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Benidipine Reduces Ischemia Reperfusion Induced Systemic Oxidative Stress through Suppression of Aldosterone Production in Mice

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Running title: Benidipine inhibits ischemia reperfusion injury in hearts

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Abstract

Aldosterone is implicated in the pathogenesis of several cardiovascular diseases, including ischemia reperfusion (I/R) and myocardial infarction, and also causes oxidative stress and inflammation in cardiovascular systems. Benidipine, a long-acting T- and L-type calcium channel blocker, reduces infarct size following myocardial I/R in rabbits. Benidipine also inhibits the production of aldosterone *in vitro*. However, the precise mechanism of this phenomenon *in vivo* remains unknown. We therefore evaluated whether benidipine has a beneficial role through the regulation of oxidative stress in myocardial I/R. C57BL/6J mice were subjected to 30 min of left ascending coronary I/R. Benidipine was administered orally at 3 mg/kg daily for 3 weeks without any changes in hemodynamic variables. Benidipine significantly reduced infarction size ($13.4 \pm 2.5\%$) compared to controls ($25.5 \pm 3.6\%$). Urinary 8-hydroxy-2' deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, increased significantly after I/R. I/R-induced increases in 8-OHdG were significantly lower with benidipine. Local myocardial 8-OHdG was also elevated in I/R, but this augmentation was significantly suppressed with benidipine. The plasma aldosterone concentration significantly increased 2 days after I/R and remained elevated at least 7 days after I/R. Treatment with benidipine significantly decreased I/R-induced elevation of the plasma aldosterone concentration. I/R-induced markers of fibrosis in hearts also reduced in benidipine. These results suggest that the administration of benidipine reduces myocardial infarct

size as well as systemic oxidative stress after I/R. These phenomena are partially linked to reduced plasma aldosterone levels.

Keywords: ischemia reperfusion, calcium channel blocker, oxidative stress, aldosterone

Introduction

Early recanalization of an occluded coronary artery in acute myocardial infarction (MI) reduces the extent of myocardial necrosis, improves prognosis, and preserves the function of the left ventricle (LV).¹ On the other hand, opening an occluded coronary artery can sometimes induce myocardial ischemia reperfusion (I/R) injury.² A growing body of evidence demonstrates that reperfusion after ischemia results in additional cell death and enhances infarct size, called I/R injury.³ One major component of I/R injury mediating the development and progression of postischemic myocardial damage is oxidative stress, which leads to cardiomyocytes' apoptosis.⁴ It is important to block the rennin-angiotensin-aldosterone pathway after MI. Indeed, aldosterone might be essential to the pathophysiology of cardiovascular injury.⁵ Using a mineralocorticoid receptor (MR) antagonist in addition to standard therapy also appears to reduce clinical mortality,⁶ which suggests that MR activation may play a role in MI and heart failure. MR blockade is associated with decreased fibrosis, vascular inflammation, reduced oxidative stress, and I/R injury⁷ and endothelial dysfunction.⁸ Accordingly, aldosterone must be an important risk factor for cardiovascular diseases.

Benidipine, a long-acting dihydropyridine calcium channel blocker, is used widely to treat hypertension and ischemic heart disease.⁹⁻¹⁰ Benidipine evokes the vasodilatation of coronary and peripheral arteries by inhibiting calcium influx via L-type calcium channels. Benidipine also has

the effect to block T- and N-type calcium channels. This unique calcium channel blocker exhibits additional pharmacological effects, such as limiting infarct size via bradykinin and nitric oxide-dependent mechanisms in the canine heart,¹¹ and reducing the production of hydroxyl radicals in rabbits.¹² Benidipine also inhibits the production of aldosterone in human adrenocortical cells through the suppression of T-type calcium channels.¹³ However, it remains unknown whether this calcium channel blocker attenuates myocardial I/R injuries and aldosterone production in mice.

In the current study, we demonstrated that benidipine would reduce myocardial infarction size following I/R in mice. We also showed that benidipine decreased I/R-induced oxidative stress through mechanisms partially involving down-regulation in plasma aldosterone concentration.

Methods

An expanded Methods section is available in the Online Supplement.

Experimental protocol

The experiments were approved by the institutional and governmental animal research committees and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals in Kanazawa University which strictly conforms to Guide for the Care and Use of Laboratory, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Ischemia reperfusion and administration of drugs

The method for inducing myocardial I/R in mice has been described.¹⁴ Male C57BL/6J mice (BW 24.5 ± 1.86 g, 8-12 weeks of age, Charles River Laboratories) were subjected to I/R or sham operation. Sham and I/R + V (vehicle) groups were orally administered 0.3% sodium carboxymethyl cellulose (CMC)¹⁵ by gavage daily from 7 days before the operation to 14 days after it. In the I/R + Beni groups, a 3 mg kg^{-1} dose of benidipine (Kyowa Kirin, Tokyo, Japan) dissolved by CMC was administered similarly and the same surgical procedures of I/R were followed. Nifedipine (3 mg kg^{-1}) was orally administered daily from 7 days before operation in mice. Eplenerone (200 mg kg^{-1}) was administered daily from just before operation of I/R to 7 days after operation. In I/R + Beni + Aldo groups, mice received a continuous infusion of

aldosterone by micro pump at a dose of $8 \mu\text{g kg}^{-1}$ per day for 4 days. Previous reports¹⁶⁻¹⁹ and preliminary experiments indicated that this dose of these drugs does not reduce blood pressure in C57BL/6J mice (Supplementary Table 1 and 2).

Measurement of plasma aldosterone concentration (PAC), plasma renin activity (PRA)

Then blood samples were collected from the left ventricular apex in before operation and 24 h, 2, 4 and 7 days after operation, and PAC was measured by radioimmunoassay (Coat-a-count; DPC).²⁰ PRA in 24 h after operation was measured by PRA kit (TFB, Japan).²¹

Hemodynamic measurements

Systolic blood pressure, mean blood pressure, and heart rate were measured in unanesthetized mice using a computerized tail-cuff system²² (BP-98A system; Softron Co., Tokyo, Japan). The animals were placed in restraining units mounted on a warmed ($36\text{--}37^\circ\text{C}$) surface (THC-2; Softron Co.), and their tails were passed through a cuff. Each session included more than 10 tail-cuff measurements.

Determination of myocardial apoptosis

Myocardial apoptosis was determined by TdT-mediated dUTP nick end labeling (TUNEL) staining using an *in situ* apoptosis detection kit (Takara, Shiga, Japan) 24 h after operating. The positively stained nuclei were counted under 200 x magnifications and the ratio of positively stained apoptotic cells to all cells was evaluated.

Assessment of myocardial oxidative stress

Myocardial oxidative stress was assessed by an immunohistochemical method using the mouse ImmunoCruz™ staining system (sc-2050; Santa Cruz Biotechnology, Inc. CA, USA) for 8-hydroxy-2'-deoxyguanosine (8-OHdG)²³ 24 h after the operation.

Urine collection and assessment of systemic oxidative stress

The urine samples, collected using metabolic cages for mice (Shinano, Japan) in before operation and 24 h, 2, 4, 7 and 14 days after operation, were stored at –80°C until use. We performed an enzyme-linked immunosorbent assay (ELISA) to detect urinary 8-OHdG using the New 8-OHdG Check ELISA Kit (Japan Institute for the Control of Aging, Shizuoka, Japan).

Isolation of mRNA and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using RNeasy (Qiagen) from heart according to the protocol of the manufacturer. Total RNA (1µg) was used to generate cDNA using Rever Tra Ace (Toyobo, Osaka, Japan) according to the protocol of the manufacturer. Real-time Quantitative PCR analysis was performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster, CA). The following primers and TaqMan probes (Applied Biosystems) were used: interleukin 6 (Il6 Mm99999064_m1), tumor necrosis factor (Tnf, ID# Mm00443258_m1), collagen, type I, alpha1 (Colla1, ID# Mm00801666_g1), alpha smooth muscle actin (Acta2, ID# Mm01546133_m1), gp91phox (cybb, ID# Mm00432775_m1), p22phox (cyba, ID#

Mm00514478_m1), p47phox (ncf1, ID# Mm00447921_m1), p67phox(ncf2, ID# Mm00726636_s1), Mineralocorticoid receptor (MR) (nr3c2, ID# Mm01241596_m1). Glyceraldehyde-3-phosphate Dehydrogenase (Gapdh, ID# Mm99999915_g1) were used as an endogenous control.

Statistical analysis

The values, represented as the mean \pm standard error for each group of mice, were assessed by ANOVA with a subsequent Bonferroni's post hoc test for multiple comparisons. The significance of differences between two groups was determined using the Student's t test. Associations of urinary 8-OHdG and FS were assessed by Pearson correlation coefficient. Statistical significance was established at a value of $p < 0.05$. All statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA).

Results

Hemodynamic parameters

Before the operation, the administration of benidipine did not affect heart rate, systolic/mean blood pressure, and urine volume (Table 1). The heart rate and urine volume did not differ significantly at each time point among the three groups throughout the protocol. In mice subjected to I/R, the systolic and mean blood pressures 2 and 4 days after operation were significantly lower than those of the sham groups, with or without benidipine. These results indicate that there were no significant changes in hemodynamic parameters during the oral administration of benidipine.

Effect of benidipine on myocardial infarct size and left ventricular function after I/R

To examine the effects of benidipine on cardioprotection during I/R injury, we applied 30 min of ischemia and a subsequent 24 h of reperfusion to I/R mice. The mean AAR in the left ventricular (LV) area was no different with or without benidipine (Figure 1B). I/R significantly increased IA/AAR after 24 h of reperfusion compared to the sham mice, but the administration of benidipine significantly attenuated I/R-induced increases in IA/AAR (Figures 1A, 1C). To examine the effect of L-type calcium channel blocker on I/R injury, nifedipine, a pure L-type calcium channel blocker, was administrated. The administration of nifedipine did not affect the heart rate and systolic and mean blood pressures after I/R. IA/AAR after 24 h of reperfusion was

similar in I/R+Nife compared to IR+V groups (Supplemental Figure 1).

Echocardiographic measurements showed that I/R resulted in LV dilatation and decreased fractional shortening (%FS), compared to sham mice. The administration of benidipine significantly attenuated I/R-induced increases in LVEDD (Figure 1D) as well as decreases in %FS (2.69 ± 0.07 versus 3.15 ± 0.07 mm, $p < 0.01$; 41.2 ± 0.9 versus 33.3 ± 0.8 %, $p < 0.01$; respectively; Figure 1E). Taken together, these results suggest that benidipine, not nifedipine, attenuates I/R-induced myocardial infarct size and LV dysfunction.

Benidipine attenuates apoptosis following I/R

I/R injury is associated with increased apoptosis in the myocardium. To determine the extent of apoptosis, TUNEL-positive nuclei were counted among 5,000 nuclei in the AAR of each mouse. The number of TUNEL-positive myocytes was significantly higher in I/R+V groups than in sham mice ($9.0 \pm 0.9\%$ versus $1.0 \pm 0.3\%$, $p < 0.0001$; Figures 2A and 2B), suggesting that apoptosis is enhanced during cardiac I/R. The number of TUNEL-positive myocytes was also induced in I/R+Beni groups, but I/R-induced TUNEL-positive myocyte was significantly lower than in I/R+V groups ($6.0 \pm 1.3\%$ versus $9.0 \pm 0.9\%$, $p < 0.05$). Protein levels of total bax were increased and protein levels of bcl-2 were decreased in I/R+V groups (Figure 2C). I/R-induced changes of bax and bcl-2 were not observed in I/R+Beni groups. Furthermore, the level of eNOS phosphorylation was increased after I/R. I/R-induced eNOS phosphorylation was higher in

I/R+Beni than in I/R+V (Supplementary Figure 2). These results suggest that benidipine inhibits cardiac myocyte apoptosis induced by I/R.

Myocardial oxidative stress after I/R

To examine oxidative stress after I/R in hearts, we evaluated the myocardial levels of 8-OHdG, a sensitive indicator of oxidative DNA damage. As shown in Figure 3, I/R significantly increased 8-OHdG expression in the AAR ($12.4 \pm 1.3\%$ versus $2.7 \pm 0.4\%$, $p < 0.0001$). The administration of benidipine attenuated this response ($6.7 \pm 0.4\%$ versus $12.4 \pm 1.3\%$, $p < 0.05$). The expression of gp91phox, NAD(P)H oxidase, mRNA was decreased in I/R+Beni compared to I/R+V (Supplementary Figure 3). These results suggest that benidipine attenuates cardiac oxidative stress after I/R.

Aldosterone plasma levels after I/R

Plasma aldosterone levels were markedly elevated after I/R, with a peak at 2 days that gradually declined thereafter but remained elevated for at least 7 days (Figure 4A). Plasma renin activities were significantly increased in both I/R+V and I/R+Beni groups compared to sham (Supplementary Table 3). Administration of benidipine in this model was not associated with an increase in serum renin activities. I/R was not affected the levels of serum potassium. Interestingly, the administration of benidipine significantly inhibited this response 2 and 4 days after I/R. The administration of nifedipine did not affect aldosterone levels after I/R (Figure 4B).

These results suggest that benidipine inhibits aldosterone production in response to I/R.

Systemic oxidative stress and cardiac remodeling after I/R

We estimated the ratio of urinary 8-OHdG to creatinine (Cr), a marker of systemic oxidative DNA damage induced by reactive oxygen species. Urinary 8-OHdG/Cr levels increased significantly in mice subjected to I/R compared to sham mice after operation (days 1, 2, 4, and 7).

Benidipine significantly attenuated this response (Figure 5A), as did the administration of eplerenone, a selective mineral corticoid receptor antagonist (Figure 5B). Furthermore, continuous infusion of aldosterone abolished the benidipine's inhibition of I/R induced urinary 8-OHdG/Cr levels. These results suggest that benidipine attenuates cardiac and systemic oxidative stress after I/R through aldosterone production. We also examined the effect of benidipine administration for cardiac inflammation and remodeling after I/R. We isolated heart tissues 4 days after I/R for quantitative real-time PCR. The mRNA expression level of mineralocorticoid receptor (MR) was no changes in both I/R+V and I/R+Beni compared to sham groups (Supplementary Figure 4). Multiple markers of inflammation and fibrotic remodeling were elevated in I/R compared to sham. I/R-induced mRNA upregulations of IL6, TNF α , Col1a1 and Acta2 were decreased in I/R+Beni groups (Figure 5C, 5D, 5E and 5F). In aldosterone infusion mice, mRNA upregulations of only Col1a1 was significantly elevated. Continuous infusion of aldosterone partially reduced the beneficial effect of benidipine. These results indicate

that administration of benidipine inhibited I/R-induced cardiac inflammation and fibrotic remodeling, partially through the production of aldosterone.

Discussion

In this study, the long-acting dihydropyridine calcium channel blocker benidipine, when administered at a dose that did not decrease blood pressure, reduced the myocardial infarct size, apoptosis of cardiac myocytes, and myocardial 8-OHdG in I/R. These results suggest that the cardioprotective effects of benidipine may be independent of its anti-hypertensive effects.

This study demonstrated the following major findings. First, benidipine reduces infarct size and may be improve cardiac dysfunction following I/R. Second, benidipine reduces the plasma aldosterone concentration that increases due to I/R. Finally, benidipine inhibits I/R-induced increases in urinary 8-OHdG and cardiac inflammatory responses and fibrotic remodeling. Thus, these results suggest that benidipine may be a useful cardioprotective agent against oxidative stress and could potentially serve as a therapeutic strategy for I/R injury.

It was previously demonstrated that benidipine limits I/R injury *in vivo*. In canine ischemia reperfused hearts, benidipine increased the nitric oxide (NO) and bradykinin (BK) levels of coronary venous blood and limited infarct size.¹¹ Wang *et al.* reported that benidipine reduced myocardial infarct size by reducing hydroxyl radicals and inducing the production of NO in rabbits.¹² Moreover, benidipine has been shown to reduce postischemic myocardial apoptosis by inhibiting caspase-3 activation.²⁴ In the current study, we demonstrated that the oral administration of benidipine reduces myocardial infarct size. Expression of bax protein was

increased and bcl2 protein was decreased in hearts after I/R. These changes in apoptotic regulating proteins did not occur by administration of benidipine. Consequently, the number of TUNEL positive cells was smaller in benidipine treatment. We also demonstrated that benidipine increased eNOS activity in hearts. Based on our results, we suggest that the reduction of hydroxyl radicals may play one of the important key roles in the cardioprotective effect of benidipine.

Benidipine treatment (3 mg kg⁻¹ per day) did not affect to blood pressure and heart rate. Even after I/R, these parameters showed no difference with or without benidipine. Consistent with previous work,¹⁶ these results suggest that the cardioprotective effect of benidipine is likely to be independent of blood pressure, because the dose of benidipine used in this study did not significantly affect systemic blood pressure.

The generation of free radicals and oxidants is associated with the exacerbation of reperfusion injury in myocardial I/R.²⁵ 8-OHdG is a sensitive and integral marker of oxidative damage to DNA. Cordis *et al.* reported that 8-OHdG is formed at the onset of reperfusion in the myocardium.²⁶ Benidipine treatment reduces the level of hydroxyl radicals in reperfused myocardium in rabbit.¹¹ In the current study, 8-OHdG accumulated in mouse hearts 24 h after reperfusion, but benidipine treatment reduced 8-OHdG levels. Benidipine also suppressed increases of gp91phox, one of the important components of NAD(P)H oxidase, after I/R. In addition, urinary 8-OHdG, a marker of systemic oxidative stress, was elevated in both the early (1

and 2 days after reperfusion) and late phases (4 and 7 days after reperfusion), and benidipine reduced urinary 8-OHdG levels in both phases. We suggests that reduction of myocardial oxidative stress is one of the mechanism through which benidipine decreases infarct size, and that myocardial oxidative stress reflect systemic oxidative stress in the early phase after reperfusion.

Aldosterone, an antidiuretic hormone, plays an important role in regulating electrolyte composition by promoting sodium retention and potassium excretion²⁷ and MR antagonist regressed hypertrophy, fibrosis and diastolic dysfunction in human and experimental hypertensive heart disease²⁸⁻²⁹. Excess of mineralocorticoid causes cardiac hypertrophy, fibrosis and diastolic dysfunction³⁰⁻³¹. In the current study, we found for the first time that the plasma concentration of aldosterone increased after I/R partially through the upregulation of plasma rennin activity and benidipine treatment effectively inhibited this response *in vivo*. Aldosterone is produced mainly by the adrenal cortex in response to increased potassium or angiotensin II.³² Benidipine inhibits angiotensin II-induced aldosterone production by regulating T-type calcium channels.¹³ The synthesis of aldosterone requires the aldosterone synthetase CYP11B2.³³ Several reports has been shown that there are CYP11B2 mRNA expressions and activities in hearts and vessel walls,³⁴ however, results regarding the synthesis of aldosterone in the heart and peripheral vasculature are contradictory.³⁵ We tried to examine the mRNA expression levels of CYP11B2 in the heart, aorta, and kidney using quantitative real-time PCR, but we could not detect the expression of CYP11B2

in any tissues (data not shown). Furthermore, we found that the administration of nifedipine, a pure L-type calcium channel blocker, did not inhibit I/R-induced aldosterone production. Based on these results, we speculate that benidipine inhibits aldosterone synthesis in the adrenal gland by blocking T-type calcium channels. At present, however, this hypothesis remains to be tested.

Oxidative stress and inflammation lead to increased cardiovascular morbidity and mortality.³⁶

Much evidence indicates that aldosterone and/or MR activation result in oxidative stress and vascular inflammation. Aldosterone itself enhances NAD(P)H oxidase activation,³⁷ a major source of reactive oxygen species, and the MR blocker inhibits angiotensin II-induced activation of NAD(P)H oxidase activity and myocardial interstitial fibrosis after MI. This study demonstrates the I/R-induced enhancement of urinary 8-OHdG in the late phase and elevation of PAC. Interestingly, the elevation in urinary 8-OHdG was reduced by both benidipine and eplerenone in the late phase after reperfusion. Continuous infusion of aldosterone was abolished beneficial effects of benidipine after I/R. These results suggest that MR regulates I/R-induced increases in 8-OHdG. Benidipine also reduced I/R-induced inflammatory and fibrotic responses in hearts. These effects partially canceled by continuous infusion of aldosterone. In addition, benidipine also appears to have renoprotective and vascular endothelial protective effects³⁸. These results further suggest that the inhibition of aldosterone production and oxidative stresses contributes to the cardioprotective effect of benidipine and that benidipine provides a beneficial

effect to multiple organs following cardiac I/R.

In conclusions benidipine, calcium channel blocker, reduces I/R injury by inhibiting cardiac myocyte apoptosis, aldosterone production, oxidative stress, and inflammation in mice. Our results suggest that benidipine, as a T-type calcium channel blocker, has additional benefits for cardiovascular disease beyond its anti-hypertensive effects. Thus benidipine will be able to potentially serve as a therapeutic strategy for I/R injury.

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Disclosures

None.

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Figure legends

Figure 1. (A) Representative examples of myocardial infarction stained with Evans blue (EB)/triphenyl tetrazolium chloride (TTC) 24 h after ischemia reperfusion. Benidipine decreases infarct size. EB-stained areas (purple staining) indicate nonischemic regions; TTC-stained areas (red staining) indicate ischemic but viable tissue; EB/TTC stained negative (white) areas indicate myocardium infarcts. (B) Area at risk (AAR) and infarct area (IA) of the I/R + V and I/R + Beni groups. There was no significant difference in the AAR between the two groups. Infarct size was significantly reduced in the I/R + Beni group compared to that of the I/R + V group (C). LV, left ventricular area. N = 4 per group. Data are expressed as mean \pm SEM. (D), (E) Effect of benidipine on myocardial function 7 days after ischemic reperfusion as determined by echocardiography. LVEDD, left ventricular end-diastolic diameter; FS, fractional shortening. N = 10 per group. Data are expressed as mean \pm SEM.

Figure 2. (A) Representative examples of myocardial apoptotic cells by TdT-mediated dUTP nick end labeling (TUNEL) staining of the area at risk (AAR) 24 h after operation. There were fewer TUNEL-positive cells (dark brown) in the I/R + Beni group than in the I/R + V group. There was a significant difference in the TUNEL-positive cells among the three groups (B). Myocardial apoptosis was significantly lower in the I/R + Beni group than the I/R + V group. (C)

Immunoblots with anti-bax and anti-bcl2 antibodies are shown. N = 6–7 per group. Data are expressed as mean \pm SEM.

Figure 3. (A) Representative examples of myocardial 8-hydroxydeoxyguanosine (8-OHdG) staining by an immunohistochemical method at the area at risk (AAR) 24 h after operation. There were fewer 8-OHdG-positive cells (brown) in the I/R + Beni group than in the I/R + V group. There was a significant difference in the number of 8-OHdG-positive cells among the three groups (B). N = 5 per group. Data are expressed as mean \pm SEM.

Figure 4. (A) Effect of benidipine on plasma aldosterone concentration (PAC), from before I/R to 7 days after I/R (N = 5 per group). (B) PAC in the I/R group that was administered nifedipine (3 mg/kg/day from 7 days before) compared to three other groups on day 2. Nifedipine did not significantly inhibit the elevation of PAC-induced I/R (N = 5). Data are expressed as mean \pm SEM. $^{\S}P < 0.05$ versus sham group, $^{\dagger}p < 0.05$ versus I/R + V group.

Figure 5. (A) Effect of benidipine on systemic oxidative stress before I/R to 14 days after I/R as determined by urinary 8-OHdG/Cr levels (n = 9–10 per group). $^{\S}P < 0.05$ versus the sham group, $^{\S\S}p < 0.05$ versus sham group, $^{\dagger}p < 0.05$ versus I/R + V group, $^{\dagger\dagger}p < 0.01$ versus I/R + V group. (B)

The urinary 8-OHdG/Cr level on day 4. Eplerenone significantly inhibited the elevation of I/R-induced urinary 8-OHdG/Cr levels (n = 10). Continuous infusion of aldosterone canceled the suppression of benidipine in I/R-induced urinary 8-OHdG/Cr levels. Inflammatory cytokines and fibrotic markers expression. Myocardial mRNA levels of IL6 (C), TNF α (D), Collagen Ia (E), and α SMA (F). Data are expressed as mean \pm SEM.

Figure 1.

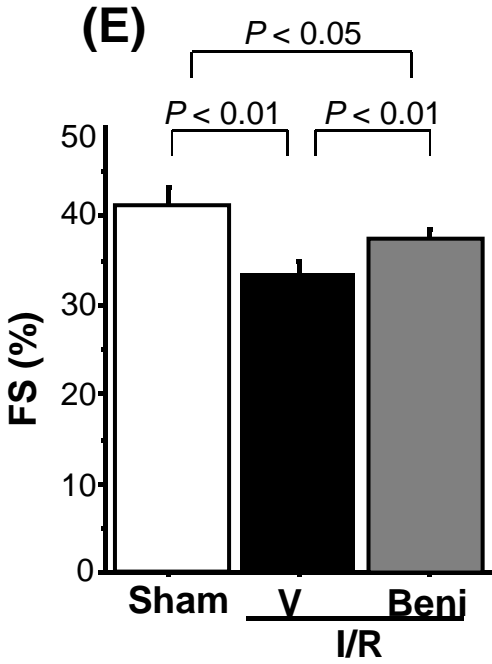
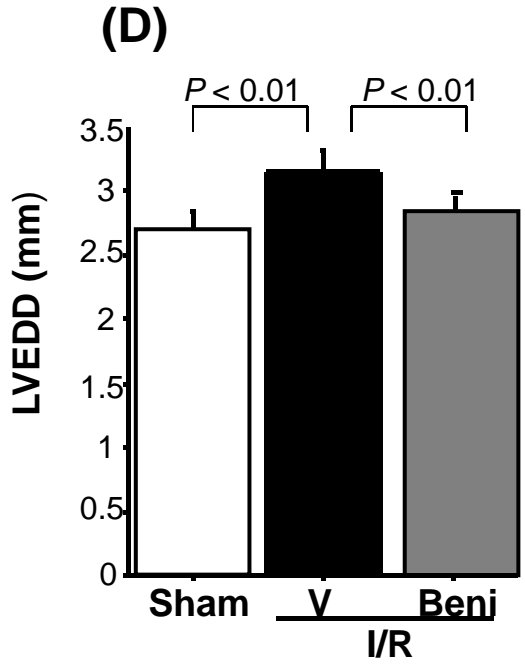
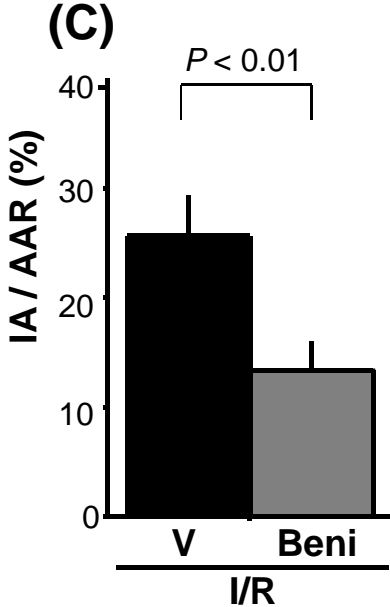
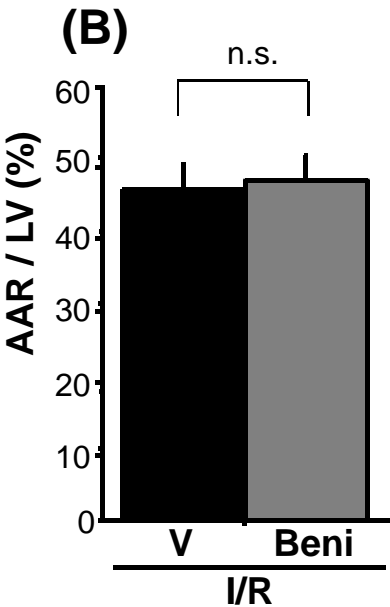
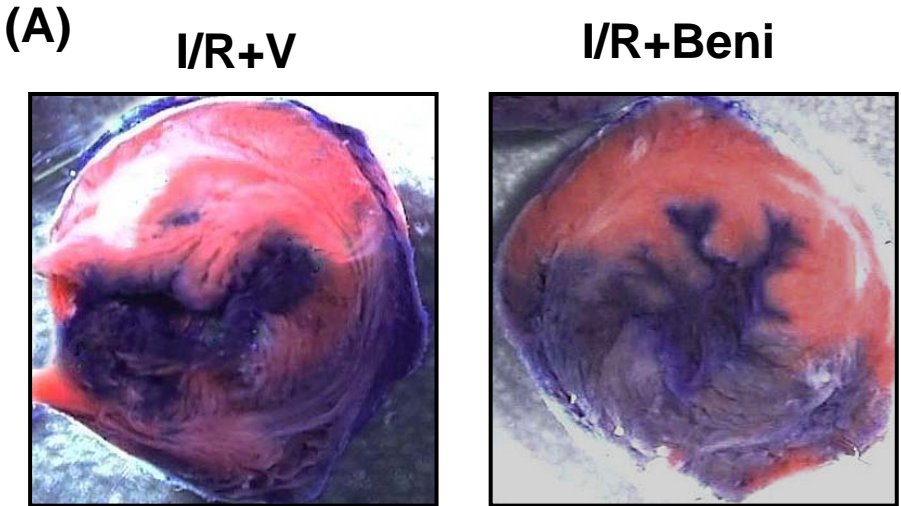
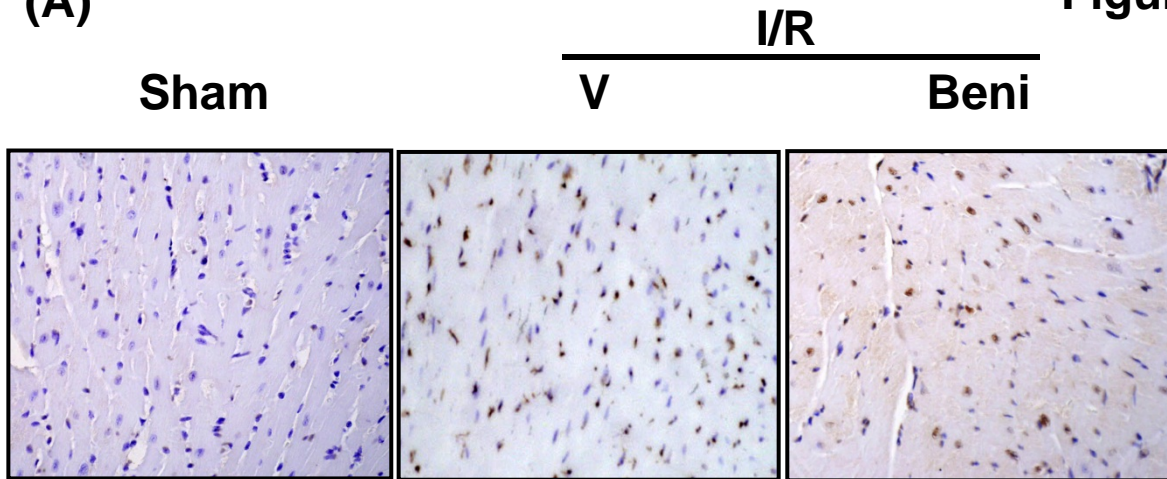
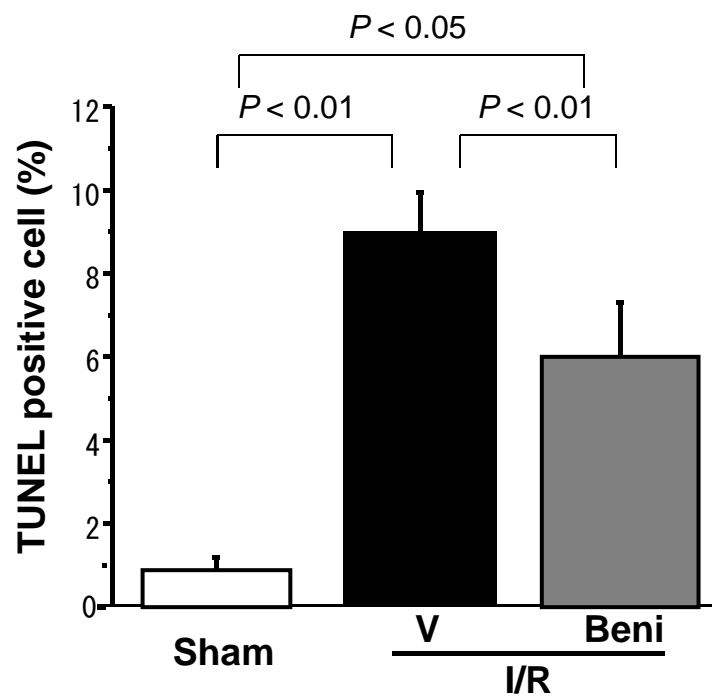


Figure 2.

(A)



(B)



(C)

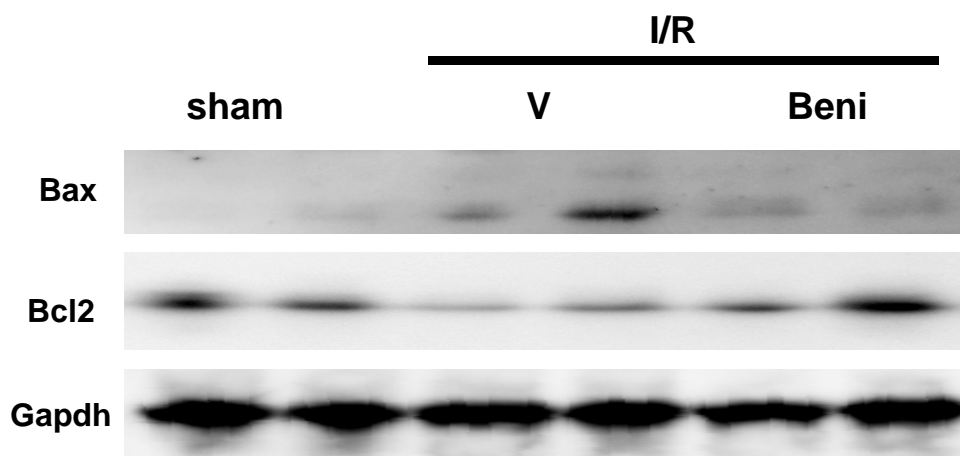


Figure 3.

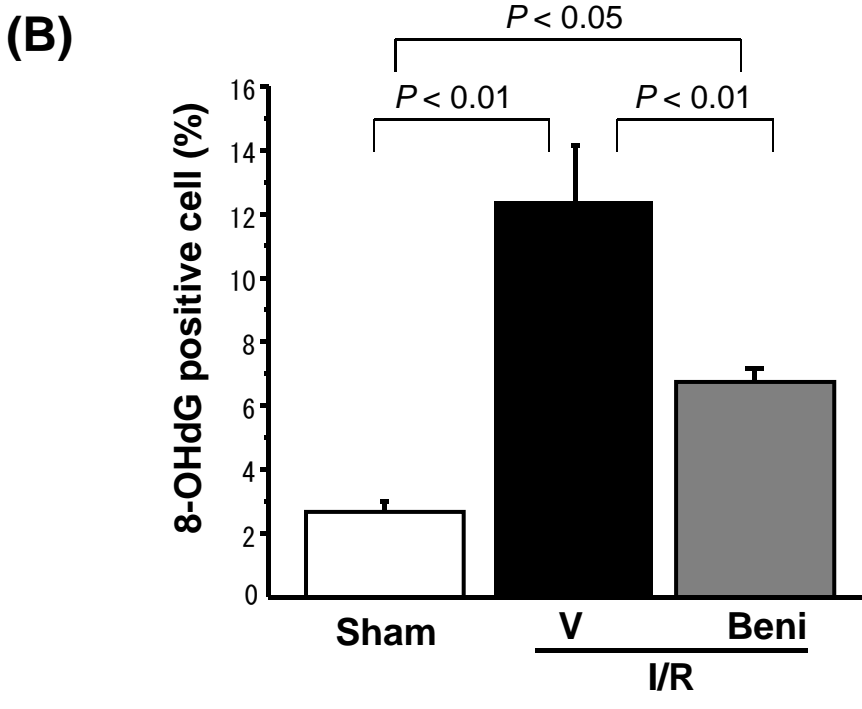
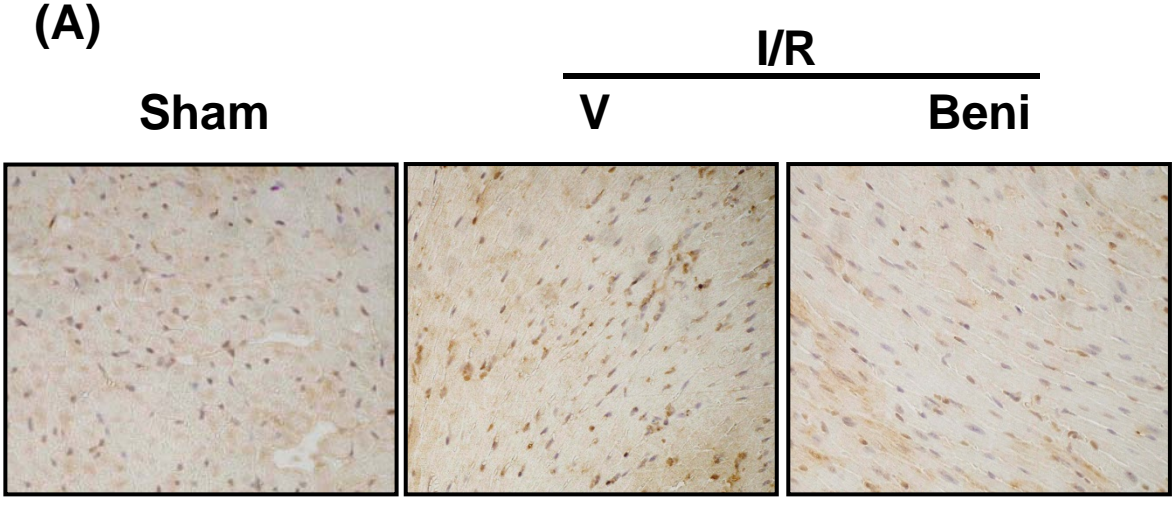
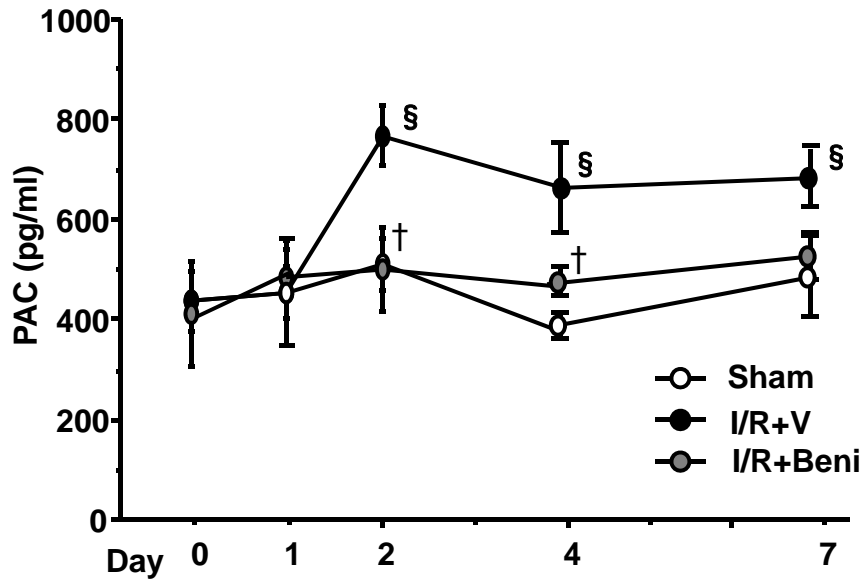


Figure 4.

(A)



(B)

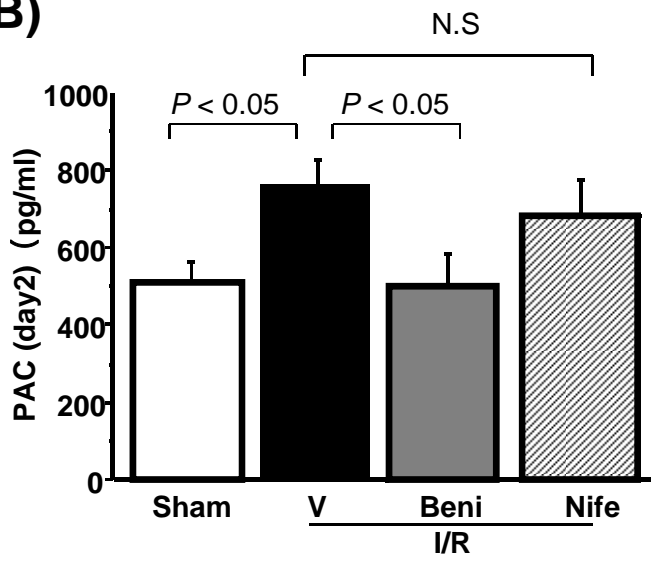
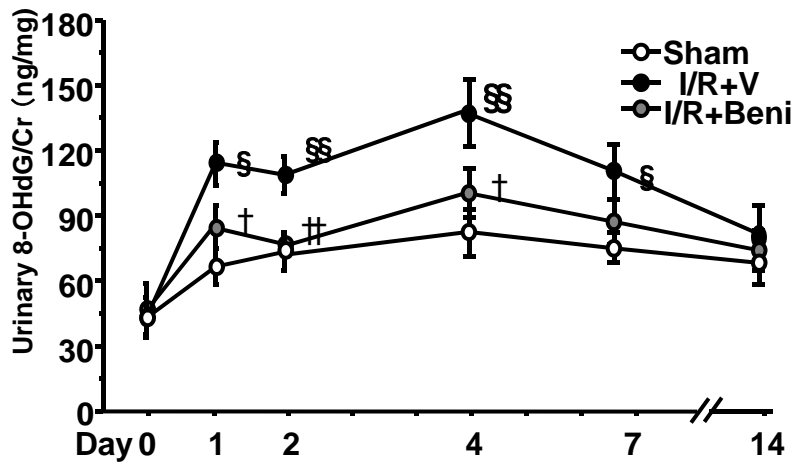
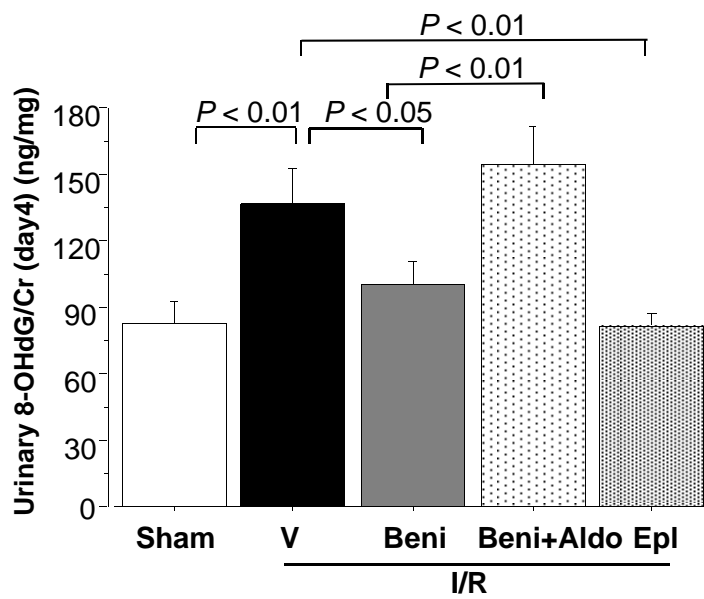


Figure 5.

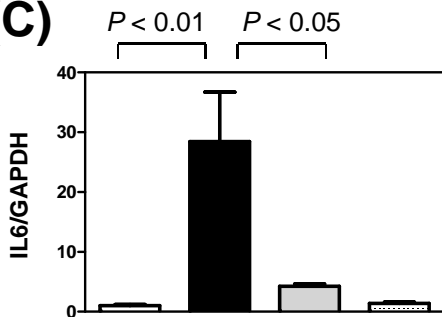
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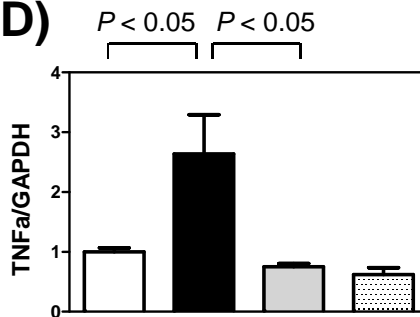
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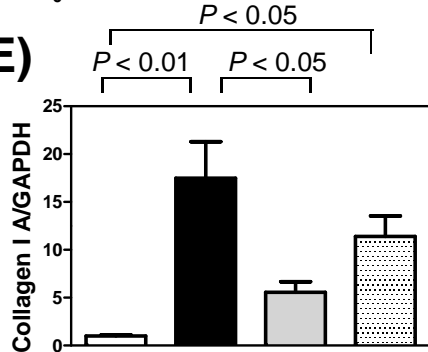
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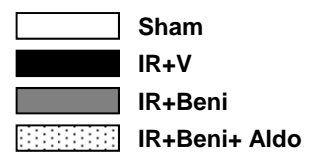
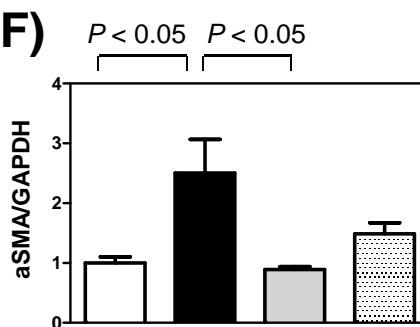
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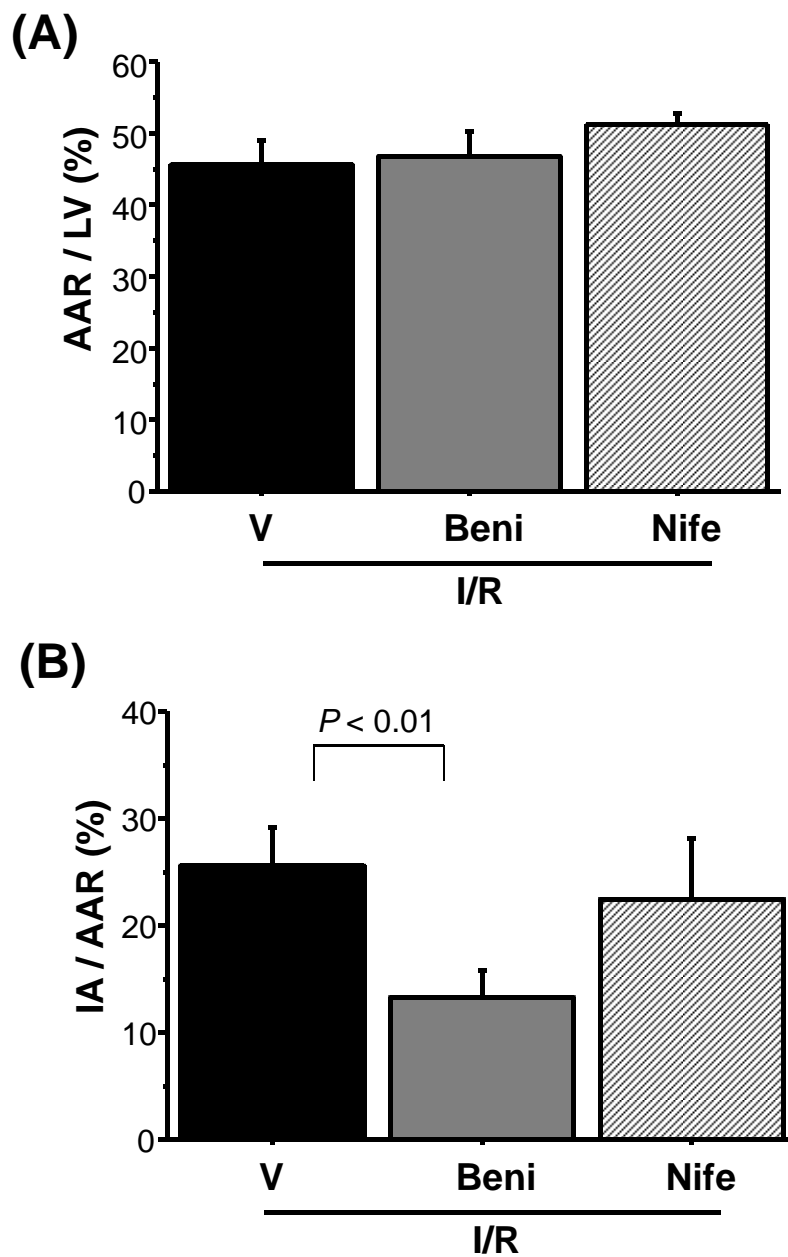
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(F)



Supplementary Figure 1



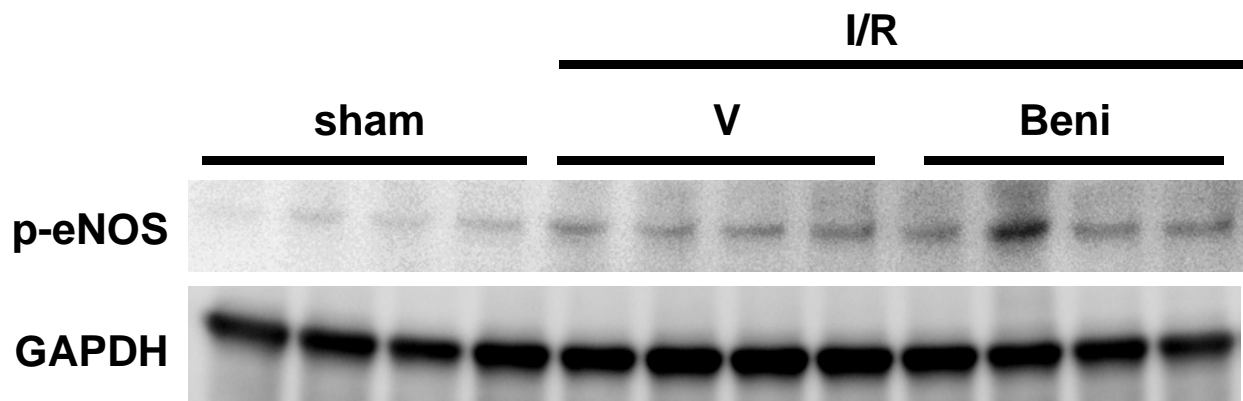
Supplementary Figure 1.

Area at risk (AAR) and infarct area (IA) of the I/R+V, I/R+Beni and I/R+Nife groups.

(A) There was no significant difference in the AAR among three groups.

(B) Infarct size was significantly reduced in the I/R+Beni compared to that of the I/R+V and I/R+Nife groups. Data are expressed as mean \pm SEM.

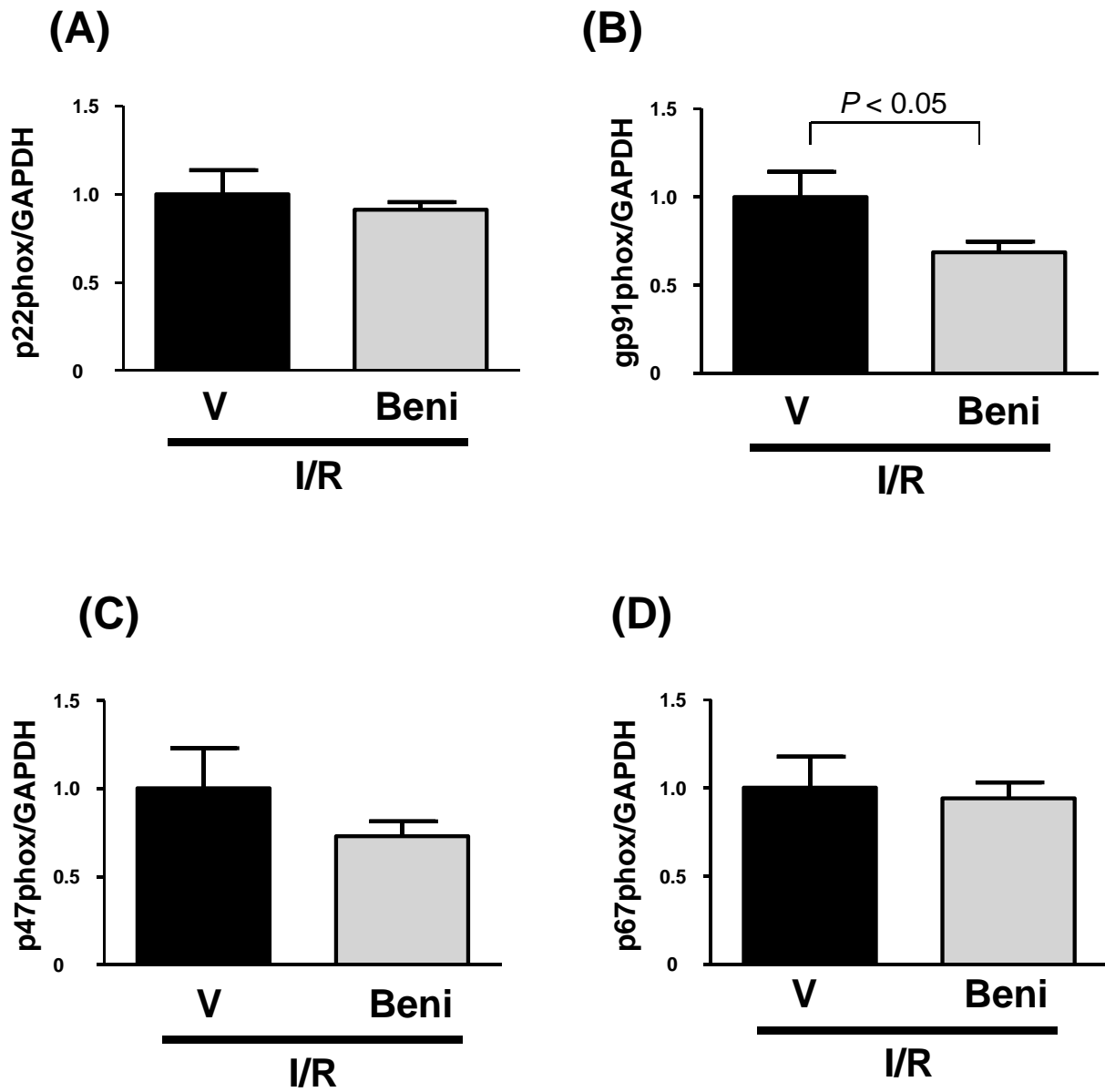
Supplementary Figure 2



Supplementary Figure 2.

Benidipine reduced I/R-induced phosphorylation of eNOS in hearts.
Immunoblot analysis of phosphorylated eNOS are shown.

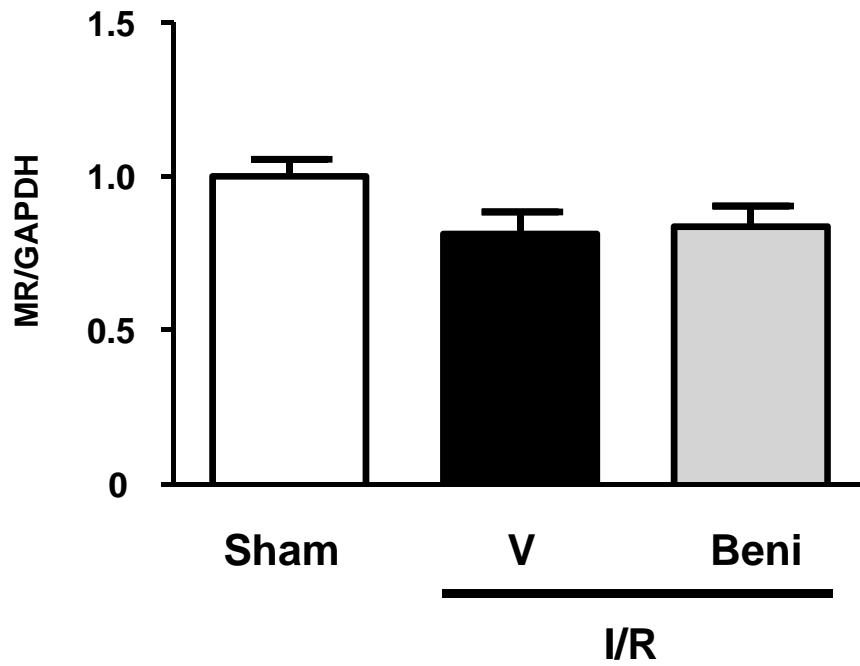
Supplementary Figure 3



Supplementary Figure 3.

mRNA expression of NAD(P)H oxidase was determined by Taqman RT-PCR. A, p22phox; B, gp91phox; C, p47phox; D, p67phox. Data are expressed as mean \pm SEM.

Supplementary Figure 4



Supplementary Figure 4.
mRNA expression of mineralocorticoid receptor (MR) was determined by Taqman RT-PCR.
Data are expressed as mean \pm SEM.

Supplementary Table 1. Hemodynamic parameters before I/R

Group	Before Operation
Heart rate (beats/min)	
Sham	594 ± 21
I/R+Beni	622 ± 12
I/R+Nife	631 ± 24
I/R+Epl	546 ± 22
Systolic blood pressure (mmHg)	
Sham	114 ± 3
I/R+Beni	110 ± 2
I/R+Nife	113 ± 2
I/R+Epl	115 ± 3
Mean blood pressure (mmHg)	
Sham	78 ± 2
I/R+Beni	79 ± 2
I/R+Nife	77 ± 3
I/R+Epl	91 ± 3*

Values are expressed as mean ± SEM (N=9 to 10/group)

* $P < 0.05$ vs sham group , ** $P < 0.01$ vs sham group

Supplementary Table 2. Hemodynamic parameters 4 days after I/R

Group	Day4
Heart rate (beats/min)	
Sham	605 ± 31
I/R+V	606 ± 16
I/R+Beni	590 ± 18
I/R+Beni+Aldo	601 ± 13
Systolic blood pressure (mmHg)	
Sham	110 ± 2
I/R+V	105 ± 2*
I/R+Beni	102 ± 1**
I/R+Beni+Aldo	102 ± 2**
Mean blood pressure (mmHg)	
Sham	79 ± 3
I/R+V	74 ± 3
I/R+Beni	74 ± 3
I/R+Beni+Aldo	74 ± 2

Values are expressed as mean ± SEM (N=9 to 10/group)

* $P < 0.05$ vs sham group , ** $P < 0.01$ vs sham group

Supplementary Table 3.**Serum potassium levels and plasma renin activity after I/R**

Group	Day 1
<hr/>	
Serum K (mEq/L)	
Sham	4.43 ± 0.17
I/R	4.63 ± 0.17
I/R+Beni	4.61 ± 0.26
PRA (ng/mL/hr)	
Sham	8.0 ± 1.29
I/R	19.5 ± 1.32**
I/R+Beni	19.0 ± 3.08**

Values are expressed as mean ± SEM (N=6 to 8/group)
* $P < 0.05$ vs sham group , ** $P < 0.01$ vs sham group
