



Original Article

Chronic dosing with metformin plus bosentan decreases *in vitro* pulmonary artery contraction from isolated arteries in adults with pulmonary hypertension

Shutan Liao^{1,2}, Dongsheng Li³, Zheng Hui², Craig S McLachlan¹, Yang Zhang^{3*}

¹Rural Clinical School, University of New South Wales, Sydney, New South Wales, Australia

²The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

³The First Affiliated Hospital of Nanchang University, Nanchang, China

Article info

Article History:

Received: 27 November 2018
Accepted: 1 July 2019
published: 26 August 2019

Keywords:

Bosentan
Metformin
Pulmonary Arterial Hypertension
Congenital Heart Defect
Vessel Reactivity

Abstract

Introduction: Pulmonary arterial hypertension (PAH) specific drug therapy using bosentan has significantly improved quality of life and survival, although PAH is still an incurable disease. Recent studies suggest metformin may have additional treatment benefits in PAH. We therefore investigated *in vitro* pulmonary artery reactivity after combination therapy of bosentan and metformin in PAH patients as compared with bosentan monotherapy in a prospective, randomized study.

Methods: Adult patients with PAH associated with congenital heart defects (PAH-CHD) were randomised to receive bosentan (initially at 62.5 mg twice daily for 4 weeks and then 125 mg twice daily) for 3 months with or without the combination treatment of metformin (500 mg twice daily). Vessel reactivity of isolated pulmonary arteries was examined using a wire myograph.

Results: Phenylephrine (PE)-induced contractions of arteries in patients received combination therapy were significantly attenuated at concentrations of 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M, compared to those received bosentan monotherapy. After denudation, PE-induced contractions at concentrations of 3×10^{-6} M and 10^{-5} M were significantly decreased in the combination therapy group. AMP-activated protein kinase (AMPK) inhibitor compound C abrogated the inhibitory effects of metformin on PE-induced contractility. AMPK and eNOS phosphorylation in the pulmonary arteries of patients treated with combination therapy was increased compared to monotherapy ($P < 0.05$).

Conclusion: Adding metformin to bosentan therapy in patients with PAH-CHD decreased *in vitro* pulmonary artery contraction induced by PE, which is possibly related to increased AMPK phosphorylation.

Please cite this article as: Liao S, Li D, Hui Z, McLachlan CS, Zhang Y. Chronic dosing with metformin plus bosentan decreases *in vitro* pulmonary artery contraction from isolated arteries in adults with pulmonary hypertension. *J Cardiovasc Thorac Res* 2019;11(3):189-195. doi: 10.15171/jcvtr.2019.32.

Introduction

Pulmonary arterial hypertension (PAH) with increased pulmonary vascular resistance (PVR) is a common complication of congenital heart defects (CHD).¹ Over 7% of CHD patients have possible PAH and approximately 100 per million general adult population suffer from this disease.² The development of PAH in CHD patients leads to markedly increased morbidity and mortality.^{3,4} Currently, there are no curative treatments for PAH associated with CHD (PAH-CHD) other than heart-lung transplantation. Therefore, management focus on slowing down the PAH progress and improving quality of life and heart function before transplantation proceeds. Selected patients may benefit from shunt closure with correctable defects, although this approach is still questionable.⁵ With

the advent of PAH-specific drugs, in particular bosentan-an dual endothelin receptor (ER) antagonist that targets the endothelin (ET) pathway and stimulates vascular smooth muscle cell (VSMC) relaxation, the “treat and repair approach” has been proposed to re-evaluate the operability in PAH-CHD patients, previously thought to be unsuitable candidates for surgery.⁶ Pulmonary reactivity response to vasodilators, namely acute vasoreactivity testing, remains one of the gold standards to assess prognosis and indication for specific PAH therapy, and to assess the operability of PAH-CHD, given the existence of other more readily attainable prognostic factors.⁷ Multiple mechanistic pathways, including the ET, nitric oxide (NO) and prostacyclin pathways, are involved in the pathogenesis of PAH. These pathways promote

*Corresponding Author: Yang Zhang, Email: 87297950@qq.com

© 2019 The Author (s). This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

vascular constriction of pulmonary arteries and hence increase vascular resistance and pressure. Combining drugs that target more than one pathway is an attractive option for treatment as additional benefits compared with monotherapy had been observed in previous studies.⁸⁻¹⁰ As a well-known antihyperglycemic agent, metformin improves insulin resistance in type 2 diabetes, and has been shown to reduce cardiovascular disease risks in those patients.¹¹ Animal studies reveal that metformin reverses the development of experimental PAH in rats,^{12,13} suggesting that metformin may have additional treatment benefits in PAHs. Indeed, metformin increases the NO concentration and endothelial NO synthase (eNOS) expression and reduces the ET-1 concentration in the insulin-resistant human endothelial cells.¹⁴ Through AMP-activated protein kinase (AMPK), metformin not only promotes phosphorylation and activation of eNOS,^{15,16} but also decreases cytotoxic peroxynitrite, a well-known vasoconstrictor, accompanied by enhanced NO release and reduced nitroxidative stress in obese rats.¹⁷ Moreover, metformin-induced AMPK activation suppresses rat VSMC contraction,¹⁸ as well as exaggerates phenylephrine (PE)-induced AMPK phosphorylation and attenuates contractile response in endothelium-denuded rat aorta.¹⁹ Song et al also demonstrated that the activity of AMPK negatively suppresses pulmonary VSMC proliferation and has possible utility in modulating pulmonary vascular remodelling.²⁰

We previously found that combination therapy with bosentan and metformin in PAH-CHD patients provides additional improvements in important outcomes such as exercise capacity and pulmonary hemodynamics, compared with bosentan alone.²¹ Therefore, the current study aimed to investigate the vessel reactivity (induced constriction) of isolated pulmonary arteries from PAH-CHD patients after combination therapy of bosentan and metformin as compared with bosentan monotherapy and explore the possible mechanism of action of metformin in those patients.

Material and Methods

In accordance with the Declaration of Helsinki, all study protocols were approved by the Institutional ethics committee of Nanchang University on human research, and written informed consent was obtained from all patients for the use of their tissue. Between May 2016 and December 2017, 93 adult patients (18-65 years) firstly diagnosed with PAH-CHD at our hospital were enrolled. The inclusion and exclusion criteria have been described previously.²¹

Treatment

All enrolled patients were randomised to receive bosentan (initially at 62.5 mg twice daily for 4 weeks and then 125 mg twice daily) for 3 months with or without the combination treatment of metformin (500 mg twice

daily). At 3 months follow-up, right heart catheterization and acute vasoreactivity testing were performed to assess the eligibility for shunt closure.^{22, 23} Selected patients, assessed by our heart surgery team, underwent operations with biventricular circulation.

Wire myography

Lung tissue biopsy was taken from the left upper lobe before cardiopulmonary bypass intraoperatively and placed in ice-cold physiological saline solution (PSS; mmol/L; 119 NaCl, 25 NaHCO₃, 4.69 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 5 glucose, 0.034 EDTA; pH 7.4). The small pulmonary arteries (internal diameter ≈250 μm) were dissected and carefully cleaned of adherent connective tissue and cut into 2 mm segments. 16 artery segments (8 of them had mechanical removal of endothelium using the steel wire) from each patient were mounted onto an isometric wire myograph system (610M wire myography; Danish Myotechniques, Aarhus, Denmark). The vessels were bathed in PSS, gassed with 5% CO₂/95% air, maintained at a temperature of 37°C, and allowed to equilibrate for 30 min before normalization to an internal diameter of 0.9 of L3.67kPa using normalization software (Myodata, Danish Myotechnologies, Aarhus, Denmark), which has been shown to be optimal in our preliminary study using the method described previously.²⁴ Vessel viability was assessed by exposure to high-potassium PSS (KPSS, mmol/L; 12.45 NaCl, 25 NaHCO₃, 120 KCl, 2.4 MgSO₄, 1.6 CaCl₂, 1.18 KH₂PO₄, 5 glucose, 0.034 EDTA; pH 7.4), followed, after washout with PSS, by 1 μM PE. The constriction by PE was allowed to plateau, then 1 μM the endothelial dependent vasodilator acetylcholine (ACh) was added to test the integrity of the endothelium or ensuring complete denudation of artery rings as described previously.²⁵ A concentration-response curve to PE or ET1 (0.1 nM to 10 μM) was performed. A NOS inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME; Abcam, UK; 40 μM), was used to blocking NOS in the endothelium. An AMPK inhibitor, compound C (Abcam, UK; 40 μM), was used to test the effect of AMPK activation by metformin on vessel tone.¹⁹ Arteries were preincubated with either L-NAME or compound C for 30 min before a second concentration-response curve was performed. Contractile responses to PE or ET1 are shown as a percentage of the contraction induced by KPSS.

Western blotting

Whole-cell lysates were made from homogenized isolated pulmonary artery samples with radio-immunoprecipitation assay buffer (Abcam, UK) containing a protease inhibitor cocktail (Roche, UK). Protein concentrations were determined through a Direct Detect Spectrometer (Merck, Germany). 20 μg protein from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting with antibodies specifically recognizing

human AMPK and phosphorylated AMPK α (p-AMPK) (Cell Signaling Technology, US), as well as eNOS, phosphorylated eNOS (p-eNOS), ERA, ERB and GAPDH (Abcam, UK). Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescence on a BioRad Chemidoc MP system.

Statistical analysis

Data are expressed as means \pm SEM and were compared using one-way ANOVA or two-way repeated-measures ANOVA with Bonferroni post hoc testing where appropriate. Sigmoidal curve fitting was performed on wire myography concentration-response curve data using GraphPad Prism software 7.0 (San Diego, CA). A p-value of < 0.05 was accepted as statistically significant.

Results

After 3 months of treatments, 20 of 28 patients with bosentan monotherapy and 18 of 24 patients with combination therapy were eligible for operations with biventricular circulation. 7 patients with monotherapy and 3 patients with combination therapy were excluded from the statistical analysis due to either unsuccessful

dissection of suitable pulmonary arteries or poor response of isolated arteries to vessel constrictor or dilator.

Adding metformin to bosentan therapy attenuates *in vitro* PE-induced contractility in pulmonary arteries from PAH-CHD patients

Both PE and ET1 induced dose-dependent constrictions in isolated pulmonary arteries with intact endothelium. These arteries were examined following prior treatments with either bosentan monotherapy or bosentan with metformin as combination therapy (Figure 1A and B). PE-induced contractions of arteries in patients received combination therapy were significantly attenuated at concentrations of 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M, compared to those received monotherapy (Figure 1A). Combination treatment had no effects on ET1-induced contractions (Figure 1B).

To determine the potential mechanisms of metformin-improved NO production on pulmonary artery response, vessels were preincubated with L-NAME for 30 min to block the endothelial NOS activity. We found that L-NAME preincubation increased the contractions to PE or ET1 by decreasing the NO bioavailability. However, blocking eNOS by L-NAME did not alter differential responses

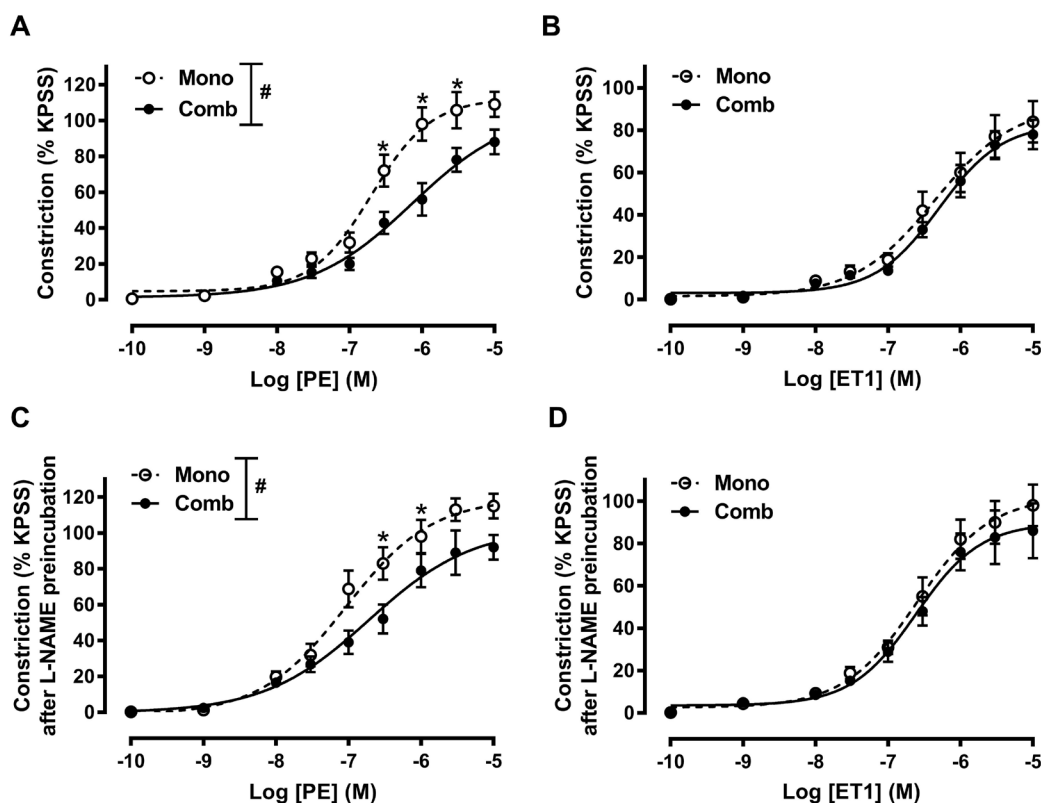


Figure 1. The reactivity of pulmonary artery with intact endothelium after bosentan monotherapy and bosentan/metformin combination therapy for 3 months. (A) and (B) Concentration-response curves for PE or ET-1 induced constriction of isolated pulmonary arteries. (C) and (D) Concentration-response curves for PE or ET-1 induced constriction of isolated pulmonary arteries after 30 min L-NAME preincubation. Constriction of pulmonary arteries in response to PE was significantly lower in combination therapy compared with monotherapy before or after L-NAME preincubation. Data are expressed as means \pm SEM. Mono, bosentan monotherapy, $n = 13$; Comb, bosentan/metformin combination therapy, $n = 15$; # $P < 0.05$; two-way ANOVA; * $P < 0.05$; two-way ANOVA with Bonferroni post hoc test.

to PE between arteries in patients with monotherapy or combination therapy (Figure 1C).

Compound C prevents the inhibitory effect of metformin on PE-induced contractility

To determine the direct effect of metformin on VSMCs, we tested the vascular response to vessel constrictors on endothelium-denuded pulmonary arteries. Both PE and ET1 induced dose-dependent constrictions on endothelium-denuded pulmonary arteries (Figure 2). After denudation, PE-induced pulmonary artery contractions at concentrations of 3×10^{-6} M and 10^{-5} M were significantly decreased in the combination therapy group, compared to the monotherapy groups (Figure 2A). There were no significant differences in ET1-induced contractions (Figure 2B). Interestingly, preincubating the rings with compound C abrogated the inhibitory effect of metformin on PE induced contractility (Figure 2C).

Increased AMPK and eNOS phosphorylation after adding metformin to bosentan therapy

To confirm the activity of AMPK and eNOS after metformin treatment, western blotting was performed. Western blotting revealed that increased levels of p-AMPK and p-eNOS in the pulmonary arteries of patients treated with combination therapy, compared to bosentan monotherapy (Figure 3A and B). However, there were no significant differences in the pulmonary protein levels of AMPK, eNOS, ERA and ERB between the two treatment

regimens (Figure 3 A and B).

Discussion

In the present study, we compared the pulmonary artery response after 3 months treatment of bosentan monotherapy vs combination bosentan/metformin therapy and found that pulmonary artery contraction induced by PE was decreased after adding metformin to bosentan therapy in PAH-CHD patients. This is significant because PAH is characterized by progressive inflammation and vessel wall remodelling leading to increased vasoconstriction and increased pulmonary artery resistance.^{26, 27}

The beneficial effects of metformin on the vascular function has been suggested in clinical observations,²⁸ although the molecular mechanisms are still unclear. In rats, oral administration of metformin diminished vascular reactivity to catecholamine constrictor both with and without the endothelium.^{29, 30} In humans, metformin improves vascular function in patients presented with insulin resistance.^{31, 32} We found that increased phosphorylation of AMPK and eNOS after chronic metformin treatment, which is consistent with results from previous translational studies with metformin treatments.³³⁻³⁵ The AMPK-eNOS-NO pathway is therefore considered to be the main pathway attributing to the regulative effect of metformin on vascular function.³⁶ AMPK is a heterotrimeric enzyme comprising a catalytic subunit and two regulatory subunits. The catalytic subunit

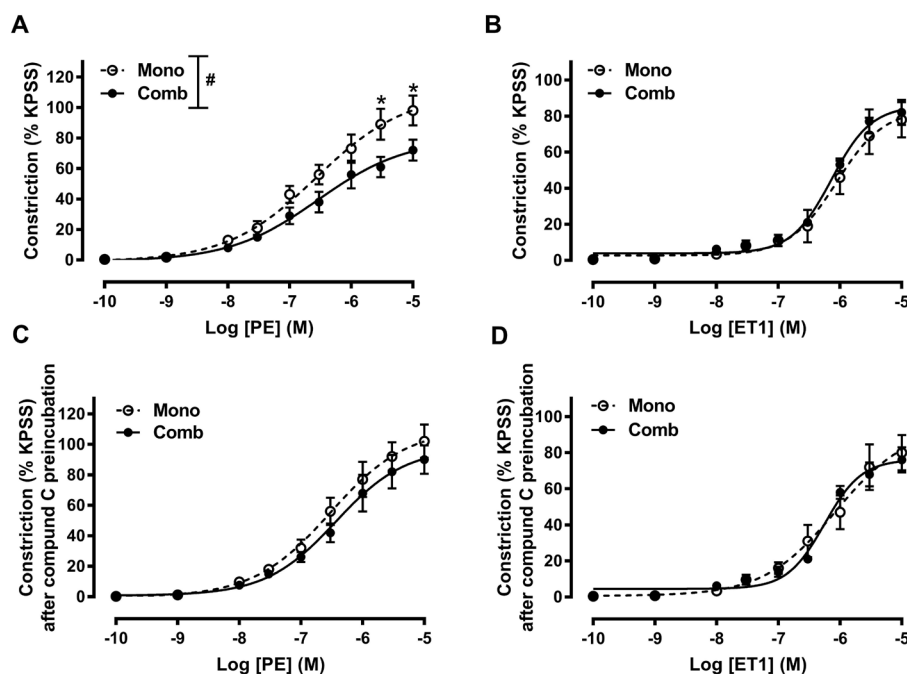


Figure 2. The reactivity of endothelium-denuded pulmonary artery after bosentan monotherapy and bosentan/metformin combination therapy for 3 months. (A) and (B) Concentration-response curves for PE or ET-1 induced constriction of isolated pulmonary arteries. (C) and (D) Concentration-response curves for PE or ET-1 induced constriction of isolated pulmonary arteries after 30 min compound C preincubation. Constriction of pulmonary arteries in response to PE was significantly lower in combination therapy compared with monotherapy before compound C preincubation. Compound C abrogated the inhibitory effect of metformin on PE induced contractility. Data are expressed as means \pm SEM. Mono, bosentan monotherapy $n = 13$; Comb, bosentan/metformin combination therapy, $n = 15$; # $P < 0.05$; two-way ANOVA; * $P < 0.05$; two-way ANOVA with Bonferroni post hoc test.

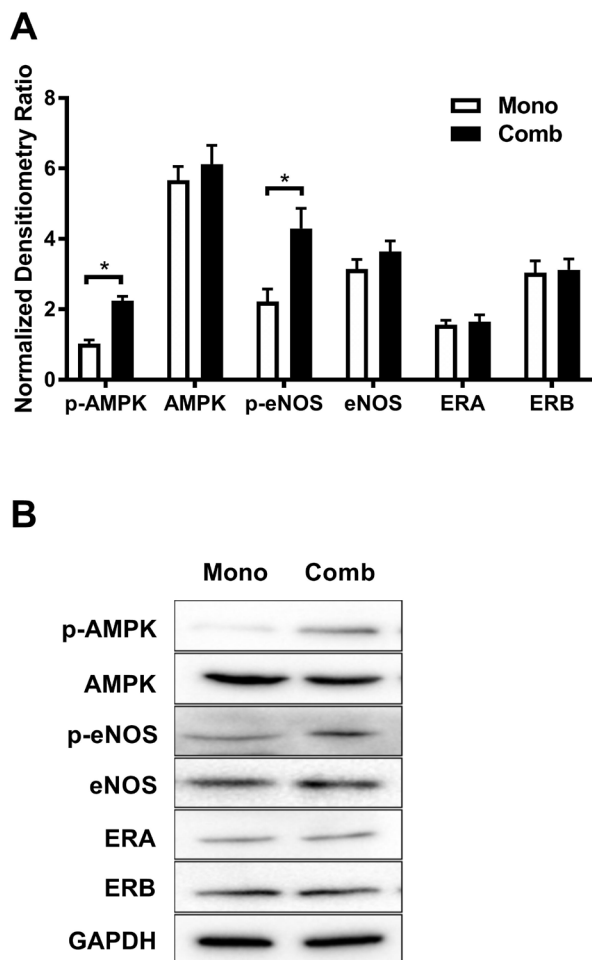


Figure 3. Pulmonary artery protein expressions after bosentan monotherapy and bosentan/metformin combination therapy for 3 months. (A) Protein levels of p-AMPK, AMPK, p-eNOS, eNOS, ERA and ERB. There were increased levels of p-AMPK and p-eNOS in the pulmonary arteries of patients treated with combination therapy, compared to bosentan monotherapy. (B) representative immunoblots of the corresponding proteins. Data are expressed as means \pm SEM. Mono, bosentan monotherapy; Comb, bosentan/metformin combination therapy; * $P < 0.05$; one-way ANOVA.

consists of an N-terminal catalytic kinase domain and a C-terminal regulatory domain. The phosphorylation of the kinase domain by upstream kinases is required for AMPK activation.³⁷ AMPK not only is involved in the regulation of cellular and organ metabolism,³⁸ but also plays a regulatory role over vascular structure and function and is essential for the maintenance of cardiovascular health.³⁹ Activation of AMPK by metformin stimulates NO synthesis in vascular endothelial cells by increasing phosphorylation and activation of eNOS^{15, 40} and reduces mitochondrial reactive oxygen species, which diminish the bioavailability of NO.⁴¹ However, blocking the eNOS or removing the endothelium did not fully reverse the inhibitory effect on vessel constriction by metformin in our study. A direct effect of metformin on VSMCs is suspected. Indeed, it is reported that the activation of

AMPK by metformin suppresses VSMC contraction by inhibiting myosin light chain (MLC) kinases and MLC phosphorylation in rats,¹⁸ reduces VSMC proliferation and migration,⁴² and attenuates elevation of intracellular Ca^{2+} levels in VSMCs.^{43,44} A direct effect via AMPK on smooth muscle cell wall of the artery is supported in our study, where we found that the AMPK inhibitor compound C reversed the inhibitory effect on PE-induced contraction by metformin on endothelium-denuded pulmonary arteries. Consistent with this, metformin increases AMPK phosphorylation and attenuates contractile responses in endothelium-denuded rat aorta,¹⁹ suggesting a potential role of AMPK as an intermediary signalling component for metformin action in pulmonary artery response.

It is worthy to note that compound C is the primary reagent used as an AMPK inhibitor and has been widely used in biochemical *in vitro* and some *in vivo* experiments. However, Compound C unfortunately inhibits several other kinases much more potently than AMPK and is therefore highly non-specific, and its inhibitory effects seem to be dose-dependent.^{45,46} Furthermore, ET1 is a strong vasoconstrictor and is one of key mediators of PAH development.⁴⁷ It is reported that the activation of AMPK by metformin suppresses ET1 induced pulmonary artery VSMC proliferation in rats,⁴⁸ and inhibits *ET1* expression at the transcriptional and translational level in the aorta.⁴⁹ Additional effects of metformin on the ET pathway and/or other pathways may also contribute to the differential vascular responses we observed.

Conclusions

In summary, our findings suggest that adding metformin to bosentan therapy in patients with PAH-CHD decreased *in vitro* pulmonary artery contraction induced by PE, which is possibly related to increased AMPK phosphorylation. Our findings demonstrate the efficacy of metformin clinically in diseased pulmonary arteries.

Competing interests

The authors report no relationships that could be construed as a conflict of interest.

Ethical approval

In accordance with the Declaration of Helsinki, all study protocols were approved by the Institutional ethics committee of Nanchang University on human research, and written informed consent was obtained from all patients for the use of their tissue.

Acknowledgments

The authors gratefully acknowledge the financial support from The Limingzhang Sciences Foundation (2014-778903). Shutan Liao is funded under an NHMRC Development Grant.

References

- Gatzoulis Ma, Alonso-Gonzalez R, Beghetti M. Pulmonary Arterial Hypertension In Paediatric And Adult Patients With Congenital Heart Disease. *Eur Respir Rev* 2009;

- 18(113): 154-61. doi: 10.1183/09059180.00003309
2. Van Riel Ac, Schuurin Mj, Van Hensen Id, Zwinderman Ah, Cozijnsen L, Reichert Cl, Et Al. Contemporary Prevalence Of Pulmonary Arterial Hypertension In Adult Congenital Heart Disease Following The Updated Clinical Classification. **Int J Cardiol** 2014; 174(2): 299-305. doi: 10.1016/J.ijcard.2014.04.072
 3. Barst Rj, Ivy Dd, Foreman Aj, Mcgoon Md, Rosenzweig Eb. Four- And Seven-Year Outcomes Of Patients With Congenital Heart Disease-Associated Pulmonary Arterial Hypertension (From The Reveal Registry). **Am J Cardiol** 2014; 113(1): 147-55. doi: 10.1016/J.amjcard.2013.09.032
 4. Kaemmerer H, Gorenflo M, Hoepfer M, Huscher D, Ewert P, Pittrow D. [Pulmonary Arterial Hypertension In Patients With Congenital Heart Disease: Current Issues And Health Care Situation]. **Dtsch Med Wochenschr** 2013; 138(23): 1247-52. doi: 10.1055/S-0033-1343189
 5. Gatzoulis Ma, Beghetti M, Landzberg Mj, Galie N. Pulmonary Arterial Hypertension Associated With Congenital Heart Disease: Recent Advances And Future Directions. **Int J Cardiol** 2014; 177(2): 340-7. doi: 10.1016/J.ijcard.2014.09.024
 6. Hu Z, Xie B, Zhai X, Liu J, Gu J, Wang X, Et Al. Midterm Results Of "Treat And Repair" For Adults With Non-Restrictive Ventricular Septal Defect And Severe Pulmonary Hypertension. **J Thorac Dis** 2015; 7(7): 1165-73. doi: 10.3978/J.issn.2072-1439.2015.07.06
 7. Apitz C, Hansmann G, Schranz D. Hemodynamic Assessment And Acute Pulmonary Vasoreactivity Testing In The Evaluation Of Children With Pulmonary Vascular Disease. Expert Consensus Statement On The Diagnosis And Treatment Of Paediatric Pulmonary Hypertension. The European Paediatric Pulmonary Vascular Disease Network, Endorsed By Ishlt And Dgpk. **Heart** 2016; 102 Suppl 2: Ii23-9. doi: 10.1136/Heartjnl-2014-307340
 8. Kemp K, Savale L, O'callaghan Ds, Jais X, Montani D, Humbert M, Et Al. Usefulness Of First-Line Combination Therapy With Epoprostenol And Bosentan In Pulmonary Arterial Hypertension: An Observational Study. **J Heart Lung Transplant** 2012; 31(2): 150-8. doi: 10.1016/J.healun.2011.11.002
 9. Sitbon O, Jais X, Savale L, Cottin V, Bergot E, Macari Ea, Et Al. Upfront Triple Combination Therapy In Pulmonary Arterial Hypertension: A Pilot Study. **Eur Respir J** 2014; 43(6): 1691-7. doi: 10.1183/09031936.00116313
 10. Humbert M, Barst Rj, Robbins Im, Channick Rn, Galie N, Boonstra A, Et Al. Combination Of Bosentan With Epoprostenol In Pulmonary Arterial Hypertension: Breathe-2. **Eur Respir J** 2004; 24(3): 353-9. doi: 10.1183/09031936.04.00028404
 11. Abbasi F, Chu Jw, Mclaughlin T, Lamendola C, Leary Et, Reaven Gm. Effect Of Metformin Treatment On Multiple Cardiovascular Disease Risk Factors In Patients With Type 2 Diabetes Mellitus. **Metabolism** 2004; 53(2): 159-64.
 12. Agard C, Rolli-Derkinderen M, Dumas-De-La-Roque E, Rio M, Sagan C, Savineau Jp, Et Al. Protective Role Of The Antidiabetic Drug Metformin Against Chronic Experimental Pulmonary Hypertension. **Br J Pharmacol** 2009; 158(5): 1285-94. doi: 10.1111/J.1476-5381.2009.00445.X
 13. Dean A, Nilsen M, Loughlin L, Salt Ip, Maclean Mr. Metformin Reverses Development Of Pulmonary Hypertension Via Aromatase Inhibition. **Hypertension** 2016; 68(2): 446-54. doi: 10.1161/Hypertensionaha.116.07353
 14. Chen H, Li J, Yang O, Kong J, Lin G. Effect Of Metformin On Insulin-Resistant Endothelial Cell Function. **Oncol Lett** 2015; 9(3): 1149-53. doi: 10.3892/Ol.2015.2883
 15. Morrow Va, Foufelle F, Connell Jm, Petrie Jr, Gould Gw, Salt Ip. Direct Activation Of Amp-Activated Protein Kinase Stimulates Nitric-Oxide Synthesis In Human Aortic Endothelial Cells. **J Biol Chem** 2003; 278(34): 31629-39. doi: 10.1074/Jbc.m212831200
 16. Chen Z, Peng Ic, Sun W, Su Mi, Hsu Ph, Fu Y, Et Al. Amp-Activated Protein Kinase Functionally Phosphorylates Endothelial Nitric Oxide Synthase Ser633. **Circ Res** 2009; 104(4): 496-505. doi: 10.1161/Circresaha.108.187567
 17. Sambe T, Mason Rp, Dawoud H, Bhatt Dl, Malinski T. Metformin Treatment Decreases Nitroxidative Stress, Restores Nitric Oxide Bioavailability And Endothelial Function Beyond Glucose Control. **Biomed Pharmacother** 2018; 98: 149-56. doi: 10.1016/J.biopha.2017.12.023
 18. Sung Jy, Choi Hc. Metformin-Induced Amp-Activated Protein Kinase Activation Regulates Phenylephrine-Mediated Contraction Of Rat Aorta. **Biochem Biophys Res Commun** 2012; 421(3): 599-604. doi: 10.1016/J.bbrc.2012.04.052
 19. Pyla R, Osman I, Pichavaram P, Hansen P, Segar L. Metformin Exaggerates Phenylephrine-Induced Ampk Phosphorylation Independent Of Camkkbeta And Attenuates Contractile Response In Endothelium-Denuded Rat Aorta. **Biochem Pharmacol** 2014; 92(2): 266-79. doi: 10.1016/J.bcp.2014.08.024
 20. Song Y, Wu Y, Su X, Zhu Y, Liu L, Pan Y, Et Al. Activation Of Ampk Inhibits Pdgf-Induced Pulmonary Arterial Smooth Muscle Cells Proliferation And Its Potential Mechanisms. **Pharmacol Res** 2016; 107: 117-24. doi: 10.1016/J.phrs.2016.03.010
 21. Liao S, Li D, Hui Z, Mclachlan Cs, Zhang Y. Metformin Added To Bosentan Therapy In Patients With Pulmonary Arterial Hypertension Associated With Congenital Heart Defects: A Pilot Study. **Erj Open Research** 2018; 4(3). doi: 10.1183/23120541.00060-2018
 22. Lopes Aa, O'leary Pw. Measurement, Interpretation And Use Of Haemodynamic Parameters In Pulmonary Hypertension Associated With Congenital Cardiac Disease. **Cardiol Young** 2009; 19(5): 431-5. doi: 10.1017/S1047951109990771
 23. Kozlik-Feldmann R, Hansmann G, Bonnet D, Schranz D, Apitz C, Michel-Behnke I. Pulmonary Hypertension In Children With Congenital Heart Disease (Pah-Chd, Pphvd-Chd). Expert Consensus Statement On The Diagnosis And Treatment Of Paediatric Pulmonary Hypertension. The European Paediatric Pulmonary Vascular Disease Network, Endorsed By Ishlt And Dgpk. **Heart** 2016; 102 Suppl 2: Ii42-8. doi: 10.1136/Heartjnl-2015-308378
 24. Angus Ja, Wright Ce. Techniques To Study The Pharmacodynamics Of Isolated Large And Small Blood Vessels. **J Pharmacol Toxicol Methods** 2000; 44(2): 395-407.
 25. Baranowska-Kuczko M, Kozłowska H, Kozłowski M, Schlicker E, Kloza M, Surazynski A, Et Al. Mechanisms Of Endothelium-Dependent Relaxation Evoked By

- Anandamide In Isolated Human Pulmonary Arteries. **Naunyn Schmiedebergs Arch Pharmacol** 2014; 387(5): 477-86. doi: 10.1007/S00210-014-0961-9
26. Low A, George S, Howard L, Bell N, Millar A, Tulloh Rmr. Lung Function, Inflammation, And Endothelin-1 In Congenital Heart Disease-Associated Pulmonary Arterial Hypertension. **J Am Heart Assoc** 2018; 7(4). doi: 10.1161/Jaha.117.007249
 27. Tuder Rm. Pulmonary Vascular Remodeling In Pulmonary Hypertension. **Cell Tissue Res** 2017; 367(3): 643-9. doi: 10.1007/S00441-016-2539-Y
 28. Grant Pj. Beneficial Effects Of Metformin On Haemostasis And Vascular Function In Man. **Diabetes Metab** 2003; 29(4 Pt 2): 6S44-52.
 29. Verma S, Bhanot S, Mcneill Jh. Decreased Vascular Reactivity In Metformin-Treated Fructose-Hypertensive Rats. **Metabolism** 1996; 45(9): 1053-5.
 30. Lobato Ns, Filgueira Fp, Hagihara Gn, Akamine Eh, Pariz Jr, Tostes Rc, Et Al. Improvement Of Metabolic Parameters And Vascular Function By Metformin In Obese Non-Diabetic Rats. **Life Sci** 2012; 90(5-6): 228-35. doi: 10.1016/J.lfs.2011.11.005
 31. Joya-Galeana J, Fernandez M, Cervera A, Reyna S, Ghosh S, Triplitt C, Et Al. Effects Of Insulin And Oral Anti-Diabetic Agents On Glucose Metabolism, Vascular Dysfunction And Skeletal Muscle Inflammation In Type 2 Diabetic Subjects. **Diabetes Metab Res Rev** 2011; 27(4): 373-82. doi: 10.1002/Dmrr.1185
 32. Romualdi D, Costantini B, Selvaggi L, Giuliani M, Cristello F, Macri F, Et Al. Metformin Improves Endothelial Function In Normoinsulinemic Pcos Patients: A New Prospective. **Hum Reprod** 2008; 23(9): 2127-33. doi: 10.1093/Humrep/Den230
 33. Cao X, Li H, Tao H, Wu N, Yu L, Zhang D, Et Al. Metformin Inhibits Vascular Calcification In Female Rat Aortic Smooth Muscle Cells Via The Ampk-Enos-No Pathway. **Endocrinology** 2013; 154(10): 3680-9. doi: 10.1210/En.2013-1002
 34. Zhang Cx, Pan Sn, Meng Rs, Peng Cq, Xiong Zj, Chen Bl, Et Al. Metformin Attenuates Ventricular Hypertrophy By Activating The Amp-Activated Protein Kinase-Endothelial Nitric Oxide Synthase Pathway In Rats. **Clin Exp Pharmacol Physiol** 2011; 38(1): 55-62. doi: 10.1111/J.1440-1681.2010.05461.X
 35. Yu Jw, Deng Yp, Han X, Ren Gf, Cai J, Jiang Gj. Metformin Improves The Angiogenic Functions Of Endothelial Progenitor Cells Via Activating Ampk/Enos Pathway In Diabetic Mice. **Cardiovasc Diabetol** 2016; 15: 88. doi: 10.1186/S12933-016-0408-3
 36. Salt Ip, Hardie Dg. Amp-Activated Protein Kinase: An Ubiquitous Signaling Pathway With Key Roles In The Cardiovascular System. **Circ Res** 2017; 120(11): 1825-41. doi: 10.1161/Circresaha.117.309633
 37. Hawley Sa, Davison M, Woods A, Davies Sp, Beri Rk, Carling D, Et Al. Characterization Of The Amp-Activated Protein Kinase Kinase From Rat Liver And Identification Of Threonine 172 As The Major Site At Which It Phosphorylates Amp-Activated Protein Kinase. **J Biol Chem** 1996; 271(44): 27879-87.
 38. Hardie Dg. Ampk: Positive And Negative Regulation, And Its Role In Whole-Body Energy Homeostasis. **Curr Opin Cell Biol** 2015; 33: 1-7. doi: 10.1016/J.ceb.2014.09.004
 39. Ewart Ma, Kennedy S. Ampk And Vasculoprotection. **Pharmacol Ther** 2011; 131(2): 242-53. doi: 10.1016/J.pharmthera.2010.11.002
 40. Davis Bj, Xie Z, Viollet B, Zou Mh. Activation Of The Amp-Activated Kinase By Antidiabetic Drug Metformin Stimulates Nitric Oxide Synthesis In Vivo By Promoting The Association Of Heat Shock Protein 90 And Endothelial Nitric Oxide Synthase. **Diabetes** 2006; 55(2): 496-505.
 41. Zou Mh, Kirkpatrick Ss, Davis Bj, Nelson Js, Wiles Wgt, Schlattner U, Et Al. Activation Of The Amp-Activated Protein Kinase By The Anti-Diabetic Drug Metformin In Vivo. Role Of Mitochondrial Reactive Nitrogen Species. **J Biol Chem** 2004; 279(42): 43940-51. doi: 10.1074/Jbc.m404421200
 42. Vignetti D, Clerici M, Deleonibus S, Karousou E, Viola M, Moretto P, Et Al. Hyaluronan Synthesis Is Inhibited By Adenosine Monophosphate-Activated Protein Kinase Through The Regulation Of Has2 Activity In Human Aortic Smooth Muscle Cells. **J Biol Chem** 2011; 286(10): 7917-24. doi: 10.1074/Jbc.m110.193656
 43. Sharma Rv, Bhalla Rc. Metformin Attenuates Agonist-Stimulated Calcium Transients In Vascular Smooth Muscle Cells. **Clin Exp Hypertens** 1995; 17(6): 913-29.
 44. Chen Xl, Panek K, Rembold Cm. Metformin Relaxes Rat Tail Artery By Repolarization And Resultant Decreases In Ca²⁺ Influx And Intracellular [Ca²⁺]. **J Hypertens** 1997; 15(3): 269-74.
 45. Dasgupta B, Seibel W. Compound C/Dorsomorphin: Its Use And Misuse As An Ampk Inhibitor. **Methods Mol Biol** 2018; 1732: 195-202. doi: 10.1007/978-1-4939-7598-3_12
 46. Bain J, Plater L, Elliott M, Shpiro N, Hastie Cj, Mclauchlan H, Et Al. The Selectivity Of Protein Kinase Inhibitors: A Further Update. **Biochem J** 2007; 408(3): 297-315. doi: 10.1042/Bj20070797
 47. Guignabert C, Tu L, Girerd B, Ricard N, Huertas A, Montani D, Et Al. New Molecular Targets Of Pulmonary Vascular Remodeling In Pulmonary Arterial Hypertension: Importance Of Endothelial Communication. **Chest** 2015; 147(2): 529-37. doi: 10.1378/Chest.14-0862
 48. Wu Y, Liu L, Zhang Y, Wang G, Han D, Ke R, Et Al. Activation Of Ampk Inhibits Pulmonary Arterial Smooth Muscle Cells Proliferation. **Exp Lung Res** 2014; 40(5): 251-8. doi: 10.3109/01902148.2014.913092
 49. Tang St, Su H, Zhang Q, Tang Hq, Wang Cj, Zhou Q, Et Al. Sitagliptin Inhibits Endothelin-1 Expression In The Aortic Endothelium Of Rats With Streptozotocin-Induced Diabetes By Suppressing The Nuclear Factor-Kappab/Ikappabalpha System Through The Activation Of Amp-Activated Protein Kinase. **Int J Mol Med** 2016; 37(6): 1558-66. doi: 10.3892/Ijmm.2016.2578