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Short communication

Digoxin increases hydrogen sulfide concentrations in brain, heart and kidney tissues in mice

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Abstract:
The interest in digoxin has recently increased due to the expanding knowledge regarding endogenous cardiac glycosides and a potential oncological application of this drug. Hydrogen sulfide (H_2S), a crucial co-modulator of various physiological processes, is involved in the pathophysiology of different disorders and may be useful in the treatment of some diseases. The interaction between cardiac glycosides and H_2S is unknown. The aim of the study is to assess the influence of digoxin on H_2S tissue concentrations in mouse brain, heart and kidney. Thirty male BALB/c mice were given intraperitoneal injections of digoxin at 0.5 mg/kg body weight (b.w.) per day (group D1, n = 10) or 1 mg/kg b.w. per day (group D2, n = 10). The control group (n = 10) received physiological saline. Free H_2S tissue concentrations were measured *via* the Siegel spectrophotometric modified method. There was a significant, progressive increase in the H_2S concentrations for both the low and high digoxin doses in the brain (7.7% and 8.5%, respectively), heart (by 6.0% and 22.1%, respectively) and kidney (by 7.6% and 13.0%, respectively). This report shows that digoxin administration is followed by an increase in the free H_2S concentrations in mouse brain, heart and kidney tissues.

Key words:
hydrogen sulfide, cardiac glycosides, digoxin, heart, mice

Abbreviations: Akt – protein kinase B, CTS – cardiotonic steroids, ERK – extracellular signal-regulated protein kinase, H_2S – hydrogen sulfide, K_{ATP} – ATP-sensitive potassium channels, NO – nitric oxide, PI3K – phosphoinositide 3-kinase, PKC – protein kinase C

Introduction

Recent studies have shifted the perspective on hydrogen sulfide (H_2S) from a dangerous industrial and environmental toxin to a crucial co-regulator of various physiological processes in mammals [15]. Moreover, H_2S has been shown to be involved in the development of different clinical disorders in many branches of medicine [18]. The importance of H_2S is so pervasive that several pharmaceutical companies are already working on H_2S-based agents to treat cardiovascular diseases and other disorders [24].

Plant extracts containing cardiac glycosides were used by the ancient Egyptians, Romans and Syrians as emetics and heart tonics, and medieval warriors added

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it to their arrows to poison targets. In the twentieth century, cardiac glycosides were established as an important agent in the treatment of heart failure [21]. In the era of evidence-based medicine, cardiac glycosides were pushed aside following the release of the Digitalis Investigation Group results [1]. However, these compounds were later resurrected with clinical *post-hoc* reanalysis (low digoxin concentrations significantly reduced mortality and hospitalizations in chronic heart failure patients) [2, 25]. They are again extensively researched with new vistas in oncology, endogenous synthesis (endogenous cardiotonic steroids – CTS) discovery and complex physiological actions explored [17, 39].

The interaction between cardiac glycosides and endogenous H_2S is unknown. The aim of this study is to assess the influence of digoxin on endogenous H_2S concentrations in mouse brain, heart and kidney tissues.

Materials and Methods

Animals

Thirty BALB/c strain male mice (8–9 week old individuals) weighing approximately 20 g were involved in the study. The animals were housed under standard laboratory conditions and had free access to water and food. They were kept at 22–24°C with a light/dark cycle of 12 h (8 am – 8 pm, and 8 pm – 8 am, respectively).

Study design

An injectable solution of purified cardiac glycoside digoxin (Digoxin WZF, Polfa Warszawa, Poland) was used. Intraperitoneal injections of 0.5 mg per kg b.w. of digoxin (group D1, n = 10) or 1 mg per kg b.w. of digoxin (group D2, n =10) were given daily for 5 consecutive days at the same time of the day (10:00 am) in 0.2 ml of saline solution. The control group (n = 10) received physiological saline at the same rate and volume. The individuals were randomly assigned to each group. The animals tolerated the applied doses of digoxin well and remained in good condition throughout the duration of the experiment. Measurements of the free H_2S concentrations were performed using the modified method of Siegel [28, 30].

The study was 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 samples preparation

Two hours after the last injection, the animals were killed by cervical dislocation, and their brains, hearts, and kidneys were quickly removed, and homogenized with 0.01 mol/l sodium hydroxide (NaOH) at a ratio of 1:4 for brain, 1:5 for kidney and 1:10 for heart and frozen. Then, 50% trichloroacetic acid (TCA) was added (0.5 ml to 2 g of brain 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), and the suspension was shaken and centrifuged. Subsequently, 1.5 ml brain 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-phenyldiamine sulfate in 7.2 mol/l hydrochloric acid (HCl). Then, 0.15 ml or 0.075 ml portions of 0.03 mol/l iron(III) chloride ($FeCl_3$) in 1.2 mol/l HCl were added, respectively. After 20 min in darkness, the content was shaken for 1 min 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 plotted with an iodometrically determined 0.0001 mol/l sodium sulfide (Na_2S) solution. For all groups of the animals, four concurrent analyses of each tissue type were performed.

Statistical analysis

Statistical analysis was performed within the R Environment using Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

Results and Discussion

There was a significant progressive increase in the H_2S concentration along with the increasing digoxin doses as compared to the control group in the brain (D1 by 7.7%, D2 by 8.5%), heart (D1 by 6.0%, D2 by

Tab. 1. Hydrogen sulfide (H_2S) concentrations in mouse brain, heart and kidney tissues following the administration of 0.5 mg/kg b.w. per day or 1 mg/kg b.w. per day digoxin (groups D1 and D2, respectively). The results are presented as the mean values ± SD

H_2S tissue concentration (μg/g)	Control group (n = 10)	D1 (n = 10)	p (control *vs.* D1)	D2 (n = 10)	p (control *vs.* D2)
Brain	2.60 ± 0.05	2.80 ± 0.03	$p < 0.001$	2.82 ± 0.04	$p < 0.001$
Heart	11.44 ± 0.13	12.12 ± 0.22	$p < 0.001$	13.97 ± 0.02	$p < 0.001$
Kidney	6.67 ± 0.06	7.18 ± 0.14	$p < 0.001$	7.54 ± 0.08	$p < 0.001$

22.1%) and kidney (D1 by 7.6%, D2 by 13.0%). The free H_2S tissue levels are presented in Table 1.

H_2S is formed from L-cysteine in several enzymatic reactions catalyzed by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST), in addition to non-enzymatic pathways in many tissues [27]. Cytoplasmatic bound sulfur is postulated to absorb and store exogenously applied and endogenously produced H_2S, which is then released in the presence of physiologic concentrations of glutathione and cysteine in slightly alkaline conditions. The second form of sulfur storage is an acid-labile sulfur which resides in iron-sulfur clusters of non-heme iron sulfur proteins: iron-sulfur complex of enzymes involved in oxidative phosphorylation localized primarily in mitochondria [14]. The method applied in our experiment determines the free H_2S tissue concentrations.

H_2S is lipophilic, freely permeates plasma membranes and participates in the sulfhydration of numerous proteins thus altering their function. Sulfhydration in an important physiological signal and a prominent post-translational modification [9]. The cardioprotective action of H_2S is comprised of numerous intracellular mechanisms, including adenosine triphosphate (ATP)-sensitive potassium channels (K_{ATP}) stimulation, inhibition of L-type calcium channels, an influence on extracellular signal-regulated protein kinases (ERKs), phosphoinositide 3'-kinase (PI3K)/Akt (protein kinase B), and protein kinase C (PKC) [13, 18, 32]. H_2S also exerts some anti-inflammatory effects under certain conditions by reducing the NF-κB complex activation [22, 29]. Moreover, H_2S interacts with the carbon monoxide (CO) and nitric oxide (NO) systems in a complex manner that includes affecting each other's synthesis and biological responses within target tissues and organs. All three of these gases bind to hemoglobin and temper mitochondrial oxidative phosphorylation by inhibiting cytochrome c oxidase [16].

CTS form a mammalian and human class of heterogeneous steroid hormones synthesized in adrenal glands with the secretion controlled by the hypothalamus, midbrain and sympathetic nervous system. This group includes cardenolides, like ouabain or endogenous digoxin, and bufadienolides, like marinobufagenin, telocinobufagin and 19-norbufalin [11, 26]. CTS target Na^+/K^+-ATPase as a receptor, and play an important role in the regulation of renal sodium transport and arterial pressure, cell growth and differentiation, apoptosis, fibrosis, the modulation of immunity and of carbohydrate metabolism and the control of various central nervous functions and are believed to participate in the complex pathophysiology of cardiovascular diseases [3]. The action of digoxin and other cardiac glycosides in heart failure is reportedly based on the Na^+-lag hypothesis. This hypothesis suggests that the inhibition of Na^+/K^+-ATPase leads to local rise in intracellular Na^+ concentration with a subsequent increase in the intracellular Ca^{2+} level resulting in positive inotropic effects on the myocardium [4]. This concept seems to contradict the modern strategy of heart failure therapy that is based on avoiding intracellular Ca^{2+} concentration augmentation which aggravates heart failure through altered protein expression and apoptosis [26]. In addition, other known mechanisms of cardiac glycosides, including the inhibition of the activated neuroendocrine system, primarily the adrenergic and renin-angiotensin-aldosterone systems, cannot explain the effects of the hormones in heart failure [10]. Numerous experiments have shown that the Na^+ pump is not necessary for the inotropic effects of cardiac glycosides [20]. Na^+/K^+-ATPase acts as a signalosome located in caveolar structures that contain different proteins, including membrane Ca^{2+}-ATPase, L-type Ca^{2+} channels, Na^+/Ca^{2+} exchanger and NO synthase, and interact with the sarcoplasmic reticulum (SR) peripheral proteins [7]. The interaction of cardiac glycosides with Na^+/K^+-ATPase

leads to conformational changes that are recognized by neighboring proteins, leading to the stimulation of different pathways of signal transduction, like the Ras-Raf-MEK-ERK cascade, PI3K/Akt, ERKs, NF-κB complex, PKC activity and Ca^{2+} as a second messenger [26, 41]. The activation of ERKs and the increase in intracellular Ca^{2+} concentration result in K_{ATP} opening [33].

As we have demonstrated, digoxin administration leads to increases in H_2S tissue concentrations, especially in the heart. H_2S has been shown to attenuate left ventricular dysfunction, prevent malfunctional remodeling and reduce mortality in mouse models of chronic heart failure, which was associated with decreased oxidative and proteolytic stress, a reduced level of apoptosis, fibrosis and mitochondrial dysfunction [6, 19]. Exogenous H_2S administration (with Na_2S as a donor) significantly reduced the infarct size in different murine and rat models of myocardial ischemia-reperfusion [6, 8]. H_2S increases blood flow in models of permanent ischemia, exerts pro-angiogenic action, increases endothelial cell growth and migration, enhances wound healing and induces neovascularization and collateral vessel growth in peripheral artery disease, which might play an important role in chronic heart failure, especially for an ischemic background [5, 23, 34]. The gasotransmitter was also protective in ischemia-reperfusion injury in the kidney [12].

Our study provides evidence that digoxin interferes with endogenous H_2S, resulting in increased tissue bioavailability. This makes the drug effects even more complex, given the multidirectional actions of H_2S. It is unknown whether H_2S mediates any of digoxin's effects or to what extent cardiac glycoside biology is dependent on the messenger, because research dedicated to this issue has never been done. The interaction mechanisms are obscure, and it is unknown whether, and in what manner, digoxin affects H_2S production and/or release. Interestingly, crucial aspects of physiology, such as blood pressure control, salt metabolism, cardiac function, kidney proliferation and central nervous functions, are regulated by H_2S and CTS and are affected by digoxin, and the involvement of other molecular features, like Ca^{2+}, NO, ERKs, PI3K/Akt, PKC, are K_{ATP} is common [3, 18, 40]. Furthermore, H_2S has been shown to be involved in the actions of other drugs, including aspirin, the angiotensin-converting enzyme inhibitor ramipril, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitor atorvastatin and paracetamol [31, 35–38]. Our results, in combination with the beneficial effects of H_2S in experimental animal models of heart failure and favorable clinical data regarding digoxin effects in chronic heart failure, strongly encourage further research into the role of H_2S donors and H_2S releasing agents in this disease [24].

In conclusion, exogenous digoxin has an impact on endogenous sulfur metabolism in different mouse organs, which is reflected by increases in free H_2S concentrations in mouse kidney, brain and heart tissues.

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Conflict of interests:
None declared.

References:

1. The effect of digoxin on mortality and morbidity in patients with heart failure. The Digitalis Investigation Group. N Engl J Med, 1997, 336, 525–533.
2. Ahmed A, Aban IB, Weaver MT, Aronow WS, Fleg JL: Serum digoxin concentration and outcomes in women with heart failure: A bi-directional effect and a possible effect modification by ejection fraction. Eur J Heart Fail, 2006, 8, 409–419.
3. Bagrov AY, Shapiro JI, Fedorova OV: Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. Pharmacol Rev, 2009, 61, 9–38.
4. Blaustein MP, Zhang J, Chen L, Hamilton BP: How does salt retention raise blood pressure? Am J Physiol Regul Integr Comp Physiol, 2006, 290, R514–523.
5. Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC: The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. Cardiovasc Res, 2007, 76, 29–40.
6. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A, Lefer DJ: Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation, 2010, 122, 11–19.
7. Daniel EE, El-Yazbi A, Cho WJ: Caveolae and calcium handling, a review and a hypothesis. J Cell Mol Med, 2006, 10, 529–544.
8. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X et al.: Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci USA, 2007, 104, 15560–15565.
9. Gadalla MM, Snyder SH: Hydrogen sulfide as a gasotransmitter. J Neurochem, 2010, 113, 14–26.
10. Gheorghiade M, Ferguson D: Digoxin. A neurohormonal modulator in heart failure? Circulation, 1991, 84, 2181–2186.

11. Goto A, Ishiguro T, Yamada K, Ishii M, Yoshioka M, Eguchi C, Shimora M, Sugimoto T: Isolation of a urinary digitalis-like factor indistinguishable from digoxin. Biochem Biophys Res Commun, 1990, 173, 1093–1101.
12. Hosgood SA, Nicholson ML: Hydrogen sulphide ameliorates ischaemia-reperfusion injury in an experimental model of non-heart-beating donor kidney transplantation. Br J Surg, 2010, 97, 202–209.
13. Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK, Bian JS: Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways. Pflugers Arch, 2008, 455, 607–616.
14. Ishigami M, Hiraki K, Umemura K, Ogasawara Y, Ishii K, Kimura H: A source of hydrogen sulfide and a mechanism of its release in the brain. Antioxid Redox Signal, 2009, 11, 205–214.
15. Kimura H: Hydrogen sulfide: its production, release and functions. Amino Acids, 2010, 136, 335–339.
16. Li L, Hsu A, Moore PK: Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation – a tale of three gases. Pharmacol Ther, 2009, 123, 386–400.
17. Lopez-Lazaro M: Digoxin, HIF-1, and cancer. Proc Natl Acad Sci USA, 2009, 106, E26.
18. Łowicka E, Bełtowski J: Hydrogen sulfide (H_2S) – the third gas of interest for pharmacologists. Pharmacol Rep, 2007, 59, 4–24.
19. Mishra PK, Tyagi N, Sen U, Givvimani S, Tyagi SC: H_2S ameliorates oxidative and proteolytic stresses and protects the heart against adverse remodeling in chronic heart failure. Am J Physiol Heart Circ Physiol, 2010, 298, H451–456.
20. Noble D: Mechanism of action of therapeutic levels of cardiac glycosides. Cardiovasc Res, 1980, 14, 495–514.
21. Norn S, Kruse PR: Cardiac glycosides: From ancient history through Withering's foxglove to endogeneous cardiac glycosides (Danish). Dan Medicinhist Arbog, 2004, 119–132.
22. Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, Jeon SB et al.: Hydrogen sulfide inhibits nitric oxide production and nuclear factor-κB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med, 2006, 41, 106–119.
23. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG et al.: Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci USA, 2009, 106, 21972-21977.
24. Predmore BL, Lefer DJ: Development of hydrogen sulfide-based therapeutics for cardiovascular disease. J Cardiovasc Transl Res, 2010, 3, 487–498.
25. Rahimtoola SH: Digoxin and reduction in mortality in systolic and diastolic heart failure at low serum digoxin concentrations. Eur Heart J, 2006, 27, 1630.
26. Schoner W, Scheiner-Bobis G: Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. Am J Physiol Cell Physiol, 2007, 293, C509–536.
27. Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K, Kimura H: 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. Antioxid Redox Signal, 2009, 11, 703–714.
28. Siegel LM: A direct microdetermination for sulfide. Anal Biochem, 1965, 11, 126–132.
29. Sivarajah A, Collino M, Yasin M, Benetti E, Gallicchio M, Mazzon E, Cuzzocrea S et al.: Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. Shock, 2009, 31, 267–274.
30. Somogyi E, Piotrowska J, Rzeszutko W: An effect of some parameters of the determination of the hydrogen sulfide in pig liver and brain on the obtained results (Polish). Farm Prz Nauk, 2008, 5, 33–36.
31. Srebro Z, Somogyi E, Wiliński B, Góralska M, Wiliński J, Sura P: Aspirin augments the concentration of endogenous hydrogen sulfide in mouse brain and liver. Folia Med Cracov, 2006, 47, 87–91.
32. Sun YG, Cao YX, Wang WW, Ma SF, Yao T, Zhu YC: Hydrogen sulphide is an inhibitor of L-type calcium channels and mechanical contraction in rat cardiomyocytes. Cardiovasc Res, 2008, 79, 632–641.
33. Tian J, Liu J, Garlid KD, Shapiro JI, Xie Z: Involvement of mitogen-activated protein kinases and reactive oxygen species in the inotropic action of ouabain on cardiac myocytes. A potential role for mitochondrial K(ATP) channels. Mol Cell Biochem, 2003, 242, 181–187.
34. Wang MJ, Cai WJ, Li N, Ding YJ, Chen Y, Zhu YC: The hydrogen sulfide donor NaHS promotes angiogenesis in a rat model of hind limb ischemia. Antioxid Redox Signal, 2010, 12, 1065–1077.
35. Wiliński B, Wiliński J, Somogyi E, Góralska M, Piotrowska J: Paracetamol (acetaminophen) decreases hydrogen sulfide tissue concentration in brain and increases in the heart, liver and kidney in mice. Folia Biol (Kraków), 2011, 59, 41–44.
36. Wiliński B, Wiliński J, Somogyi E, Góralska M, Piotrowska J: Ramipril affects hydrogen sulfide generation in mouse liver and kidney. Folia Biol (Kraków), 2010, 58, 177–180.
37. Wiliński B, Wiliński J, Somogyi E, Piotrowska J, Góralska M: Atorvastatin affects the hydrogen sulfide tissue concentration in mouse kidneys and other organs. Pharmacol Rep, 2011, 63, 184–188.
38. Wiliński J, Somogyi E, Góralska M, Wiliński B, Czarnecka D: Ramipril enhances the endogenous hydrogen sulfide tissue concentration in mouse heart and brain. Folia Med Cracov, 2008, 49, 123–130.
39. Winnicka K, Bielawski K, Bielawska A: Synthesis and cytotoxic activity of G3 PAMAM-NH(2) dendrimer-modified digoxin and proscillaridin A conjugates in breast cancer cells. Pharmacol Rep, 2010, 62, 414–423.
40. Xia M, Chen L, Muh RW, Li PL, Li N: Production and actions of hydrogen sulfide, a novel gaseous bioactive substance, in the kidneys. J Pharmacol Exp Ther, 2009, 329, 1056–1062.
41. Xie Z, Askari A: Na^+/K^+-ATPase as a signal transducer. Eur J Biochem, 2002, 269, 2434–2439.

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