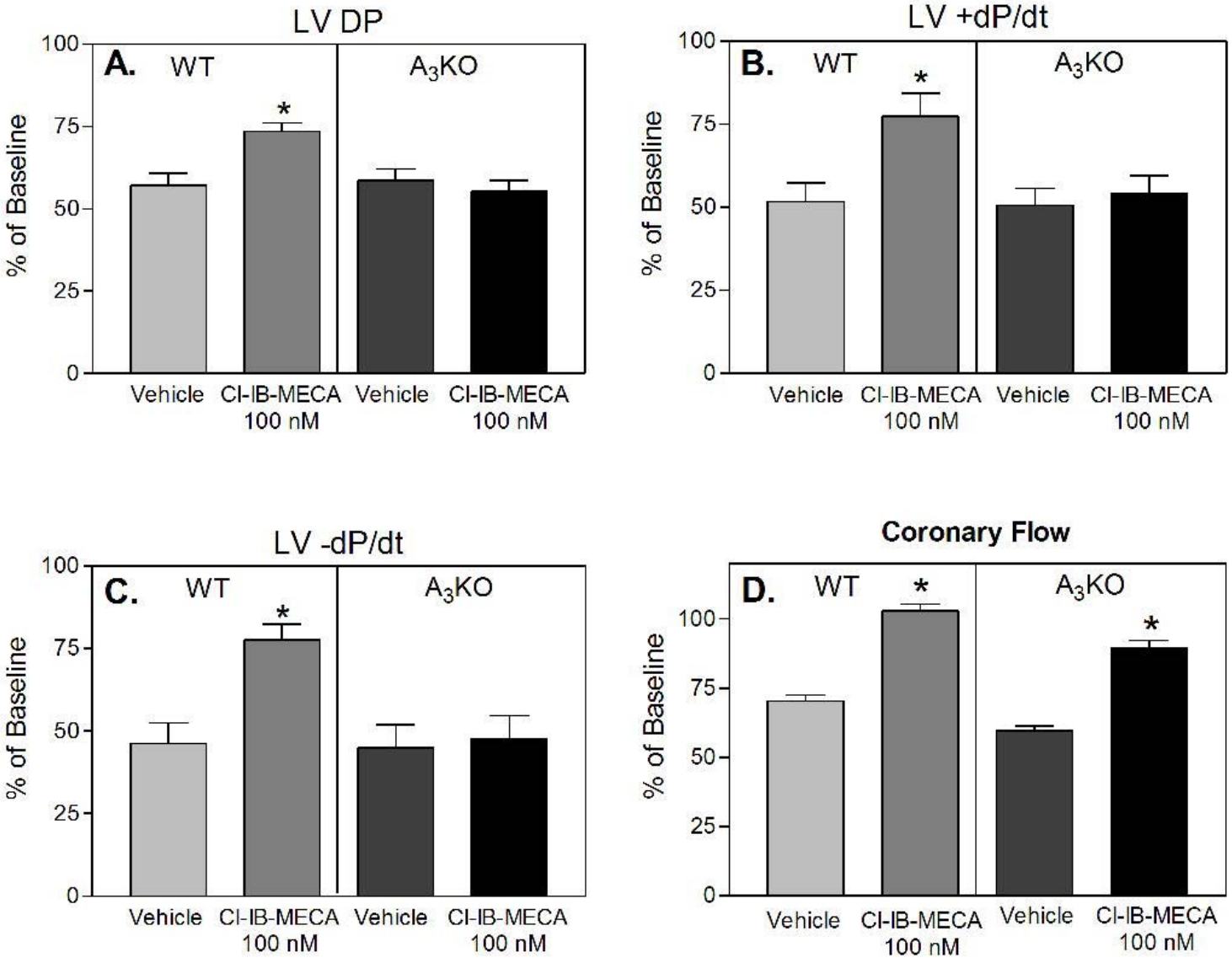

Figure 7

JPET Fast Forward. Published on September 19, 2006 as DOI: 10.1124/jpet.106.111351

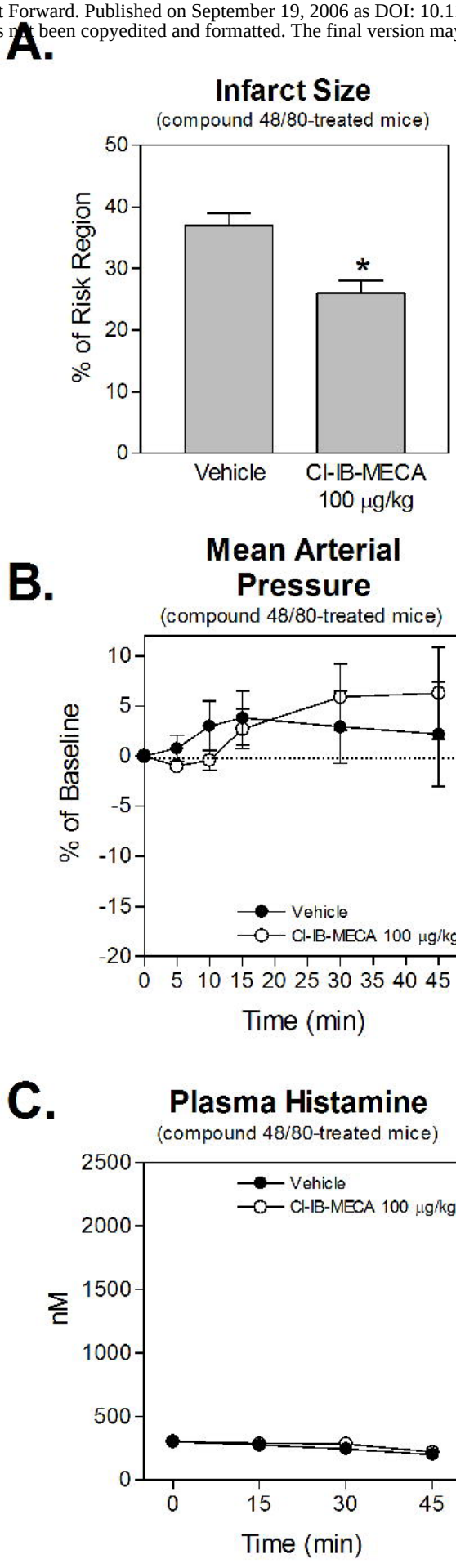

Figure 8

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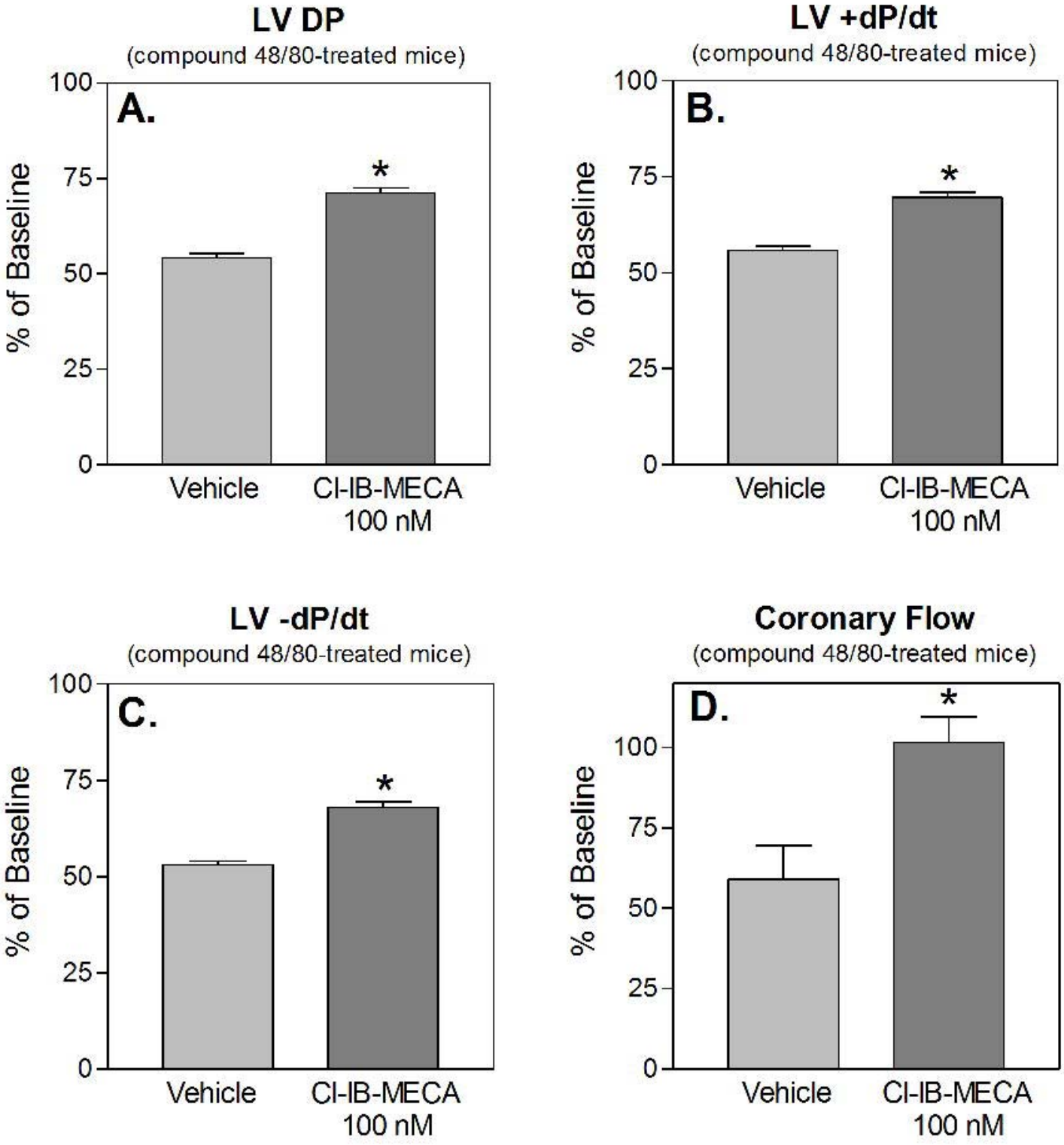

Figure 9