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REVIEW

HYDROGEN SULFIDE: SYNTHESIS AND FUNCTION IN THE ADIPOSE TISSUE

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Abstract

Apart from nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) is the third gaseous mediator in mammals. H_2S is synthesized from L-cysteine by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), or by sequential action of alanine aminotransferase and 3-mercaptopyruvate sulfurtransferase. In the cardiovascular system, H_2S is involved in the regulation of vascular tone and blood pressure, inhibits atherogenesis, and protects myocardium from ischemia-reperfusion injury. Recent studies indicate that H_2S is synthesized also in the adipose tissue. Hydrogen sulfide produced in periadventitial adipose tissue (*tunica adiposa*) of the blood vessels induces vasodilation by activating K^+ channels in smooth muscle cells. On the other hand, H_2S inhibits basal and insulin-stimulated glucose uptake in visceral adipose tissue, and may be involved in the pathogenesis of insulin resistance. H_2S production in periadventitla adipose tissue is stimulated by vasoconstrictors and aortic banding-induced hypertension and downregulated by aging. H_2S signaling in adipose tissue may be affected by pharmacotherapy. Lipid-soluble statins (3-hydroxy-3-methylglutarylcoenzyme A reductase inhibitors) increase H_2S level in periadventitial adipose tissue and thus augment its anticontractile effect on the blood vessels. This effect of statins results from the depletion of ubiquinone – a component of mitochondrial respiratory chain – and the impairment of mitochondrial H_2S oxidation.

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Introduction

It was realized during the last two decades that the family of endogenous regulatory mediators includes not only complex organic compounds such as peptides/proteins, amines (serotonin, catecholamines, histamine), purines (adenosine, ATP), steroids, etc., but also simple inorganic molecules such as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S); now referred to as "gasotransmitters", because in nature they exist in gaseous form (1). The first member of gasotransmitter family, nitric oxide, was identified in 1980s, initially as the endothelium-derived relaxing factor. Now, it is evident that NO is ubiquitously generated in various tissues by three isoforms of NO synthase, and is involved in the regulation of vascular tone, myocardial contractility, renal function, neurotransmission, inflammatory response and many other processes (2). Soon thereafter, it was realized that carbon monoxide (CO), synthesized from heme by heme oxygenases (HO), is not only a metabolic waste product but also an endogenous gasotransmitter. CO shares with NO

not only the main mechanism of action, i.e. stimulation of soluble guanylyl cyclase, but also many biological activities such as vasorelaxation, neurotransmission, anti-inflammatory effect etc. (3). H_2S is the youngest member of gasotransmitter family. It was first suggested in 1996 by prof. Hideo Kimura that endogenously generated H_2S is a neurotransmitter/neuromodulator (4). This hypothesis was later confirmed by many studies and now it is evident that H_2S is as ubiquitous as NO and CO.

Both NO (5, 6) and CO (7, 8) are synthesized in the adipose tissue. H_2S research is a rapidly developing field, and, although we still know much less about H_2S than about its two older cousins, several recent studies have demonstrated that H_2S is also synthesized in the adipose tissue. Thus, we can classify NO, CO and H_2S as the new family of adipose tissue-derived mediators ("adipomediators") which, we suggest, should be referred to as "adipogasotransmitters". The purpose of this review is to describe the current state of knowledge about role of H_2S in adipose tissue and its link with obesity/metabolic syndrome.

Chemical properties, synthesis and metabolism of H_2S

H_2S is the colorless flammable gas with a strong odor of rotten eggs, soluble in both water and organic solvents. In aqueous solutions, H_2S dissociates into HS^- and H^+ ions with a pK_a of 6.76. At physiological pH of 7.4, about 18.5% of H_2S exists as the undissociated form and the rest as the hydrosulfide anion (HS^-). Undissociated H_2S is lipophilic and easily permeates plasma membranes (9).

In nature, H_2S is formed during anerobic bacterial digestion of organic substrates, originates in inorganic reactions in volcanic gases, and during chemical or enzymatic transformation of sulfur-containing components of food. High amounts of H_2S are generated in the colon by commensal bacteria by reduction of alimentary sulfates. Like NO and CO, H_2S is toxic at high concentrations and shares with them the main mechanism of toxicity – inhibition of cytochrome c oxidase (mitochondrial complex IV) (10). In fact, H_2S is actually a more potent inhibitor of cytochrome c oxidase than cyanide. NO inhibits cytochrome c oxidase at physiological concentrations, and through this mechanism reduces some detrimental effects of hypoxia by decreasing mitochondrial oxygen consumption (11). It is unclear if endogenous H_2S is generated in amounts sufficient to inhibit mitochondrial respiration.

H_2S is synthesized by most tissues in our body; the highest amounts are generated in the central nervous system, liver and kidney. There are three pathways of endogenous H_2S formation (12): (*i*) desulfhydration of L-cysteine by cystathionine β-synthase (CBS, EC 4.2.1.22), (*ii*) desulfhydration of L-cysteine by cystathionine γ-lyase (CSE, EC 4.4.1.1), and (*iii*) transamination reaction between L-cysteine and α-ketobutyrate catalyzed in mitochondria by cysteine aminotransferase (identical with aspartate aminotransferase) to form aspartate and 3-mercaptopyruvate, followed by decomposition of the latter to pyruvate and H_2S by 3-mercaptopyruvate sulfurtransferase (3-MST).

CBS and CSE are pyridoxal 5'-phosphate (vitamin B_6)-dependent enzymes, which act sequentially in the transsulfuration pathway to convert L-homocysteine to L-cysteine with L-cystathionine as the intermediate (13). Thus, both enzymes are necessary for efficient homocysteine metabolism. Desulfhydration of L-cysteine to H_2S is an additional activity of CBS and CSE which, at least under V_{max} conditions, is much lower than their primary canonical activities in the transsulfuration pathway. There are several different mechanisms of H_2S synthesis from L-cysteine by both CBS and CSE (see ref. (14) for details). Recent studies indicate that H_2S may be synthesized also directly from homocysteine, at least by CSE (14). At physiological concentrations of these aminoacids, about 70% of H_2S is synthesized from cysteine and the remaining 30% from homocysteine; the contribution of homocysteine increases in hyperhomocysteinemia. The third, 3-MST-dependent pathway, was until now observed only in vitro in the nervous system (15) and in endothelial cells of some species (e.g. rat and human but not mouse) (16), and its contribution to overall H_2S formation is unknown.

Recently, it was demonstrated that CBS and CSE may be released to the blood by at least two cell types, endothelial cells and hepatocytes, and can generate H_2S extracellularly from homocysteine circulating in the blood (17). Interestingly, extracellularly formed H_2S protects endothelial cells from various insults such as serum starvation, hypoxia/reoxygenation or hyperhomocysteinemia-induced injury.

The level of endogenous H_2S is controversial. In studies in which colorimetric methods of H_2S assay were applied, values between 10 and 50 μM in plasma and even more in some tissues were reported. However, more recent studies suggest that most of this H_2S is not free but bound as the so-called sulfane sulfur, i.e. sulfur atoms bound only to other sulfur atoms, mostly in persulfide groups (-SSH) of protein cysteine residues. H_2S may be released from sulfane sulfur by reducing agents such as synthetic thiol-reducing dithiotreitol as well as by endogenous glutathione; this reaction is especially efficient at alkaline pH (18). Thus, sulfane sulfur may be a reservoir of preformed H_2S in tissues. Another form of H_2S storage is acid labile sulfur, i.e. iron-sulfur clusters of proteins, from which H_2S is released at pH<5.4. Because most of iron-sulfur clusters are contained in mitochondrial proteins and intramitochondrial pH is >7.4, acid-labile sulfur is unlikely to be a physiologically relevant H_2S store, although may be released during tissue processing before colori-

metric H_2S assay (12). The level of free H_2S measured by more specific electrochemical methods is in the low micromolar or even nanomolar range (19).

Apart from protein binding, the main reason of low steady-state H_2S concentration in tissues is its rapid metabolism. H_2S may be oxidized spontaneously by molecular oxygen and/or reactive oxygen species. Some of the products such as sulfite (SO_3^{2-}) may have their own regulatory role, e.g. as a vasodilator or phagocyte-derived bactericidal agent (20, 21). However, most of H_2S is oxidized enzymatically in mitochondria. The discovery of mitochondrial H_2S oxidation is one of the most fascinating recent breakthroughs in biology. Since the discovery of role of mitochondria in cell energetic it was widely accepted that mitochondria can produce ATP only by oxidizing organic substrates. In the classic pathway of mitochondrial respiration, electrons are transferred from NADH to ubiquinone (coenzyme Q) by mitochondrial complex I (NADH:ubiquinone oxidoreductase) or from succinate to ubiquinone by complex II (succinate:ubiquinone oxidoreductase). Then, reduced coenzyme Q (ubiquinol) donates electrons to cytochrome c in the reaction catalyzed by complex III (ubiquinol:cytochrome c reductase). Finally, electrons are transferred from cytochrome c to molecular oxygen by cytochrome c oxidase (complex IV). H_2S is the first and the only currently known inorganic substrate for eukaryotic mitochondria which can provide energy for ATP synthesis (22). The mechanism of H_2S oxidation was recently deciphered (23). H_2S is first oxidized to elemental sulfur by sulfide:quinone oxidoreductase (SQR), which transfers electrons to ubiquinone where they enter the mitochondrial respiratory chain. Further steps of H_2S oxidation are catalyzed by sulfur dioxygenase (elemental sulfur to sulfite) and sulfite oxidase (sulfite to sulfate), with sulfate (SO_4^{2-}) being the final product. Efficient H_2S oxidation is especially vital for colonic epithelial cells which are exposed to high amounts of H_2S of bacterial origin. However, in other cells H_2S oxidation is also very effective and regulates its level. Under hypoxic conditions, H_2S oxidation is compromised and H_2S-mediated signaling is augmented (19); it is suggested that H_2S operates as an "oxygen sensor" and mediates many biological effects of hypoxia such as vasorelaxation (24), stimulation of arterial chemoreceptors (25) or regulation of sodium transport in the kidney (26). Protein binding and rapid oxidation shorten the half-life of H_2S making it a locally acting auto- and paracrine mediator rather than a circulating hormone.

The most specific signaling mechanism triggered by H_2S is stimulation of ATP-sensitive potassium channels (K_{ATP}). H_2S activates these channels by converting extracellular cysteine thiol groups (-SH) to persulfide groups (-SSH) (27). Some other signaling mechanisms have been suggested, however, most of them occur only at high supraphysiological gas concentrations. In contrast to NO and CO, H_2S does not stimulate soluble guanylyl cyclase. Many biological effects of endogenous H_2S have been described such as vasorelaxation, inhibition of atherogenesis, myocardial protection against ischemia-reperfusion injury, regulation of inflammatory reaction, neurotransmission and regulation of renal function (28). The link between H_2S and adipose tissue is just an emerging field of H_2S research.

Synthesis of H_2S in adipose tissue

Both CBS and CSE are expressed in perirenal; epididymal and perivascular white adipose tissue, as well as in brown adipose tissue in the rat (29,30). In addition, the recent study (31) indicates that homocysteine is produced in adipose tissue in high amounts. Thus, adipose tissue contains the whole machinery required for the transsulfuration pathway of homocysteine metabolism, as well as for the generation of H_2S by desulfhydration of either cysteine or homocysteine. In 2009 Fang *et al* (30) first demonstrated that incubation of homogenates of rat periaortic adipose tissue (PAT) with cysteine in the presence of pyridoxal 5'-phosphate results in H_2S formation. H_2S production in PAT was similar to aortic wall with removed PAT, and was inhibited by 65-75% with CSE inhibitors, propargylglycine or β-cyano-L-alanine. These CSE inhibitors were slightly more potent (inhibition of H_2S production by >80%) in the aortic wall without PAT. These data suggest that CSE is the main source of H_2S in both vascular smooth muscle cells and perivascular adipose tissue. In addition, the expression of CSE in PAT was demonstrated by Western blotting, and CSE protein was found in PAT adipocytes by immunohistochemistry (30). H_2S concentration measured by sensitive sulfur electrode was 2-fold higher in the incubation medium of PAT+ than of PAT- rat aortic rings. The same group (29) demonstrated H_2S production from L-cysteine by epididymal, perirenal and brown adipose tissue. In that study (29), both CBS and CSE transcripts were found in these fat pads by real-time PCR, however, propargylglycine and β-cyano-L-alanine inhibited H_2S synthesis by >80% confirming that CSE is a predominant source of H_2S. Both CSE expression and H_2S synthesis were also observed in cultured rat epididymal adipocytes and preadipocytes, and H_2S production from cysteine was by about 30% higher in mature fat cells than in preadipocytes (29). These data demonstrate that CSE-H_2S pathway exists in fat cells. It should be noted that 3-MST dependent pathway of H_2S production could not be detected in these studies (29, 30), because α-ketoglutarate – an obligatory cosubstrate for cysteine aminotransferase – was not added. Thus, it cannot be excluded that 3-MST dependent pathway of H_2S production is also operative in adipose tissue.

Perivascular adipose tissue-derived H_2S as a vasodilator

Demonstration of H_2S synthesis in periaortic adipose tissue led to the question about its function. The natural hypothesis was that PAT-derived H_2S might be involved in the regulation of vascular tone. H_2S produced in vascular smooth muscle and endothelial cells dilates blood vessels by activating ATP-sensitive potassium channels (K_{ATP}) in smooth muscle cells and inducing cell hyperpolarization (28). Intravenously administered H_2S or its donors decrease blood pressure in experimental animals, and deficiency of endogenous H_2S has been implicated as a pathogenic factor in arterial hypertension (28).

Although neglected in most studies concerning the regulation of vascular tone, perivascular adipose tissue is an integral part of the vascular wall and thus should rather be referred to as periadventitial adipose tissue or *tunica adiposa* (32). It was first demonstrated in 1991 that rat aortic rings with PAT are less responsive to constricting effect of norepinephrine than aortic rings without PAT, however, the effect was initially attributed to norepinephrine uptake by adipocytes or sympathetic endings localized in PAT (33). In 2002, Lohn *et al* demonstrated that vasoconstricting effect of angiotensin II, serotonin and phenylephrine (α_1-adrenergic agonist) were also smaller in PAT+ than in PAT-aortic rings, and suggested that periadventitial adipose tissue secretes humoral relaxing factor which they named adipose tissue-derived relaxing factor (ADRF) (34). It was demonstrated that ADRF activity is not accounted for by nitric oxide, cyclooxygenase- or cytochrome P450-dependent arachidonate derivatives and adenosine. Furthermore, vasodilating effect of ADRF was abolished by high, depolarizing, extracellular K^+ concentrations, suggesting the involvement of potassium channels. In addition, the effect of ADRF was at least partially attenuated by K_{ATP} channel blocker, glibenclamide. Apart from rat aortic rings, subsequent studies demonstrated the anticontractile effect of periadventitial fat on peripheral arteries, which play more significant role in the regulation of systemic vascular resistance than conduit vessels such as aorta (35). These data led Fang *et al* to suggest that PAT-derived H_2S may function as at least one of ADRFs (30). They confirmed that the increase in aortic tension induced by serotonin or phenylephrine was lower in PAT+ than in PAT-rings, and that in PAT-rings this anticontractile effect could be mimicked by exogenous H_2S.

The mixture of L-cysteine and pyridoxal 5'-phosphate (but neither of these compounds alone) augmented, whereas CSE inhibitors, propargylglycine or β-cyano-L-alanine, abolished the anticontractile effect of PAT, while having no effect on vascular tone of PAT- rings. Interestingly, contractile responses of PAT+ and PAT- rings were not different after pretreatment with propargylglycine. The anticontractile effect of PAT was not affected by endothelial removal or NO synthase inhibitor, L-NAME, but was abolished by K_{ATP} channel blocker, glibenclamide. Moreover, transfer of incubation/culture medium from PAT+ aortic rings or isolated periadventitial adipocytes to PAT- rings reduced constricting effect of phenylephrine, serotonin or angiotensin II, and this effect could not be observed if donor PAT was preincubated with CSE inhibitors before medium collection. Taken together, these results indicate that H_2S, produced in PAT by CSE, reduces vasoconstriction by activating K_{ATP} channels in smooth muscle cells.

Subsequently, Schleifenbaum *et al* (36) have demonstrated that the presence of PAT also impairs serotonin-induced contractility of rat mesenteric artery. In contrast to aortic rings, the anticontractile effect of PAT on the mesenteric artery was not affected by K_{ATP} channel antagonist, glibenclamide, but was reduced by nonspecific inhibitor of voltage-sensitive K^+ channels (K_v), 4-aminopyridine, as well as by the specific antagonist of $K_v7.x$ (KCNQ) channels, XE991. In contrast, XE991 had no effect on serotonin-induced contraction of mesenteric artery rings with removed PAT. Similarly to aorta, anticontractile effect of PAT on the mesenteric artery was abolished by CSE inhibitors. In addition, NaHS relaxed mesenteric artery rings without PAT, and this effect was inhibited by XE991. Taken together, these data indicate that PAT-derived H_2S reduces vascular tone also in small resistance arteries, however, in contrast to aorta its effect on smooth muscle cells is not mediated by K_{ATP} but rather by KCNQ channels. In addition, those authors (36) demonstrated that the other gasotransmitter, carbon monoxide, does not mediate PAT-induced inhibition of vasoconstriction because this effect was not reduced by heme oxygenase inhibitors. It was also demonstrated that KCNQ channel activators such as retigabine or VRX0621688 induced more prominent vasorelaxation of PAT- rings or PAT+ rings treated with CSE inhibitor in comparison to PAT+ rings not treated with CSE inhibitors. These results indicate that KCNQ channel-mediated vasorelaxing mechanism is "saturated" by PAT-derived H_2S under physiological conditions. Thus, KCMQ channel activators might be especially useful vasodilators when CSE-H_2S pathway in PAT is impaired.

Regulation of H_2S in periadventitial adipose tissue by hemodynamic factors

Given the role of PAT-derived H_2S in the regulation of vascular tone, it is interesting if and how the CSE-H_2S pathway in PAT is modulated by hemodynamic factors. Fang *et al* (30) have demonstrated that phenylephrine, serotonin and angiotensin II increased H_2S production from L-cysteine in isolated PAT. In contrast, these vasoconstrictors reduced H_2S production in aortic rings without PAT. Because stimulation of H_2S release was

observed in isolated PAT without adjacent aortic wall, it could not result from vasoconstriction itself but rather from the direct effect of these mediators on adipose cells.

In experimental hypertension induced in the rat by constriction of the abdominal aorta, H_2S synthesis and CSE expression in the aortic wall without PAT was unchanged in comparison to control normotensive animals, however, H_2S production and CSE expression in PAT increased by 70% and 130%, respectively. Plasma H_2S level was also slightly higher in hypertensive animals. Thus, CSE-H_2S system in PAT could be a back-up vasodilatory mechanism, which is up-regulated in response to both acute effect of vasoconstrictors and chronic hypertension. The mechanism of CSE-H_2S up-regulation in PAT in hypertensive rats is unclear, but the effect could partially results from higher concentration of angiotensin II in this model.

Interestingly, transplantation of PAT from healthy donor rats to the stenotic area of the abdominal aorta of hypertensive rats decreased systolic and diastolic blood pressure in hypertensive animals, but this effect could not be reproduced by transplantation of subcutaneous adipose tissue (30). Consequently, transplantation of PAT (but not of subcutaneous adipose tissue) reduced myocardial hypertrophy as well as decreased angiotensin II concentration in plasma and aortic wall of hypertensive animals. Although it was not examined if these effects were mediated by PAT-derived H_2S, this possibility is likely since H_2S was recently demonstrated to suppress renin-angiotensin system by inhibiting renin secretion (37) and angiotensin-converting enzyme activity (38).

Aging is well-known to exert detrimental effects on endothelium-dependent vasorelaxation. The effect of age on CSE-H_2S system in periadventitial adipose tissue was also examined (30). In the rat, H_2S production in PAT decreased between 6 and 12 months by 35%-64% in comparison to 1-month old animals. Surprisingly, CSE expression markedly increased in PAT in age-dependent manner starting from 2 months. It could be hypothesized that up-regulation of CSE is a negative feedback response to H_2S deficiency. However, CSE expression was markedly higher already in 2-month old in comparison to 1-month old rats, whereas H_2S production in 2-month old rats was still normal (30). The alternative explanation is that during ageing CSE becomes "dysfunctional", i.e. H_2S production is reduced despite greater amount of enzymatic protein. The similar, although less pronounced, age-dependent decrease in H_2S production despite concomitant up-regulation of CSE was also observed in rat aortic rings without PAT (30). Interestingly, although CSE expression increased between 2 and 12 months of age also in epidydimal and perirenal fat pads, this was accompanied by parallel increase in H_2S production (29). Thus, although CSE expression during aging behaves similarly in PAT and in other parts of visceral fat tissue, H_2S production changes in opposite directions. The reason for this difference between PAT and epidydimal/perirenal fat is unclear.

H_2S in adipose tissue as the regulator of insulin sensitivity

In freshly isolated rat epidydimal adipocytes, H_2S in solution (10-1000 μM) reduced basal and insulin-stimulated uptake of glucose as well as of non-metabolizable 2-deoxyglucose in a time and concentration-dependent manner (29). Although H_2S concentration used in this study was relatively high, the effect seems to be physiologically relevant because was reproduced when adipocytes were incubated in the presence of cysteine and pyridoxal 5'-phosphate to increase endogenous H_2S formation. Moreover, either propargylglycine or β-cyano-L-alanine not only abolished cysteine+pyridoxal phosphate-induced reduction of basal or insulin-stimulated glucose uptake, but also reduced baseline H_2S production in adipocytes and stimulated glucose uptake either in the absence or in the presence of insulin. These data indicate that H_2S produced under physiological conditions regulates glucose uptake and insulin sensitivity of adipocytes. Interestingly, the effect of H_2S on glucose uptake was not inhibited by K_{ATP} channel antagonist, glibenclamide, but was abolished by phosphoinositide 3-kinase (PI3-K) inhibitor, LY294002 (29). Although H_2S has been demonstrated to stimulate PI3-K in other tissues, this result is surprising since PI3-K is also a main insulin-triggered signaling mechanism. The exact intracellular mechanism through which H_2S impairs glucose uptake in adipocytes is not clear, however, it was recently noted that H_2S inhibits cyclic AMP and cyclic GMP-degrading phosphodiesterases (39). Since both these cyclic nucleotides stimulate lipolysis in adipocytes (40) and enhanced lipolysis is associated with reduced glucose uptake, this mechanism may contribute to H_2S-induced insulin resistance.

In primary culture of epidydimal rat adipocytes, high concentrations of glucose reduced H_2S production in a time- and concentration-dependent manner (29). The effect of glucose was not reproduced by mannitol thus indicating that it does not result from hyperosmolality but rather is specific for glucose. Thus, the negative feedback regulatory mechanism between glucose and H_2S may exist in adipose tissue, with H_2S inhibiting glucose uptake and glucose inhibiting CSE-H_2S pathway. Furthermore, CSE expression and H_2S production in adipose tissue was up-regulated in rats fed high fructose diet for 12 weeks, which is a widely used experimental model of insulin resistance (29). In addition, the significant negative correlation between H_2S production and insulin-stimulated glucose uptake in the adipose tissue was observed. This observation suggests that CSE-H_2S

system in adipose tissue may contribute to insulin resistance in the metabolic syndrome (29).

Effect of obesity/metabolic syndrome on H_2S signaling system

As noted above, high-fructose feeding is associated with the up-regulation of CSE-H_2S system in the epididymal and perirenal adipose tissue. High fructose diet results in hypertriglyceridemia, insulin resistance, hyperinsulinemia and, according to some studies, arterial hypertension (41). Later, progressive deterioration of carbohydrate metabolism is observed such as impaired glucose tolerance or even fasting hyperglycemia. Consistently with results observed in visceral adipose tissue, it was found higher expression and activity of CBS and CSE in the liver in Zucker diabetic fatty (ZDF) rats at the 5th week of age when these animals are insulin resistant and hyperinsulinemic but still nonrmoglycemic (42).

However, the effect of metabolic syndrome on CSE and CBS expression/activity is controversial. Experimental obesity induced in the rat by high fat and high sucrose feeding resulted in the reduction of CBS expression and activity in the liver, which was accompanied by about 50% elevation of plasma homocysteine (43). Most experimental data indicate that insulin down-regulates the expression of CBS and CSE in the liver (44,45), and insulin deficiency in streptozotocin-induced diabetes is associated with the elevation of these enzymes, reduction of plasma homocysteine, and overproduction of H_2S (46,47). Thus, hyperinsulinemia could result in the down-regulation of H_2S production if insulin sensitivity is preserved. Recently, Whiteman *et al* (48) compared plasma H_2S levels in 11 lean, 16 overweight non-diabetic and 11 overweight type 2 diabetic males. Plasma H_2S was lower in normoglycemic overweight than in lean volunteers, and was negatively correlated with markers of insulin sensitivity such as HOMA-index or insulin tolerance test. However, the strongest negative correlations were observed between H_2S and measures of central adiposity such as waist circumference and waist-to-hip ratio (r=-0.65). Multiple regression analysis demonstrated that high waist circumference was an independent predictor of low plasma H_2S level even after adjusting for insulin resistance. In overweight diabetic patients plasma H_2S was even lower than in overweight non-diabetic subjects (48). Although plasma H_2S originates from various sources apart from adipose tissue, these results would be consistent with the downregulation of H_2S producing enzymes in obesity.

Adipopharmacology of H_2S

PAT-derived H_2S may be involved not only in the regulation of vascular tone but may also inhibit atherogenesis. Indeed, H_2S exerts many potentially antiatherogenic effects such as inhibition of vascular smooth muscle cell proliferation, antiplatelet activity, inhibition of LDL oxidation and antiinflammatory effect (28). Recently, it has been demonstrated that H_2S inhibits atherogenesis in apolipoprotein E knockout mice (49). Thus, enhancing H_2S production by PAT and/or other parts of the vascular wall could be a useful therapeutic approach in cardiovascular disorders. Unfortunately, currently available inorganic H_2S donors such as NaHS, Na_2S or Ca_2S, although widely used in experimental studies, are unsuitable for therapy because are unstable (undergo spontaneous oxidation in solutions in vitro) and release H_2S rapidly and in high amounts *in vivo* (50). Some new organic H_2S donors such as GYY4137 have been synthesized which release H_2S slowly and in moderate amounts. They exert a more persistent effect on vascular tone and blood pressure (51). However, these compounds are still in the early stage of preclinical development, and their potential use in therapy is a matter of future. Thus, the effect of drugs currently used in cardiovascular disorders on H_2S signaling is highly interesting.

Recently, we examined the effect of statins on H_2S production in the vascular wall. Statins are competitive inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol synthesis, which converts HMG-CoA to mevalonate. Statins reduce plasma LDL cholesterol and are very effective in primary and secondary prevention of ischemic heart disease (52). Apart from affecting cholesterol metabolism, statins inhibit synthesis of many other active products of the mevalonate cascade including: (*i*) farnesylpyrophosphate – a substrate for protein farnesyltransferase which attaches farnesyl group to small GTP-binding Ras proteins; farnesylpyrophosphate is also necessary for the synthesis of heme A which is a specific component of cytochrome c oxidase, (*ii*) geranylgeranylpyrophosphate – a substrate for protein geranylgeranyltransferase which prenylates small GTP-binding Rho proteins, (*iii*) dolichol, involved in protein glycosylation, (*iv*) coenzyme Q (CoQ, ubiquinone) – a component of mitochondrial respiratory chain, (*v*) intermediates in cholesterol synthesis and hydroxylated cholesterol derivatives (oxysterols), which are endogenous agonists of the nuclear receptor – liver X receptor (LXR), and (*vi*) isopentenyladenosine required for selenoprotein synthesis. Reduction of these mevalonate metabolites is responsible for the so-called pleiotropic effects of statins, independent from the decrease in blood cholesterol. Statins can be classified according to their solubility into hydrophilic (pravastatin, rosuvastatin) and lipophilic (fluvastatin, simvastatin, atorvastatin, pitavastatin). Hydrophilic statins poorly permeate plasma membranes and are considered more liver-specific because are effectively transported to hepatocytes by organic anion transporters. Consequently, although hydrophilic statins affect the lipid profile, they posses

less peripheral effects. In contrast, lipophilic statins permeate effectively both to hepatocytes and to peripheral tissues. Previous studies have demonstrated that (especially lipophilic) statins have many effects on adipose tissue function (reviewed in 52).

We used two representant statins: hydrophilic pravastatin and lipophilic atorvastatin, and administered them for 3 weeks to healthy normolipidemic rats at doses which exerted comparable effects on plasma lipids (53). Then, we examined H_2S formation catalyzed by tissue (aortic media and PAT) homogenates under optimal conditions (saturating L-cysteine and pyridoxal 5'-phosphate concentrations). We found that only atorvastatin, but not pravastatin, increased H_2S production in PAT, whereas neither statin had any effect in the aortic media. Both statins increased H_2S production in the liver. Thus, we identified new pleiotropic, lipid-independent, effect of statins in the vascular wall which may contribute to beneficial impact of these drugs on atherogenesis and vascular tone.

In our initial experiments we used post-nuclear tissue homogenates which contain both cytosol (where CSE is localized) and mitochondria. Thus, it is unclear if the increase in net H_2S production resulted from increased synthesis or reduced mitochondrial oxidation. To address this issue, we measured H_2S production separately also in post-mitochondrial supernatants. We found that H_2S formation in post-mitochondrial supernatants of both PAT and liver was higher than in post-nuclear supernatant. That the difference in H_2S production between post-mitochondrial and post-nuclear supernatants indeed represents mitochondrial H_2S oxidation was confirmed by two observations: (*i*) this difference was abolished to virtually zero if the measurement was performed in carefully deoxygenated buffer to stop mitochondrial H_2S oxidation, and (*ii*) the difference was markedly inhibited by myxothiazole (mitochondrial complex III inhibitor) and potassium cyanide (cytochrome c oxidase inhibitor) but not by rotenone – inhibitor of complex I which is not involved in H_2S oxidation. Atorvastatin increased net H_2S production only in post-nuclear but not in post-mitochondrial supernatant and thus reduced the estimated mitochondrial H_2S oxidation in PAT. Similarly, both statins had the same effect in the liver. In support of these results, we found that oxidation of NaHS in vitro by isolated liver mitochondria was reduced in statin-treated in comparison to control rats. We could not obtain sufficient amount of isolated mitochondria from PAT to measure NaHS oxidation in vitro, however, taken together these results indicate that statins reduce mitochondrial H_2S oxidation but have no effect on cytosolic H_2S synthesis. Consistently with this conclusion, statins had no effect on CSE activity toward cystathionine in either PAT or the liver.

Next, we asked which products of the mevalonate cascade are responsible for the effect of statins on H_2S. To answer this question, we supplemented statin-treated rats with various mevalonate products or the mevalonate itself at doses which, according to previous studies, restored their levels to control values (54). We found that only mevalonate itself, farnesol and coenzyme Q_9 (the major coenzyme Q species in the rat) normalized mitochondrial H_2S oxidation in the liver in atorvastatin or pravastatin-treated rats and in PAT in atorvastatin-treated rats. These data suggest that statins inhibit H_2S oxidation by suppressing coenzyme Q synthesis, because mevalonate and farnesol, as well as exogenous coenzyme Q_9, correct reduced CoQ concentration in statin-treated rats. In contrast, squalene and geranylgeraniol, which are not CoQ precursors, failed to normalize H_2S production (53, 54). Similarly, synthetic LXR agonist, TO901317, also did not restore mitochondrial H_2S oxidation in statin-treated rats. In addition, incubation of liver mitochondria isolated from atorvastatin-treated rats with exogenous CoQ_9 *in vitro* normalized NaHS oxidation. Supplementation of CoQ_9 either *in vivo* or *in vitro* in rats not treated with statins had no effect on H_2S oxidation, although increased tissue and plasma CoQ_9 above control levels. These data indicate that physiological CoQ concentration (until compromised by statin treatment) is sufficient to support optimal H_2S oxidation.

Consistently with these results, no effect on H_2S oxidation was observed in animals receiving more specific inhibitors of the mevalonate cascade acting distally from mevalonate and having no effect on CoQ such as zaragozic acid (squalene synthase inhibitor), perillic acid (protein farnesyl- and geranylgeranyltransferase inhibitor), fasudil (which inhibits Rho-activated protein kinase) or farnesylthiosalicylic acid, an inhibitor of farnesylated Ras proteins. Neither of these inhibitors, in contrast to atorvastatin, affected coenzyme Q synthesis and H_2S level in the liver and periaortic adipose tissue.

Statins have been demonstrated to reduce CoQ in plasma and tissues of both experimental animals and humans (55), and statin-induced CoQ deficiency is implicated in the pathogenesis of some adverse effects of these drugs such as hepato- and myotoxicity. The results obtained by us indicate that CoQ depletion may also contribute to some desirable effects of statins.

Obviously, coenzyme Q is involved not only in H_2S oxidation but also in electron transfer from organic substrates to molecular oxygen. Thus, the question appears if statins specifically reduce mitochondrial oxidation of H_2S or affect also oxidation of organic substrates? In the latter case, statins would compromise ATP production and induce cell energy deficit. Data about the effect of statins on mitochondrial respiration are scarce and mainly obtained using models of statin-induced myopathy and/or hepatotoxicity. No previous studies addressed the effect of

statins on mitochondrial function in adipose tissue. Because we could not obtain sufficient amount of mitochondria from PAT, we isolated mitochondria from the liver of statin-treated rats and measured oxidation of NaHS as well as of organic substrate of complex II, succinate. We measured two markers of mitochondrial function: ATP production and mitochondrial membrane potential ($\Delta\psi_m$). $\Delta\psi_m$ is potential difference across inner mitochondrial membrane, between mitochondrial matrix and mitochondrial intermembrane space (negative potential in matrix). During electron transport through the mitochondrial respiratory chain, protons (H^+) are also transferred from matrix to intermembrane space. The resulting H^+ gradient provides energy for ATP synthesis and makes mitochondria the most negatively charged organelles in the cell with $\Delta\psi_m$ from -150 to -180 mV. Thus, $\Delta\psi_m$ is a global marker of electron transport efficacy. We measured $\Delta\psi_m$ in suspended liver mitochondria by lipophilic cationic fluorescent probe, JC-1 (54). This probe accumulates in negatively charged space of mitochondrial matrix and changes not only the intensity but also the character of fluorescence in a concentration-dependent manner. In diluted solutions, JC-1 exists as monomers which, when excited with the wavelength of 488 nm, emit green light at 535 nm. When JC-1 concentration increases, aggregates are formed which exhibit maximal emission within the orange range (595 nm). The ratio between intensity of orange-to-green fluorescence increases very sharply with increasing JC-1 concentration which, inside mitochondria, is proportional to $\Delta\psi_m$ (56). To measure $\Delta\psi_m$, we incubated suspended liver mitochondria with 1 μM JC-1 in the presence of either succinate or NaHS, and then measured fluorescence at both wavelengths to calculate this ratio. We found that $\Delta\psi_m$ measured in the presence of succinate was similar in control and statin-treated rats. In contrast, $\Delta\psi_m$ measured in the presence of NaHS was significantly lower in statin-treated in comparison to control group (54). In addition, the highly significant correlation between $\Delta\psi_m$ in the individual samples and NaHS oxidation (the rate of decrease in NaHS concentration) was observed. Similar results were obtained when ATP synthesis by isolated mitochondria was assessed. These results indicate that statins specifically reduce H_2S oxidation while having no effect on oxidation of organic substrates. We suggest that this specificity is accounted for by different K_m values of SQR vs. complex I/complex II for coenzyme Q, however, this hypothesis requires further research. Because under physiological conditions H_2S constitutes only a minor fraction of mitochondrial substrates, this effect of statins is unlikely to impair ATP production and cell energy status but is related only to H_2S signaling.

Conclusions and future directions

The most important messages resulting from studies performed so far are as follows: (*i*) H_2S is synthesized in adipose tissue, mainly by CSE, (*ii*) H_2S produced by periadventitial adipose tissue is a vasodilator; it induces hyperpolarization of adjacent vascular smooth muscle cells in large conduit and small resistance arteries by activating K_{ATP} and KCNQ channels, respectively, (*iii*) in visceral adipose tissue, H_2S inhibits basal and insulin-stimulated glucose uptake and thus may be involved in insulin resistance, (*iv*) CSE-H_2S system in the adipose tissue is regulated by physiological (e.g. vasoconstrictors, aging), pathological (hyperglycemia, hypertension, fructose-induced metabolic syndrome) and pharmacologic (statins) factors, and (*v*) apart from CSE expression and activity, H_2S level in the adipose tissue is dependent on its mitochondrial oxidation and thus may be affected by factors such as coenzyme Q availability.

Several important issues for future research emerge from data presented above. First, our study with statins was performed only on periadventitial adipose tissue surrounding the aorta. This tissue contains many brown adipocytes which are very rich in mitochondria. We estimated that about 50% of synthesized H_2S in this tissue is immediately oxidized. Other parts of visceral and subcutaneous adipose tissue (with the exception of brown adipose tissue itself) contain much less or no brown adipocytes and thus the relative contribution of mitochondrial oxidation to H_2S signaling is unclear. If mitochondrial oxidation plays an important role in the regulation of H_2S level not only in periadventitial but also in other fat pads, H_2S might be up-regulated by adipose tissue hypoxia which is observed in obesity (57). In view of possible role of H_2S in inducing insulin resistance (29) and a well-documented role of adipose tissue hypoxia in insulin resistance (57), this possibility becomes highly interesting.

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