

Aliskiren and Valsartan Reduce Myocardial AT1 Receptor Expression and Limit Myocardial Infarct Size in Diabetic Mice

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Abstract

Purpose We assessed the ability of Aliskiren (AL), a direct renin inhibitor, and Valsartan (VA), an angiotensin receptor blocker, to limit myocardial infarct size (IS) in mice with type-2 diabetes mellitus.

Methods Db/Db mice, fed Western Diet, received 15-day pretreatment with: 1) vehicle; 2) AL 25 mg/kg/d; 3) AL 50 mg/kg/d; 4) VA 8 mg/kg/d; 5) VA 16 mg/kg/d; 6) AL 25+VA 16 mg/kg/d; or 7) AL 50+VA 16 mg/kg/d. Mice underwent 30 min coronary artery occlusion and 24 h reperfusion. Area at risk (AR) was assessed by blue dye and IS by TTC staining. Protein expression was assessed by immunoblotting.

Results IS in the control group was $42.9 \pm 2.1\%$ of the AR. AL at 25 ($21.9 \pm 2.9\%$) and 50 mg/kg/d ($15.5 \pm 1.3\%$) reduced IS. VA at 16 mg/kg/d ($18.8 \pm 1.2\%$), but not at 8 mg/kg/d ($35.2 \pm 4.0\%$), limited IS. IS was the smallest in the AL50+VA16 group ($6.3 \pm 0.9\%$). Both AL and VA reduced myocardial AT1R levels, without affecting AT2R

levels, and increased the expression of Sirt1 and PGC-1 α with increased phosphorylation of Akt and eNOS.

Conclusions AL, dose dependently limited myocardial IS in mice with type-2 diabetes mellitus. At doses shown to limit IS in non-diabetic animals, VA failed to reduce IS in Db/Db mice. However, at higher dose (16 mg/kg/d), VA reduced IS. Both drugs reduced the expression of AT1R and increased myocardial levels of the longevity genes Sirt1 and PGC-1 α along with increased Akt and eNOS phosphorylation.

Key words Ischemia-reperfusion injury · Infarct size · Angiotensin receptor blocker · Direct renin inhibitor · Type-2 diabetes mellitus · Sirt1

Introduction

Several studies have suggested that angiotensin converting enzyme inhibitors (ACE-I) [1–14] and angiotensin receptor blockers (ARB) [1, 2, 4, 14–22] protect the heart against ischemia-reperfusion injury and reduce myocardial infarct size (IS) in various experimental animal models. It has been suggested that the protective effects of both ACE-Is [9–11, 13, 23] and ARBs [17, 19, 21] are dependent on Bradykinins. ACE-Is inhibit the degradation of bradykinins, whereas ARBs causes unopposed activation of the angiotensin-2 type 2 (AT-2) receptors with subsequent release of bradykinins.

The effects of aliskiren (AL), a nonpeptide direct renin inhibitor [24], on ischemia-reperfusion injury is unknown. Aliskiren does not increase bradykinins levels [25]; therefore, it is plausible that the effect of aliskiren on ischemia-reperfusion injury could differ from that of ACE-Is or ARBs.

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In addition, most of the above mentioned studies were conducted in young healthy animals. It has been suggested that although a large number of novel cardioprotective strategies have been discovered in a research setting, their translation into the clinical setting has been mostly disappointing. The reason for this failure can be attributed to a number of factors, including the inadequacy of the animal ischemia-reperfusion injury models used in preclinical cardioprotection studies [26]. Animals with type-2 diabetes mellitus (T2DM) have reduced protection against ischemia-reperfusion injury by ischemic preconditioning [27–31], postconditioning [32] and pharmacological conditioning by erythropoietin [33]. A recent study has suggested that a 14 day pretreatment with losartan (3 mg/kg/d) or valsartan (2 or 10 mg/kg/d) failed to limit IS in Otsuka–Long–Evans–Tokushima fatty (OLETF) diabetic rats, although the ARBs restored the protective effects of erythropoietin (EPO) and [D-Ala2, D-Leu5]-enkephalin acetate (DADLE) (a δ -opioid receptor agonist) [27].

Activation of phosphoinositide 3 kinase (PI3K)/Akt pathway with downstream activation of endothelial nitric oxide synthase (eNOS) and p70 ribosomal S6 protein kinase (P70S6K) is essential for mediating the cardioprotective effects of ischemic- and various forms of pharmacological preconditioning [34]. Akt is activated by phosphorylation at Ser-473 and Thr-308 by mTORC2 and PDK1, respectively [35]. Activated Akt phosphorylates eNOS. We have chosen to assess myocardial levels of Ser-1177 phosphorylated eNOS as an indicator for activation by Akt [36].

We assessed the effects of oral aliskiren (AL) and high-dose valsartan (VA) (an ARB), alone and in combination; on myocardial IS in mice with T2DM. In addition, we explored the effects of AL and VA on myocardial activation of Akt and eNOS, known mediators of myocardial protection against ischemia-reperfusion injury [34]. We also assessed the effects of AL and VA on myocardial expression of Silent Information Regulator 1 (Sirt1) and Peroxisome Proliferator-activated Receptor- γ Co-activator-1 α (PGC-1 α), factors with major effects on cardiac metabolism and survival [37–39], and on the expression of the angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors.

Methods

Male db/db mice were purchased from Charles River Laboratories (Wilmington, MA) and received humane care in compliance with ‘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). The protocol was approved by the University of Texas Medical Branch IACUC.

Materials

Anti-Akt, anti-phospho-Akt^{Thr308} and anti-Ser-473 phospho-Akt^{Ser473} antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-Sirt1, anti-Angiotensin II Type 1 receptor (AT1R), anti-Angiotensin II Type 2 receptor (AT2R) and PGC-1 α antibodies were purchased from Abcam Inc. (Cambridge, MA). Anti-Phospho-eNOS^{Ser1177} and anti-eNOS antibodies were purchased from Cell Signaling (Beverly, MA). Anti- β -actin antibodies were purchased from Sigma (St Louis, MO). Valsartan and aliskiren were provided by Novartis Pharma AG (Basel, Switzerland).

Treatment

Six week old Db/Db mice were fed Western Diet for 2 weeks. Thereafter, Western diet was mixed with: 1) vehicle (control); 2) AL 25 mg/kg/d (AL25); 3) AL 50 mg/kg/d (AL50); 4) VA 8 mg/kg/d (VA8); 5) VA 16 mg/kg/d (VA16); 6) AL 25+VA 16 mg/kg/d (AL25+VA16); or 7) AL 50+VA 16 mg/kg/d (AL50+VA16). Treatment was continued for 15 days.

Infarct size

On the 15th day, mice were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated and ventilated (FIO₂=30%). The rectal temperature was monitored and body temperature was maintained between 36.7 and 37.3°C throughout the experiment. After a stabilization period of 10 min, systolic blood pressure was recorded using a tail cuff monitor (ADInstruments, Monrovia, Ca). The chest was opened and the left coronary artery was encircled with a suture and ligated for 30 min. Ischemia was verified by regional dysfunction and discoloration of the ischemic zone. Isoflurane (1–2.0% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. At 30 min of ischemia, the snare was released and myocardial reperfusion was verified by a change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed and the mice recovered from anesthesia. Twenty-four hours after reperfusion the mice were re-anesthetized, the coronary artery was reoccluded, Evan’s blue dye 3% was injected into the right ventricle and the mice were euthanized under deep anesthesia [40, 41].

The pre-specified exclusion criteria were lack of signs of ischemia during coronary artery ligation, lack of signs of reperfusion after release of the snare, prolonged ventricular

arrhythmia, and area at risk $\leq 10\%$ of the left ventricular weight.

Determination of area at risk (AR) and infarct size (IS)

Hearts were excised and the left ventricle was sliced transversely into 6 sections. Slices were incubated for 10 min at 37°C in 1% buffered (pH=7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC), fixed in a 10% formaldehyde and photographed in order to identify the AR (uncolored by the blue dye), the IS (unstained by TTC), and the non-ischemic zones (colored by blue dye). The area of AR and IS in each slice were determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice. The results were summed to obtain the weight of the myocardial AR and IS [40, 41].

Immunoblotting

Myocardial samples from the risk zone of the anterior wall of the left ventricular wall exposed to ischemia-reperfusion (IR), or from the anterior wall of control hearts not exposed to ischemia (sham operated mice pretreated with vehicle-control group) were homogenized in lysis buffer (in mMol): 25 Tris-HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenyl-methylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na_3VO_4 , 1% Triton X-100, 2% SDS and 1% protease inhibitor cocktail. The lysate was centrifuged at 10,000g for 15 min at 4°C. The resulting supernatants were collected. Protein (50 μg) was fractionated by SDS-PAGE (4%–20% polyacrylamide gels) and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C with primary antibodies. Bound antibodies were detected using the chemiluminescent substrate (NEN Life Science Products, Boston, MA). The protein signals were quantified with an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β -actin signal. Data are expressed as percent of the expression in the control group.

Statistical analysis

Data are presented as mean \pm SEM. The significance level α is 0.05. Data were compared using analysis of variance (ANOVA) with Sidak corrections for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

A total of 62 mice were included in the infarct size experiment, nine animals died during the initial surgical procedure, (one from the control group, one from the AL25 group, two from the

AL50 group, one from VA8 group, one from the VA16 group, two from the AL25+VA group and one from the AL50+VA16 group). Fifty three animals completed the study.

Hemodynamics

Overall, there were differences in mean heart rate among the groups (Table 1). Mean heart rate was the fastest in the VA8 group (530 \pm 32 bpm) and the slowest in the AL25+VA16 group (509 \pm 5 bpm). However, none of the differences between the individual groups was significant. Overall, there were differences among the groups in systolic blood pressure (SBP) (Table 1). SBP was 124 \pm 16 mmHg in the control group. AL and VA tended to reduce SBP. Interestingly SBP in the AL25+VA16 tended to be higher than in the AL25 alone or VA16 alone; and SBP was the highest in the AL50+VA16 group. However, none of the differences between the individual groups was statistically significant.

Infarct size

Body weight, left ventricular weight and the size of the ischemic area at risk were comparable among groups (Table 2). In contrast, there were significant differences in IS among groups (Table 2, Fig. 1). AL, dose dependently reduced IS; however, the difference between AL25 and AL50 was not significant ($p=0.518$). VA8 did not affect IS ($p=0.327$ vs. control); however VA16 significantly reduced IS ($p < 0.001$ vs. control; $p < 0.001$ vs. VA8). The combination of AL50 and VA16 tended to have additive effects. IS in the AL50+VA group was significantly smaller compared to the control, AL25, VA8 and VA16 groups, and tended to be different from the AL50 alone group ($p=0.095$).

Immunoblotting

Additional animals underwent the surgical procedure and their hearts were explanted at 24 h of reperfusion for immunoblotting ($n=4$ per group). Ischemia-reperfusion had

Table 1 Heart rate (HR) and systolic blood pressure (SBP)

Group	Heart rate (bpm)	Systolic blood pressure (mmHg)
Cont	522 \pm 12	124 \pm 16
AL25	522 \pm 8	106 \pm 4
AL50	525 \pm 5	109 \pm 5
VA8	530 \pm 32	101 \pm 3
VA16	484 \pm 13	94 \pm 10
AL25+VA16	509 \pm 5	112 \pm 4
AL50+VA16	512 \pm 7	132 \pm 4
<i>P</i> value	0.038	0.021

Table 2 Body weight (BW), left ventricular weight (mg), area at risk (mg), and infarct size (mg) of control and treated mice

Group	BW (g)	LV (mg)	AR (mg)	IS (mg)
Control <i>n</i> =9	41.6±1.8	117±3	50±1	21±1
AL25 <i>n</i> =9	42.2±1.7	114±3	46±1	10±1
AL50 <i>n</i> =8	42.9±2.1	116±4	45±2	7±1
VA8 <i>n</i> =6	40.7±1.9	116±2	46±2	16±2
VA16 <i>n</i> =7	41.2±2.7	116±5	43±4	8±1
AL25+VA16 <i>n</i> =7	40.3±2.5	116±3	41±4	6±1
AL50+VA16 <i>n</i> =7	41.3±1.7	118±2	41±1	3±1
<i>P</i> value	0.981	0.990	0.050	<0.001

no effect on myocardial AT1R levels. AL50, VA16 and AL50+VA16 significantly reduced AT1R levels, without any significant difference among the three treatment groups (Fig. 2). In contrast, AT2R levels were not affected by ischemia-reperfusion, AL50, VA16 or their combination (Fig. 2).

Ischemia-reperfusion increased myocardial levels of Ser-473 P-Akt and Thr-308 P-Akt, without altering total Akt levels (Fig. 2). AL50 and VA16 augmented Ser-473 P-Akt and Thr-308 P-Akt in the post ischemic zone. AL50+VA16 resulted in the highest levels; however, the differences between AL50+VA16 and the AL50 alone or VA16 alone were not significant (Fig. 3).

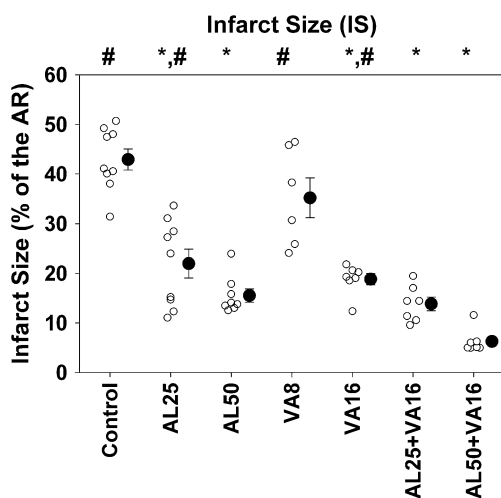


Fig. 1 Infarct size (percent of the ischemic area at risk (AR)). Overall there were significant differences among groups ($p < 0.001$). * $p < 0.001$ vs. control; # $p < 0.008$ vs. AL50+VA16

Ischemia-reperfusion increased myocardial levels of Ser-1177 P-eNOS, without affecting total eNOS levels. AL50 alone and VA16 alone caused a non-significant increase in Ser-1177 P-eNOS levels. In contrast, myocardial levels of Ser-1177 P-eNOS were significantly higher in the AL50+VA16 group than in the control and sham-operated group (Fig. 3).

Ischemia-reperfusion did not affect myocardial Sirt1 levels. AL50 and VA16 induced a significant increase in myocardial Sirt1 levels. However, the combination of AL50+VA16 did not induce it more than AL50 or VA16 alone (Fig. 4).

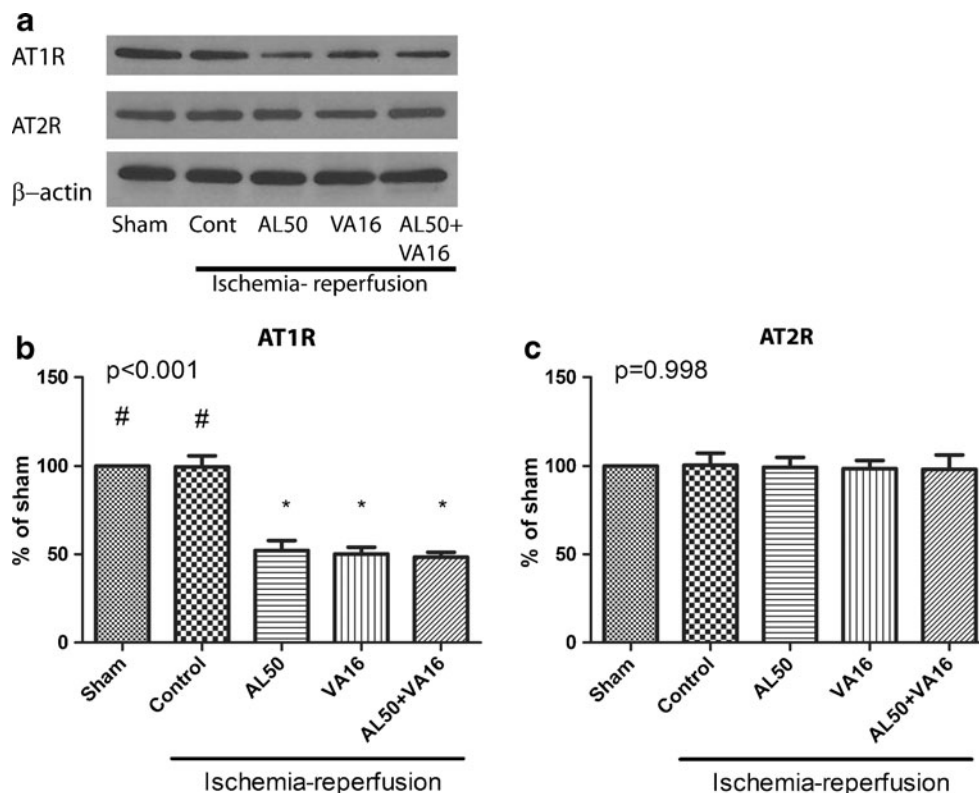
Similarly, ischemia-reperfusion did not affect myocardial PGC1 α levels. In contrast, AL50 and VA16 significantly increased PGC-1 α levels. The combination of AL50+VA16 resulted in significantly higher PGC-1 α level when compared to the other groups (Fig. 4).

Discussion

In the present study we show for the first time that AL limited myocardial IS in mice with T2DM. In addition, high dose (16 mg/kg/d), but not a lower dose (8 mg/kg/d) of VA, limited IS in mice with T2DM. Both AL and VA reduced myocardial AT1R levels without affecting AT2R levels, increased Akt phosphorylation, and increased myocardial Sirt1 and PGC-1 α levels. The combination of AL50 and VA16 tended to have additive myocardial protective effects, without additional blood pressure lowering effects.

ARBs block AT1R (that is thought to mediate most of the unwanted effects of angiotensin II) leaving the activation of AT2R (that is thought to mediate numerous beneficial effects including myocardial protection against ischemia-reperfusion injury) by angiotensin II intact [17, 20]. AL, on the other hand, by reducing renin activity is expected to reduce angiotensinogen, angiotensin I and angiotensin II levels. Thus, activation of both AT1R and AT2R are expected to be reduced [42]. However, it has been shown that the inhibitory effect of AL on plasma renin activity tends to decrease over time [43]. Moreover, at 3 weeks of treatment, the effects of AL on reducing plasma angiotensin I and angiotensin II that were seen after 1 week, were significantly attenuated. After 3 weeks of treatment, angiotensin I and II concentrations in the kidney of spontaneously hypertensive rats were comparable between the controls and AL-treated rats [43]. In contrast, cardiac concentrations of angiotensin I and II remained reduced after 3 weeks of treatment with AL [43]. This study shows that systemic inhibition of plasma angiotensin I and II levels may not fully explain the beneficial effects of AL. Inhibition of the tissue-specific renin-angiotensin systems [44] and other effects,

Fig. 2 Samples of immunoblots (a) and densitometric analyses of myocardial levels of AT1R (b) and AT2R (c) levels. * $p < 0.001$ vs. control; # $p < 0.001$ vs. AL50+VA16



such as reduction in the expression of AT1R [43], may explain the effects of AL.

Valsartan and myocardial protection against ischemia-reperfusion injury

Previous studies using non-diabetic animals have shown that VA limits IS. Jugdutt et al. reported that intravenous VA (10 mg/kg) reduced IS in rats [22, 45] and dogs [45–47]. Yang and colleagues reported that 14-day pretreatment with VA (5 and 10 mg/kg/d) dose dependently reduced IS in rats [48]. Zhao et al. found that at 2 mg/kg/d, 3-day pretreatment with VA reduced IS in mini-swines [49]. In contrast, Hotta and colleagues have recently shown that daily VA (2 mg/kg/d or 10 mg/kg/d) administered subcutaneously for 2 weeks did not affect IS in Otsuka–Long–Evans–Tokushima fatty (OLETF) rats with T2DM, although they normalized calcineurin activity and restored the response of Jak2 to erythropoietin with subsequent restoration of the IS-limiting effects of erythropoietin [27]. Our findings that at 8 mg/kg/d, VA did not affect IS in Db/Db mice support the findings of Hotta and colleagues that animals with T2DM may be more resistant to preconditioning with ARBs than nondiabetic animals. Indeed, it has been shown that preconditioning is less effective in limiting IS in diabetic animals due to higher levels of Phosphatase and Tensin Homolog on Chromosome Ten (PTEN) that prevents effective Akt phosphorylation [31,

50]. However, we are showing that with a higher dose (16 mg/kg/d), VA effectively limited IS without a major blood pressure lowering effect in Db/Db mice.

Myocardial AT1R and AT2R levels

It was shown that ARBs increase AT2R protein levels in the post-ischemic reperfused myocardial zone, without affecting AT1R protein levels in nondiabetic dogs [19, 46] and rats [22]. In contrast, we are showing that in the Db/Db mice, pretreatment with VA reduced AT1R protein levels without affecting AT2R levels. We cannot exclude the possibility that an interaction between VA and AT1R prevented accurate detection of the protein by the antibody. The lack of effect on AT2R levels in our model may be related to the fact that our animals had T2DM, although we cannot exclude species differences between mice, rats and dogs [26].

We also found that AL reduces myocardial AT1R levels, supporting the findings of van Esch and colleagues that reported that AL reduced AT1R mRNA and protein expression in kidneys of spontaneously hypertensive rats [43, 51] and in skeletal muscles of transgenic mice with increased renin activity [52].

Thus, reducing AT1R levels may partially explain the beneficial effects of VA and AL. The fact that IS was smaller in the AL50+VA16 group than in the VA16 alone and AL50 alone group, despite the fact the AT1R

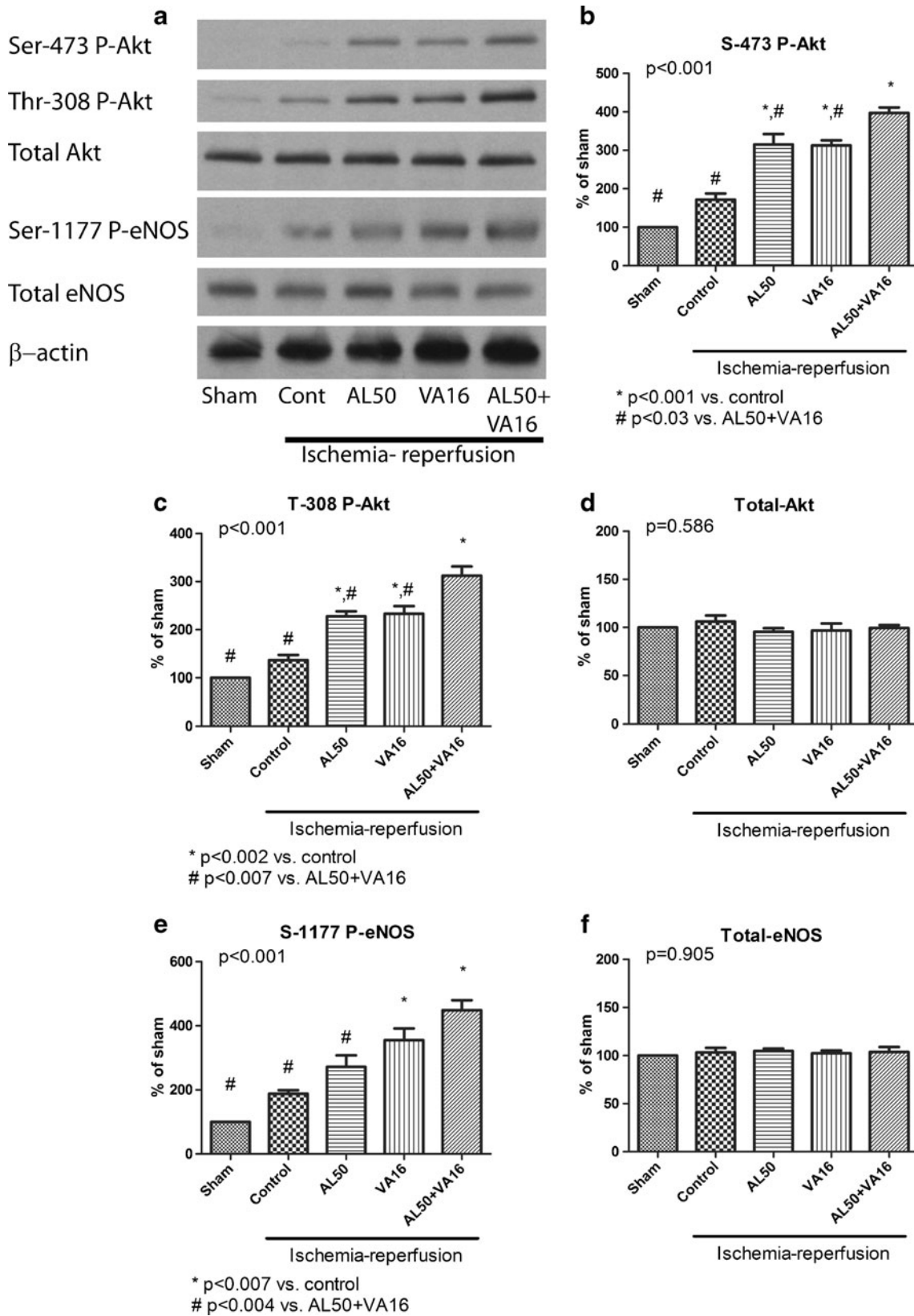
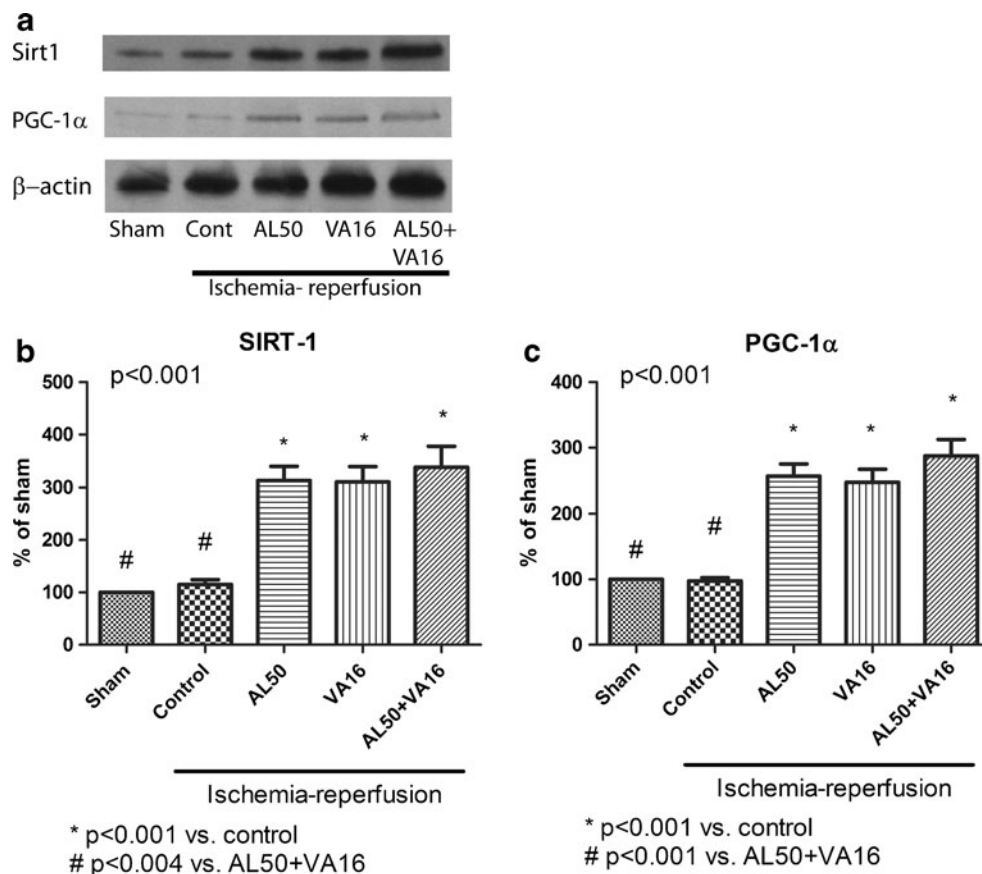


Fig. 3 Samples of immunoblots (a) and densitometric analyses of myocardial levels of Ser-473 P-Akt (b), Thr-308 P-Akt (c), total Akt (d), Ser-1177 P-eNOS (e), and total eNOS (f)

Fig. 4 Samples of immunoblots (a) and densitometric analyses of myocardial levels Sirt1 (b) and PGC-1 α (c)



levels were comparable among these three groups, suggest that this may not be the only mechanism involved.

Akt phosphorylation

Activation of the Phosphoinositide 3-kinase (PI3K)/Akt pathway is an essential component of innate protection against ischemia-reperfusion injury [53]. However, it has been shown that preconditioning is less effective in limiting IS in diabetic animals due to higher levels of Phosphatase and Tensin Homolog on Chromosome Ten (PTEN) that prevents effective Akt phosphorylation [31, 50]. Here, we show that both VA and AL augmented Akt phosphorylation at Ser-473 and Thr-308 after ischemia-reperfusion, and that the levels of P-Akt were highest in the AL50+Va16 group, in agreement with their smallest IS. Previously, we showed that azilsartan (TAK-491) reduced IS and increased Akt phosphorylation following ischemia-reperfusion in non-diabetic rats [18]. Previous studies have also shown that ARBs increase Akt phosphorylation [27, 54–56] and several studies have shown that AL increases Akt phosphorylation in various experimental models [52, 57–60]. Thus, increased phosphorylation of Akt may mediate the protective effects of VA and AL against ischemia-reperfusion injury. However, the mech-

anism(s) of Akt activation by these drugs has yet to be explored. Moreover, it was recently suggested that there might be differences among species, as blocking PI3K/Akt activation by wortmannin failed to attenuate the infarct size limiting effects of ischemic postconditioning in pigs [61].

eNOS phosphorylation

eNOS is involved in the delayed form of ischemic preconditioning [62] and is essential for the IS-limiting effects of statins [63]. We have shown that azilsartan (TAK-491) increases myocardial calcium-dependent NOS activity and eNOS phosphorylation at Ser-1177 following ischemia-reperfusion in the rat [18]. Losartan also increases eNOS phosphorylation in the rat heart [64]. In human aortic endothelial cells, valsartan induced eNOS phosphorylation is dependent on PI3K, but not on protein kinase A (PKA), protein kinase C (PKC) or adenosine monophosphate-activated protein kinase (AMPK) [54]. ARBs prevent NOS uncoupling and the production of superoxide, increasing NO availability [65]. The nonspecific NOS inhibitor (N(G)-monomethyl-L-arginine (L-NMMA) abrogates the protective effects of candesartan against ischemia-reperfusion injury in dogs [19]. Imanishi and colleagues reported that both VA and AL augmented

acetylcholine-induced NO production and eNOS phosphorylation in aortae of Watanabe heritable hyperlipidemic rabbits [59]. It was also described that VA increases the tyrosine residue phosphorylation of AT1R via activation of Src Kinase, suppressing the interaction between AT1R and eNOS and upregulating eNOS activation [54].

Here we show that AL 50 alone, or VA16 alone, caused a non-significant increase in myocardial Ser-1177 P-eNOS levels. In contrast, myocardial levels of Ser-1177 P-eNOS were significantly higher in the AL50+VA16 group than in the control and sham-operated groups, in accordance with there being a greater effect from the combination treatment group on IS. The lack of a significant effect of the ARB on eNOS phosphorylation in the present study, compared to our previous study in the rat [18], may be due to the fact that in the present study we used mice with T2DM whereas in the previous study we used nondiabetic rats. Another possible explanation is that in the previous study we assessed P-eNOS expression 4 h after reperfusion and in the present study we assessed it at 24 h. It is possible that P-eNOS levels decrease over time.

Silent Information Regulator 1 (Sirt1) levels

Sirt1 is a member of the sirtuin family of class III histone deacetylases [39]. Sirt1 is involved in gene silencing, cell survival, differentiation, metabolism, and longevity [39]. Resveratrol, by stimulating Sirt1, prolongs the lifespan of mice fed a high-calorie diet [38]. Upregulation of Sirt1 inhibits apoptosis, protects against oxidative stress in cardiac myocytes, and retards the progression of aging in the mouse heart [37]. It has been reported that Sirt1 inhibits the expression of AT1R both *in vivo* and *in vitro* [66]. Here we show that both AL and VA increased myocardial Sirt1 levels and reduced AT1R levels, in support of the previous findings.

Hyperglycemia decreases Sirt1 expression with subsequent p53 upregulation leading to endothelial senescence and dysfunction [67]. Sirt1 activation or p53 inhibition ameliorates high glucose-induced endothelial dysfunction [67]. Sirt1 upregulation by ischemic preconditioning or resveratrol protects rat brain against ischemia, and the Sirt1-specific inhibitor sirtinol abolishes the neuroprotection afforded by resveratrol [68]. Blocking Sirt1 abrogates neuroprotection against ischemia afforded by Icarin [69]. Hsu and colleagues have recently shown that cardiac-specific overexpression of Sirt1 reduced apoptosis and limited myocardial IS in the mouse [70]. In their model, Sirt1 positively regulates expression of pro-survival molecules, including manganese superoxide dismutase, thioredoxin-1, and Bcl-xL, whereas it negatively regulates the pro-apoptotic molecules Bax and cleaved caspase-3 [70]. Moreover, it was suggested that Sirt1 deacetylates Akt, leading to enhanced binding of Akt to

phosphatidylinositol 3,4,5-trisphosphate (PIP3); and therefore, enhanced activation by phosphorylation [71].

However, in the present study the AL50+VA16 combination did not induce higher levels of Sirt1 when compared to each agent alone, despite having a greater effect on IS; suggesting that upregulation of Sirt1 is not the sole mechanism contributing to myocardial protection by VA and AL.

Peroxisome Proliferator-activated Receptor- γ Co-activator-1 α (PGC-1 α)

Here we show that both AL and VA significantly increased myocardial PGC-1 α levels. The combination of AL50+VA16 resulted in significantly higher PGC-1 α levels when compared to all the other groups, in concordance with the effect on IS. PGC-1 α is a transcriptional co-activator that coordinately regulates the expression of distinct sets of metabolism-related genes in different tissues [72]. Sirt1 controls PGC-1 α expression in skeletal muscle of mice [72]. It has been shown that intracoronary cyclosporine reduces myocardial IS in pigs [73, 74] and increases myocardial PGC-1 α levels in pigs [73]. It was reported that Icarin reduces brain infarct size in the mouse and upregulates Sirt1 and PGC-1 α levels [69]. Blocking Sirt1 or inhibiting PGC-1 α with siRNA abrogated the neuroprotective effects of Icarin [69]. Ischemic preconditioning and diazoxide, a mitochondrial ATP-sensitive K channel opener, increases myocardial levels of PGC-1 α [75]. However, Lynn and colleagues reported opposite findings. They found that in an isolated heart model of cardiac-restricted inducible PGC-1 α transgenic mouse, overexpression of PGC-1 α increased necrosis and decreased recovery of function following reperfusion [76]. They reported that PGC-1 α overexpression induced the expression of adenine nucleotide translocase 1 (ANT1) that mediates apoptosis [76]. Indeed, Rasbach and Schnellmann reported that overexpression of PGC-1 α before oxidant exposure increased death of renal proximal tubular cells *in vitro*. However, overexpressing PGC-1 α after oxidant injury accelerated recovery of mitochondrial function [77]. Thus, the role of PGC-1 α in modulating ischemia-reperfusion injury is yet to be determined.

In conclusion, in the present study we show that AL, dose dependently limits myocardial IS in mice with T2DM. At doses shown to limit IS in non-diabetic animals, VA failed to reduce IS in Db/Db mice. However, at higher dose (16 mg/kg/d), VA reduced IS. Both AL and VA reduced myocardial AT1R levels and increased the expression of the longevity genes Sirt1 and PGC-1 α with increased phosphorylation of Akt. Further studies are needed to elucidate the role of Sirt1 and PGC-1 α in mediating “pleiotropic” effects of ARBs and direct renin inhibitors.

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