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ORIGINAL ARTICLE

Plasma Levels of Acylated Ghrelin in Children with Pulmonary Hypertension Associated with Congenital Heart Disease

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Abstract This study aims to estimate plasma levels of acylated ghrelin in children with pulmonary hypertension (PH) associated with congenital heart disease (CHD) and to correlate the levels of acylated ghrelin with endothelin-1 (ET-1), nitric oxide (NO), and clinical hemodynamic parameters. We investigated the plasma concentration of acylated ghrelin, ET-1, NO, and the hemodynamic parameters in 20 children with CHD, 20 children with PH-CHD, and 20 normal children. Plasma-acylated ghrelin and NO levels were significantly higher in CHD group than in control subjects ($P < 0.001$). Moreover, plasma-acylated ghrelin, ET-1, and NO levels were significantly elevated in PH-CHD group compared with the CHD group ($P < 0.05$). In PH-CHD children, plasma-acylated ghrelin levels correlated positively with pulmonary artery systolic pressure (PASP; $r = 0.740$, $P < 0.001$), pulmonary artery diastolic pressure (PADP; $r = 0.613$, $P = 0.004$), right ventricular systolic pressure (RVSP; $r = 0.642$, $P = 0.002$), mean pulmonary arterial hypertension (mPAP; $r = 0.685$, $P = 0.001$), right ventricle diameter (RVD; $r = 0.473$, $P = 0.035$), pulmonary artery trunk diameter (PAD; $r = 0.613$, $P = 0.004$), NO ($r = 0.463$, $P = 0.04$), and ET-1 ($r = 0.524$, $P = 0.018$). Plasma-acylated ghrelin levels were elevated both in CHD and in PH-CHD. Increased acylated ghrelin levels correlated positively with ET-1, NO, PASP, PADP, RVSP, mPAP, RVD, and PAD. Acylated ghrelin may be a new biomarker of PH-CHD.

Keywords Acylated ghrelin · Pulmonary hypertension · Congenital heart disease · Endothelin-1 · Nitric oxide

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Introduction

Pulmonary hypertension (PH) is a fatal disease characterized by a progressive increase in pulmonary vascular resistance with remodeling of the pulmonary vasculature, which previously carried a poor prognosis in children with congenital heart disease (CHD) [6, 12, 25]. Endothelial dysfunction contributes to sustained pulmonary vasoconstriction and endothelial and vascular smooth muscle cell proliferation, playing a very important role in the complex pathology of PH [4, 7]. Ghrelin, a 28-amino acid peptide released from the stomach, has recently been identified as a natural ligand of the growth hormone secretagogue receptor (GHSR); it increases food intake and effects physical development and growth [8, 36]. Ghrelin is broadly expressed in many tissues and organs [16], including cardiovascular tissues [11]. Ghrelin has two subtypes: an acylated form and a deacylated form [2, 3]. Recently, it was found that ghrelin could exert a protective effect on the cardiovascular [35, 38] and digestive systems [17], including decreasing mean arterial pressure, raising cardiac output in patients with congestive heart failure (CHF), improving left ventricle function in rats with CHF [28], and reducing hepatic injury [17]. Most importantly, exogenous ghrelin attenuated pulmonary hypertension,

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pulmonary vascular remodeling [31], and right ventricular hypertrophy and decreased both right ventricular systolic pressure (RVSP) and right ventricular diastolic pressure (RVDP) in experimental model of PH [13]. Recently, researchers found that total plasma ghrelin levels were elevated in adult patients with idiopathic pulmonary arterial hypertension, and increased plasma ghrelin levels correlated positively with pulmonary artery systolic pressure (PASP) and nitric oxide (NO) [40]. Although total plasma ghrelin levels were higher in children with CHD [1, 18, 42], none of the study subjects had PH. In addition, the acylated ghrelin which possesses biological activity has not been investigated in children with PH-CHD.

Based on these understandings, the objective of this study was to estimate plasma levels of acylated ghrelin, NO, and ET-1 in children with PH-CHD and to correlate the levels of acylated ghrelin with pulmonary artery pressure and right ventricular pressure, which have not been confirmed in the literature to date.

Materials and Methods

Study Population

This study was conducted on 20 children with CHD, 20 children with PH-CHD, and 20 healthy children. All patients' cardiac diagnoses were made on the basis of clinical and laboratory examinations (including right heart catheterization). The diagnosis of PH-CHD was established according to Stephen et al. [32] and Currie et al. [9]. This study was conducted in accordance with the Declaration of Helsinki. The local ethics committee approved the study protocol. Informed consents were obtained from the parents of the subjects.

Anthropometry

The following clinical characteristics were obtained from the patients' medical chart: age, sex, body weight (in kilograms), height (in meters), and medical history. Body mass index (BMI) was calculated as the ratio of body weight (kg) and squared height (m^2).

Echocardiography

All patients underwent a complete M-mode and 2D echocardiography examination according to the recommendations for quantification methods during the performance of a pediatric echocardiogram [24]. The right

ventricle diameter (RVD), pulmonary artery trunk diameter (PAD), and ejection fraction (EF) were measured.

Cardiac Catheterization

All patients underwent right heart catheterization under general anesthesia and systemic heparinization. Details of the procedure were described previously [10]. PASP, PADP, RVSP, RVDP, and mPAP were assessed during the process of catheterization, and the above data were retrievable from operation reports.

Blood Sampling and Hormone Assay

After overnight fasting, specimens were collected at 08–09 a.m. Blood samples for the measurement of acylated ghrelin, ET-1, and NO were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (500 U/ml), centrifuged at 4 °C and $1600 \times g$ for 15 min, and then plasma samples were stored at −80 °C until assayed. The commercially available human acylated ghrelin enzyme immunoassay kit (Cayman Chemical Co., Michigan, USA) was used to assay the acylated ghrelin levels. Plasma ET-1 levels were analyzed with ELISA kit (ET-1 kit was purchased from Shang Hai Westang Bio-Tech. CO. LTD, China). Plasma NO levels were assessed with a nitric acid reduction method according to the instructions of the NO detection kit (Jian Cheng, China).

Statistical Analyses

Statistical Package for Social Sciences (SPSS) program version 13.0 was used in the analysis of data. Quantitative data were used to describe mean ± standard error. Statistical differences between the two groups were analyzed using an independent Student's t test or Chi-square test. Correlations between variables were explored using Pearson's coefficient. To assess the relative strength of the association, a multiple linear regression analysis was performed to examine the possible factors that impact acylated ghrelin. $P < 0.05$ was considered statistically significant.

Results

Subjects

Table 1 depicts the baseline characteristics of the study population. There were no significant differences in age and sex for these three groups. Height, weight, and BMI

Table 1 Characterization of the subjects included in the study

Variable	Control ($n = 20$)	CHD group ($n = 20$)	PH-CHD group ($n = 20$)	P value (control vs. CHD)	P value (control vs. PH-CHD)	P value (CHD vs. PH-CHD)
Age (months)	35.1 ± 12.8	36.2 ± 17.9	35.9 ± 20.9	0.823	0.885	0.961
Male (n)	8 (20)	11 (20)	13 (20)	0.812	0.113	0.519
H (cm)	93.1 ± 8.3	86.5 ± 10.2	85.3 ± 11.1	0.029	0.016	0.734
Weight (kg)	14.7 ± 2.8	12.2 ± 2.9	11.8 ± 3.0	0.006	0.004	0.472
BMI (kg/m^2)	16.9 ± 0.9	16.1 ± 0.8	15.8 ± 1.2	0.009	0.001	0.323
Ventricular septal defect	–	10	9			
Patent ductus arteriosus	–	6	5			
Atrial septal defect	–	4	2			
Others	–	0	4			

Values are represented as mean $\pm$ SE, $P < 0.05$ was regarded as statistically significant

Table 2 Comparison of echocardiographic and cardiac catheterization parameters among the studied groups

Parameters	CHD ($n = 20$)	PH-CHD ($n = 20$)	P value
PASP (mmHg)	18.9 ± 3.6	47.6 ± 11.5	0.000
PADP (mmHg)	14.3 ± 2.4	27.4 ± 9.8	0.000
RVSP (mmHg)	26.5 ± 3.6	48.1 ± 11.7	0.000
RVDP (mmHg)	10.5 ± 3.7	12.9 ± 6.5	0.161
MPAP (mmHg)	16.4 ± 2.6	35 ± 10.0	0.000
RVD (mm)	8.1 ± 0.6	12.1 ± 1.1	0.000
PAD (mm)	14.0 ± 1.5	16.4 ± 1.8	0.000
EF (%)	64.8 ± 2.8	63.9 ± 2.2	0.261

Values are represented as mean $\pm$ SE, $P < 0.05$ was regarded as statistically significant

were significantly lower in both the CHD group and PH-CHD group compared with the control group, while the difference between CHD group and PH-CHD group was not significant. Table 2 shows that compared with the CHD group, the PH-CHD group had higher PASP, PADP, RVSP, mPAP, RVD, and PAD.

Differences in Acylated Ghrelin, ET-1, and NO Levels

As shown in Fig. 1, plasma-acylated ghrelin and NO levels were significantly higher both in CHD group and in PH-CHD group than in control subjects. Moreover, plasma-acylated ghrelin, ET-1, and NO levels were significantly elevated in PH-CHD group compared with the CHD group (86.8 ± 21.7 ng/ml vs. 27.4 ± 9.5, $P < 0.001$; 185.5 ± 50 pg/ml vs. 73.2 ± 18.5 pg/ml, $P < 0.001$; 67.1 ± 17.7 μmol/l vs. 53.5 ± 20 μmol/l, $P < 0.001$ respectively).
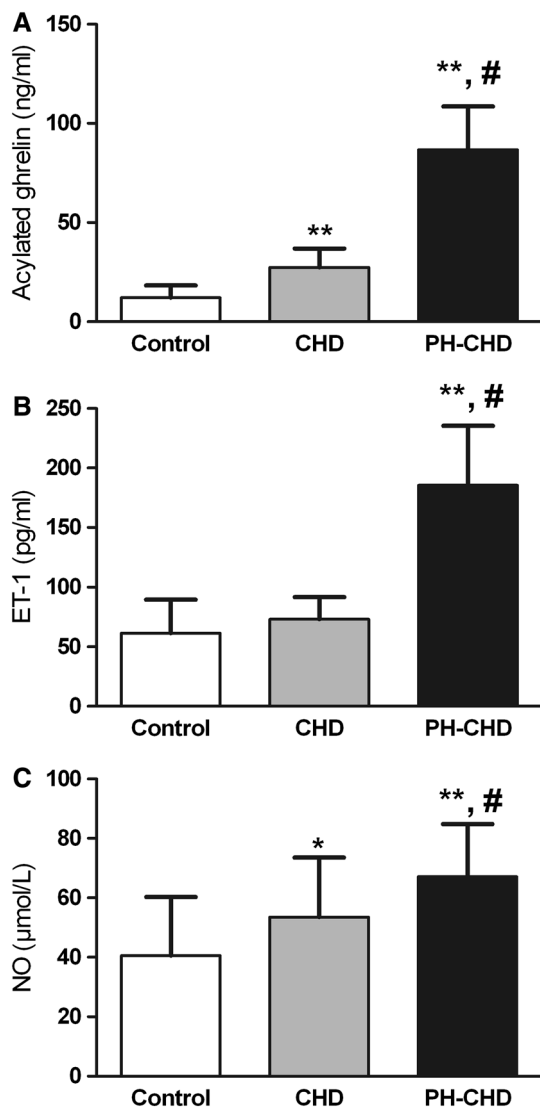
Correlations of Acylated Ghrelin, ET-1, and NO with Various Parameters

Table 3 indicates the results of Pearson's correlation of the variables in the PH-CHD patients. PASP, PADP, RVSP, mPAP, RVD, and PAD were positively correlated with plasma-acylated ghrelin levels. Plasma ET-1 levels also correlated positively with PASP and RVDP. Furthermore, PASP and RVD were found to be positively correlated with plasma NO levels. However, there were no significant correlations between plasma-acylated ghrelin levels and age, height, weight, RVDP, or EF. Additionally, both plasma ET-1 and NO levels were also positively correlated with acylated ghrelin levels, while there were no significant correlations between ET-1 and NO plasma levels.

Based on the sample correlation coefficients, the relationship between acylated ghrelin, ET-1, NO, and variables were further assessed in a multiple regression analysis. In a multiple regression model including age, weight, height, BMI, PASP, PADP, RVSP, RVDP, mPAP, RVD, PAD, EF, ET-1, and NO, none of the variables had significant correlations with acylated ghrelin.

Discussion

Although the available information confirms that the two ghrelin isoforms: acylated ghrelin and deacylated ghrelin have different metabolic effects [14], only acylated ghrelin owns physiologic activity and can promote growth hormone release and food intake [19]. So, we focused our investigation on acylated ghrelin. In this study, we demonstrated for the first time that plasma-acylated ghrelin levels were elevated in all the children with CHD. PASP, PADP, RVSP, mPAP, RVD, and PAD were positively

Fig. 1 Plasma-acylated ghrelin levels (a), plasma ET-1 levels (b), plasma NO levels (c) in the control, CHD group, and PH-CHD group. *P < 0.05 vs. control; **P < 0.01 vs. control; #P < 0.01 vs. CHD groups

Table 3 Correlations of acylated ghrelin, ET-1, and NO with various parameters in PH-CHD patients

Variable	Acylated ghrelin		ET-1		NO	
	r	P	r	P	r	P
Age	−0.148	0.533	−0.223	0.344	−0.032	0.894
H	−0.052	0.826	−0.129	0.588	0.094	0.693
Weight	−0.227	0.336	−0.170	0.475	−0.052	0.829
BMI	−0.466	0.039	−0.067	0.780	−0.437	0.054
PASP	0.740	<0.001	0.451	0.046	0.451	0.046
PADP	0.613	0.004	0.414	0.070	0.166	0.484
RVSP	0.642	0.002	0.332	0.152	0.214	0.365
RVDP	0.323	0.165	0.465	0.039	0.021	0.929
MPAP	0.685	0.001	0.351	0.129	0.385	0.094
RVD	0.473	0.035	0.093	0.697	0.564	0.010
PAD	0.613	0.004	0.373	0.106	0.209	0.377
EF	−0.188	0.428	−0.052	0.828	−0.28	0.232
ET-1	0.524	0.018	–	–	–	–
NO	0.463	0.040	0.023	0.925	–	–

group. Lund et al. [26] showed that total plasma ghrelin levels were elevated both in cachectic [5] and in non-cachectic heart failure and did not correlate with BMI. Researchers also found that increased total plasma ghrelin in adult patients with idiopathic pulmonary arterial hypertension had no correlation with BMI [40]. Obviously, malnutrition and growth retardation cannot explain this phenomenon. The mechanism for elevated acylated ghrelin levels is not well understood [3], but total plasma ghrelin levels were decreased in patients with advance cancer and weight loss compared with the health controls, and the reduction in ghrelin levels might be attribute to the severity and progression of the disease [21].

Ghrelin is implicated as a cardiovascular hormone that can directly regulate cardiopulmonary function, including regulation of blood pressure [20, 27]. Yang et al. [40] reported that elevated total plasma ghrelin levels were positively associated with PASP and RVD in adult patients with idiopathic pulmonary arterial hypertension. Interestingly, a study by Rodriguez et al. [30] showed that increased acylated ghrelin was positively correlated with blood pressure in adult patients with metabolic syndrome. We also found a similar phenomenon that children with PH-CHD had higher plasma-acylated ghrelin levels compared with the CHD, and PASP, PADP, RVSP, mPAP, RVD, and PAD were positively correlated with acylated ghrelin. It is known that patients with CHD have left-to-right shunting which causes the increased pulmonary blood flow and right ventricular dilation. PAD and RVD are important indicators for PH. Echocardiography shows that PH-CHD group had higher PAD and RVD. It is absolutely

correlated with plasma-acylated ghrelin levels in the children with PH-CHD. Additionally, both ET-1 and NO plasma levels were also positively correlated with acylated ghrelin levels.

It has been recently demonstrated that plasma ghrelin concentration was inversely correlated with BMI. Consistent with this conclusion, acylated ghrelin levels were increased and were negatively associated with BMI in all the children with CHD. Several studies agreed that increased plasma ghrelin levels were a compensatory mechanism in response to anabolic–catabolic imbalance. However, our results reveal that since there was no significant difference in BMI between CHD group and PH-CHD group, acylated ghrelin levels were significantly elevated in PH-CHD

possible that there is no difference in EF between CHD group and PH-CHD group because of the left-to-right shunting. So, acylated ghrelin may predict the severity of PH-CHD in children. However, the available data demonstrate completely opposite results in PH with atrial septal defect in adult patients [23]. The differences were completely reasonable due to the different cohorts, different assays, and different ages.

It is not completely clear, however, how ghrelin regulates PH-CHD. ET-1 contributes to endothelial dysfunction both directly, through its vasoconstrictor effects, and indirectly, through inhibition of NO production [34]. Interestingly, ghrelin, a potent physiological antagonist of ET-1 [37], was demonstrated to reverse endothelial dysfunction in adult patients with metabolic syndrome by increasing NO bioactivity [33]. In our study, both plasma ET-1 and NO levels were elevated in PH-CHD. Our study also showed that plasma ET-1 and NO levels were in accordance with increased plasma-acylated ghrelin levels. From previous findings, it is known that ghrelin participates in ET-1-mediated biological processes [4, 7]. We suppose that, in children with PH-CHD, acylated ghrelin improved endothelial dysfunction and regulated PH via decreasing the vasoconstrictor effect of ET-1 [31] and increasing NO bioactivity [22]. This conclusion is supported by recent evidence that ghrelin has the ability to activate endothelial eNOS in endothelial cells dependent on the phosphatidylinositol 3-kinase (PI3 K) pathway [39, 41]. Ghrelin also has been demonstrated to reduce chronic liver injury and fibrogenesis by increased eNOS expression [17]. Rocha-Sousa et al. speculated that ghrelin's hypotensive effect in ocular hypertension animal models may be associated with NO [29]. Additionally, ghrelin stimulates the production of NO by PI3 K, Akt, and eNOS pathways [15]. Collectively, these findings imply that elevated plasma-acylated ghrelin levels had some effect on the pulmonary vasculature. Our findings, along with those of previous studies, indicate that ghrelin may be a new therapeutic approach for PH-CHD.

Of course, more research is needed to further verify this assumption. Firstly, we need to assay plasma-deacylated ghrelin levels and total plasma ghrelin levels. Secondly, it is necessary to obtain data using increased/varied numbers, races, regions, and ages of patients. Thirdly, a study with a longer follow-up period should be executed to evaluate the relationship between acylated ghrelin and clinical hemodynamic parameters. Fourthly, more work is required to validate the supposition that acylated ghrelin is a novel prognostic biomarker, and further understanding of the pathways controlling ghrelin secretion will be useful to develop new therapeutic approaches for PH-CHD.

In conclusion, plasma-acylated ghrelin, ET-1, and NO levels are elevated in children with PH-CHD. PASP, PADP, RVSP, MPAP, RVD, PAD, plasma NO, and ET-1 levels were positively correlated with acylated ghrelin. Acylated ghrelin may predict the severity of PH-CHD and be a new therapeutic approach for PH-CHD.

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