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Vitamin D3 (Cholecalciferol) Boosts Hydrogen Sulfide Tissue Concentrations in Heart and other Mouse Organs

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Vitamin D3 is a crucial co-regulator of bone growth and remodeling, neuromuscular function, inflammation, proliferation, differentiation and apoptosis of cells. Intensive research on endogenous sulfur metabolism has revealed that hydrogen sulfide (H_2S) is an important modulator of various physiological processes in mammals. Noteworthy, these compounds are perceived as potential agents in the treatment of numerous disorders, including cardiovascular diseases and different types of cancer. The interaction between vitamin D3 and H_2S is unknown. The aim of the study is to assess the influence of cholecalciferol (vitamin D3, calcitriol) on H_2S tissue concentrations in mouse brain, heart and kidney. Twenty four SJL mice were given intraperitoneal injections of cholecalciferol at 10000 IU/kg body weight (b.w.) per day (group A, n = 8) or 40000 IU/kg b.w. per day (group B, n = 8). The control group (n = 8) 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 concentration along with the rising cholecalciferol doses as compared to the control group in the heart (by 29.6% and by 74.1%, respectively). Higher vitamin D3 dose caused H_2S accumulation in the brain (by 10.9%) and in the kidney (by 10.1%). Our study has proven that cholecalciferol affects H_2S tissue concentration in different mouse organs.

Key words: Hydrogen sulfide, cholecalciferol, calcitriol, vitamin D, heart, mice.

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Vitamin D is a group of fat-soluble secosteroids known for years to be implicated in the regulation of calcium and phosphate homeostasis. Interestingly, studies in recent decades have shown that the action of vitamin D3, the most important pleiotropic vitamin D form in humans and most vertebrates, involves other crucial processes such as modulation of neuromuscular function, inflammation, proliferation, differentiation and apoptosis of cells (LISS & FRISHMAN 2012). Analogically, intensive research on endogenous sulfur metabolism has revealed that hydrogen sulfide (H_2S), a dangerous industrial and environmental toxin, is an important physiological co-regulator in mammals (KIMURA 2011). What vitamin D3 and H_2S have in common is that they are both perceived as potential agents in the treatment of disorders of many branches of medicine, including cardiovascular diseases and different types of cancer (LEE *et al.* 2011; PREDMORE & LEFER 2010; WELSH 2012).

The interaction between vitamin D3 (cholecalciferol, calcitriol) and H_2S is unknown. The aim of this study is to assess the influence of cholecalciferol on endogenous H_2S concentrations in mouse brain, heart and kidney tissues.

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Material and Method

Animals

Twenty four SJL male mice (7-8 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.

Study design

An injectable solution of vitamin D3 (Devikap, Medana Pharma, Poland) was used. Intraperitoneal injections of 10000 IU per kg b.w. of cholecalciferol (group A, n = 8) or 40000 IU per kg b.w. of cholecalciferol (group B, n = 8) were given daily for 5 consecutive days at the same time of day (10:00 am) in 0.2 ml of saline solution. The control group (n = 8) received physiological saline at the same rate and volume. Individuals were randomly assigned to each group. The animals tolerated the applied doses of cholecalciferol well and remained in good condition throughout the duration of the experiment. Measurements of free H_2S concentrations were performed using the modified method of Siegel (SIEGEL 1965; SOMOGYI *et al.* 2008).

The study was performed in accordance to the guidelines for the care and use of laboratory animals accepted by the 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. The brain, heart and kidney tissues of each animal were quickly removed, homogenized with 0.01 M sodium hydroxide (NaOH) and frozen. Each tissue was combined with NaOH in different proportions (brain: 1 to 4, kidney: 1 to 5 and heart: 1 to 10). Then 50% trichloroacetic acid (TCA) was added to the samples. The TCA solution (0.5 ml) was added to 2 g of brain samples in tight 3 ml capsules, and 0.25 ml was added to 1 g of heart or kidney sample in tight 2 ml capsules. These suspensions were shaken, and the resultant mixture was 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 M N,N-dimethyl-p-phenyldiamine sulfate in 7.2 M hydrochloric acid (HCl), and 0.15 ml or 0.075 ml of 0.03 M iron (III) chloride ($FeCl_3$), respectively, was then added in 1.2 M HCl portions. After 20 min in the dark, the contents were shaken for 1 min with 1 ml of chloroform.

H_2S tissue concentration measurements

Absorbance was measured at 650 nm with a Varian Cary 100 spectrophotometer. A standard curve was prepared with an iodometrically determined 0.0001 M sodium sulfide (Na_2S) solution. Four concurrent analyses of every analyzed tissue type were performed for each group of animals.

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

There was a significant progressive increase in the H_2S concentration along with the rising cholecalciferol doses as compared to the control group in the heart (by 29.6% and by 74.1%, respectively). Higher vitamin D3 dose caused tissue H_2S accumulation in the brain (by 10.9%) and in the kidney (by 10.1%). The free H_2S tissue levels are presented in Table 1.

Table 1

Hydrogen sulfide (H_2S) tissue concentrations in mouse brain, heart and kidney following the administration of 10000 IU/kg b.w. per day or 40000 IU/kg b.w. per day of cholecalciferol (groups A and B, respectively)

Tissue type	H_2S tissue concentration [μg/g]			ANOVA
	Control group (n = 8)	Group A (n = 8)	Group B (n = 8)	
Brain	2.21 ± 0.02	2.25 ± 0.04	2.49 ± 0.07†	F(2, 9) = 40.5; P<0.001
Heart	11.00 ± 0.18	14.26 ± 0.11*	19.15 ± 0.44†	F(2, 9) = 854.2; P<0.001
Kidney	6.35 ± 0.10	6.44 ± 0.15	6.99 ± 0.11†	F(2, 9) = 31.8; P<0.001

Statistical significance: *p<0.001 for Control group vs D1 group; †P<0.001 for Control vs D2 group.

H_2S is formed from cysteine and homocysteine in several enzymatic reactions catalyzed by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT), and in non-enzymatic pathways in many tissues (KIMURA 2011). Enzymes participating in its production have been localized i.a. in heart, brain, liver, kidney, lung, vessels including aorta and portal vein, pancreatic islets, placenta, thymus and testis. Apart from free H_2S presence in tissues, organized stores for the messenger in cells have been identified. Cytoplasmatic bound sulfane sulfur is postulated to absorb and store exogenously applied and endogenously produced H_2S, which releases H_2S under reducing conditions – the presence of glutathione and cysteine at pH 8.4. The second H_2S store consists of mitochondria acid-labile sulfur – sulfur atoms of redox reactions catalyzed by enzymes of the respiratory chain. H_2S is released below the critical pH 5.4 achieved locally in those organelles (ISHIGAMI *et al.* 2009; OGASAWARA *et al.* 1994).

The method applied in our experiment traces the changes of free H_2S upon certain interventions. The sampled tissues contain all sorts of H_2S. Immediate alkalization prevents loss of free H_2S through binding it into salt. Obviously, under these circumstances hydrolysis and the transformation of other forms of sulfane into free H_2S might take place, but rapid freezing efficiently limits the process. Alkalization and refrigeration are necessary for maintaining free H_2S until the moment of analysis. Acidification of a sample during the process of H_2S analysis releases the bound into salt, but initially free, H_2S. It should be noted that samples were not incubated for a sufficient amount of time in temperatures above zero that would allow other forms of H_2S to transform into free H_2S, as applied in the study of Ishigami and colleagues (2009) to determine acid-labile sulfur. Such a process can not be excluded but its impact on the final results is eliminated by the use of blank determinations.

H_2S is lipophilic, freely permeates plasma membranes, has pronounced reducing activity and participates in the sulfhydration of numerous proteins, altering their function, posing an important physiologic signal (GADALLA & SNYDER 2010). The gasotransmitter interacts with carbon monoxide (CO) and nitric oxide (NO) in a number of ways (LI *et al.* 2009). H_2S has multidirectional actions (Table 2) and co-modulates various physiological and pathophysiological processes of different systems in mammals (LI *et al.* 2011; LI *et al.* 2009; ŁOWICKA & BEŁTOWSKI 2007; WILIŃSKI *et al.* 2010).

Vitamin D3 exerts a large number of biological effects reaching beyond calcium and phosphate metabolism, comprising modulation of immunity, inflammation, cell differentiation and proliferation or insulin secretion (DI ROSA *et al.* 2011). Moreover, cholecalciferol has been shown to be implicated in the pathophysiology of such diseases as hypertension, heart failure, chronic kidney disease, diabetes and dementia (VAIDYA & WILLIAMS 2011). These disorders are characterized by endogenous sulfur metabolism perturbances and are extensively studied as concerns the role of H_2S in

Table 2

Biological actions of hydrogen sulfide in mammals

protein sulfhydration	interaction with nitric oxide (NO) and carbon monoxide (CO)	maintaining protein –SH groups in the reduced state (reducing activity)
potassium channel (KATP) stimulation	metabolic inhibition (cytochrome c blockade)	smooth muscle cell hyperpolarization
reaction with reactive oxygen and nitrogen species (ROS and RNS)	stimulation of cysteine transport to the cell and reduced glutathione (GSH) synthesis	impact on pro-inflammatory cytokine production
TRP channel stimulation (i.a. TRPV1 channel opening)	inhibition of L-type, T-type and M-type calcium channels	enhancement of NMDA receptor activity
impact on protein kinase C (PKC)	influence on extracellular signal-regulated kinases	inhibition of phosphodiesterases
CFTR channel activation	impact on phosphoinositide 3'-kinase (PI3K)/Akt (protein kinase B)	leukocyte-endothelial cell interaction decrease
modification of matrix metalloproteinases activity (i.a. MMP-2 and MMP-9)	effect on NF-E2-related factor-2 (Nrf-2)	inhibition of angiotensin-converting enzyme (ACE)
membrane potential stabilization	influence on NF-κB complex activation	promotion of Ca^{2+} influx into astrocytes

CFTR – cystic fibrosis transmembrane conductance regulator, NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells, NMDA – N-Methyl-D-aspartate, TRP channel – transient receptor potential channel, TRPV1 – transient receptor potential vanilloid.

their treatment (ŁOWICKA & BEŁTOWSKI 2007). In its biological action vitamin D3 binds to specific receptors (VDR) and Retinoid X receptor-α (RXR-α) in the nucleus of various cells of the body, altogether binding to a specific DNA sequence, then the complex regulates the expression of different genes. Additionally, 'cross-talking' to other signaling pathways and some intracellular 'rapid' signaling pathways have been identified (ARANDA & PASCUAL 2001). They explain the complex outcome to a certain extent, but some biological effects of cholecalferol are possibly mediated by different messangers. Various features and molecular aspects of vitamin D3 and H_2S are common such as production at the inflammation site, the involvement of calcium channels, NO, renin-angiotensin-aldosterone system, prostaglandin E and extracellular signal-regulated kinases (ERKs), antioxidant properties, matrix metalloproteinase activity regulation or impact on cholesterol metabolism (DI ROSA *et al.* 2011; KIMURA 2011; LAGGNER *et al.* 2007; VAIDYA & WILLIAMS 2011; WILIŃSKI *et al.* 2010). Experimental data on anti-atherosclerotic, hypotensive, anti-proliferative and anti-cancer effects also connect both compounds (KRISHNAN & FELDMAN 2011; ŁOWICKA & BEŁTOWSKI 2007; MASON *et al.* 2003; PEI *et al.* 2011). The interaction mechanisms remain obscure with NO as a probable link between vitamin D and endogenous sulfur biology, as the impact of cholecalciferol on nitric oxide synthase was revealed (ROCKETT *et al.* 1998). It is worth mentioning that other drugs were also shown to affect the complex sulfur metabolism and H_2S including acetylsalicylic acid, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitor atorvastatin, ACE inhibitor ramipril, digoxin and paracetamol and the non-selective β-blocker carvedilol (SREBRO *et al.* 2006, WILIŃSKI *et al.* 2011a; WILIŃSKI *et al.* 2011b; WILIŃSKI *et al.* 2011c; WILIŃSKI *et al.* 2011d; WILIŃSKI *et al.* 2010).

In conclusion, our study reveals the existence of a link between vitamin D biology and endogenous sulfur metabolism, appraised by H_2S bioavailability. The presented results are highly indicative but less conclusive. The involvement of H_2S throws some light on the complexity of vitamin D biology. The impact of cholecalciferol on CBS, CSE and 3MST gene expression has never been explored. The biological effects of vitamin D3 following the inhibition of H_2S production have not been assessed. These issues and the role of vitamin D and H_2S in cardiology, nephrology and neurology are promising fields for future research.

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