

# Chapter 9

## Cardiovascular Protection with Vanadium Compounds

Kohji Fukunaga and Md Shenuarin Bhuiyan

**Abstract** Protein kinase B/Akt plays a critical role in the regulation of cardiac hypertrophy, angiogenesis and apoptosis. The evidences that elevation of Akt in cardiomyocytes *in vivo* and *in vitro* protects against apoptosis after ischemia/reperfusion injury provide possibility that agents targeting Akt activation become a novel therapeutic strategy for limiting myocardial injury following ischemia. Vanadium compounds inhibiting protein tyrosine phosphatases are potent activator of the Akt signaling pathways and elicit cardioprotection in heart ischemia/reperfusion injury along with cardiac functional recovery in rats. In addition, vanadium compounds has strong anti-hypertrophic in the pressure overload-induced hypertrophy in ovariectomized and aortic-banded rats. The elevation of Akt activity and Akt-dependent eNOS phosphorylation are central roles on vanadium compound-induced anti-hypertrophy and heart failure in the ovariectomized and aortic-banded rats. Taken together, vanadium compounds are potential therapeutics for ischemia/reperfusion-induced myocardial injury and heart failure associated with hypertension in the postmenopausal women.

**Keywords** Cardiovascular disease • Hypertrophy • Endothelial nitric oxide synthase • Postmenopausal women • Protein kinase B • Protein tyrosine phosphatase • Vanadium compounds

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## 9.1 Introduction

In the cardiovascular system, protein kinase B (PKB)/Akt plays an important role in the regulation of cardiac hypertrophy, angiogenesis, and apoptosis [1–5]. The Akt subfamily comprises three mammalian isoforms, PKB $\alpha$ /Akt1, PKB $\beta$ /Akt2 and PKB $\gamma$ /Akt3, which are products of distinct genes and share a conserved structure that includes three functional domains: an N-terminal pleckstrin homology domain, a central kinase domain, and a C-terminal regulatory domain containing the hydrophobic motif phosphorylation site [FxxF(S/T)Y] [6]. Among the three Akt genes, only Akt1 and Akt2 are highly expressed in the heart. Consistent with the general trophic function of Akt, the Akt1 whole-genome-knockout mice weigh approximately 20% less than wild-type littermates and have a proportional reduction in size of all somatic tissues, including the heart [7, 8]. In contrast, Akt2-knockout mice have only a modest reduction in organ size [9]. Thus, data from Akt-knockout mice support a critical role specifically for Akt1 in normal growth of the heart [10]. The observation that acute activation of Akt in cardiomyocytes *in vivo* and *in vitro* protects against apoptosis after ischemia/reperfusion injury provide possibility that agents targeting Akt activation become a novel therapeutic strategy for limiting myocardial injury following ischemia [11].

## 9.2 Cardioprotection with Vanadium Compound in Myocardial Infarction

### 9.2.1 Vanadium Compounds as Akt Activator

Vanadium compounds are potent activator of the Akt signaling pathways [12–15] and elicit cardioprotection in heart ischemia/reperfusion injury along with cardiac functional recovery in rats [1, 2, 15, 16]. Vanadium compounds inhibit protein tyrosine phosphatases [12, 13] and promote an increase in protein tyrosine phosphorylation, leading to the upregulation of Akt [12, 14]. An increase in tyrosine phosphorylation in the heart via increased tyrosine kinase activity has been implicated in the signal transduction pathway of cardioprotection by ischemic preconditioning [17–19] which is the most potent endogenous mechanism to limit myocardial infarct size. Among several oxidation states of vanadium II to V, vanadium ion in living cells exists exclusively as vanadyl (IV) cation and a small amount as vanadate (V) anion [20]. Furthermore, vanadyl (VO<sup>2+</sup>) compounds, of oxidation state IV, under physiological conditions are subject to oxidation by a variety of oxidants, including molecular oxidant and vanadate compounds, of oxidation state V are thought to undergo reduction to state IV in the cell [21–24]. To develop a novel therapeutic drug to protect cardiomyocytes from heart diseases, we selected a novel vanadyl (IV) compound having the VO<sup>2+</sup> chelate, bis(1-oxy-2-pyridinethiolato) oxovanadium(IV), [VO(OPT)], which is a potent activator of the Akt signaling pathway both *in vivo* and *in vitro* [12–14, 25].

### 9.2.2 Pharmacotoxicity of Vanadium Compounds

The toxicity of vanadium compounds is low. The most common toxic effects reported for inorganic vanadium compounds are diarrhea, decreased food uptake, dehydration and reduced body weight gain [14] which can, however, be corrected by adding sodium chloride to drinking water, adjusting the pH of the solution to neutrality and by gradually increasing the dose of vanadium [26, 27]. Organic vanadium compounds were much safer than inorganic vanadium salts and do not cause any gastrointestinal discomfort, hepatic or renal toxicity [25, 28]. Along with the previous studies, we also did not find any gastrointestinal, hepatic or renal toxicity in the VO(OPT) treated rats [29, 30].

Recent work has demonstrated that vanadium compounds inhibited serum- and growth factor-stimulated mitogenesis [31, 32] and possess anti-tumor activity [33, 34]. Many other studies have, however, failed to detect any change in the levels of urea, creatinine, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, indices of kidney and liver functions [35–37]. Moreover, no significant changes in the histopathology of several tissues, including the liver, spleen, stomach, heart and lung, have been observed among control and vanadyl sulfate-treated animals [38]. Electron microscopic examination of *ob/ob* mice treated with vanadate for 47 days revealed no sign of hepatotoxicity [27].

In patients treated with vanadium salts, gastrointestinal discomfort was the most common toxic effect, which could be corrected by decreasing the dose level [39]. Moreover, clinical studies have been of short duration (up to 6 weeks) and utilized lower doses than those administered in animal experiments; thus, the long-term toxicity of vanadium in humans remains to be explored. Clearly, at present, there is no consensus on the toxic effects of vanadium compounds, and detailed and systematic investigations are needed to evaluate the toxicity of various vanadium compounds before undertaking long-term clinical trials in humans. It should be noted that use of chelating agents and organo-vanadium compounds, such as VO(OPT), have shown significantly reduced vanadium toxicity and may serve as more potent cardioprotective agents than inorganic vanadium salts.

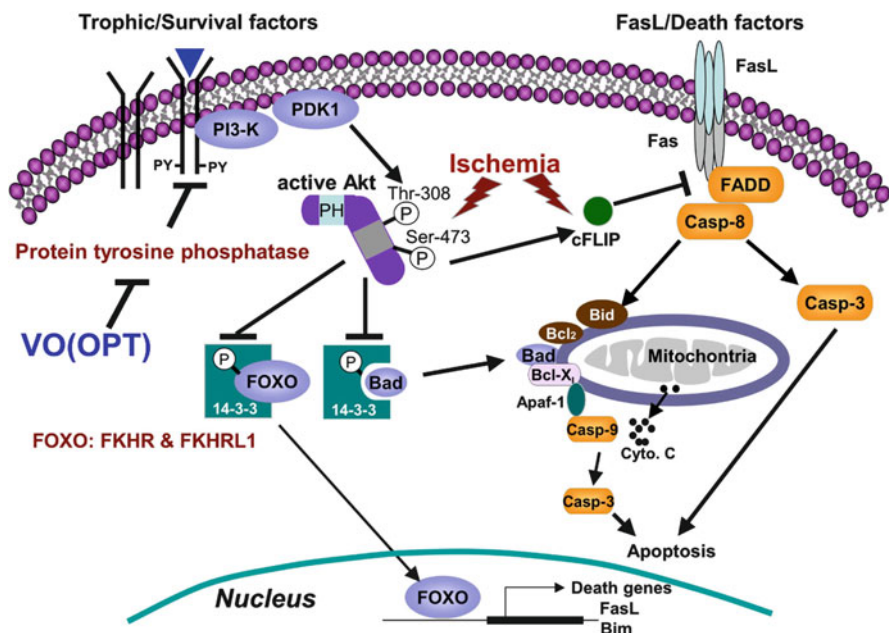
### 9.2.3 Cardioprotection of VO(OPT) in Ischemia/Reperfusion Injury

We tested whether VO(OPT) treatment has cardioprotective effect against myocardial ischemia/reperfusion injuries in rats. Rats were subjected to 30 min ischemia followed by 24 h reperfusion to define the cytoprotective effect of VO(OPT) (0.5 and 1.25 mg V/kg) on myocardial infarct size. The infarct sizes in the VO(OPT) treated group ( $53 \pm 7\%$  and  $37 \pm 2\%$  in 0.5 and 1.25 mg V/kg, respectively) were significantly smaller than that in the vehicle group ( $67 \pm 4\%$ ) [1]. This observation indicated that VO(OPT) has cardioprotective effect on ischemia/reperfusion induced myocardial infarction.

Sodium orthovanadate restores ischemia-induced decrease in Akt phosphorylation on Ser-473 in the gerbil hippocampus, thereby rescuing hippocampal neurons from ischemia-induced cell death [40]. Akt activation has been shown to reduce cardiomyocyte apoptosis, thereby preventing myocardial injury after transient ischemia [11]. We confirmed that post-treatment with VO(OPT) significantly rescues decreased Akt activity after myocardial ischemia/reperfusion and the preserved Akt activity possibly accounts for the VO(OPT)-induced cytoprotective action in cardiomyocytes. Activated Akt is believed to suppress apoptosis through phosphorylation of several substrates, including the Bcl-2 family member, Bad [41] and Forkhead transcription factors (FOXO) [42, 43]. We confirmed downstream targets of Akt to mediate anti-apoptotic signaling in cardiomyocytes. Significant decrease in phosphorylation of Bad and Forkhead transcription factors (FKHR and FKHL1) are closely correlated with decreased Akt activity following myocardial ischemia/reperfusion injury. Phosphorylation of Bad and Forkhead transcription factors were markedly potentiated in cardiomyocytes by treatment with VO(OPT), similar to its response to Akt activity. These results suggest that both Bad and FOXOs are Akt targets and mediate cardioprotective action by inhibiting them following treatment with VO(OPT). Like FKHR, FKHL1 has been shown to induce apoptosis in the brain [44] and fibroblasts through up-regulation of the Fas ligand expression and activation of the death receptor pathway [45]. We also found significantly increased expression of both Fas ligand and Bim after myocardial ischemia/reperfusion and significant inhibition by treatment with VO(OPT) [1].

Recent findings showed that FKHL1 is regulated by Akt activity in endothelial cells and that FKHL1 dephosphorylation promotes apoptosis by negatively regulating FLIP expression [46]. Moreover, Akt was found to rescue endothelial cells from Fas/Fas ligand-mediated cell death through up-regulation of FLIP level [47]. Similar with the previous studies, we found significant FLIP degradation after ischemia/reperfusion [48, 49] and treatment with VO(OPT) significantly increased FLIP expression in cardiomyocytes. The increased FLIP expression was correlated with decreased Fas ligand expression in VO(OPT)-treated group compared with vehicle-treated group. Taken together, inactivation of FKHR and FKHL1 by treatment with VO(OPT) after ischemia/reperfusion likely promotes expression of FLIP, thereby inhibiting cardiomyocyte [1] apoptosis

Cardiomyocyte apoptosis is one of the major contributors in the development of myocardial infarct [50], which is related to the pathogenesis of heart failure after ischemia. Accumulating evidences indicate that apoptosis, different type of cell death form necrosis, plays essential role in cardiomyocyte death after ischemia/reperfusion [50]. Therefore, we examined whether VO(OPT) has anti-apoptotic effects in cardiomyocytes by measuring Caspase 3, Caspase 7 and Caspase 9 processing as a marker of apoptosis. Interestingly, short-term infusion of VO(OPT) significantly reduced cardiomyocyte apoptosis after ischemia/reperfusion as indicated by dose-dependent inhibition of Caspase-3, -7 and -9 processing [3]. The results confirm anti-apoptotic effects of VO(OPT) against myocardial ischemia/reperfusion injury.



**Fig. 9.1** Putative mechanism of myocardial protection by VO(OPT). Binding of trophic/survival factors to tyrosine kinase receptors activate Akt through PI3K and phosphatidylinositol-dependent kinase-1(PDK1) activation. Ischemia/reperfusion caused inactivation of Akt, thereby promoting apoptotic pathways including the Bad, Forkhead transcription factors (FOXO; FKHR and FKHL-1) and FLIP via the Fas pathways. Treatment with VO(OPT) activates and/or preserves Akt activity after ischemia-reperfusion leading to the phosphorylation and thereby inactivation of Bad and Forkhead transcription factors thus preventing apoptosis by Fas ligand and Bim. VO(OPT) treatment also preserves ischemia-reperfusion induced breakdown of FLIP and thereby preventing cell death via Fas pathways. Therefore, the cytoprotective action of VO(OPT) is mediated by the Akt-Forkhead transcription factor-FLIP mediated pathway

VO(OPT) treatment protects the heart from ischemia/reperfusion-induced cardiac injury, thereby improving cardiac contractile dysfunction in rats. VO(OPT) induced cardioprotection is mainly elicited by Akt activation after myocardial ischemia/reperfusion. Akt activation induces phosphorylation of proapoptotic protein Bad, thereby reducing mitochondria-dependent apoptosis. Moreover, VO(OPT) treatment abolished dephosphorylation of forkhead transcription factors after ischemia/reperfusion injury, thereby inhibiting expression of Fas ligand and Bim. Furthermore, VO(OPT) treatment after ischemia/reperfusion promoted expression of FLIP through Akt activation, thereby further inhibiting activation of Fas/Fas-ligand intracellular signal. Taken together, VO(OPT) treatment at reperfusion is likely beneficial as a cardioprotective drug in subjects undergoing reperfusion therapy following a myocardial infarction (Fig. 9.1).

## 9.3 Cardioprotection of Vanadium Compounds in the Postmenopause

### 9.3.1 Cardiovascular Diseases in the Postmenopausal Women

Menopause is the permanent cessation of menstruation and ovarian follicular production of estrogens and progesterone. The mean age at menopause is 51 years, and 95% of women reach menopause between the ages of 45 and 55 [51]. Over the past 20 years, numerous observational, retrospective, interventional, and meta-analytic studies [52] as well as studies using animal models have supported the hypothesis that ovarian steroids exert important protective actions in women and the absence of sex hormones after menopause makes postmenopausal women more vulnerable than younger premenopausal women to cardiovascular diseases (CVD). Indeed, CVD is the leading cause of morbidity and mortality among postmenopausal women in westernized societies [53] and accounts for nearly half of all deaths in women [54]. The Framingham study showed that the incidence of CVD is higher among postmenopausal than in premenopausal women, even among women of the same age [55]. Women who also experience early menopause, both naturally and surgically (bilateral oophorectomy), also have increased risk of coronary events [56].

Approximately 1 in 8 women above age 55 years has undergone bilateral oophorectomy before reaching natural menopause [56, 57]. Bilateral oophorectomy may be performed for a benign disease or for prophylaxis against ovarian cancer, and is usually performed along with hysterectomy (in nearly 90% of cases) [58]. Of the more than 600,000 hysterectomies performed annually in the United States, approximately half include bilateral oophorectomy [59]. In addition, the practice of prophylactic oophorectomy has increased over time and became more than doubled between 1965 and 1990 [60]. Meanwhile, reports now link premenopausal oophorectomy with serious health consequences including premature death, cardiovascular and neurologic disease, and osteoporosis in addition to menopausal symptoms, psychiatric symptoms, and impaired sexual function [61]. The preponderance of evidence suggests that bilateral oophorectomy is associated with increased cardiovascular risk and premature death, and that oophorectomy at a young age further increases this risk [62]. Estrogen therapy started early after surgical or natural menopause at a young age appears to reduce this risk [62–65].

Clinical and experimental studies have established that sex influences the patterns of LV hypertrophy [66]. In response to pressure overload, such as hypertension or aortic stenosis, human male hearts exhibit LV dilatation or eccentric hypertrophy, whereas female hearts tend to maintain normal chamber size but develop increased wall thickness, consistent more with concentric hypertrophy [67]. Evidences of sex hormones influence on the patterns of hypertrophy is revealed from studies demonstrating that physiological replacement of  $17\beta$ -estradiol, the main circulating

form of estrogen in premenopausal women, to ovariectomized female mice limits pressure overload–induced LV hypertrophy [68, 69].

Despite well established models of myocardial hypertrophy and heart failure using rodents, there has been lack of suitable animal models to study postmenopausal hypertension and hypertrophic cardiac remodeling. Although nonhuman primates, sheep, rabbits, mice and rats have all been used as models of various menopausal changes [70], progress in elucidating the mechanisms responsible for postmenopausal hypertension and hypertrophic remodeling has been hampered by the lack of a suitable animal model. Therefore, despite impressive progress in the diagnosis and treatment of hypertrophy, more research on postmenopausal cardiac decompensation under stress is absolutely needed, and adequate animal models are critical to fill the gap between basic science discovery and clinics. An ideal model of postmenopausal cardiac hypertrophy and heart failure should meet various requirements to mimic a complex syndrome characterized by cardiac, hemodynamic and neurohumoral alterations as close as possible. We focused on the molecular mechanisms that contribute to cardioprotective effects of estrogen on LV hypertrophy in response to pressure overload in a rat model of postmenopausal phenotypes to develop novel therapeutic strategy instead of hormone replacement therapy in the postmenopausal women.

### ***9.3.2 Development of Postmenopausal Cardiac Hypertrophy Models***

There have been attempts to elucidate the cardioprotective effect of estrogen in pressure overload induced hypertrophy by using ovariectomized animals [71, 72], however, these have rarely taken into account the effect of menopause on cardiac adverse remodeling and the transition to heart failure. In any case, there is no normotensive animal model that exhibits hypertrophy and decompensation with postmenopausal phenotypes. Female spontaneously hypertensive rats (SHR) represent some postmenopausal phenotypes as they stop cycling at age 10–12 months and have low estradiol levels comparable to postmenopausal women [73]. However, the sex difference in blood pressure no longer exists because of the increase in blood pressure in old females, whereas blood pressure in male SHR remains fairly stable after age 8 months [73]. Dahl salt-sensitive (DS) hypertensive rats also represent some characteristics of postmenopause as they exhibit increases in blood pressure with aging [74]. When the young female DS rats are ovariectomized and fed a high-salt diet, the blood pressure increases to higher levels than in intact females [74]. Still it is not known at what age these animals cease cycling and what happens to their blood pressure after cessation of cycling. In addition to the rat models, the follicular-stimulating hormone receptor knockout mouse also exhibits some of the characteristics of postmenopausal women [75]. These mice have low plasma estradiol levels, have functionally active estrogen receptors, increased testosterone



levels, hypertension, hypercholesterolemia, and weight gain when compared with their wild-type counterparts [75]. However, these animals did not exhibit increased oxidative stress or endothelial dysfunction at age of 14–16 weeks, factors common to postmenopausal women [75].

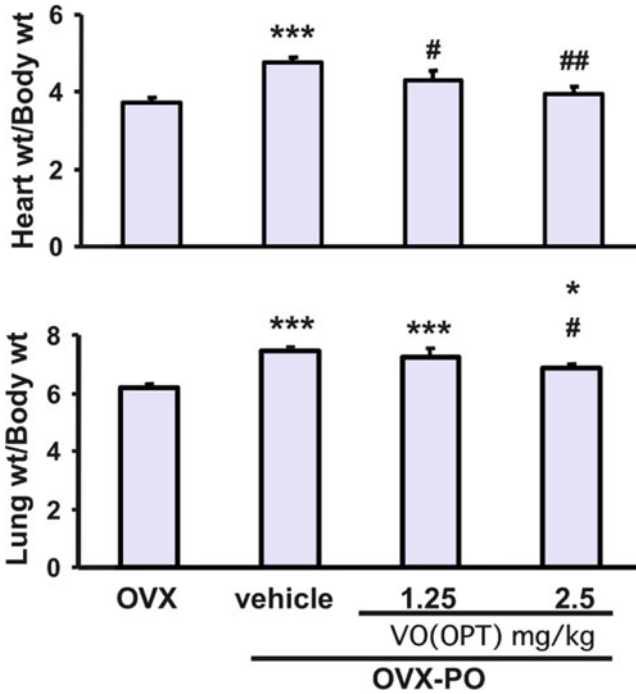
To produce left ventricular pressure overload in the ovariectomized rats, we introduced a model of transverse aortic constriction in the abdominal aorta between the right and left renal arteries [2, 4, 76]. Serum estrogen level was significantly decreased following ovariectomy (OVX) compared with the sham rats (sham:  $56.6 \pm 8.3$  pg/ml; OVX:  $3.6 \pm 2.3$  pg/ml). Pressure overload (PO)-induced hypertrophy had no effect on the serum estrogen level as seen both in the PO ( $60.8 \pm 13.6$  pg/ml) and OVX-PO ( $3.4 \pm 1.2$  pg/ml) group rats. Following ovariectomy, body weight (BW) was significantly increased compared to sham animals. Significant increases in LV weight were also seen in the OVX-PO group compared to sham, OVX and PO groups without significant changes in right ventricle weight.

### 9.3.3 *Anti-hypertrophic Effect of VO(OPT)*

Consistent with my previous observation, LV weight and LW significantly increased in the ovariectomized pressure overloaded (OVX-PO) group compared with the OVX group, without significant changes in right ventricle weight [2]. VO(OPT) (2.5 mgV/kg, p.o.) treatment on the sham and OVX rats have no effect on the morphometric parameters [4]. VO(OPT) treatment significantly and dose-dependently decreased the elevated LV weights and lung weight (LW). Notably, the ratio of HW to BW markedly increased in OVX-PO group compared with OVX group. VO(OPT) (2.5 mg/kg) treatment significantly inhibited the elevated the HW/BW ratio ( $P < 0.01$  vs. OVX-PO) (Fig. 9.2). The LW/BW ratio was also significantly increased in OVX-PO group compared with OVX group. VO(OPT) treatment dose-dependently decreased the elevated LW/BW ratio. Moreover, oral treatment with VO(OPT) (2.5 mg V/kg) for 14 days have no effect on HW/BW ratio and HW/LW ratio in sham-operated animals [4].

Since treatment with VO(OPT) (1.25 and 2.5 mg V/kg) restored HR and MABP, we evaluated LV functions in OVX-PO heart with or without treatment of VO(OPT). Consistent with my previous observation [2], left ventricular end diastolic pressure (LVEDP) was significantly increased in the OVX-PO group compared with OVX group. VO(OPT) treatment dose-dependently restored elevated LVEDP. Similarly, left ventricular developed pressure (LVDP) significantly increased in OVX-PO group and VO(OPT) treatment dose-dependently restored elevated LVDP. The rate of LV contraction (+dp/dt) and relaxation (−dp/dt) also significantly increased in OVX-PO [2] and VO(OPT) treatment dose-dependently restored the elevated LV contraction (+dp/dt) and relaxation (−dp/dt). In agreement with earlier studies [29, 30], the treatment with VO(OPT) containing 2.5 mg V/kg resulted in slight decrease in body weight and food intake compared to vehicle treated rats but these changes are not significant [4].

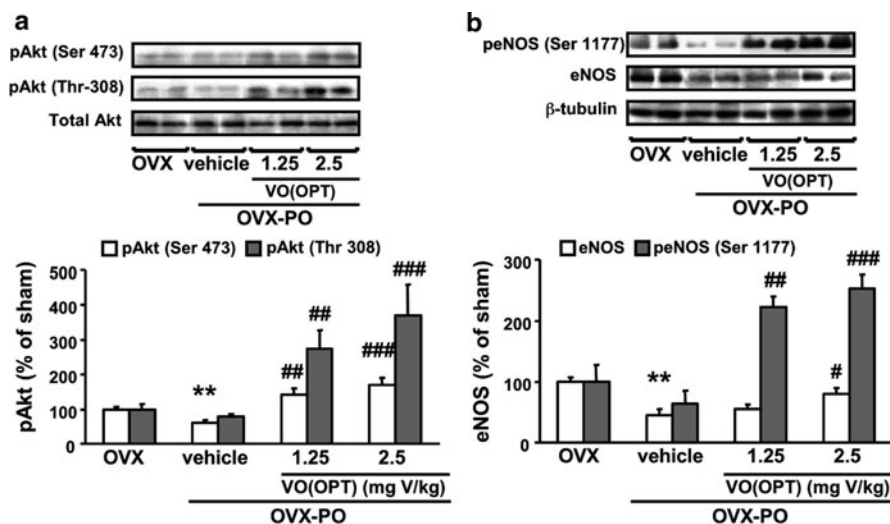




**Fig. 9.2** Effect of VO(OPT) on heart weight (HW)/body weight (BW) (a) and lung weight (LW)/body weight BW (b) ratio. Each bar represents the mean  $\pm$  S.E.M. \*,  $P < 0.05$  and \*\*\*,  $P < 0.001$  versus OVX group; #,  $P < 0.05$  and ##,  $P < 0.01$  versus OVX-PO-vehicle treated group (Modified from [4])

### 9.3.4 Impaired Cardiac Akt/eNOS Signaling in Postmenopause

Akt phosphorylation at Ser-473 was significantly reduced in the hearts of OVX-PO rats compared with the OVX group, indicating that Akt signaling is markedly impaired in pressure overload-induced heart failure concomitant with severe cardiac hypertrophy. Akt Thr-308 phosphorylation also decreases only in the OVX-PO group. 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 left ventricular Akt activity. Heart weigh/body weight ratio is increased time dependently from 1 to 4 weeks following PO in OVX rats [4]. LV Akt phosphorylation at Ser 473 was increased 1 week after PO, thereafter decreased time dependently with significant decreased level observed 4 weeks after PO in OVX rats. On the contrary, no significant change was observed in the total Akt level [4]. Importantly, VO(OPT) treatment markedly and dose-dependently increased Akt activity as assessed by increased phosphorylation at Ser 473 and at Thr 308 (Fig. 9.3).

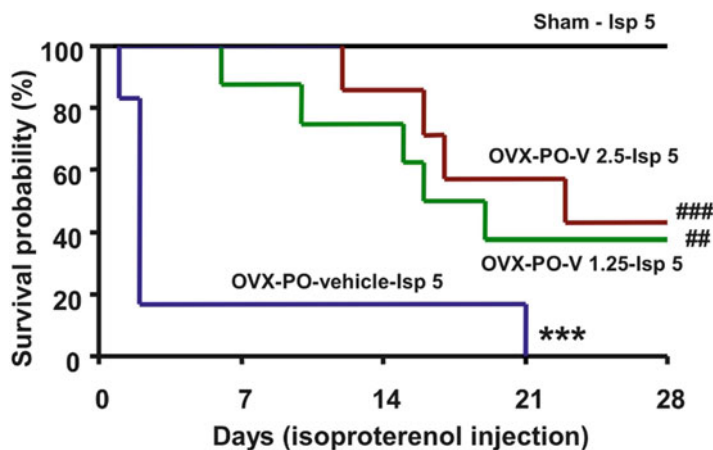


**Fig. 9.3** Effects of VO(OPT) on Akt and eNOS phosphorylation in the left ventricle. Representative Western blot analysis using cell extracts from OVX ( $n = 8$ ), OVX-PO-vehicle ( $n = 8$ ), OVX-PO-VO(OPT) containing 1.25 mg/kg vanadium (V 1.25) ( $n = 8$ ) and OVX-PO-VO(OPT) containing 2.5 mg/kg vanadium (V 2.5) ( $n = 8$ ) treated hearts probed with phospho-Akt (Ser-473) and phospho-Akt (Thr-308) (a) and eNOS and phospho-eNOS (Ser 1179) (b). Data are expressed as percentages of the value of OVX rats. Each column represents the mean  $\pm$  S.E.M. \*\*,  $P < 0.01$  versus the OVX group; ##  $P < 0.01$  and ###  $P < 0.001$  versus the OVX-PO-vehicle treated group (Modified from [4])

Since eNOS is physiological substrate for Akt in human vascular endothelial cells [77], we determine 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 Ser 1179 decreased time dependently following PO-treatment with significant decreased level observed 4 weeks after PO in OVX rats [4]. Consistent with our previous observation [2], we also observed severe impairment of eNOS expression following OVX-PO treatment. I here found a slight but not significant reduction of eNOS phosphorylation at Ser 1179. Notably, VO(OPT) treatment dose-dependently increased eNOS phosphorylation (Fig. 9.3). VO(OPT) also significantly increased eNOS expression.

### 9.3.5 Prevention of $\beta$ -Adrenergic Induced Heart Attack with VO(OPT)

$\beta$ -adrenoceptor ( $\beta$ -AR) agonists differently affect heart rate during the estrous cycle in female rats and in ovariectomized rats with or without estrogen replacement [78]. Ovariectomy also increases susceptibility to the effects of  $\beta$ -AR agonists



**Fig. 9.4** Kaplan-Meier survival analysis following chronic isoproterenol treatment. Five mg/kg isoproterenol (Isp 5) was administered daily to sham, sham-PO, OVX, OVX-PO-vehicle, OVX-PO-VO(OPT) containing 1.25 mg/kg vanadium (V 1.25) and OVX-PO-VO(OPT) containing 2.5 mg/kg vanadium (V 2.5) treated groups. Survival over 28 days was monitored. \*\*\*,  $P < 0.001$  versus the sham group; ##,  $P < 0.01$  and ###,  $P < 0.001$  versus the OVX-PO-vehicle treated group (Modified from [4])

[79]. Importantly, Kam et al. [80] demonstrated that  $\beta$ -AR stimulation with isoproterenol led to a significantly greater increase in electrical stimulation-induced  $\text{Ca}^{2+}$  elevation,  $\text{Ca}^{2+}$ -uptake through cardiac L-type  $\text{Ca}^{2+}$  channels, heart rate and contractility in hearts of ovariectomized rats compared to sham rats. These responses were rescued by estrogen replacement. Several studies hypothesized that excessive adrenergic activation could initiate the progression from compensated LV hypertrophy in hypertension to cardiac dysfunction and that this effect is primarily through adverse LV remodeling.

Similar with aortic banding [2], chronic administration of  $\beta$ -AR agonist to rats led to adverse cardiac injury through impaired Akt-eNOS signaling pathways and ovariectomy further aggravated the cardiac injury suggesting the cardioprotective role of ovarian hormones [4]. Kaplan-Meier survival data indicate that chronic  $\beta$ -adrenergic stimulation with isoproterenol has no effect on the survival of sham and pressure overloaded female rats (Fig. 9.4). Remarkably, treatment with isoproterenol on the ovariectomized rats tended to increase mortality with a survival rate of 84% at 28 days. Interestingly, the most significant mortality occurred in the OVX-PO rats with a survival rate of 0% at 21 days following chronic  $\beta$ -adrenergic stimulation (Fig. 9.4) [4]. These observations indicate that decompensation against cardiac stress such as isoproterenol treatment occurs only in OVX and OVX-PO rats. Treatment with the Akt activator VO(OPT) dose dependently increased survival following acute cardiac stress caused by chronic  $\beta$ -adrenergic stimulation [1, 3], suggesting that cardiac remodeling and recovered cardiac functions by

VO(OPT) treatment contributes the reduced mortality (Fig. 9.4). Thus, simultaneous impairment of Akt-eNOS signaling by ovariectomy contributed to cardiac decompensation during PO-induced hypertrophy, deteriorated heart functions and thereby increased mortality during chronic  $\beta$ -adrenergic stimulation. Potentiation of the Akt and eNOS signaling pathways by treatment with VO(OPT) after OVX-PO likely contributes to increased survival following acute cardiac stress caused by chronic  $\beta$ -adrenergic stimulation. These results contribute to our understanding of the mechanisms underlying increased cardiac injuries in postmenopausal women by chronic  $\beta$ -adrenergic activation and bear relevance to further clinical management strategies to prevent adverse cardiac remodeling.

## 9.4 Crosstalk of Estrogen Receptor and Akt/eNOS Signaling

### 9.4.1 *Cardioprotection Through Estrogen Receptor*

Both *in vivo* and *in vitro* studies indicates that estrogen have cardioprotective effects. However, despite these positive results, hormone replacement therapy has not been shown to consistently lower blood pressure in postmenopausal women. Moreover, in women who have experienced surgical menopause, estrogen replacement therapy also did not result in significant sustained reductions in blood pressure. The mechanisms responsible for increased prevalence of cardiovascular diseases in postmenopausal women are complex and multifaceted. They are not nearly so simple as a reduction in estradiol. We focused on evaluating the molecular mechanisms responsible cardiac injuries in postmenopausal women and characterized the postmenopausal OVX-PO model to provide a suitable animal model to quantitatively evaluate some of the mechanisms and hoped that the information could be extrapolated to women.

Ovarian hormones are believed to possess cardiovascular protective effects, and they seem to play a role in the gender-related differences in the development of hypertension in experimental models [81, 82]. Naturally, ovariectomy causes a significant reduction in estradiol and progesterone levels [74, 83]. Comparison of estradiol levels with MABP in DS rats suggests that the OVX-induced increase in MABP is associated with decreased levels of plasma estrogen because estradiol replacement was able to prevent the OVX-induced hypertension [74]. Moreover, estradiol supplementation did not markedly alter circulating progesterone levels in both young and aged DS rats [74]. Furthermore, progesterone levels did not correlate with the difference in MABP between the OVX and OVX-estrogen groups in DS rats [74]. This lack of effect of progesterone supports previous reports in deoxycorticosterone salt hypertension showing that progesterone had no effect on the development of hypertension in OVX rats [82].

### ***9.4.2 Stimulation of Akt/eNOS Signaling Through Estrogen Receptor***

Signaling through phosphatidylinositol 3-kinase (PI3K)/Akt pathway is important for the physiological growth and inhibition of pathological hypertrophy [11, 84, 85]. 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 Akt signaling pathway [86]. It is evident that the E2-activated PI3K/Akt pathway functions as one of the acute nongenomic actions of E2 in various types of cells [87]. Studies indicate that young women possess higher levels of nuclear-localized phospho-Akt (Serine 473) relative to comparably aged men or postmenopausal women [88] and Phospho-Akt (Serine 473) is also localized to the nucleus of cultured cardiomyocytes after exposure to E2 [88]. Previous studies have demonstrated that OVX-reduced Akt activation occurs with decreased myocyte contractile function and impaired intracellular calcium handling, whereas E2-upregulated Akt is associated with restored cardiac contractility and intracellular calcium homeostasis [89]. Moreover, activation of the PI3K/Akt pathway is required for E2-suppressed apoptosis and E2-protected myocardial function in the heart following ischemia [68]. Estrogen receptor  $\alpha$  mediates increased activation of PI3K/Akt signaling and improved myocardial function in female hearts following acute ischemia [90]. Recent study also suggests that E2-mediated improvement in cardiac function following trauma-hemorrhage is mediated by an increase in cardiac Akt activation [91]. In endothelial cells, estrogen receptors localize to caveolae and their stimulation activate eNOS activity via PI3K/Akt signaling [92, 93]. Strikingly, Grasselli et al. [94] demonstrated that estrogen receptor  $\alpha$  and eNOS form a complex and translocate to nucleus to bind to the estrogen response element in the promoter region of telomerase catalytic subunit gene, thereby leading to the increase telomerase activity. Since the telomerase activity is critical to determine the lifespan of a cell. This is the first report to define enhancement of telomerase activity by eNOS activation and NO production.

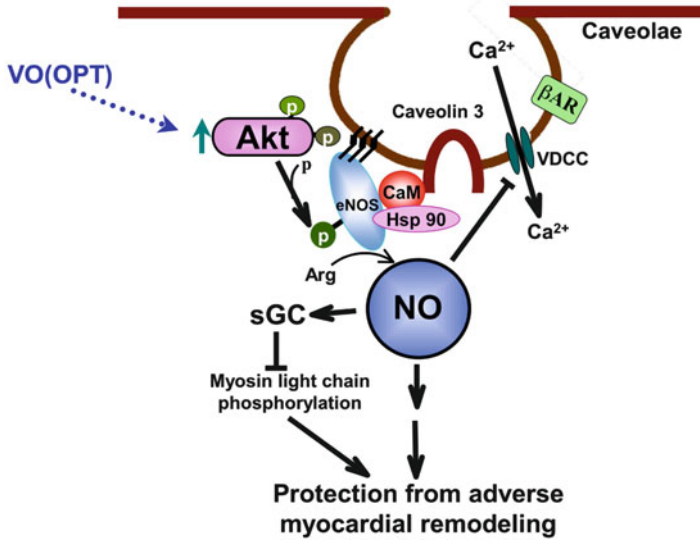
### ***9.4.3 Stimulation of Nitric Oxide Production Through Estrogen Receptor***

Men and postmenopausal women may have less endogenous nitric oxide (NO) production, shown by less vasoconstriction following inhibition of NO synthase by L-N-monomethyl-arginine (L-NMMA), than premenopausal women. After estrogen therapy, L-NMMA resulted in greater constriction consistent with estrogen restoring vascular NO activity to levels seen in premenopausal women [95]. Other studies have shown improvement in endothelial function with estrogen therapy

with a greater improvement in hypertensive postmenopausal women [96]. Acute estrogen deprivation after oophorectomy in healthy women results in impaired endothelium-dependent vasodilatation as a result of reduced NO availability [97]. Estrogen therapy improves endothelium-dependent vasodilatation after oophorectomy and also natural menopause [98]. However, among a broader sample of postmenopausal women, HRT results in improved flow-mediated dilation only among women with no cardiovascular risk factors [99, 100]. Studies indicate that estrogen activates eNOS activity through Akt pathway, thereby promoting NO production in heart [87]. Moreover, estrogen mediated Akt activation results in eNOS activation in cultured human endothelial cells [77] and in intact elastic and muscular arteries in vivo [101]. Upregulation of PI3K/Akt by administration of E2 results in endothelial nitric oxide synthase activation via a transcription-independent mechanism [77]. Localization and activity of eNOS are regulated by making a complex with a chaperone protein, heat shock protein 90 and caveolin-3 in cardiomyocytes, especially in caveolae [102]. I also found significantly increased expression of the eNOS regulatory proteins like HSP-90 and caveolin-3 in the hearts of the OVX rats and pressure overload downregulated their expression on the OVX-PO rats [2]. More extensive studies are required to determine temporal changes and immunohistochemical localization of HSP-90 and eNOS after OVX-PO.

Under physiological conditions, myosin light chain (MLC) phosphorylation correlates with increased maximum tension or  $dp/dt$  values [103]. In the postmenopausal hypertrophy rat model, I found that phosphorylation was markedly increased only in OVX-PO rats. By contrast, total MLC content was significantly decreased in OVX-PO rats. The increased MLC phosphorylation/total MLC ratio was likely associated in part with an increase in  $\pm dp/dt_{max}$  in heart contractile function [2]. Nitric oxide in vascular smooth muscle activates soluble guanylyl cyclase (sGC) to increase cGMP formation, thereby leading to a decrease in  $[Ca^{2+}]_i$  with subsequent inhibition of myosin light chain phosphorylation and contraction [104, 105]. My data suggest that ovariectomy followed by pressure overload subsequently increases MLC phosphorylation accompanied by increased cardiac contractility, as observed particularly in OVX-PO rats (Fig. 9.5).

Detailed *in vivo* and *in vitro* studies suggest that underlying mechanisms of estrogen mediated cardioprotection against myocardial hypertrophy are multi-fold. Estradiol-mediated activation of PI3K signaling blocks angiotensin II- or endothelin-1-initiated cardiomyocyte hypertrophy in vitro via upregulation of the gene encoding modulatory calcineurin-interacting protein (MCIP), a calcineurin antagonist. 17 $\beta$ -estradiol stimulates both the transcriptional transactivation and mRNA stability of the MCIP gene. Interestingly, Estradiol-induced PI3K signaling to MCIP up-regulation does not occur through AKT, as indicated by silencing Akt [106]. As the MCIP1 gene utilizes four different promoters, in a cell context-specific fashion, and so a detailed *in vivo* analysis is required to further clarify this effect.



**Fig. 9.5** Putative mechanism of VO(OPT) mediated cardioprotection. In normal female heart, eNOS is localized to the caveolae through its interaction with caveolin 3 and compartmentalized with L-type  $Ca^{2+}$  channel and b-adrenergic receptor. Activation of Akt signaling ultimately leads to eNOS phosphorylation, eNOS activation and thereby activates nitric oxide signaling pathways. The resulting combination of these effects subsequently confers ventricular dilation and cardioprotection. Pressure overload-induced hypertrophy severely impairs eNOS and Akt signaling pathways, thereby imbalances the NO mediated cardioprotective action. Treatment with VO(OPT) activate the Akt activity and enhances Akt mediated eNOS activity and subsequently confers cardioprotection against myocardial hypertrophy

### 9.5 Conclusion

Despite numerous studies on postmenopausal cardiac remodeling in hypertension and hypertrophy, we are just beginning to understand the pathophysiology of postmenopausal cardiovascular diseases. Particularly in light of the disappointing cardioprotective results obtained in several clinical trials with hormone replacement therapy, the mechanisms underlying estrogen-mediated cardioprotective action merit further study. In normal female heart, estrogen-mediated increased Akt-eNOS signaling restores the impaired nitric oxide signaling pathways and subsequently confers cardioprotection. In our postmenopausal model of cardiac hypertrophy by ovariectomy and pressure overload, impaired Akt-eNOS signaling imbalances the physiological protective mechanism by nitric oxide. Therefore, menopause likely accounts for cardiac decompensation against chronic stress through impairment of functions of eNOS and Akt signaling, and showed increased mortality following acute cardiac stress caused by chronic  $\beta$ -adrenergic stimulation. The novel model of postmenopausal cardiac decompensation using OVX-PO rats and elucidation of the mechanisms of detrimental cardiac remodeling are attractive for testing



cardioprotective drugs in hypertension-induced cardiac injury in postmenopausal women. Furthermore, Akt/eNOS signaling is central dogma of cardiac remodeling following ischemia and hypertension and vanadium compound therapy instead of HRT is clinically relevant for cardioprotection not only in ischemic injury but also postmenopausal heart disease.

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