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The Protein Tyrosine Kinase Inhibitor Genistein Suppresses Hypoxia-Induced Atrial Natriuretic Peptide Secretion Mediated by the PI3K/Akt-HIF-1 α Pathway in Isolated Beating Rat Atria

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3 **Beating Rat Atria**

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15 **Running Title:** Protein Tyrosine Kinases Regulate Hypoxia-induced ANP Secretion

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Abstract

Background: Genistein, an isoflavonoid that can inhibit protein tyrosine kinase (PTK) phosphorylation, was proved to play pivotal roles in the signal transduction pathways of hypoxic disorders. In this study, we established a rat model of isolated beating atrium and investigated the regulator role of genistein and its downstream signaling pathways in acute hypoxia-induced ANP secretion.

Methods: Radio-immunoassay was used to detect the ANP content in the atrial perfusates. Western blot analysis was used to determine the protein level of hypoxia-inducible factor-1 α (HIF-1 α), and GATA₄ in the atrial tissue.

Results: The results showed that acute hypoxia substantially promoted ANP secretion, whereas this effect was partly attenuated by the PTKs inhibitor genistein (3 μ M). By western blotting

analysis, we found that hypoxia-induced the increase in phosphorylation of Akt and transcriptional factors, including HIF-1 α , were also reversed by genistein. The perfused HIF-1 α inhibitors rotenone (0.5 μ M) or CAY10585 (10 μ M) plus genistein significantly abolished the enhanced ANP secretion induced by hypoxia. Additionally, the perfused PI3K/Akt agonist IGF-1 (30 μ M) also abolished ANP secretion induced by genistein as well as inhibited expression of HIF-1 α .

Conclusions: In summary, our data suggested that acute hypoxia markedly increased ANP secretion by PTKs through the PI3K/HIF-1 α depended pathway.

Keywords: Atrial Natriuretic Peptide; Hypoxia; Hypoxia Inducible Factor-1 α ; Protein tyrosine kinases; Genistein

Introduction

Atrial natriuretic peptide (ANP) is one of the cardiac hormones synthesized and primarily stored in the cardiac atria with multiple functions on cardio-related disease (Cannone et al., 2019; Zhou et al., 2014). A previous study demonstrated that under hypoxic conditions, ANP plays an important role by protecting cardiomyocytes from hypertrophy, fibrosis, and heart failure (Casserly et al., 2010). Hence studies are increasing accumulated to elaborate what stimulus and cellular signaling pathway involved in during ANP release. Notably, hypoxia was recognized as a potent trigger of ANP secretion as reported by multiple studies (Lu, 2011; Xiao and Bariqige,

2016). However, the mechanism responsible for hypoxia-induced ANP secretion has not yet been entirely elucidated.

Protein tyrosine kinases (PTKs) are critical enzymes required for the phosphorylation of proteins and their mutant or affected by cellular stress may lead to dysregulation of PTK-mediated intracellular signaling pathway. They have been known to play an important role in regulating gene expression and signaling pathway changes in cells responding to hypoxia stress (Gluck et al., 2015; Zhang et al., 2004b), which can result in many disorders, including myocardial function under normal and hypoxic conditions (Li et al., 2008; Tsibulnikov et al., 2018; Wijetunge and Hughes, 2007). However, the involved PTKs in the regulation of ANP release are not well known. Genistein, a non-selective tyrosine kinase inhibitor (Lopez et al., 2015), has been used widely to define the role of PTKs in physiological and pathological changes of heart in the level of cells or tissues or human (Bai and Wang, 2019; De Gregorio et al., 2017; Meng et al., 2019). Therefore, we selected genistein and explored its role in ANP secretion in isolated perfused atria.

Recent studies have shown that the PTK signaling pathway is closely related to activation of hypoxia-inducible factor-1 α (HIF-1 α) (Mabjeesh et al., 2003; Maebayashi Asanuma et al., 2014; Nilsson et al., 2010), a transcriptional factor that activated in response to hypoxic stimulation, and is involved in hypoxia-induced pulmonary hypertension (Pullamsetti et al., 2012). Moreover, phosphoinositide-3 kinase (PI3K)/Akt signaling has been recognized as an important pathway downstream to PTKs (Lemmon and Schlessinger, 2010). Genistein treatment can induce inhibition of the PI3K/Akt-dependent pathway in the cardiovascular system (Kuriyama et al., 2014). Considering the finding in our previous study that hypoxia-induced ANP secretion could be elicited via a signaling cascade from MAPK and PI3K to HIF-1 α (Zhang et al., 2013), we

speculated that PI3K/Akt-HIF-1 α pathway may participate in the regulatory role genistein on ANP secretion.

In the present study, we treated isolated perfused rat atria with genistein and investigated its role in hypoxia-induced atrial ANP secretion. We also assessed whether PTKs and their possible downstream signaling pathways are activated during hypoxia-induced atrial ANP secretion in the perfused beating rat atria.

Material and Methods

Animals

Sprague-Dawley rats (8-10 weeks, weighing 348 ± 20 g) were purchased from the Institute of Laboratory Animal Resources of Yanbian University. Before experiments, animals were kept in a room at 25 °C with free-feeding and drinking device. Any operation protocols on animals complied with the approval in the Committee on the Ethics of Animal Experiments at Yanbian Medical University of Science and Technology and were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

In vitro atria perfusion preparation

Beating atria were isolated from rats and perfused *in vitro* according to protocols in an earlier report with minor modification on procedures (Gao et al., 2010). In brief, rats were executed by decapitation and then the hearts were instantly excised. The prepared 36.5°C normal saline washed off the blood from the heart. The left atria were separated and ligated on a cannulus using suture. The fixed atria were beating and continuously perfused with HEPES-buffered saline in an organ chamber at 36.5°C, with simultaneous electrical stimulation at 1.5 Hz (duration, 0.3 ms; and

voltage, 30–40 V). The pericardial buffer solution, as well as the perfusate, was oxygenated via silicone tube coils. For observing intra-atrial pressure, a pressure transducer (YLJ100, Chengdu Instruments, China) was connected between the beating atria and a signal acquisition system (RM6240C, Chengdu Instruments, China). Before drug treatment, functional parameters were stabilized by 70 min perfusion to atria with HEPES-buffered saline. The control samples began to be collected and were sampled every 2 min for 12 min. Then HEPES-buffered saline contained N₂ was used to perfuse atria for 48 min during which hypoxia samples were sampled every 2 min. All samples were collected at 4°C. Atrial tissues were collected immediately after the perfusion protocols, frozen, and preserve at -80°C.

Acute hypoxia atrial model preparation and experimental protocols

Three different experimental conditions were tested and evaluated in this study. In **Protocol 1**, atria were beating HEPES buffer containing N₂ as the hypoxia experiment for three cycles after one cycle of normoxia control. In **Protocol 2**, atria were with one cycle of normoxia control, one cycle of normoxia perfusion in presence of genistein (an inhibitor of PTKs; 3 µM), and following with three cycles of hypoxia in presence of genistein. In **Protocol 3**, atria were with one cycle of normoxia control, one cycle of normoxia perfusion in presence of genistein or together with rotenone or CAY10585 (HIF-1α inhibitor, 0.5 µM and 10 µM, respectively) and IGF-1 (an agonist for Akt, 30 µM), and following with three cycles of hypoxia in presence of these agents. All samples to be tested will be collected every 2 minutes. Each protocol used 6 rats.

Radioimmunoassay for measuring ANP concentrations

Specific radioimmunoassays were used to measure the ANP levels of the perfusates as described in an earlier study (Cho et al., 1993). The amount of secreted immunoreactive ANP was

represented as ng/min/g (ng/ perfusion time/weight of wet atrium). The secreted ANP were processed ANP.

Western blot analysis

Tissues were homogenized in pre-cooled RIPA Lysis Buffer (P0013B, Beyotime, China) and subjected to extraction of total proteins using centrifugation. Protein concentrations were determined by the BCA method. For all samples, the equal amount of proteins was loaded and separated using SDS-PAGE (8% gel). After gel electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) filter membrane (0.45 mm, Millipore, USA). To prevent nonspecific staining, the membrane was treated with a blocking buffer containing 5% non-fat dry milk in TBST buffer. The target proteins were blotted with primary antibodies against HIF-1 α , GAPDH (ab84593, ab65979; and ab9485; diluted 1:1000 by Tris Buffer Saline or TBS; Abcom, Hong Kong), and *p*-Akt (sc-7976; diluted 1:500 by TBS; Santa Cruz Biotechnology, USA) and incubated overnight at 4°C. The following day blotted proteins were incubated with IgG horseradish peroxidase-linked secondary antibodies (diluted 1:1000 by TBS). Protein blotted in the membrane was visualized after being infiltrated with enhanced chemiluminescence plus reagent (ECL kit, Ewbio, China).

Statistical analysis

A software of GraphPad Prism 5.0 was used for statistics and analysis of data. Difference analysis between or among groups was performed using one-way ANOVA followed by Dunnett's multiple comparison tests or an unpaired *t*-test. All data were presented as mean $\pm$ SEM. A statistical significance was reasonable when the P-value was less than 0.05.

Results

PTK inhibitor genistein suppressed hypoxia-induced ANP secretion from atria

Rat atria secreted a higher level of ANP concentration under acute hypoxia stimulation (6.24-fold; $P < 0.001$ vs. normoxia control) as shown in Fig. 1Aa and Ba. ANP secretion peaked in the last period of the third experimental cycle (approximately 26 min after hypoxia) and then mildly dropped, maintaining high levels overall. Under such hypoxia, atria showed significantly lower atrial dynamics ($P < 0.001$ vs. normoxia control), as shown in Fig. 1Ab and Bb. These data indicated a successful acute hypoxia model of beating atrium made by *in vitro* perfusion method.

To explore the action of genistein, an inhibitor for PTKs, on hypoxia-induced atrial ANP secretion, this agent was added into perfusate buffer and observe its effect on ANP release from hypoxic atria. The result showed that genistein treatment significantly reduced the concentration of ANP in atrial perfusion ($P < 0.001$ vs. normoxia control, Fig. 1Aa and 1Ba vs. hypoxia alone, Fig. 1Ba) but exert no effect on atrial pulse pressure suppressed by hypoxia ($P < 0.001$ vs. normoxia control, Fig. 1Ab and Bb; $P > 0.05$ vs. hypoxia alone, Fig. 1Bb).

PTK inhibitor genistein suppressed PI3K/Akt signaling in hypoxia-induced atrial tissue

To investigate the downstream signaling pathway by which PTKs participate in atrial ANP secretion induced by hypoxia, the relative expression levels of phosphorylated Akt and HIF- α were determined using Western blot. The data are shown in Fig. 2A indicated that acute hypoxia significantly increased Akt phosphorylation ($P < 0.001$ vs. control) in rat atrial tissue; whereas this increase was markedly attenuated or abolished after genistein treatment ($P < 0.001$ vs. hypoxia alone). Besides, the relative expression level of HIF-1 α induced by hypoxia was also significantly reduced after genistein treatment ($P < 0.01$ vs. hypoxia alone).

Activation of PI3K/Akt signaling alleviated genistein-induced the reduction of ANP secretion

To determine the key role of PI3K/Akt signaling in the genistein-induced reduction of the ANP secretion under hypoxia, we examined the ANP secretion in rat atrial perfusate after PI3K/Akt agonist IGF-1 and genistein co-treatment. The result showed that co-perfusion of IGF-1 and genistein abrogated the inhibitory role of genistein on ANP secretion and substantially enhanced ANP level (Fig. 3A) without changing the reduced atrial pulse pressure by hypoxia ($P < 0.001$ vs. control; $P > 0.05$ vs. hypoxia alone, Fig. 3B). Western blot results showed that IGF-1 treatment reversed genistein-induced HIF- α and p-AKT expression under hypoxia (Figure 3C). Our previous work has proved that the specific inhibitor of Akt (LY294002) inhibits hypoxia-induced ANP secretion (Zhang et al., 2013). Therefore, we examined the expression of Akt phosphorylation and found that it was reduced more by LY294002 under hypoxia (Supplement 1). To further confirm PI3K/AKT signaling pathway was affected by genistein, we examined the pAKT expression in atria treated with genistein and specific inhibitor of Akt (LY294002) and found that pAKT in genistein group expresses the same level as genistein+ LY294002 group (Figure 3D).

Inhibition of HIF-1 α increases PTK-reduced and hypoxia-induced atrial ANP secretion

To explore the involvement of HIF-1 α in PTK regulation of atrial ANP secretion under hypoxia, the specific inhibitors for HIF-1 α (rotenone, 0.5 μ M and CAY10585, 10 μ M, respectively) were used to stimulate atria together with genistein and their action on ANP release was observed. The results showed that in the presence of rotenone plus genistein, genistein-induced the decrease of ANP secretion was not changed ($P < 0.05$ vs. control period or genistein+hypoxia; Fig. 4Aa and 4Ba). Meanwhile, CAY10585 plus genistein also had no effect on atrial ANP secretion under hypoxia ($P < 0.001$) in comparison to genistein+hypoxia (Fig. 5Aa

and 5Ba). Both inhibitors did not change the decreased atrial pulse dynamics induced by hypoxia ($P < 0.001$ vs. control period; Fig. 4Ab–Bb and 5Ab–Bb). As for the relative expression level of HIF-1 α in these atrial tissues after perfusion, the increased HIF-1 α level induced by hypoxia was blocked by genistein, rotenone+genistein and CAY10585+genistein ($P < 0.05$, ns vs. control; $P < 0.05$ vs. hypoxia alone; respectively; Fig.6).

Discussion

Increased ANP release under hypoxia is an adaptive biological change of atrium during the pathogenesis of cardiopulmonary diseases and it is more relevant to understand what underlying molecular mechanism is involved to explore the effective therapeutic target for disease. The present study focused on PTKs and found that its antagonist, genistein, are the key regulators of atrial ANP secretion induced by acute hypoxia in the beating rat atria *in vitro*, with a potential downstream signaling pathway of PTKs-PI3K/Akt-HIF-1 α pathway.

PTKs are important enzymes that catalyze substrate protein phosphorylation, where they transmit extracellular signals to the intracellular space and activate various intracellular signaling pathways that regulate processes of cell growth, differentiation, metabolism, migration, and apoptosis (Amatya et al., 2019). Also, studies suggested that they control the abnormal signaling transduction of cells under ischemia conditions (Cheng et al., 2015; Mao et al., 2015; Wang et al., 2016). As reported in previous studies, hypoxia and ischemia are a strong stimulus for ANP release from cells or tissue (Xiao et al., 2012). Herein, we concentrate on the functional involvement of PTK by observing the role of its antagonist genistein in ANP release from hypoxia-conditioned atria using an *in vitro* beating atria model of rat. Our data suggested that genistein effectively suppressed hypoxia-induced ANP secretion without influence on atrial pressure

219 suggesting the negative role of genistein in hypoxia-induced ANP secretion.

220 Hypoxia-response elements have been characterized by the promoter sequence of NPs
221 genes(Arjamaa and Nikinmaa, 2011), suggesting that NPs genes are under controlled by HIF. That
222 has been demonstrated to be involved in regulating the expression of various hypoxia-dependent
223 genes. It has been demonstrated that PTKs are involved in the regulation of HIF-1 α protein
224 expression under hypoxia(Kuriyama et al., 2014), and modulate HIF-1 α activity through the
225 PI3K/Akt signaling pathway (Zhang et al., 2004a). The results of our study also show that the
226 PTK inhibitor, genistein decreased phosphorylation level of Akt and reduced hypoxia-induced
227 HIF-1 α protein expression in perfused rat atria. These data suggested that genistein inhibited PTK
228 downstream PI3K/Akt signals. This was confirmed by present data of Akt agonist combined with
229 genistein co-perfusion experiment which showed that activated Akt alleviated the inhibitory role
230 of genistein on ANP secretion, HIF-1 α expression, and p-AKT expression. We thus examined this
231 pathway in the hypoxia-induced ANP section.

232 As the tissues adapt to hypoxic conditions, HIF-1 α plays a key role in the regulation of
233 expression and transcription of multiple hypoxia-related genes including PDGF, IGF-1, EGF,
234 VEGF, and ANP (Richard et al., 1999; Shafee et al., 2009). In a previous study, we have
235 demonstrated that acute hypoxia markedly increases HIF-1 α activity and regulates
236 hypoxia-induced ANP secretion in atria (Zhang et al., 2013). The present study showed that
237 genistein alone greatly inhibited hypoxia-increased *p*-Akt and HIF-1 α expressions, and
238 significantly attenuated hypoxia-promoted atrial ANP secretion. However, genistein combined
239 with IGF-1, an agonist for PI3K/Akt, treatment, not only markedly attenuated or completely
240 blocked hypoxia-induced ANP secretion but also blocked the increase of hypoxia-induced HIF-1 α .

To determine the key role of HIF-1 α in the inhibitory role of genistein on the ANP secretion, we performed rat atrial co-perfusion experiments with genistein plus rotenone or CAY10585 (two inhibitors for HIF-1 α). The data showed that rotenone or CAY10585 did not alter the biological effect of genistein on hypoxia-induced ANP secretion. These data suggested that HIF-1 α is an effective responder for genistein in reduced ANP secretion.

In conclusion, acute hypoxia significantly promotes ANP release in beating rat atria. Inhibition of PTKs by genistein regulates ANP secretion from hypoxia-conditioned atria through PI3K/Akt-HIF-1 α signaling pathways. HIF-1 α and related hypoxia-induced signaling components adapt cells to hypoxic conditions and thus, the marked increase of ANP secretion is a functional behavior of atrial tissue under hypoxic stress.

Ethics approval and consent to participate

The experiment protocols were approved by the Committee on the Ethics of Animal Experiments at Yanbian Medical University of Science and Technology.

Consent for publication

None.

Competing interests

All authors have declared that there is no conflict of interest.

Availability of data and materials

The material and data are made available.

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Authors' Contributions

Qiu-li Zhang and Xun Cui conceived of the study, designed the study and collected the data. All authors analyzed the data and were involved in writing and revised the manuscript.

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Figure legends

Figure 1 Effect of PTK inhibitor, genistein (3 μ M) on ANP secretion from hypoxia-conditioned atria (Aa and Ba), pulse pressure (Ab and Bb). Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia. n = 6. ***P < 0.001 compared with normoxia control period; ###P < 0.001 compared with hypoxia.

Figure 2 Effect of genistein (3 μ M) on expressions of (A) p-Akt and (B) HIF-1 α in perfused atria were relative to expression level of GAPDH. Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia. n = 6. ***P < 0.001 compared with normoxia control period; ###P <

0.001 compared with hypoxia.

Figure 3 Effect of Akt activation on the inhibiting action of genistein on ANP secretion from hypoxia-conditioned rat atria. (A) Change of ANP secretion. (B) Change of pulse pressure. (C) expression levels of HIF-1 α and p-AKT were examined using western bolt assay. (D) Western bolt showed an expression level of p-AKT. n = 6. ***P < 0.001 compared with normoxia control period; ###P < 0.001 compared with genistein +hypoxia.

Figure 4 Effect of PTKs and HIF-1 α inhibitors, genistein (3 μ M) + rotenone (0.5 μ M) on ANP secretion from hypoxia-conditioned atria (Aa and Ba) and pulse pressure (Ab and Bb). Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia; Gen+ro, genistein+rotenone; Gen+ro+hy, genistein+rotenone+hypoxia. n = 6. ***P < 0.001 compared with normoxia control period; ###P < 0.001 compared with genistein+hypoxia.

Figure 5 Effect of PTKs and HIF-1 α inhibitors, genistein (3 μ M) + CAY10585(10 μ M) on ANP release from hypoxia-conditioned atria (Aa and Ba) and atrial pulse pressure (Ab and Bb). Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia; Gen+CAY, genistein+CAY10585; Gen+CAY+hy, genistein+CAY10585+hypoxia. n = 6. *P < 0.05; ***P < 0.001 compared with normoxia control period; ###P < 0.001 compared with genistein+hypoxia.

Figure 6 Effect of antagonist for HIF-1 α on HIF-1 α expression in presence of genistein in beating atria. n = 6. *P < 0.05 compared with control period; ###P < 0.05 compared with hypoxia.

Supplement 1 Western bolt showed an expression level of p-AKT.

189x151mm (300 x 300 DPI)

225x149mm (96 x 96 DPI)

69x58mm (300 x 300 DPI)

76x63mm (300 x 300 DPI)

75x64mm (300 x 300 DPI)

30x30mm (300 x 300 DPI)