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

Qiu-li Zhang
First Affiliated Hospital of Inner Mongolia University for the nationalities
Chao-chao Bian
Yanbian University
Ping Li
Yanbian University
Lan Hong
Yanbian University
Li-ping Liu
Yanbian University
Bai-ri Cui
Yanbian University Hospital
Xun Cui (cuixun_yj@163.com)
Yanbian University

Research

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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.

Aim of the stud

y: 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-1α (HIF-1α), and $GATA_4$ 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 μM). By western blotting analysis, we found that hypoxia-induced the increase in phosphorylation of Akt and transcriptional factors, including HIF-1α, were also reversed by genistein. The perfused HIF-1α inhibitors rotenone (0.5 μM) or CAY10585 (10 μM) plus genistein significantly abolished the enhanced ANP section induced by hypoxia. Additionally, the perfused PI3K/Akt agonist IGF-1 (30 μM) also abolished ANP secretion induced by genistein as well as inhibited expression of HIF-1α.

Conclusions

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

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 [1, 2]. A previous study demonstrated that under hypoxic conditions, ANP plays an important role by protecting cardiomyocytes from hypertrophy, fibrosis, and heart failure [3]. 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 [4, 5]. 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 own 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 [6, 7], which can result in many disorders, including myocardial function under normal and hypoxic conditions [8–10]. However, the involved PTKs in the regulation of ANP release is not well known. Genistein, a non-selective tyrosine kinase inhibitor [11], has been used widely to define the role of PTKs in physiological and pathological changes of heart in level of cells or tissues or human [12–14]. 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-1α (HIF-1α) [15–17], a transcriptional factor that activated in response to hypoxic stimulation, and is involved in hypoxia-induced pulmonary hypertension [18]. Moreover, phosphoinositide-3 kinase (PI3K)/Akt signaling has been recognized as an important pathway downstream to PTKs [19]. Genistein treatment can induce inhibition of the PI3K/Akt-dependent pathway in the cardiovascular system [20]. Considering to the finding in our previous study that hypoxia-induced ANP secretion could be elicited via MAPK and PI3K pathways by controlling HIF-1α [21], we speculated that the involvement of PI3K/Akt-HIF-1α pathway in 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 and were housed in mesh cages in a room maintained at 25 °C, illuminated with 12:12-h light-dark cycles, and the rats were provided with standard rodent chow and water ad libitum. The experiment protocols were approved by the Committee on the Ethics of Animal Experiments at Yanbian Medical University of Science and Technology.

Preparation of isolated perfused rat atria

Isolated perfused beating atria were prepared as described in an earlier report with a minor modification [22]. In brief, rats were executed by decapitation and then the hearts were instantly excised. After washing

with 36.5 °C normal saline, the left atria were separated and placed on a cannulus and ligated by a suture. The fixed atria were kept 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, the atrium was perfused for 70 min to stabilize functional parameters. The atrial perfusate was collected six times at 2-min intervals as the control, and the perfusate was changed to N_2-exposed HEPES buffer for establishing the hypoxic model for 48 min. All samples were collected at 4 °C. For protein analysis, atrial tissues were immediately frozen with liquid nitrogen after perfusion and stored at -80 °C.

Acute hypoxia atrial model preparation and experimental protocols

Three different experimental conditions were tested and evaluated in this study. In **Group 1**, the atria were exposed to N_2-containing HEPES buffer for the control period (n = 6). In **Group 2**, atria were exposed to N_2-containing HEPES buffer and an inhibitor of PTKs (genistein, 3 μM; n = 6) after three cycles of the control period and a cycle in the presence of genistein. **Group 3** atria were exposed to N_2-containing HEPES buffer with genistein (3 μM) in the presence of a HIF-1α inhibitor (rotenone, 0.5 μM or CAY10585, 10 μM; both n = 6), or an agonist for Akt (IGF-1, 30 μM; n = 6) for three cycles of hypoxia after the control period and a cycle in the presence of inhibitors. The inhibitors were pretreated 30 min before sample collection and the N_2-containing HEPES buffer with different agents was given after the control period.

Radioimmunoassay for measuring ANP concentrations

Specific radioimmunoassays were used to measure the ANP levels of the perfusates as described in an earlier study [23]. The amount of secreted immunoreactive ANP was represented as ng/min/g (ng/perfusion time/weight of wet atrial tissue). We observed that most of the secreted ANP were processed ANP.

Western blot analysis

The atrial tissues were homogenized in RIPA Lysis Buffer (P0013B, Beyotime Institute of Biotechnology, China) and analyzed for expression of the signal by western blot. The homogenate was heated for 5 min at 95 °C and then centrifuged for 10 min at 4 °C. The protein concentrations in the supernatant 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 [(mmol/l); Tris-HCl 20 (pH 7.6); NaCl 137; and 0.1% Tween 20]. The target proteins were blotted with primary antibodies to anti-HIF-1α, GAPDH (ab84593, ab65979; and ab9485; diluted 1:1000 by Tris Buffer

Saline or TBS; Abcom, Hong Kong), and anti-*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). After extensive washing with TBST, the target proteins were visualized using enhanced chemiluminescence plus reagent (ECL kit, Ewbio, China) according to the manufacturer's instructions. The specific protein bands were quantified using Image J software.

Statistical analysis

The statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparison tests or an unpaired *t*-test. Differences were considered to be statistically significant at $P < 0.05$. All data were presented as mean ± SEM.

Results

PTK inhibitor genistein suppressed hypoxia-induced atrial ANP secretion

ANP secretion was significantly enhanced by acute hypoxia stimulation (6.24-fold; n = 6; $P < 0.001$ vs. control) in isolated perfused beating rat atria, as shown in Fig. 1Aa and Ba. ANP secretion peaked in the last period of the third experimental cycle (i.e., the second cycle of hypoxia, approximately 26 min after hypoxia) and then slightly dropped, maintaining high levels overall. The atrial dynamics were attenuated by acute hypoxia (n = 6; $P < 0.001$ vs. control), as shown in Fig. 1Ab and Bb. These results indicated that our acute hypoxic model of isolated perfused beating rat atria was successful.

To investigate the effect of PTKs on hypoxia-induced ANP secretion, another series of experiments were performed. PTK inhibitor genistein (3 μM) significantly attenuated the effect of hypoxia-induced ANP secretion (n = 6; $P < 0.001$ vs. Control, Fig. 1Aa and 1Ba vs. hypoxia alone, Fig. 1Ba) without changing hypoxia-suppressed atrial pulse pressure (n = 6; $P < 0.001$ vs. 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 regulate hypoxia-induced ANP secretion, the phosphorylation level of Akt and expression of HIF-α were determined using Western blot. The data are shown in Fig. 2A indicated that acute hypoxia significantly increased Akt phosphorylation (n = 6; $P < 0.001$ vs. control) in rat atrial tissue; whereas this increase was markedly attenuated or abolished after genistein treatment (n = 6; $P < 0.001$ vs. hypoxia alone). In addition, hypoxia-induced HIF-1α expression was also attenuated by genistein treatment (n = 6; $P < 0.01$ vs. hypoxia alone).

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

To determine the key role of PI3K/Akt signaling in the genistein-induced reduction of the ANP section under hypoxia, we examined the ANP section in rat atrial perfusate after PI3K/Akt agonist IGF-1and 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 hypoxia-suppressed atrial pulse pressure (n = 6; $P < 0.001$ vs. control; $P > 0.05$ vs. hypoxia alone, Fig. 3B). Western blot result showed that IGF-1 treatment reversed genistein-induced HIF-α and p-AKT expression under hypoxia (Fig. 3C). Our previous work has proved that the specific inhibitor of Akt (LY294002) inhibits the hypoxia-induced ANP secretion [21]. 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 (Fig. 3D).

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

To explore the involvement of HIF-1α on PTK regulation of hypoxia-induced ANP secretion, experiments were performed with HIF-1α inhibitors rotenone (0.5 μM) or CAY10585 (10 μM). In the presence of rotenone plus genistein, genistein-induced the decrease of ANP secretion was not changed (n = 6; $P < 0.05$ vs. control period or genistein + hypoxia; Fig. 4Aa and 4Ba). Meanwhile, CAY10585 plus genistein also had no effect hypoxia-induced ANP secretion (n = 6; $P < 0.001$) in comparison to genistein + hypoxia (Fig. 5Aa and 5Ba). Both inhibitors did not change hypoxia-suppressed atrial dynamics (n = 6; $P < 0.001$ vs. control period; Fig. 4Ab–Bb and 5Ab–Bb). By result of western blot, hypoxia significantly increased HIF-1α protein levels protein in atrial tissues (n = 6, $P < 0.05$ vs. control; Fig. 6). The enhancement was blocked by genistein, rotenone + genistein and CAY10585 + genistein (n = 6, 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 acute hypoxia-induced ANP secretion in the isolated perfused beating rat atria, with a potential downstream signaling pathway of PTKs-PI3K/Akt-HIF-1α 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 [24]. Also, studies suggested that they control the abnormal signaling transduction of cells under ischemia conditions [25–27]. As reported in previous studies, hypoxia and ischemia are a strong stimulus for ANP release from cells or tissue [28]. Herein, we focus on the functional involvement of PTK by observing the role of its antagonist genistein in hypoxia-induced ANP process in rat isolated perfused beating atria. Our data suggested that genistein effectively suppressed hypoxia-induced ANP section without influence on atrial pressure suggesting the negative role of genistein in hypoxia-induced ANP secretion.

Hypoxia-response elements have been characterized from the promoter sequence of NPs genes[29], suggesting that NPs genes are under controlled by HIF. That has been demonstrated to be involved in regulating the expression of various hypoxia-dependent genes. It has been demonstrated that PTKs are involved in the regulation of HIF-1α protein expression under hypoxia[20], and modulate HIF-1α activity through the PI3K/Akt signaling pathway [30]. Results of our study also show that the PTK inhibitor, genistein decreased phosphorylation level of Akt and reduced hypoxia-induced HIF-1α protein expression in perfused rat atria. These data suggested that genistein inhibited PTK downstream PI3K/Akt signals. This was confirmed by present data of Akt agonist combined with genistein co-perfusion experiment which showed that activated Akt alleviated the inhibitory role of genistein on ANP secretion, HIF-1α expression and p-AKT expression. We thus examined this pathway in hypoxia-induced ANP section.

As the tissues adapt to hypoxic conditions, HIF-1α plays a key role in the regulation of expression and transcription of multiple hypoxia-related genes including PDGF, IGF-1, EGF, VEGF, and ANP [31, 32]. In a previous study, we have demonstrated that acute hypoxia significantly increases atrial HIF-1α activity and regulates hypoxia-induced ANP secretion [21]. The present study showed that genistein alone greatly inhibited hypoxia-increased *p*-Akt and HIF-1α expressions, and significantly attenuated hypoxia-promoted atrial ANP secretion. However, genistein combined with IGF-1, an agonist for PI3K/Akt, treatment, not only markedly attenuated or completely blocked hypoxia-induced ANP secretion but also blocked the increase of hypoxia-induced HIF-1α. To determine the key role of HIF-1α in the inhibitory role of genistein on the ANP section, we performed rat atrial co-perfusion experiments with genistein plus rotenone or CAY10585 (two inhibitors for HIF-1α). 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-1α is an effective responder for genistein in reduced ANP secretion.

In conclusion, acute hypoxia significantly promotes ANP secretion in beating rat atria. Inhibition of PTKs by genistein regulates hypoxia-induced ANP secretion through PI3K/Akt-HIF-1α signaling pathways. HIF-1α 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.

Declarations

Compliance with Ethical Standards

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

Acknowledgments

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Figures

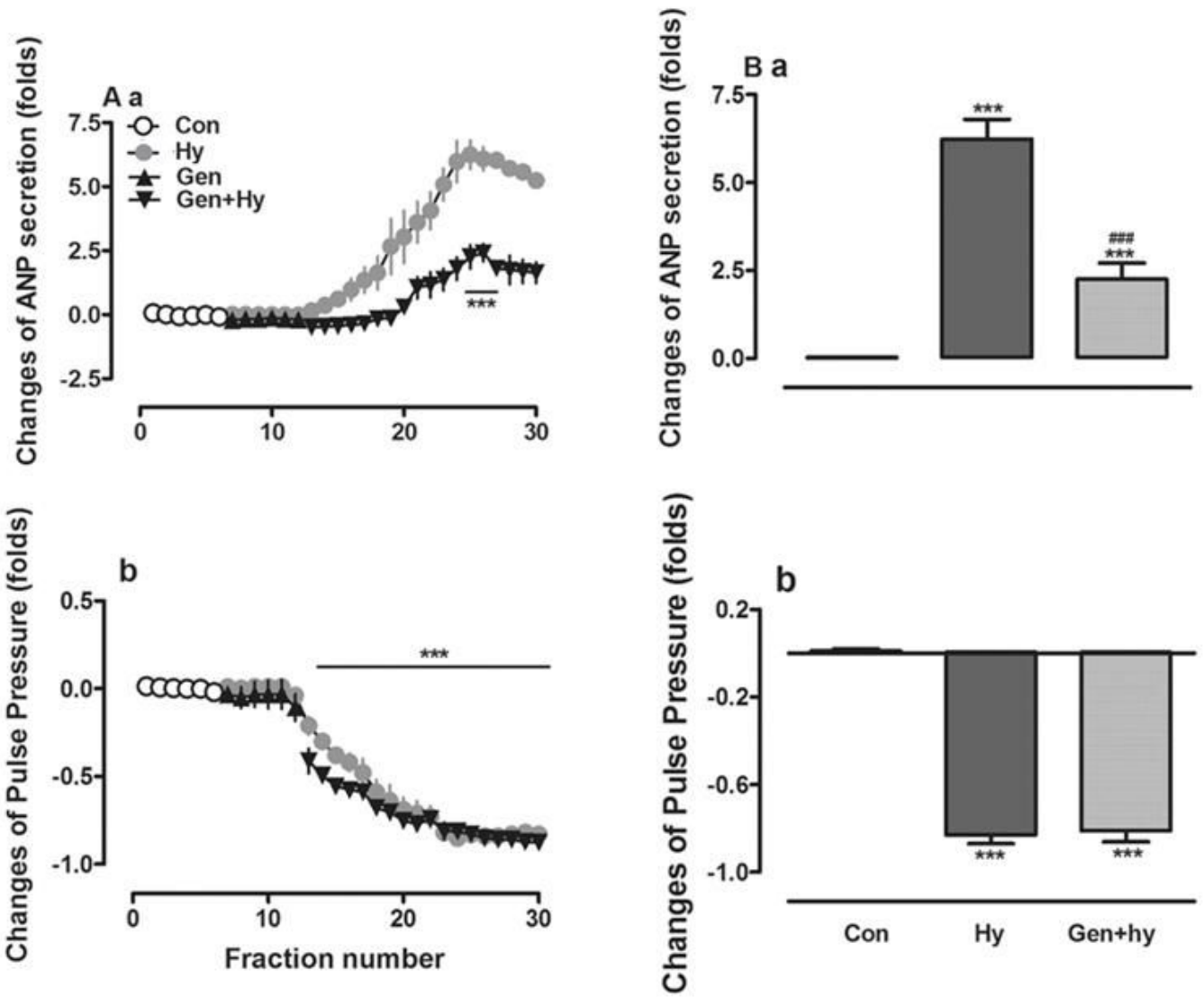

Figure 1

Effect of PTK inhibitor, genistein (3 μmol/L) on hypoxia-induced ANP secretion (Aa and Ba), pulse pressure (Ab and Bb). Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia. Data are mean±SE (n = 6). ***P < 0.001 vs. control period; ###P < 0.001 vs. hypoxia alone.

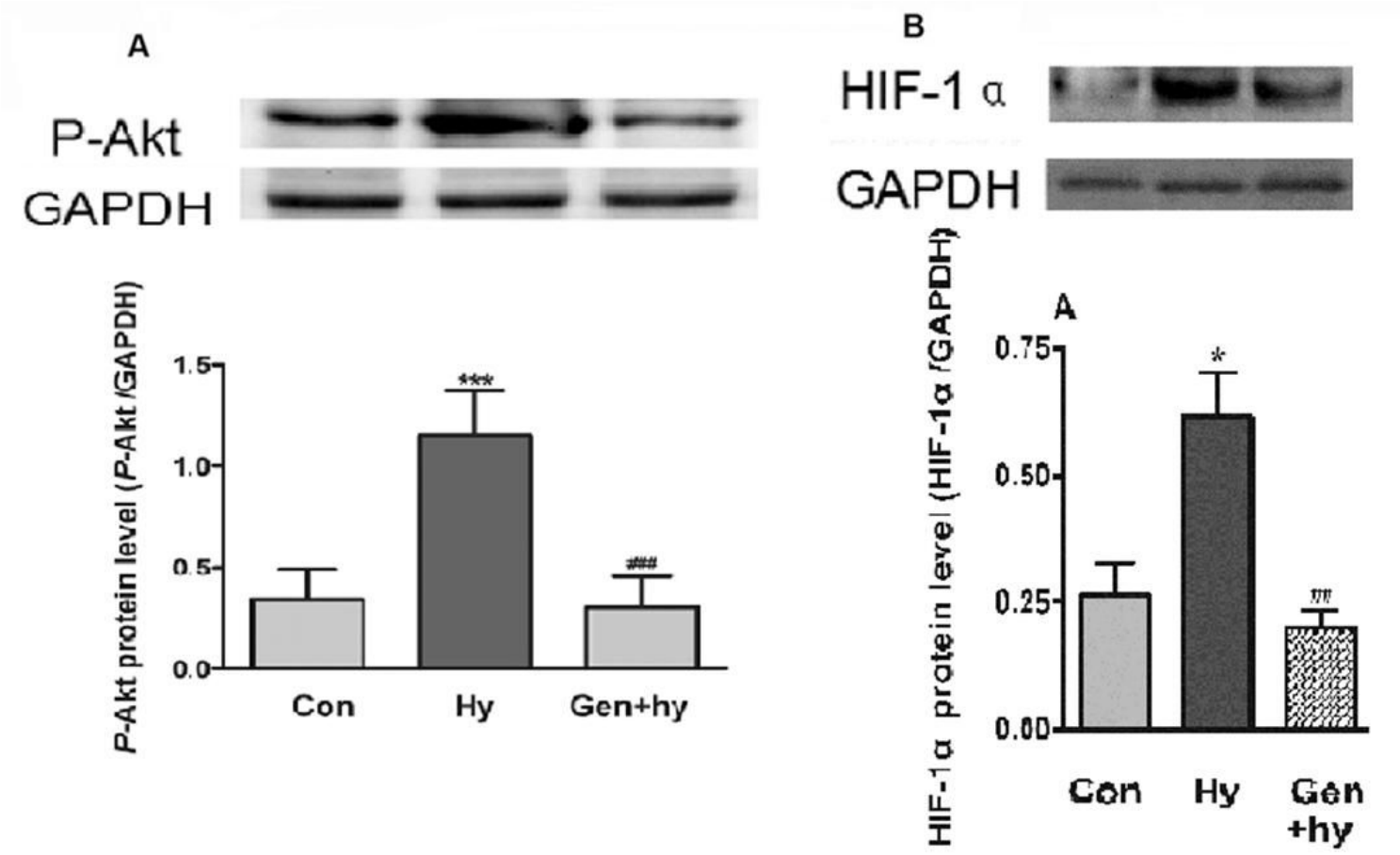

Figure 2

Effect of inhibitors of PTKs, genistein (3 μmol/L) on expressions of (A) p-Akt and (B) HIF-1α in perfused atria were relative to expression level of GAPDH. Con, control; Hy, hypoxia alone; Gen, genistein; Gen+hy, genistein+hypoxia. Data are mean±SE (n = 6). ***P < 0.001 vs. control period; ###P < 0.001 vs. hypoxia.

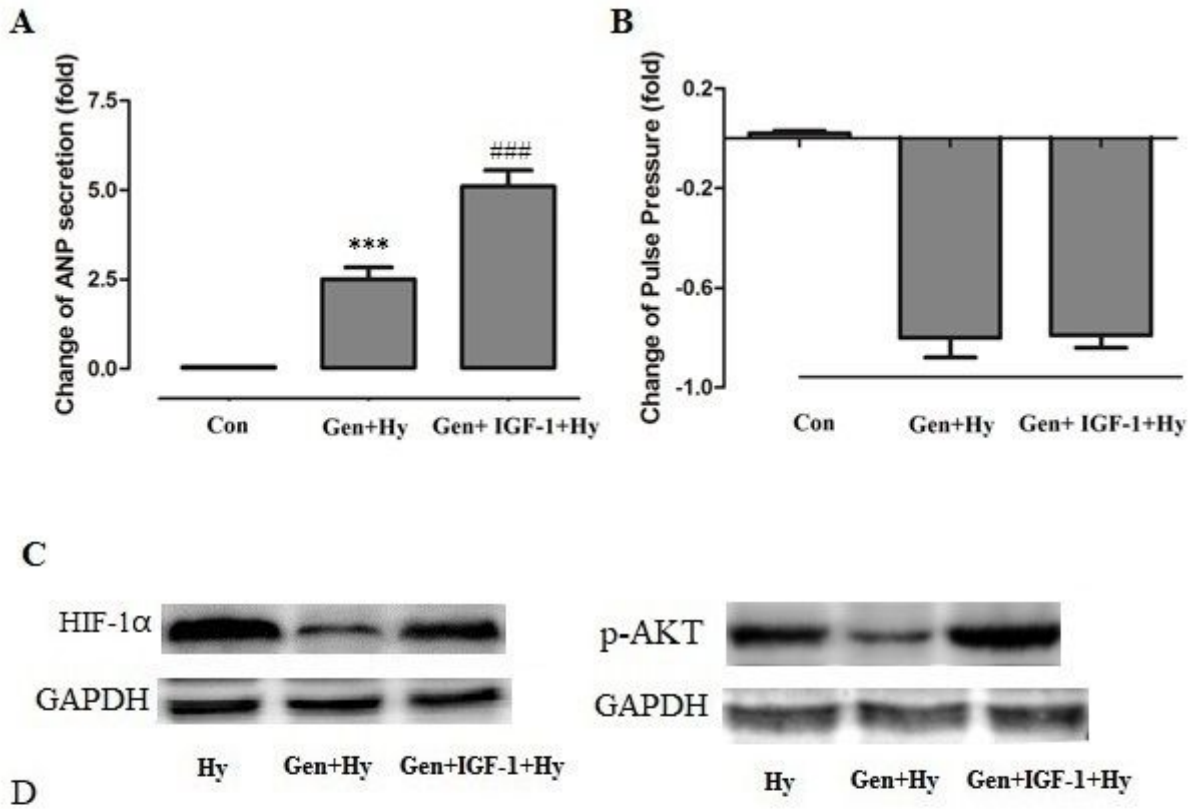

Figure 3

Effect of Akt activation on inhibitory action of genistein on hypoxia-induced ANP secretion in paced isolated rat atria. (A) Change of ANP secretion. (B) Change of pulse pressure. (C) Western bolt showed expression level of HIF-1α and p-AKT. (D) Western bolt showed expression level of p-AKT. Data are mean±SE (n = 6). ***P < 0.001 vs. control period; ###P < 0.001 vs. genistein +hypoxia.

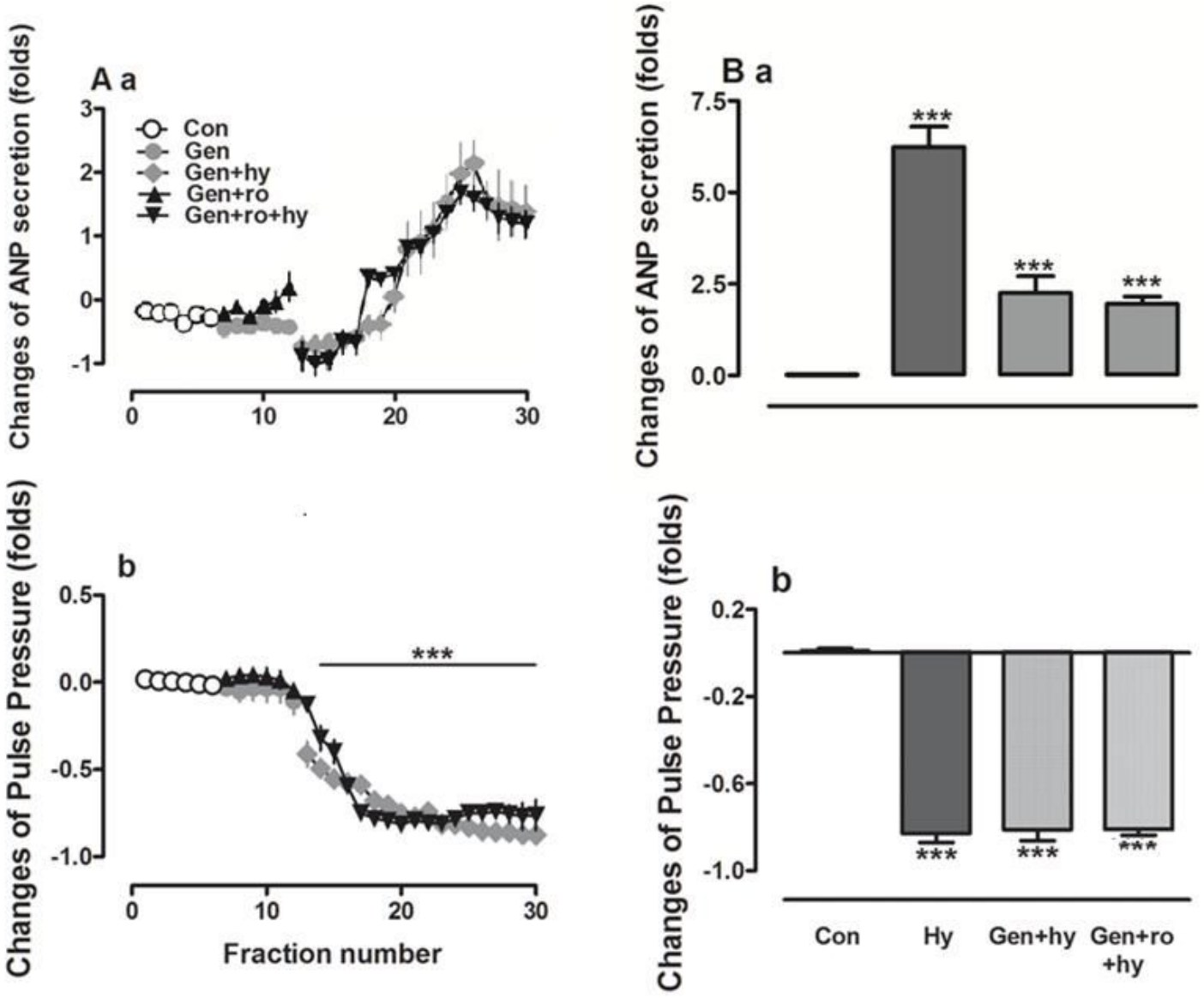

Figure 4

Effect of PTKs and HIF-1α inhibitors, genistein (3 μmol/L) + rotenone (0.5 μmol/L) on hypoxia-induced ANP secretion (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. Data are mean±SE (n = 6). ***P < 0.001 vs. control period; ###P < 0.001 vs. genistein+hypoxia.

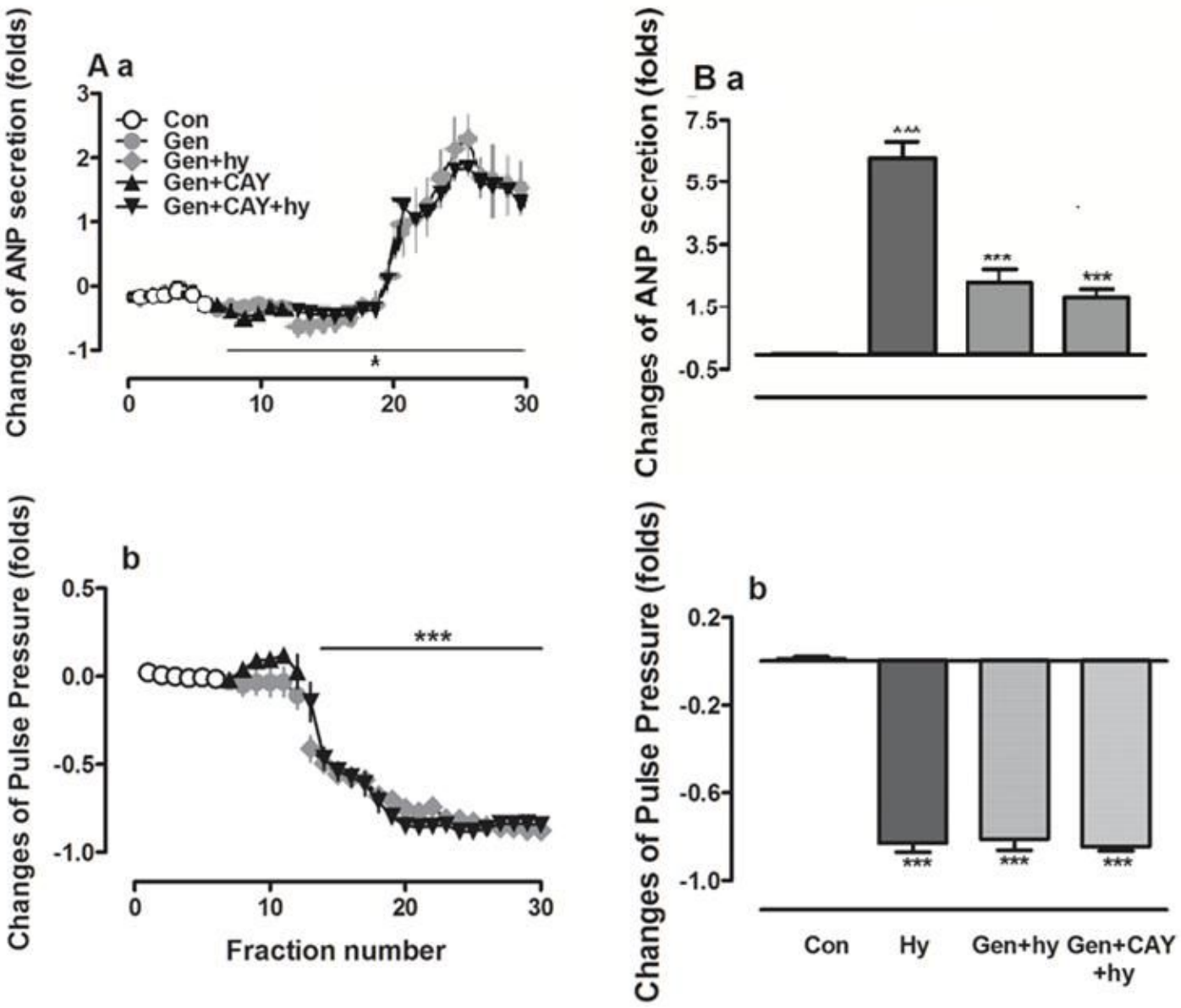

Figure 5

Effect of PTKs and HIF-1α inhibitors, genistein (3 μmol/L) + CAY10585(10 μmol/L) on hypoxia-induced ANP secretion (Aa and Ba) and 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. Data are mean±SE (n = 6). *P < 0.05; ***P < 0.001 vs. control period; ###P < 0.001 vs. genistein+hypoxia.

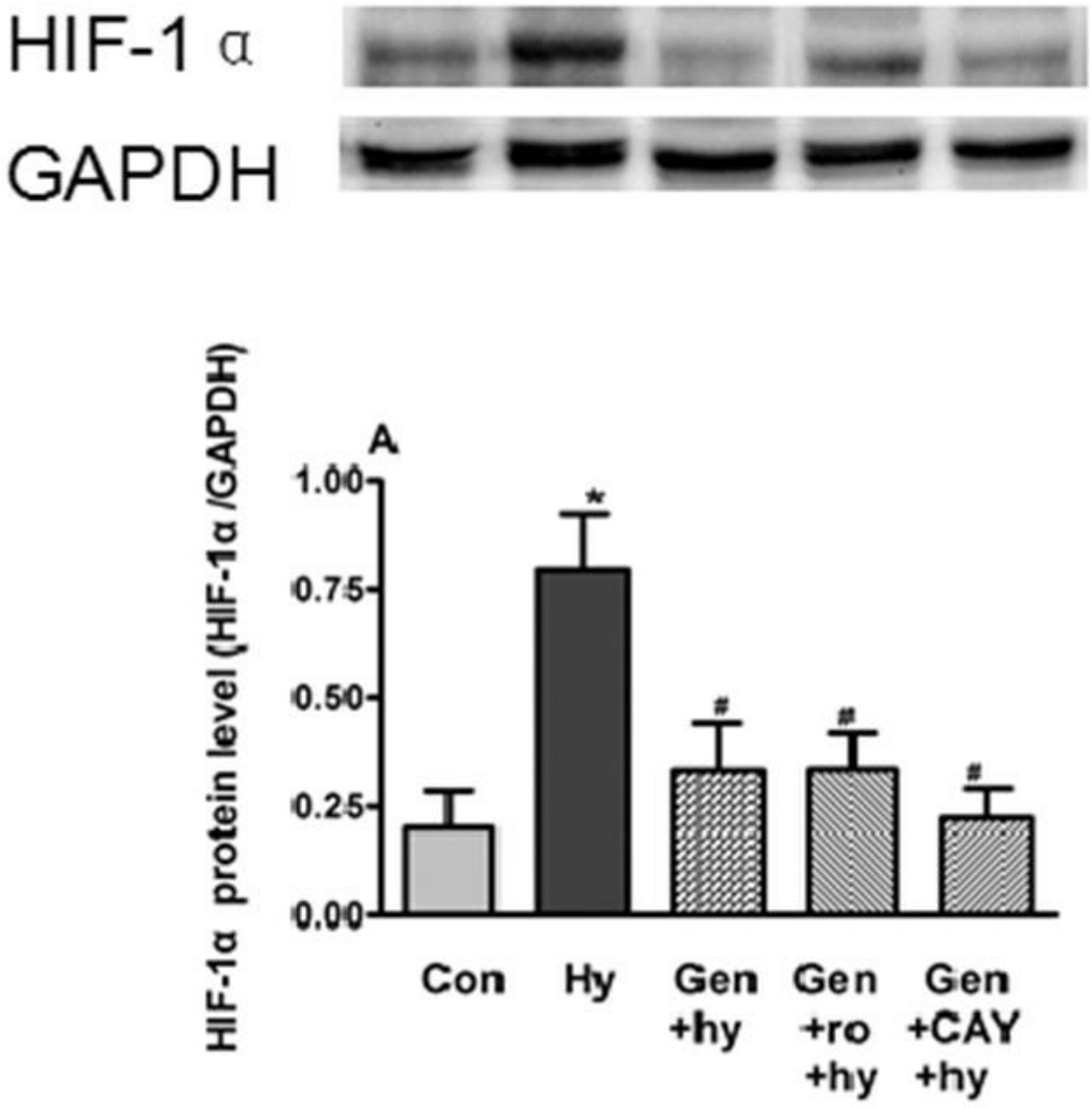

Figure 6

Effect of antagonist for HIF-1α on HIF-1α expression in presence of genistein in beating atria. Data are mean±SE (n = 6). *P < 0.05 vs. control period; ###P < 0.05 vs. hypoxia.

Supplementary Files

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