Published in final edited form as:

J Cardiovasc Pharmacol. 2017 February ; 69(2): 93–100. doi:10.1097/FJC.0000000000000446.

Nitrite Prevents Right Ventricular Failure and Remodeling Induced by Pulmonary Artery Banding

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Abstract

Background—Nitrite has been shown to reduce right ventricle (RV) remodeling in experimental pulmonary hypertension. However, whether this effect is due to a reduction in RV afterload (i.e., reduction in pulmonary artery pressure) or a direct effect on the RV itself remains unanswered. We hypothesize that nitrite has direct effects on RV remodeling and studied its effects in mice with pulmonary artery banding (PAB).

Methods and Results—PAB decreased exercise tolerance, and reduced RV systolic and diastolic function. Nitrite treatment attenuated the decrease in RV systolic function, and improved the RV diastolic function. Nitrite treated PAB mice had similar exercise tolerance compared to control group. PAB induced RV hypertrophy and fibrosis which were associated with increased expression of phospho-AKT. Interestingly, nitrite treatment attenuated PAB-induced RV hypertrophy and reduced the expression of phospho-Akt in RV tissue from PAB mice. In neonatal rat cardiac fibroblast, nitrite also attenuated hypoxia-induced increase in expression of phospho-Akt.

Conclusion—Our study indicates that nitrite treatment has direct beneficial effects on RV and improves function and attenuates remodeling in RV exposed to chronic pressure overload. These beneficial effects, at least in part, could be due to the inhibition of the p-Akt pathways activation.

Introduction

Pulmonary hypertension (PH) is a devastating disease for which still there is unmet medical need. It is characterized by pulmonary vasoconstriction and vascular remodeling leading to increased pulmonary artery pressure that results in right ventricular (RV) remodeling and subsequent RV failure. Although the primary cause of PH is changes in the vasculature, the severity of symptoms, progression of disease and patient survival are strongly dependent on RV function [1]. Despite recent progress in therapy of this disease [2–6], mortality remains unacceptably high and development of new therapeutic modalities is warranted [7]; of

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There is no disclosure of conflict of interests for any authors

particular interest are new agents that may predominantly have beneficial effects on RV function and remodeling.

Numerous studies have shown that both dietary and exogenous Nitrite (NO_2^-) have beneficial cardiovascular effects. In this regard, nitrite regulates hypoxic vasodilation in vivo and ex vivo in mice [8] and increases cardiac output and reduces mean arterial blood pressure and systemic vascular resistance in healthy volunteers exposed to exercise [9]. Furthermore, nitrite provides cytoprotection and reduces oxidative stress in cardiac ischemia-reperfusion injury [10–12]. Finally, in rodents, nitrite can ameliorates hypoxia- and monocrotaline-induced PH [13, 14], and also normalizes PH associated with heart failure with preserved ejection fraction [15]. There is a strong line of evidence that dietary and exogenous nitrite is a nitric oxide (NO) donor, i.e. in the body it is reduced to NO by hemoglobin, myoglobin, ascorbate, polyphenols, and protons [16–24].

However, it is not clear if the NO_2^- prevents the PH-related RV remodeling because it can dilate the pulmonary vessels and thereby reduces the right heart afterload or it has direct effects on RV myocardium. Further, the potential direct effect of nitrite on right ventricular remodeling and function with continuous RV pressure overload remains unknown. Therefore, in the present study, we hypothesized that oral NO_2^- prevents right ventricular remodeling associated with RV pressure overload by directly affecting right heart. Finally, as Akt pathway has been suggested to be an important pathway in the regulation of RV hypertrophy related to PH [25, 26], in this study we investigated whether Akt contributes to the PAB-induced RV remodeling and the potential effect of nitrite on this pathway.

Materials and Methods

All experimental protocols were performed according to the University of Pittsburgh and NIH guidelines.

Animal model

Male C57BL/6 mice (in-house breeding) at twelve weeks of age underwent pulmonary artery banding (PAB) procedure as previously described [27]. Briefly, mice were anesthetized with 5% isoflurane and tracheal intubation was performed. Next, animals were placed on heating pad and ventilated with 2% isoflurane during entire procedure. A mouse ventilator (MiniVent 845, Harvard Apparatus, Holliston, MA) was used for ventilating the mice during all procedures. Ventilation volume was calculated as $\text{Volume (ml)} = 6.2 \times M_b^{1.01}$ (M_b =Animal Mass, Kg), and ventilation rate was calculated as $\text{Rate (min}^{-1}\text{)} = 53.5 \times M_b^{-0.26}$, based on provided information by the company. After sterilizing the area of surgery, thoracotomy was performed at the left 2nd intercostal space. The left lung was pushed down gently and the pericardium was removed. A tantalum clip was placed around the pulmonary artery with a diameter of 27 gauge needle. This was followed by re-expansion of lungs using positive pressure at end expiration, closure of ribs and skin with 5-0 nylon and 3-0 silk sutures, respectively, and weaning off of the animals from the respirator while lowering anesthesia, allowing the mouse respiration to take over. This is followed by the removal of anesthesia and placement of the mouse on a heated pad. The animals were allowed to full recovery before being placed back in to cages.

Nitrite dietary treatment of animals

Control and PAB groups were given regular water and the PAB+Nitrite group was given sodium nitrite solution in water (0.05%) starting immediately after the surgery for 3 weeks.

Exercise capacity test

To assess the exercise capacity, three weeks after the surgery, forced swimming test was performed as described before [28]. Briefly, a paper clip weighing 2 gram was attached to the tail to increase the resistance to swimming and mice were individually placed into beaker filled with water. The mice were allowed to swim until they appeared to be exhausted. The time at which the mouse became unable to keep the nose out of water was defined as point of exhaustion. The time that the mice could maintain swimming before exhaustion was recorded.

In vivo hemodynamics

The next day after the exercise capacity test, the mice were instrumented for in vivo assessment of RV function and pulmonary hemodynamics as described before [25]. Briefly, the mice were anesthetized with 5% isoflurane and intubated by tracheotomy. Animals were placed on heating pad with body temperature being monitored continuously and mechanically ventilated with 1–2 % isoflurane during the hemodynamics assessment. The calculation of ventilation volume and rate was adjusted as performed during PAB surgeries previously described. The abdomen was opened by a vertical incision and the diaphragm was cut open to expose the heart. A transducer of Scisense Pressure Volume Measurement System (Transonic, Ithaca, NY) was introduced into the right ventricle to continuously measure cardiac hemodynamic parameters which were detected by the utilized software (EMKA, Paris, France). Detected parameters included all pressure values along with Tau value. Additional pressure and volume parameters were also measured from which contractility index and ejection fraction were derived and calculated as below:

$$EF = ((RVEDV - RVESV) / RVEDV) * 100; \text{ Contractility Index} = dPdt_{\max} / mPAP.$$

Animal were euthanized, the right ventricle and left ventricle plus septum were collected, weighed separately and Fulton index $[RV/(LV + \text{septum})]$ was calculated. Tissue samples were snap-frozen in liquid nitrogen or placed in formalin for histology.

Histology

Heart tissues were fixed in 10% formalin for 48 hours, then embedded in paraffin, sectioned at a thickness of 5 mm, and stained with Masson's trichrome stain and hematoxylin and eosin. The slides were examined using a microscope (Zesis AX10). Area of fibrosis was measured as percent fibrotic area: Ratio of fibrotic area to total heart tissue area in each section [27, 29]. Five separate fields per heart were examined for fibrosis. H&E stained RV tissues were used to measure cardiomyocytes diameter. Using ImageJ cardiomyocytes' diameter were measured at the maximum cross-sectional area in at least 25 cells per field for four fields per sample and presented as mean.

Hypoxia treatment of the neonatal rat cardiac fibroblasts

Fibroblasts were isolated and cultured from neonatal rat hearts as described before [30, 31]. Seven 9mm petri dishes of fibroblasts were incubated in the hypoxia chamber (Heracell 150i, Thermo Scientific, MA) for 6 hours with 20% CO₂ and 1% O₂. Nitrite (10mM/ml) was added to the culture medium in 4 of the dishes treated with hypoxia. Another 3 dishes of fibroblasts cultured in normal incubator and medium were used as control. After 6 hours, fibroblasts were collected for western blot experiment.

Western blot analysis

Right ventricular tissue or collected fibroblasts were homogenized and analyzed by western blot [26, 32–34] with anti-AKT (1:1000, Cell Signaling, Beverly, MA), anti-pAKT (1:1000, Cell Signaling, Beverly, MA) primary antibody and anti- β Actin antibody (1:1000, Cell Signaling, Beverly, MA). Expression of phosphoinositide 3-kinase (PI3K) was also measured with anti-PI3K (1:1000, Cell Signaling Beverly, MA). Blots were scanned on an Odyssey system imager, and relative band intensities were quantified by densitometry using ImageJ software (NIH), with samples normalized to the corresponding β -actin values.

Statistics

Data are expressed as means $\pm$ SEM for in vitro and vivo studies. End-point comparisons with three groups were performed using a one-way ANOVA (Prism Software, version 6; GraphPad). $P < 0.05$ is considered as significant difference.

Results

Nitrite treatment reverses decreased exercise capacity induced by PAB

Swimming test was performed 3 weeks after PAB procedure in 3 groups of control, PAB stress and PAB mice supplemented with dietary nitrite for 3 weeks (PAB+Nitrite). PAB-mediated pressure overload significantly decreased the swimming ability in PAB group compared with control group while treatment with nitrite maintained the exercise capacity at the normal level (Fig. 1).

Nitrite treatment prevents deterioration of RV function induced by PAB

As expected, when compared to control, PAB induced a significant increase in RV max pressure, an indicator of increased afterload. Interestingly, nitrite treatment did not significantly attenuate the PAB-mediated increase in RV max pressure (Fig 2A). PAB deteriorated RV systolic and diastolic function, evidenced by decreased ejection fraction (EF) and contractility index (CI) (Fig 2 B&C), and prolonged Tau value (Fig 2 E), while the RV end diastolic pressure (RVEDP) was not significantly changed (Fig 2 D). PAB+Nitrite group significantly increased CI and showed a tendency of elevated EF compared to PAB group, suggesting that deterioration on systolic function was partially restored by nitrite treatment (Fig 2 B&C). Interestingly, in contrast to RV max pressure, RVEDP was not significantly increased with 3 weeks of PAB and it was significantly decreased in response to the nitrite treatment regardless (Fig. 2D). Furthermore, prevention of increase in Tau value with nitrite treatment suggests that nitrite improved diastolic function in PAB mice (Fig. 2E).

No change was observed among the three groups in left ventricular functions including maximal pressure, ejection fraction, and tau (data not shown) or left ventricular end diastolic pressure (Supplemental Fig. 2).

Nitrite treatment prevents right ventricular remodeling induced by PAB

Fulton index [RV/(LV+septum) mass ratio] was used to assess the isolated right ventricular hypertrophy. As expected, PAB-mediated pressure overload significantly increased Fulton index when compared to control and importantly, nitrite prevented PAB-induced RV hypertrophy (Fig. 3A), suggesting that nitrite prevents RV remodeling.

To further assess cellular hypertrophy, diameter of right ventricular cardiomyocytes from control, PAB and PAB+Nitrite mice was measured. Our analysis revealed that cardiomyocytes were enlarged post-PAB compared with control. However, no cell enlargement reversal was observed with the nitrite treatment (Fig. 3B–C), suggesting contribution of other cardiac cell types as the source of tissue remodeling affected by nitrite dietary. To determine whether nitrite prevents cardiac remodeling contributed by cardiac fibroblasts, we stained the RV tissues with Trichrome and measured fibrosis levels. The pressure overload induced by PAB significantly increased fibrosis in the RV tissue. However, the preventive treatment with nitrite almost abolished PAB-induced fibrosis (Fig. 4). These results suggest that nitrite treatment attenuates RV remodeling by preventing cardiac tissue fibrosis, but not cardiomyocytes hypertrophy.

Nitrite diet regulates Akt signaling in cardiac tissue fibroblast post-PAB

RV tissues from all three groups were analyzed for phosphorylated and total Akt protein expression. Total Akt expression level was not changed with PAB or nitrite treatment when compared with control WT untreated mice. However, the expression of phosphorylated Akt was significantly increased with PAB stress (Fig. 5) and more importantly, phospho-Akt expression was maintained at control level with nitrite treatment, suggesting that regulating phospho-Akt pathway might be a mechanism by which nitrite prevents the RV tissue fibrosis induced by PAB pressure overload. Interestingly, the expression of PI3K was not changed in either PAB group or PAB+Nitrite group compared to controls (Supplement Fig. 1) suggestive of involvement of other pathway/s regulating Akt signaling at this time point of PAB stress.

To further confirm the direct effect of nitrite on cardiac fibroblasts' Akt signaling, neonatal rat cardiac fibroblasts were incubated in hypoxia, treated with nitrite and analyzed for Akt activation levels. Similar to the RV tissue, phospho-Akt expression was significantly upregulated by hypoxia, while treatment of nitrite restored its expression to control levels (Fig. 6).

Discussion

Clinically, pulmonary hypertension (PH) can result in right ventricular remodeling and subsequently its failure followed by decreased physical capacity. In this study, we used mice pulmonary arterial banding (PAB) model to mimic the constant right ventricular pressure overload in pulmonary hypertensive patients. We here show that the observed decrease in

exercise capacity due to RV pressure overload, was reversed to normal level with dietary nitrite. Importantly, RV function failure induced by pressure overload was prevented by nitrite treatment. Further, we show that dietary nitrite does not alleviate right ventricular remodeling by preventing cardiomyocytes hypertrophy but rather attenuating cardiac fibrosis through Akt signaling. Taken together, these findings demonstrate the potential treatment effect of nitrite on RV remodeling associated pressure overload-mediated cardiac dysfunction.

PH patients suffer from decreased physical ability associated with altered RV function [1]. Cardiovascular beneficial effect of dietary nitrite has been explored in the recent years [8, 9]. These findings inclined us to investigate whether dietary nitrite improves physical ability in mice that have undergone pulmonary arterial banding (PAB), a model for PH. Therefore, a day before terminal hemodynamic measurement of RV function, we exposed the animals to the forced swimming test (FST) to assess their exercise capacity. The FST has been used to assess rodents' skeletal muscle and heart function [28, 35, 36]. In our experiment, the mice with PAB displayed significantly reduced exercise tolerance, while nitrite treatment improved the exercise capacity to the level seen in control animals. Although the improvement might be explained by anti-fatigue effects of nitrite, our detailed cardiac hemodynamic analysis shows that nitrite can enhance exercise capacity by directly effecting altered cardiac function mediated by RV pressure overload.

We found that nitrite treatment reserves the RV function. The RV systolic and diastolic function was deteriorated by PAB as measured by decreased contractility index, ejection fraction and increased Tau (time constant). As expected, nitrite treatment improved contractility index and Tau significantly. Nitrite treatment also significantly decreased RVEDP compared with both control and PAB groups. It is reported that right atrial pressure is equivalent to RVEDP in the absence of the tricuspid valve abnormality [37]. However, right atrial pressure itself adjusts to arterial pressure with time, which by itself also affects left ventricular pressure (LVEDP). Importantly, our experiments show no changes in LVEDP mediated by 3 weeks of PAB stress suggestive of no significant variation in systemic blood pressure with PAB nor PAB+Nitrite groups (Supplemental Fig. 2). Our findings indicate that the enhanced cardiac function with nitrite treatment is due to alteration of right ventricular tissue and its remodeling rather than its effect on the vasculature in our model of right ventricular pressure overload. As expected, the RV max pressure was not changed in the animals with nitrite treatment compared with those in PAB group as the overload was introduced by pulmonary artery constriction procedure. These results suggest that nitrite has direct effects on RV function so that the exercise capacity is maintained.

As constant pressure overload is applied to the heart, ventricular remodeling develops as an important approach to regulate the heart function before it is decompensated. The Fulton Index represents a hallmark of right ventricular remodeling resulting from increased right ventricle pressure afterload [34]. Our experiments show that PAB caused significant RV remodeling, as evidenced by increased Fulton index and that nitrite prevented the occurrence of this remodeling. However, histology showed that nitrite didn't decrease the enlarged cardiomyocytes diameter with PAB. Therefore, we also examined fibrosis in RV tissues and

found that the mechanism by which nitrite prevents RV remodeling is likely through inhibition of PAB-induced fibrosis.

Akt is a serine/threonine protein kinase that regulates cardiac growth, myocardial angiogenesis, and cell death in cardiomyocytes [38]. It has been found that Akt activation is an important pathway that increases cardiac fibrosis [39–41]. Our study supports the fact that Akt pathway is activated in the right ventricular remodeling induced by pressure overload. Notably, in the present study, the treatment with nitrite inhibited the activation of Akt pathway. Considering our results showing that nitrite prevents PAB-mediated cardiac fibrosis and that Akt activation has been shown to play important role in stimulation of heart fibrosis, we postulated that nitrite's effect on preventing fibrosis is possibly mediated through Akt pathway. We then conducted additional cell culture experiments in neonatal cardiac fibroblasts and found that nitrite treatment inhibits hypoxia-induced activation of Akt in rat neonatal cardiac fibroblasts. These results suggest that fibroblasts are the targets of nitrite regulation of Akt activation. This is important as previous studies have shown hypoxia as a potent stimulus for fibroblast proliferation [42–47] and suggested Akt activation as the mechanism by which hypoxia induces proliferation [48, 49].

Conclusion

In conclusion, in the current study, we demonstrated that in PAB-mediated pressure overload nitrite treatment directly affects the RV tissue itself via preventing its remodeling and deterioration of function, rather than altering pulmonary vascular parameters that lead to RV dysfunction. Future studies on detailed mechanisms involved in nitrite-affected pathways in RV remodeling are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study is partially supported by NIH grant "P01 HL103455-06"

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Fig. 1. Nitrite treatment ameliorates swimming duration

Forced swimming test in wild type control, PAB stressed and PAB mice supplemented with dietary nitrite (PAB+Nitrite) mice. Exercise ability is measured as time for each mouse (* $p < 0.05$ compared to control; $p < 0.05$ compared to PAB; $n = 9-11$)

Fig. 2. Hemodynamics assessment of RV function with PAB pressure overload stress and Nitrite treatment

The effects of PAB and nitrite on hemodynamic parameters of RV function measured with open-chest PV Loops procedure in the mechanically ventilated mice. RV pressure induced by PAB and systolic function were measured as (A) RV max pressure, (B) ejection fraction, and (C) contractility index. RV diastolic function was measured as (D) RV end diastolic pressure and (E) Tau. Nitrite treatment partially restores the RV systolic function and improves RV diastolic function in PAB mice. (* $p < 0.05$ compared with control; $p < 0.05$ compared with PAB; $n = 6-10$).

Fig. 3. RV remodeling and histology of cardiomyocytes hypertrophy

(A) Fulton index is measured as RV/(LV+septum) mass ratio; (B) The diameter of cardiomyocytes is compared in three groups; (C) The representative histological pictures of three groups (H&E staining). (* $p < 0.05$ compared with control; $p < 0.05$ compared with PAB; $n = 5-10$)

Fig. 4. Histology of RV fibrosis

(A) The representative histological pictures of fibrosis (shown as blue) in three groups (Masson's trichrome staining). The pictures in the second row are 40x magnification of the black rectangular areas in the pictures of first row; (B) the ratio of fibrosis to total heart tissue area is compared in three groups. (* $p < 0.05$ compared with control; Δ $p < 0.05$ compared with PAB; $n = 5-10$)

Fig. 5. Nitrite regulates phospho-Akt expression in RV tissue with pressure overload from PAB mice

(A) Representative immunoblots for phospho-Akt and total Akt expression normalized to β -Actin. (B) Quantitation of phospho-Akt and total Akt normalized to β -Actin (* $p < 0.05$ compared with control; $p < 0.005$ compared with PAB; $n = 4$).

Fig. 6. Nitrite regulates phospho-Akt expression in fibroblasts treated with hypoxia

(A) Representative immunoblot of phospho-Akt, total Akt and β -Actin in rat neonatal cardiac fibroblasts. (B) Quantitation of phospho-Akt and total Akt normalized to β -Actin (* $p < 0.05$ compared with control; $\Delta p < 0.005$ compared with PAB; $n = 3-4$).