PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia biologica (Kraków), vol. 59 (2011), No 3-4

doi: 10.3409/fb59_3-4.151-155

Carvedilol Induces Endogenous Hydrogen Sulfide Tissue Concentration Changes in Various Mouse Organs*

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Accepted May 19, 2011

WILIŃSKI B., WILIŃSKI J., SOMOGYI E., PIOTROWSKA J., GÓRALSKA M., MACURA B. 2011. Carvedilol induces endogenous hydrogen sulfide tissue concentration changes in various mouse organs. Folia biologica (Kraków) **59**: 151-155.

Carvedilol, a third generation non-selective adrenoreceptor blocker, is widely used in cardiology. Its action has been proven to reach beyond adrenergic antagonism and involves multiple biological mechanisms. The interaction between carvedilol and endogenous 'gasotransmitter' hydrogen sulfide (H_2S) is unknown. The aim of the study is to assess the influence of carvedilol on the H_2S tissue level in mouse brain, liver, heart and kidney. Twenty eight SJL strain female mice were administered intraperitoneal injections of 2.5 mg/kg b.w./d (group D1, n = 7), 5 mg/kg b.w./d (group D2, n = 7) or 10 mg/kg b.w./d of carvedilol (group D3, n = 7). The control group (n = 7) received physiological saline in portions of the same volume (0.2 ml). Measurements of the free tissue H_2S concentrations were performed according to the modified method of Siegel. A progressive decline in H_2S tissue concentration along with an increase in carvedilol dose was observed in the brain (12.5%, 13.7% and 19.6%, respectively). Only the highest carvedilol dose induced a change in H_2S tissue level in the heart – an increase by 75.5%. In the liver medium and high doses of carvedilol increased the H_2S level by 48.1% and 11.8%, respectively. In the kidney, group D2 showed a significant decrease of H_2S tissue level (22.5%), while in the D3 group the H_2S concentration increased by 12.9%. Our study has proven that carvedilol affects H_2S tissue concentration in different mouse organs.

Key words: Hydrogen sulfide, carvedilol, adrenergic beta-antagonists, nitric oxide, mice.

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Carvedilol, a third generation non-selective adrenoreceptor blocker, is widely used in cardiology and general practice in the treatment of chronic diseases like congestive heart failure and arterial hypertension (CHAKRABORTY *et al.* 2010). The action of carvedilol has been proven to reach beyond adrenergic antagonism and comprises i.a. antioxidant activity, calcium channel blockade and nitric oxide (NO) production enhancement (KOSTKA-JEZIERNY & TYKARSKI 2009). On the other hand, endogenously formed 'gasotransmitter' hydrogen sulfide (H_2S) has been identified as a crucial regulator of circulatory, nervous, gastrointestinal and excretory systems. Altered production of H_2S was observed in arterial hypertension, myocardial ischemia and atherosclerosis (ŁOWICKA & BEŁTOWSKI 2007). The interaction between carvedilol and endogenous H_2S is unknown.

The aim of the study is to assess the influence of carvedilol on the endogenous tissue H_2S concentrations in mouse brain, heart, liver and kidney.

Material and Methods

Animals

Twenty eight SJL strain female mice (11-12 week old individuals) of approximately 20 g weight were involved in the study. The animals were housed under standard laboratory conditions and had free

*Supported by the grant No. K/ZBW/000175 from the Jagiellonian University Medical College.

access to water and food. They were kept at a temperature of 22-24°C with a light/dark cycle of 12 h.

Study protocol

A non-selective lipophilic β-blocker / α-1 blocker carvedilol (Avedol, Polpharma, Poland) was dissolved in physiological saline. The study design comprised intraperitoneal injections of 2.5 mg per kg of body weight of carvedilol daily (group D1, n = 7), 5 mg per kg of body weight of carvedilol daily (group D2, n = 7) or 10 mg per kg of body weight of carvedilol daily (group D3, n = 7) for 5 consecutive days at the same time of day (10:30 am), each administration consisted of 0.2 ml of the solution. The control population (n = 7) received intraperitoneally physiological saline in portions of the same volume. The individuals were randomly assigned to each group. The animals tolerated the applied doses of carvedilol well and remained in good condition till the end of the experiment. Measurements of the free tissue H_2S concentration were performed by the use of the modified method of Siegel (SIEGEL 1965; SOMOGYI *et al.* 2008). The study has been performed in accordance with the guidelines for the care and use of laboratory animals accepted by Bioethical Committee of the Jagiellonian University Medical College (Kraków, Poland).

Tissue sample preparation

Two hours after the last drug or physiological saline injection the animals were killed by cervical dislocation, their brains, hearts, livers and kidneys were quickly removed, homogenized with 0.01 mol/l sodium hydroxide (NaOH): brain tissue in proportion of 1 to 4, liver and kidney of 1 to 5 and heart of 1 to 10 and frozen. Then 50% trichloroacetic acid (TCA) was added (0.5 ml to 2 g of brain or liver samples in tight capsules of 3 ml and 0.25 ml to 1 g of heart or kidney sample in tight capsules of 2 ml), the suspension was shaken and centrifuged. Subsequently, 1.5 ml brain or liver and 0.75 ml heart or kidney supernatant samples were moved to 2 ml tight capsules with 0.15 ml or 0.075 ml of 0.02 mol/l N,N-dimethyl-p-phenyl-diamine sulfate in 7.2 mol/l hydrochloric acid (HCl), then 0.15 ml or 0.075 ml of 0.03 mol/l iron (III) chloride ($FeCl_3$) in 1.2 mol/l HCl portions were added, respectively. After 20 minutes in darkness the content was shaken for 1 minute with 1 ml of chloroform.

H_2S tissue concentration measurements

Absorbance was measured at 650 nm with the Varian Cary 100 spectrophotometer. A standard curve was prepared with an iodometrically determined 0.0001 mol/l sodium sulfide (Na_2S) solution. For each group of animals four concurrent analyses of each analyzed tissue type were performed.

Statistical analysis

Statistical analysis was performed within the R Environment by the Student's *t*-test and univariate analysis of variance (ANOVA). Statistical significance was considered when $P<0.05$.

Results and Discussion

Progressive H_2S tissue concentration decline was observed in the brain along with a rising carvedilol dose (by 12.5%, 13.7% and 19.6%, respectively). In the heart only the highest carvedilol dose induced H_2S tissue level change, but the increase was spectacular, reaching 75.5%. In the liver, medium and high doses altered the H_2S concentration – 5 mg/kg b.w./d of carvedilol increased the H_2S level by 48.1% and 10 mg/kg b.w/d by 11.8%. In the kidney, the group D2 showed a significant decrease of H_2S tissue level (22.5%), while in the D3 group H_2S concentration increased by 12.9%. In the variance analysis for each tissue type only H_2S concentration changes within the heart and brain were statistically significant (Table 1). Noteworthy, each organ has different metabolism, paracrine and endocrine regulation, and specific transmitter interactions, thus variable changes of H_2S concentrations only confirm this complexity and heterogeneity.

Carvedilol's therapeutic actions could not be fully explained by adrenoreceptor blockade. Numerous studies have provided evidence that carvedilol has various other properties including antioxidant action, calcium channel antagonism, anti-inflammatory actions: fall in interleukin-1 (IL-1), interleukin-6 (IL-6), c-reactive protein (CRP) and tumor necrosis factor-α (TNF-α), direct inhibition of transcription factors like NF-κB, low density lipoproteins (LDL) oxidation; stabilization of atherosclerotic plaques by decreasing intercellular adhesion molecule-1 (ICAM-1) and activity of matrix metalloproteinases 2 and 9 (MMP-2, MMP-9); prevention of endothelial and myocardium apoptosis, reversal of cardiac remodeling in chronic heart failure and endothelin-1 (ET-1) suppression (BELLENGER *et al.* 2004; KALINOWSKI *et al.* 2003; KOSTKA-JEZIERNY & TYKARSKI 2009; ROMEO *et al.* 2000; RUFFOLO *et al.* 1993). Moreover, the major proportion of carvedilol's biological action, especially regarding hypotensive and vascular effects, seems to be mediated by NO, whose level rises due to endothelial nitric oxide synthase (NOS) stimulation (AFONSO *et al.* 2006).

Table 1

Hydrogen sulfide (H_2S) tissue concentration in mouse brain, heart, liver and kidney following the administration of 2.5 mg/kg b.w. per day, 5 mg/kg b.w. per day or 10 mg/kg b.w. per day of carvedilol (groups D1, D2 and D3 respectively)

H_2S tissue concentration (μg/g)	Control group (n = 7)	D1 (n = 7)	D2 (n = 7)	D3 (n = 7)	ANOVA P
Brain	2.55 ± 0.04	2.23 ± 0.05**	2.20 ± 0.05**	2.05 ± 0.05***	<0.001
Heart	6.78 ± 0.06	5.91 ± 0.15	6.61 ± 0.14	11.90 ± 0.12***	<0.001
Liver	4.14 ± 0.04	4.17 ± 0.08	6.13 ± 0.14***	4.63 ± 0.10**	0.22
Kidney	8.66 ± 0.19	8.45 ± 0.17	6.71 ± 0.08***	9.78 ± 0.18***	0.21

*P<0.05 for given group vs control group, **P<0.01 for given group vs control group, ***P<0.001 for given group vs control group

H_2S is endogenously formed from L-cysteine in several enzymatic reactions catalyzed by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST), and in non-enzymatic pathways in many tissues. Cytoplasmatic bound sulfur is postulated to absorb and store exogenously applied and endogenously produced H_2S which is released from the bound sulfur pool in the presence of physiologic concentrations of glutathione and cysteine in slightly alkaline conditions (ISHIGAMI *et al*. 2009). H_2S acts as a 'gasotransmitter' and serves as a co-modulator of various physiological and pathophysiological processes such as regulation of vascular tone, myocardial contractility, neurotransmission and perception (FIORUCCI *et al.* 2006; SHIBUYA *et al.* 2009). Its biological action comprises numerous intracellular mechanisms including adenosine triphosphate (ATP)-sensitive potassium channels (K_{ATP}) stimulation, sulfhydration of different proteins and maintaining protein–SH groups in the reduced state, reaction with reactive oxygen and nitrogen species (ROS and RNS) (ŁOWICKA & BEŁTOWSKI 2007; SUN *et al.* 2008). H_2S interacts with carbon monoxide (CO) and nitric oxide (NO) in a number of ways including affecting each other's synthesis and biological responses within target tissues. All these three gases bind to haemoglobin and impede mitochondrial oxidative phosphorylation by inhibiting cytochrome c oxidase (LI *et al.* 2009). Analogically to carvedilol, H_2S decreases IL-6, TNF-α levels, reduces the activation of NF-κB complex and the activity of MMP-2 and MMP-9 (OH *et al.* 2006; SEN *et al.* 2009; SODHA *et al.* 2009).

Our study has shown that carvedilol's action involves H_2S, probably via its production rate alteration and release with possible NO share as one of the mechanisms. Some of the biological effects of the beta-blocker and H_2S are common. It is unknown whether H_2S mediates any of them and to what extent carvedilol's biology is dependent on the messenger, since research dedicated to the issue has not been done. In the heart, the cardioprotective effects of H_2S have been demonstrated to involve opening of K_{ATP} channels as well as effects of preserving mitochondrial structure and function (ELROD *et al.* 2007). It might be an accessory effect to NO beneficial impact on intracellular transcription factors resulting in increased expression of cardioprotective proteins like superoxide dismutase (SOD), inducible NOS (iNOS), cyclooxygenase-2 (COX-2) and heat shock proteins (DAWN & BOLLI 2002). In hepatology, carvedilol appears to be a potentially viable option for treating portal hypertension along with angiotensin-converting enzyme inhibitors (ACEI) (HEMSTREET 2004). H_2S regulates perfusion pressure in normal and cirrhotic liver in a NO-independent manner; NO and H_2S are released by different cellular sources and their hemodynamic effects involve different cellular targets. H_2S generation in cirrhosis is decreased due to a reduced expression/activity of CSE in hepatic stellate cells (HSC) – one of the main sources of H_2S in the liver (FIORUCCI *et al.* 2005). In our previous studies a tissue specific ACEI ramipril also enhanced the H_2S tissue level in the liver, heart and kidney (WILIŃSKI *et al.* 2010; WILIŃSKI *et al.* 2008). In kidneys H_2S has been recognized as an important regulator of renal function affecting both vascular and tubular actions (XIA *et al.* 2009). Some studies with carvedilol demonstrate attenu-

ated increases in albuminuria as well as reduction in cardiovascular events in chronic kidney disease patients with hypertension (BAKRIS *et al.* 2006). Carvedilol also protected against the renal mitochondrial toxicity induced by cisplatin and daunorubicin-induced cardiotoxicity and nephrotoxicity in rats (AROZAL *et al.* 2010; RODRIGUES *et al.* 2010).

H_2S acts as a neuromodulator as well as an intracellular messenger in the central nervous system. Its perturbed metabolism has been investigated in many neurological disorders including Alzheimer's disease (QU *et al.* 2008). Recently, carvedilol was found to improve neuronal transmission and attenuate brain oligomeric beta-amyloid content and cognitive deterioration in two mouse models of Alzheimer's disease (WANG *et al.* 2010). Analogically, non-steroidal anti-inflammatory drugs were also demonstrated to exert some anti-amyloidogenic effects and to affect H_2S biology (BILSKA *et al.* 2010; SREBRO *et al.* 2006).

In conclusion, carvedilol affects the H_2S tissue concentrations in mouse brain, heart, liver and kidney. The involvement of H_2S makes the biological action of carvedilol more complex and opens new fields for investigation for both of them in neurology, cardiology, hepatology and nephrology. Several biotechnology companies are already developing H_2S-based therapeutic compounds, and there are ongoing clinical trials investigating the therapeutic potential of H_2S (PREDMORE & LEFER 2010).

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