Activation of Endothelial Nitric Oxide Synthase by a Vanadium Compound Ameliorates Pressure Overload-Induced Cardiac Injury in Ovariectomized Rats

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Abstract—We here investigated the effect of bis(1-oxy-2-pyridinethiolato) oxovanadium (IV), [VO(OPT)], against myocardial hypertrophy and cardiac functional recovery in pressure overload–induced hypertrophy in ovariectomized female rats and defined mechanisms underlying its cardioprotective action. Wistar rats subjected to bilateral ovariectomy were further treated with abdominal aortic stenosis. VO(OPT) (containing 1.25 and 2.50 mg of vanadium per kg) was administered orally once a day for 14 days starting from 2 weeks after aortic banding. Treatment with VO(OPT) significantly inhibited pressure overload–induced increase both in the heart weight:body weight ratio and the lung weight:body weight ratio. VO(OPT) also attenuated hypertrophy-induced impaired left ventricular end-diastolic pressure, left ventricular developed pressure, and left ventricular contractility ($\frac{\Delta p}{dt}_{max}$). VO(OPT) treatment significantly restored pressure overload–induced impaired endothelial NO synthase activity with concomitant increased phosphorylation of endothelial NO synthase (Ser1179). Moreover, VO(OPT) treatment significantly restored pressure overload–induced reduced Akt activity, as indicated by increased phosphorylation at Ser473 and at Thr308. Treatment with VO(OPT) also secondarily inhibited calpastatin and dystrophin breakdown and decreased myosin light chain phosphorylation. Finally, VO(OPT) treatment significantly attenuated mortality after repeated isoproterenol administration in pressure overloaded–ovariectomized rats. Taken together, VO(OPT) attenuates cardiac myocytes hypertrophy in vivo in pressure overload–induced hypertrophy in ovariectomized rats and prevents the process from hypertrophy to heart failure. These effects are mediated by inhibition of calpastatin and dystrophin breakdown in addition to increased Akt and endothelial NO synthase activities. (Hypertension. 2009;53:57-63.)

Key Words: myocardial hypertrophy $\blacklozenge$ protein kinase B (Akt) $\blacklozenge$ endothelial nitric oxide synthase (eNOS) $\blacklozenge$ ovariectomy $\blacklozenge$ dystrophin

Left ventricular (LV) hypertrophy is an independent risk factor for the development of heart failure.1 Although LV hypertrophy is an adaptive response to pressure and volume overload, this process becomes maladaptive without drug therapy. The pathological cardiac hypertrophy, in turn, triggers the development of heart failure.2 The search for novel drug treatment has directed attention to cardiac hypertrophy as cardioprotection.2

Signaling through the phosphatidylinositol 3-kinase/Akt pathway is important for the physiological growth and inhibition of pathological hypertrophy.3–5 Moreover, physiological hypertrophy induced by exercise training also requires the activation of myocardial Akt. By contrast, pathological hypertrophies induced by pressure overload cause an inactivation of the Akt signal pathway.6 We have also reported that pressure overload (PO)–induced hypertrophy in ovariectomized (OVX) female rats markedly reduces both endothelial NO synthase (eNOS) protein expression and its activity with concomitantly marked reduced Akt activity.7

Inhibition of eNOS induces myocardial hypertrophy in rats.8 Likewise, NO has antihypertrophic effects, thereby inhibiting cardiac remodeling.9,10 Increased eNOS activity by angiotensin-converting enzyme inhibitors,11 statins,12,13 and estrogens14 elicits an improvement of cardiac remodeling. Moreover, bovine eNOS is phosphorylated on Ser1179 (1177 for the human eNOS) by the Ser/Thr kinase Akt15,16 with concomitant increased NO production even at low calcium concentrations. Indeed, statins activate the serine/threonine kinase Akt15,16 in endothelial cells, thereby enhancing eNOS phosphorylation and increasing NO.3 However, drugs targeting activation of Akt have not been developed in the hypertrophy-induced cardiac remodeling. Thus, we hypothesize that stimulator of phosphatidylinositol 3-kinase/Akt signaling likely inhibits cardiac remodeling via upregulation of eNOS in the cardiomyocytes. We17–19 and others20 have shown that vanadium compounds are potent Akt activators with cytoprotective action.
against myocardial ischemia/reperfusion injury. In addition, we recently introduced a novel vanadyl (IV) compound having VO\(^{2+}\) chelate, bis(1-oxy-2-pyridinethiolato) oxovanadium (IV), [VO(OPT)], as potent activator of protein kinase B/Akt, thereby protecting cardiomyocytes from ischemia/reperfusion injury in rats\(^7\) and in mouse brain ischemia.\(^{21}\) Consistent with our findings, the vanadyl (IV) form of vanadium possesses cardioprotective action\(^22\) and antihypertensive action in spontaneous hypertensive rats and in fructose-induced hypertensive rats\(^{23–25}\) through unknown mechanisms.

In the present study, we evaluated the effect of continuous treatment with VO(OPT) in PO-induced cardiac injury induced by transverse aortic constriction in OVX female rats.\(^7\) To observe the cardioprotective effect of the Akt activator VO(OPT) in the hypertension-induced cardiac remodeling, we used the OVX PO-induced hypertrophy model, because that model causes reproducible hypertrophy with the severe impairment of Akt and eNOS signaling.\(^7\) We also defined the molecular mechanism underlying VO(OPT)-mediated cardioprotection in LV hypertrophy and its remodeling. We especially focused on VO(OPT)-mediated activation of the myocardial Akt and eNOS signaling after PO-induced hypertrophy. Finally, we confirm VO(OPT)-induced inhibition of cardiac remodeling rescue rats from isoproterenol-induced cardiac arrest.

Materials and Methods

For detailed Materials and Methods, please see the online data supplement, available at http://hyper.ahajournals.org.

Results

Effect of VO(OPT) Treatment on Morphometric Changes

Averages of heart weights (HWs; including both left and right ventricle), lung weights (LWs), and body weights (BWs) are presented in Table S1. Consistent with our previous observation, LV weight \((P<0.01\) versus OVX) and LW \((P<0.001\) vs OVX) significantly increased in the OVX pressure overloaded (OVX-PO) group compared with the OVX group, without significant changes in right ventricle weight.\(^7\) Treatment with VO(OPT) \((2.50\) mg of vanadium per kg, PO) on the sham and OVX rats had no effect on the morphometric parameters (Table S2). Treatment with VO(OPT) significantly and dose-dependently decreased the elevated LV weights and LWs. Notably, the ratio of HW:BW markedly increased in the OVX-PO group \((P<0.001\) versus OVX) compared with the OVX group. Treatment with VO(OPT) \((2.50\) mg of vanadium per kg) significantly restored the elevated the HW:BW ratio \((P<0.01\) versus OVX-PO; Figure 1A). Increases in the LW:BW ratio also significantly increased in the OVX-PO group \((P<0.001)\) compared with the OVX group. VO(OPT) treatment dose-dependently decreased the elevated LV:BW ratio \((P<0.01\) versus OVX-PO for 1.25 mg and \(P<0.001\) versus OVX-PO for 2.50 mg of vanadium; Figure 1B). Moreover, oral treatment with VO(OPT) \((2.50\) mg of vanadium per kg) for 14 days had no effect on the HW:BW ratio (Figure S1A) or the HW:LW ratio (Figure S1B) in sham-operated animals.

Effect of VO(OPT) Treatment on Hemodynamic Parameters

We documented previously that acute treatment with VO(OPT) by intraperitoneal administration has no effect on mean arterial blood pressure (MABP) and heart rate (HR).\(^{18}\) Here we tested continuous oral administration (14 days) of VO(OPT) on HR and MABP on both sham and OVX animals. VO(OPT) treatment \((2.50\) mg of vanadium per kg) slightly decreased HR in both sham and OVX rats, but changes were not significant (Figure S2A). VO(OPT) treatment \((2.50\) mg of vanadium per kg) slightly decreased MABP in sham rats, but a significant decrease in MABP was observed in OVX rats (Figure S2B). Consistent with a previous observation,\(^7\) OVX-PO treatment significantly increases both in HR \((P<0.01\) versus OVX; Figure 2A) and in MABP \((P<0.01\) versus OVX; Figure 2B) compared with OVX rats. VO(OPT) treatment dose-dependently and significantly reduced HR \((P<0.05\) versus OVX-PO for 1.25 mg and \(P<0.05\) versus OVX-PO for 2.50 mg of vanadium; Figure 2A) and MABP \((P<0.05\) versus OVX-PO for 1.25 mg and \(P<0.001\) versus OVX-PO for 2.50 mg of vanadium; Figure 2B).

Because treatment with VO(OPT) \((1.25\) and \(2.50\) mg of vanadium per kg) restored HR and MABP, we evaluated LV functions in OVX-PO heart with or without treatment of VO(OPT). Consistent with our previous observation,\(^7\) LV end-diastolic pressure was significantly increased in the OVX-PO group \((P<0.001\) versus OVX) compared with the OVX group. VO(OPT) treatment dose-dependently restored elevated LV end-diastolic pressure \((P<0.001\) versus OVX-PO for 1.25 mg and \(P<0.001\) versus OVX-PO for 2.50 mg of vanadium; Figure 2C). Similarly, LV developed
pressure significantly increased in the OVX-PO group (P<0.001 versus OVX), and VO(OPT) treatment dose-dependently restored elevated LV developed pressure (P<0.01 versus OVX-PO vehicle for 1.25 mg and P<0.001 versus OVX-PO vehicle for 2.50 mg of vanadium; Figure 2D). The rates of LV contraction (+dp/dt) and relaxation (−dp/dt) also significantly increased in OVX-PO (P<0.001 versus OVX; Figure 2E), and VO(OPT) treatment dose-dependently increased eNOS expression after OVX-PO treatment (P<0.01 for 1.25 mg and P<0.001 versus OVX-PO for 2.50 mg of vanadium; Figure 2E). In agreement with earlier studies, the treatment with VO(OPT) containing 2.50 mg of vanadium per kg resulted in slight decrease in body weight and food intake compared with vehicle-treated rats (Figure S3), but these changes are not significant.

**Effect of VO(OPT) Treatment on Akt Activity**

To define the role of Akt activity in cardiac hypertrophy and heart failure, we evaluated the time course of cardiac hypertrophy, heart failure, and the LV Akt activity. HW:BW and LW:BW were increased time dependently from 1 to 4 weeks after PO in OVX rats (Figure S4A and S4B). LV Akt phosphorylation at Ser473 was increased 1 week after PO; thereafter, it decreased time dependently, with a significantly decreased level observed 4 weeks after PO in OVX rats. On the contrary, no significant change was observed in the total Akt level (Figure S4A). We also documented previously that OVX-PO treatment severely decreased Akt phosphorylation at Ser473 in female rats. Akt phosphorylation at Thr308 also slightly decreased in the OVX-PO group, but the change was not significant (Figure 3). Importantly, VO(OPT) treatment markedly and dose-dependently increased Akt activity as assessed by increased phosphorylation at Ser473 at Ser1179 decreased time dependently after PO; treatment with a significantly decreased level observed 4 weeks after PO in OVX rats (Figure S5B). Consistent with earlier studies, the treatment with VO(OPT) containing 2.50 mg of vanadium per kg resulted in slight decrease in body weight and food intake compared with vehicle-treated rats (Figure S3), but these changes are not significant.

**Effect of VO(OPT) Treatment on eNOS Expression and Its Activity**

Because eNOS is a physiological substrate for Akt in human vascular endothelial cells, we determined whether VO(OPT)-induced Akt activation results in increased eNOS phosphorylation and its activity. The time course studies revealed that both eNOS and Akt-mediated eNOS phosphorylation at Ser1179 decreased time dependently after PO treatment with a significantly decreased level observed 4 weeks after PO in OVX rats (Figure S5B). Consistent with our previous observation, we also observed severe impairment of eNOS expression after OVX-PO treatment (P<0.01 versus OVX). We found here a slight but not significant reduction of eNOS phosphorylation at Ser1179. Notably, VO(OPT) treatment dose-dependently increased eNOS phosphorylation (P<0.01 for 1.25 mg and P<0.001 for 2.5 mg of vanadium; Figure 4A and 4B). VO(OPT) also significantly increased eNOS expression (P<0.05 for 2.5 mg of vanadium; Figure 4A and 4C). These results suggest that VO(OPT) treatment preferentially activates eNOS activity through Akt-dependent phosphorylation at Ser1179.

**Figure 2.** Effect of VO(OPT) on hemodynamic measurements. A, HR. B, MABP. C, Left ventricular end diastolic pressure (LVEDP). D, Left ventricular developed pressure (LVDP). E, Rates of left ventricular contraction (+dp/dt) and relaxation (−dp/dt). Each bar represents the mean±SEM for 5 experiments. *P<0.05, **P<0.01, and ***P<0.001 vs the OVX group; †P<0.05, ††P<0.01, and †††P<0.001 vs the OVX-PO-vehicle–treated group.
Effects of VO(OPT) Treatment on Heat Shock Protein-90 and Caveolin-3 Expression

Localization and activity of eNOS are regulated by making a complex with a chaperone protein, heat shock protein-90 (HSP-90) and caveolin-3 in cardiomyocytes, especially in caveolae.49 Because the eNOS protein level was reduced by the OVX-PO group, we assessed whether HSP-90 and caveolin-3 levels are changed by OVX-PO and whether VO(OPT) treatment affects their expression. OVX-PO treatment slightly but not significantly decreased HSP-90 expression. VO(OPT) treatment tended to recover HSP-90 expression in the LV (Figure 5A). Similarly, OVX-PO treatment did not affect the caveolin-3 expression (Figure 5B). Taken together, downregulation of eNOS was not attributable to changes in HSP-90 and caveolin-3 levels as a binding partner of eNOS in caveolae of cardiomyocytes. We documented previously a significant and marked increase in myosin light chain (MLC) phosphorylation (Ser19) in the OVX-PO group when its phosphorylation was expressed by the ratio of the MLC chain (MLC) phosphorylation (Ser19) in the OVX-PO group previously a significant and marked increase in myosin light chain (MLC) phosphorylation (Ser19) in the OVX-PO group.

Effect of VO(OPT) Treatment on Calpastatin and Dystrophin Levels

Because increased MLC phosphorylation suggested increased intracellular Ca\(^{2+}\) concentration or inhibition of MLC phosphatase activity, we next investigated activation of calpain in OVX-PO LV muscles. OVX-PO treatment significantly induced dystrophin breakdown, which is a substrate for calpain in the pathological conditions (P<0.05 versus OVX; Figure 6A). More importantly, VO(OPT) treatment (2.5 mg of vanadium per kg) significantly inhibited calpastatin and dystrophin breakdown (calpastatin: P<0.01 versus OVX-PO; dystrophin: P<0.05 versus OVX-PO; Figure 6A and 6B).

VO(OPT) Treatment Protects Against Isoproterenol-Induced Heart Failure

We recently found repeated β-adrenergic stimulation in OVX-PO female rats, dramatically increased in mortality rate.7 Kaplan-Meier survival plots are shown in Figure 7. Repeated treatment with isoproterenol (5 mg/kg for 28 days) had no effect on sham rats but markedly increased in mortality after OVX-PO treatment with a survival rate of 0% at 21 days. VO(OPT) treatment significantly reduced the mortality (P<0.01 versus OVX-PO, P<0.001 for 1.25 and 2.5 mg of vanadium; Figure 7). Taken together, reduced mortality by VO(OPT) treatment suggest that inhibition of decompensatory signaling pathways indeed protects the heart from the cardiac arrest under pathological conditions such as OVX-PO.

Discussion

We defined here a novel cardioprotective mechanism of an organic vanadyl chelate, VO(OPT), through eNOS upregula-
HSP-90, and caveolin-3 in cardiomyocytes, especially in caveolae.30 We did not find any significant changes in HSP-90 in the OVX-PO group; thus, HSP-90 level is not the primary target for VO(OPT)-induced cardioprotection. Likewise, the caveolin-3 level was not affected by VO(OPT) treatment. Thus, eNOS phosphorylation is a predominant mechanism in VO(OPT)-induced cardioprotection. Because HSP-90 level tended to decrease after OVX-PO and increase by VO(OPT) treatment, more extensive studies are required to determine temporal changes and immunohistochemical localization of HSP-90 and eNOS after OVX-PO.

We also previously documented increased dystrophin breakdown after OVX-PO in female rats2 and after prolonged stimulation with endothelin-1 in cultured cardiomyocytes.31 The marked breakdown of dystrophin suggests involvement of calpain in OVX-PO–induced cardiac injury. We also found significant decrease in calpastatin levels after OVX-PO. Because VO(OPT) treatment significantly inhibited both calpastatin and dystrophin, break down was only by high dose of VO(OPT) (2.50 mg of vanadium per kg), suggesting that the inhibition of calpastatin and dystrophin breakdown is a consequence of the cardioprotection by VO(OPT).

Because overexpression of activated Akt in the heart in transgenic mice has been shown to promote cardiac hypertrophy using PO-induced cardiac hypertrophy in OVX female rats. We documented previously that VO(OPT) elicits cardioprotective action through Akt activation using ischemia/reperfusion-induced heart injury in the rat.18 We recently introduced a novel heart injury model using OVX-PO female rats, which is attractive for testing cardioprotective drugs in hypertension-induced cardiac injury in postmenopausal women.7 The most striking phenomenon in the hearts of the OVX-PO female rats was impairment of Akt and eNOS signaling.7 Because VO(OPT) is a potent activator of phosphatidylinositol 3-kinase/Akt signaling through upregulation of tyrosine kinase receptor signaling,16,30 we speculated that VO(OPT) rescues the heart from hypertrophy-induced heart failure.

Like the single treatment,18 the continuous oral treatment with VO(OPT) for 14 days dose-dependently increased Akt activity. Akt can directly phosphorylate recombinant eNOS or eNOS in situ, at serine1177 (human)/1179 (bovine),16,30 thereby enhancing the eNOS activity. Interestingly, the continuous VO(OPT) treatment for 14 days not only increased Akt-mediated eNOS phosphorylation on Ser1179 but also enhanced eNOS protein expression at the high dose (2.50 mg of vanadium per kg). Localization and activity of eNOS are regulated by making a complex with a chaperone protein, HSP-90, and caveolin-3 in cardiomyocytes, especially in caveolae.30

Figure 5. Effects of VO(OPT) on HSP-90 (A), caveolin-3 expression (B), and MLC phosphorylation (C) in the left ventricular cell extracts from OVX (n=8), OVX-PO-vehicle (n=8), vanadium (V) 1.25 (n=8), and V 2.50 (n=8)–treated hearts. Data are expressed as percentages of the value of OVX rats. Each column represents the mean±SEM for 5 experiments. *P<0.05 and **P<0.01 vs the OVX group; †P<0.05, ††P<0.01, and †††P<0.001 vs the OVX-PO–vehicle–treated group.

Figure 6. Effects of VO(OPT) on calpastatin (A) and dystrophin (B) levels in the left ventricular cell extracts from OVX (n=8), OVX-PO-vehicle (n=8), vanadium (V) 1.25 (n=8), and V 2.50 (n=8)–treated hearts. Data are expressed as percentages of the value of sham-operated rats. Each column represents the mean±SEM of 5 experiments. *P<0.05 and **P<0.01 vs the OVX group; †P<0.05 and ††P<0.01 vs the OVX-PO–vehicle–treated group.

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VO(OPT) (10 mg of vanadium per kg) for 21 days has no effect on blood urea nitrogen and body weight in OVX-PO–induced hypertrophy in female rats. Lack of estrogen by OVX and PO leads to both reduction of eNOS expression and Akt phosphorylation. Under these conditions, Akt activation by VO(OPT) suppressed OVX-PO–induced hypertrophy in female rats. The inhibition of cardiac hypertrophy by OVX-PO was closely associated with recovery of HR, MABP, and contractile heart functions. The increased eNOS expression and/or phosphorylation likely mediate the recovery heart from OVX-PO–induced cardiac injury.

Finally, Kaplan-Meier survival data in our study clearly indicate that significantly increased mortality occurs only in the OVX-PO group after chronic β-adrenergic stimulation by isoproterenol (5 mg/kg). VO(OPT) treatment dose-dependently increased survival after acute cardiac stress caused by chronic β-adrenergic stimulation, suggesting that cardiac remodeling and recovered cardiac functions by VO(OPT) treatment contribute to the reduced mortality. This is a supportive observation of cardioprotective effects of VO(OPT) in the treatment of cardiac injury in postmenopausal women.

Previous study indicates that oral administration of VO(OPT) (10 mg of vanadium per kg) for 21 days has no effect on blood urea nitrogen and body weight in streptozotocin-induced diabetic rats. In the present series of experiments, none of the rats died in any of the studies conducted, and no gastrointestinal, hepatic, or renal toxicity was observed after 14 days of VO(OPT) treatment, as reported previously. Moreover, VO(OPT)-treated rats continue to gain weight throughout the experimental period, as reported previously. This suggests that the reduced weight gain caused by VO(OPT) administration is attributable to the reduced food and fluid intake.

In conclusion, the most important observation presented here is that simultaneous severe reduction of eNOS and Akt activity in OVX-PO female rats likely triggers compensatory hypertrophy with increased heart contractility (Figure S6). Potentiation of the Akt and eNOS signaling pathways, along with inhibition of dystrophin breakdown, by treatment with VO(OPT) after OVX-PO likely contributes to increased survival after acute cardiac stress caused by chronic β-adrenergic stimulation (Figure S6). VO(OPT) likely accounts for the antihypertrophic effect and cardiac remodeling to rescue cardiomyocytes from heart failure through activation of Akt and eNOS signaling pathways.

**Perspectives**

We have reported previously that, in the PO-induced hypertrophy, the decreased eNOS and Akt activities are linked to functional impairment and adverse cardiac remodeling in OVX female rats. The present study revealed that the VO(OPT) treatment can attenuate the PO-induced cardiac hypertrophy through the activation of Akt and eNOS signaling pathways. Moreover, we have for the first time revealed that VO(OPT), one of the Akt activators, prevents cardiac hypertrophy and dysfunction in the pressure-overloaded OVX female rats and elucidates the possible intracellular mechanisms of the VO(OPT)-mediated antihypertrophic stimulation. These observations open a new therapeutic perspective for intervention in the hypertrophic process and, at the same time, modulation of the Akt pathway may provide novel therapeutic targets for which a new class of antihypertrophic drugs can be designed.

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**Disclosures**

None.

**References**


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