Published in final edited form as:
Microcirculation. 2014 February ; 21(2): 104–111. doi:10.1111/micc.12083.

Contribution of hydrogen sulfide to the control of coronary blood flow

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Abstract

This study examined the mechanisms by which H_2S modulates coronary microvascular resistance and myocardial perfusion at rest and in response to cardiac ischemia. Experiments were conducted in isolated coronary arteries and in open-chest anesthetized dogs. We found that the H_2S substrate L-cysteine (1-10 mM) did not alter coronary tone of isolated arteries *in vitro* or coronary blood flow *in vivo*. In contrast, intracoronary (ic) H_2S (0.1-3 mM) increased coronary flow from 0.49 ± 0.08 to 2.65 ± 0.13 ml/min/g (P□0.001). This increase in flow was unaffected by inhibition of Kv channels with 4-aminopyridine (P=0.127) but was attenuated (0.23 ± 0.02 to 1.13 ± 0.13 ml/min/g) by the K_{ATP} channel antagonist glibenclamide (P□0.001). Inhibition of NO synthesis (L-NAME) did not attenuate coronary responses to H_2S. Immunohistochemistry revealed expression of cystathionine gamma-lyase (CSE), an endogenous H_2S enzyme, in myocardium. Inhibition of CSE with β-cyano-L-alanine (10 μM) had no effect on baseline coronary flow or responses to a 15 sec coronary occlusion (P=0.82). These findings demonstrate that exogenous H_2S induces potent, endothelial-independent dilation of the coronary microcirculation predominantly through the activation of K_{ATP} channels, however, our data do not support a functional role for endogenous H_2S in the regulation of coronary microvascular resistance.

Keywords

coronary circulation; reactive hyperemia; K channels

Introduction

The endogenous gasotransmitter hydrogen sulfide (H_2S) is known to exert a variety of effects on the cardiovascular system (14, 37, 41). In particular, H_2S has been shown to be a vasodilator in multiple vascular beds (10, 17, 22, 39, 44, 45) and to influence the physiologic regulation of vascular tone (15, 40) and blood pressure (44, 46). Other studies have also demonstrated that H_2S acts as a negative inotrope (14, 37, 41). Thus, H_2S could protect the heart from ischemic injury by mediating the balance between myocardial oxygen delivery and metabolism. The potential cardioprotective actions of H_2S are supported by data indicating that exogenous administration of H_2S donors protects against a loss of

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contractile function and diminishes myocardial infarct size/necrosis in animal models of ischemia reperfusion injury (6, 19, 32, 47). Although these protective effects are associated with improvements in coronary endothelial dependent and independent microvascular reactivity (32), the direct effects of H_2S on the coronary circulation have not been specifically examined.

This investigation was designed to elucidate the mechanisms by which H_2S influences myocardial perfusion and to define the role of H_2S in the regulation of coronary microvascular resistance at rest and in response to a brief episode of cardiac ischemia. Experiments tested the hypothesis that H_2S induces dose-dependent coronary vasodilation via endothelial-dependent production of nitric oxide (NO) (1, 11), and that endogenous production of H_2S contributes to the control of coronary blood flow in normal and/or ischemic hearts. Additional studies were also conducted to examine whether H_2S elicits increases in coronary blood flow through activation of ATP-sensitive K^+ (K_{ATP}) channels or voltage-dependent K^+ (K_v) channels, both of which have been shown to modulate vascular responses to H_2S in non-coronary vascular beds (28, 36, 45, 49) and in response to myocardial ischemia (5, 7, 12). Findings from this investigation provide novel insight in to the mechanisms and functional significance of H_2S in the regulation of coronary blood flow *in vivo*.

Methods

This investigation was approved by the IUPUI Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85–23, Revised 1996). All animals studied were lean mongrel dogs weighing between 20 and 30 kg. Following completion of experimental protocols, hearts were fibrillated and excised as recommended by the American Veterinary Medical Association Guide on Euthanasia (June 2007).

Immunohistochemistry

Immunohistochemical (IHC) analyses were performed in conjunction with Indiana University Health Pathology Laboratory (Indianapolis, IN). Briefly, liver and cardiac tissues were harvested immediately post mortem, rinsed in saline and transferred to 10% formalin. Formalin fixed tissues were then exposed to primary IgG antibodies against cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) at manufacturer recommended concentrations (Sigma Aldrich, St. Louis, Missouri). Slides were imaged at 10× magnification on a Nikon Eclipse 80i microscope and images captured with a Nikon DS-Fi1 and associated Nikon Elements software.

Isometric tension studies

Canine hearts were excised upon sacrifice and the aorta cannulated to perfuse the coronary tree with 4°C, Ca^{2+}-free Krebs solution (131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) in order to rinse the excised heart of blood and blood proteins. After perfusion, coronary arteries were grossly dissected from the heart, and further isolated from surrounding myocardium and adventitia using a dissecting

microscope. Following adventitial removal, arteries were cut into 3 mm rings and mounted in water-jacketed organ baths filled with a Ca^{2+}-containing Krebs solution (131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, 4 mM $CaCl_2$) at 37°C. Optimal length (passive tension) was assessed by contractions of isolated arteries to 60 mM KCl. Passive tension was increased in gram increments until there was <10% change in active tension development to 60 mM KCl (typical optimal passive tension equaled ~4 g). Once optimal passive tension was obtained, arteries were then pre-constricted with 1 μM U46619 and stimulated with either NaHS (1-10 mM) or the H_2S substrate L-cysteine (1-10 mM). Changes in vascular tone were measured as a percent change from maximal tension developed in response to 1 μM U46619.

Surgical preparation

Dogs were initially sedated with morphine (3 mg/kg, subcutaneously) and anesthetized with α-chloralose (100 mg/kg, intravenously). The animals were then intubated and mechanically ventilated (Harvard respirator) with oxygen-supplemented room air. A catheter was placed into the thoracic aorta via the right femoral artery to measure aortic blood pressure and heart rate. The left femoral artery was catheterized to supply blood to an extracorporeal perfusion system used to perfuse the left anterior descending (LAD) artery at a controlled pressure (100 mmHg). A catheter was also inserted into the right femoral vein for injection of supplemental anesthetic, heparin and sodium bicarbonate. Arterial blood gases were analyzed periodically throughout the experimental protocol and adjustments were made as needed to maintain blood gas parameters within normal physiological limits. A left lateral thoracotomy was performed to expose the heart, and the LAD was isolated distal to its first major diagonal branch. Following heparin administration (500 U/kg, intravenously), the LAD was cannulated with a stainless steel cannula connected to an extracorporeal perfusion system. Coronary perfusion pressure (CPP) was regulated by a servo-controlled roller pump, held constant at 100 mmHg. Coronary blood flow was continuously measured by an inline Transonic Systems flow transducer (Ithaca, NY, USA). Data were continuously recorded on IOX data acquisition software from Emka Technologies (Falls Church, VA, USA).

Experimental Protocol

Following coronary cannulation, hemodynamic parameters were allowed to stabilize for 30 min. Basal coronary blood flow and hematocrit were then determined, and based on these parameters, an aqueous solution of NaHS was infused into the LAD perfusion line at controlled rates in order to achieve coronary plasma NaHS concentrations of 100μM, 300 μM, 1 mM, and 3mM. Animals were also subjected to an L-cysteine dose response curve (100 μM, 300 μM, 1 mM and 3 mM). Coronary flow responses as well as heart rate and blood pressure were monitored throughout the course of the dose response curves. Following a 15 min washout period, animals were then subjected to an identical NaHS dose response curve in the presence of the NO synthase inhibitor L-NG-Nitroarginine methyl ester (L-NAME, ~35 μg/ml, ic), the general K_v channel blocker 4-aminopyridine (4AP, 0.3mM, ic) or the K_{ATP} channel inhibitor glibenclamide (3mg/kg, iv). In a subset of animals (n = 3), coronary reactive hyperemic responses were measured following a 15 second occlusion of the LAD in the absence and presence of the CSE enzyme inhibitor β-cyano L-alanine (BCA, 10μM, ic). Hyperemic responses were measured until coronary flow reached

baseline values. All drugs (Sigma Aldrich, St Louis, MO, USA) with the exception of glibenclamide (dissolved in equal parts of ethanol, propylene glycol, 1N NaOH) were dissolved in saline and infused in to the coronary perfusion line. Data on the systemic hemodynamic effects of each of these drugs are provided in **Table 1**.

Statistical analyses

Data are presented as mean ± SE. Statistical comparisons were made by a one-way or two-way repeated measures analysis of variance (ANOVA) as appropriate (Sigma Plot 11.0 Software). If statistical differences ($P < 0.05$) in these analyses were noted, a Student-Newman-Keuls multiple comparison test was performed. Reactive hyperemic volumes were calculated as area under the curve using Prism software (GraphPad Software).

Results

Tissue Immunohistochemistry

Consistent with previous findings (2, 16), liver tissue sections stained positive for both of the H_2S producing enzymes cystathionine γ-lyase (CSE) (**Figure 1A**) and cystathionine β-synthase (CBS) (**Figure 1B**). However, expression of these enzymes within liver vasculature was relatively modest (see inset). Left ventricular myocardial tissue also stained positive for CSE (**Figure 1C**) and CBS (**Figure 1D**). However, CSE and CBS were not prominently expressed in the coronary vasculature (see inset).

Isometric Tension Studies

Isometric tension recordings were performed on isolated coronary artery rings pre-constricted with the thromboxane A_2 mimetic U46619 (1 μM). In these pre-contracted rings, administration of the H_2S substrate L-cysteine (1-10 mM; n = 3) tended to increase isometric tension ~15% (**Figure 2A**) while NaHS (1-10 mM; n = 3) tended to diminish active tension development ~5% (**Figure 2B**). However, neither L-cysteine ($P = 0.27$) nor NaHS ($P = 0.44$) significantly altered coronary artery tension relative to U46619 treatment alone.

Effects of H_2S on Coronary Blood Flow In Vivo

Intracoronary administration of L-cysteine (0.1-3.0 mM) had no effect on baseline coronary blood flow (**Figure 2C**). In contrast, infusion of NaHS (n = 5) dose-dependently increased coronary blood flow from 0.49 ± 0.09 ml/min/g at baseline to 2.65 ± 0.15 ml/min/g at the highest (3 mM) concentration of NaHS (**Figure 2D**; $P < 0.001$). This vasodilator response was not significantly diminished by inhibition of NO synthase with L-NAME (**Figure 3A**; n = 3) or by blockade of K_v channels with 4AP (**Figure 3B**; n = 3). However, administration of the K_{ATP} channel antagonist glibenclamide significantly impaired coronary vasodilation to 3mM H_2S by ~ 70% ($P < 0.001$) (**Figure 3B**; n = 5).

Inhibition of H_2S Producing Enzyme CSE

Administration of the CSE enzyme inhibitor BCA (10 μM; n = 3) had no effect on baseline hemodynamic parameters (**Table 1**). The effect of CSE inhibition on the coronary blood

flow response to a 15 sec coronary artery occlusion (i.e. coronary reactive hyperemia) is shown in **Figure 4**. BCA did not significantly affect the reactive hyperemic response as evidenced by no alterations in the peak vasodilatory response, flow volume of repayment or in the repayment of the coronary flow debt following the inhibition of CSE ($P = 0.82$) (**Table 2**).

Discussion

This study was designed to delineate the mechanisms by which H_2S modulates coronary microvascular resistance and myocardial perfusion at rest and in response to transient cardiac ischemia. The major novel findings of the investigation include: 1) prominent expression of the H_2S producing enzymes CBS and CSE in canine myocardium; 2) infusion of the key H_2S substrate L-cysteine failed to significantly alter coronary vascular tone of isolated conduit arteries *in vitro* or microvessels *in vivo*; 3) intracoronary administration of H_2S dose-dependently increases coronary blood flow (~ 5 fold) via activation of K_{ATP} channels; 4) H_2S mediated coronary vasodilation occurs independent of endothelial NO production or K_v channel activation and is largely absent in conduit coronary arteries; 5) inhibition of endogenous CSE has no effect on the regulation of coronary blood flow at rest or in response to a brief coronary artery occlusion. Taken together, these findings indicate that exogenous H_2S induces potent dilation of the canine coronary microcirculation predominantly through a K_{ATP} channel dependent (NO independent) mechanism. However, our data support no functional role for endogenous H_2S in the regulation of baseline coronary resistance or ischemic coronary vasodilation.

Functional expression of H_2S producing enzymes in canine hearts

Our IHC studies demonstrate the prominent expression the H_2S producing enzymes (CBS and CSE) in canine myocardium (**Figure 1**). This finding is consistent with other studies which have documented the presence of CSE in rat and mouse liver and cardiac tissue (14, 16, 34, 46), and expression of CBS in rodent hearts (9, 35). IHC also revealed relatively low levels of CSE and CBS expression in the liver and cardiac microcirculation (**Figure 1C & 1D**); which is consistent with little/no effect of the H_2S substrate L-cysteine on tone of isolated coronary arteries (**Figure 2A**) or on coronary blood flow *in vivo* (**Figure 2C**). This lack of a coronary response to L-cysteine is in contrast with the recent findings of Leffler *et al.* who documented dose-dependent dilation to L-cysteine in cerebral pial arterioles in newborn swine (22); i.e. differences in the functional relevance of endogenous H_2S production likely exist between the cerebral and coronary circulation.

Although H_2S is known to be a vasodilator in a variety of vascular beds (10, 17, 31, 36, 49), primarily in the cerebral circulation (22, 23, 25, 26, 39), no study has directly examined coronary vasodilation in response to NaHS administration *in vivo*. We found that NaHS significantly increased coronary blood flow in a concentration dependent manner (**Figure 2D**). Interestingly, our findings indicate marked regional differences in coronary conduit vs. microvascular responsiveness to H_2S as isolated coronary arteries responded only ~5% to 3 mM NaHS while coronary flow increased ~500% in response to the same concentration of NaHS.

Mechanism of H_2S-mediated coronary vasodilation

Earlier studies in peripheral arteries suggest that H_2S mediated dilation occurs via an endothelial-dependent mechanism (10, 48) that converges on the activation of smooth muscle K_{ATP} (22, 25, 28, 29, 49, 50) and/or K_{Ca} channels (17, 26). However, the pathways responsible for the effects of H_2S in the coronary circulation have not been delineated. In the current study, we found that intracoronary administration H_2S (plasma concentration 3 mM) induced an ~5-fold increase in coronary blood flow (**Figure 2D**). This increase in coronary flow is not related to endothelial-production of NO as administration of the NOS inhibitor L-NAME, at a dose we previously demonstrated to attenuate NO-mediated coronary vasodilation in dogs (5, 20), did not diminish the coronary response to H_2S (**Figure 3A**). Infusion of the voltage-dependent K^+ channel antagonist 4-aminopyridine (4AP) also had no effect on H_2S mediated coronary vasodilation (**Figure 3B**). However, inhibition of K_{ATP} channels with glibenclamide significantly reduced the increase in coronary blood flow to H_2S by nearly 70% (**Figure 3B**). Therefore, our findings demonstrate that exogenous H_2S acts as a potent endothelial-independent vasodilator in the coronary circulation predominantly via activation of smooth muscle K_{ATP} channels. Given the prominent role of K_v and K_{ATP} channels in the regulation of coronary blood flow (3, 4, 13, 43), and the relatively modest effect of K_{Ca} channel inhibition on coronary responses *in vivo* (7, 8, 21) we elected to focus the present studies on H_2S mediated activation of coronary K_v and K_{ATP} channels. Since earlier studies in other vascular beds have documented a role for K_{Ca} channels in H_2S-induced dilation (18, 24, 27, 42), further systematic experiments are needed to specifically examine the contribution of specific K_{Ca} channels (BK_{Ca}, IK_{Ca}, SK_{Ca}) to H_2S mediated increases in coronary blood flow.

Role of endogenous H_2S in control of coronary blood flow

As outlined above, the lack of a coronary response to the H_2S substrate L-cysteine (**Figure 2**) does not support an active role for endogenous H_2S in the regulation of coronary microvascular resistance. However, alterations in the physiologic state of the myocardium, such as ischemia, have been shown to increase endogenous production of H_2S and limit myocardial ischemia-reperfusion injury (38). In order to examine the role of H_2S in ischemic coronary vasodilation, coronary reactive hyperemia studies were conducted in the absence and presence of the CSE inhibitor BCA (30, 33). Findings from these experiments indicate that 10 μM BCA, an effective dose capable of inhibiting liver H_2S synthesizing activity (30), had no effect on baseline coronary blood flow (**Table 1**) or on any aspect of the coronary reactive hyperemic response (**Figure 4**); i.e. peak vasodilator response or the overall debt to repayment ratio (**Table 2**). Therefore, although there is evidence to support a role for endogenous H_2S in mitigating myocardial ischemia-reperfusion injury (19, 32, 38, 51), there are little/no data to support that endogenous H_2S is an active regulator of coronary vasomotor tone at rest or following a brief episode of myocardial ischemia.

Conclusions

Findings from this investigation are the first to show that exogenous H_2S induces potent, endothelial NO-independent dilation of the canine coronary microcirculation, predominantly through the activation of K_{ATP} channels. Despite the pronounced effects of exogenous H_2S,

our data do not support a functional role for endogenous H_2S in the regulation of baseline coronary resistance or ischemic coronary vasodilation. Such findings do not negate prior studies regarding the cardioprotective effects of endogenous H_2S in the ischemic heart (38), but rather indicate that H_2S-related improvements in cardiac function and ischemic injury are not mediated by alterations in myocardial perfusion per se. Therefore, although exogenous H_2S demonstrates the capacity for robust coronary dilator responses, there was no demonstrable physiologic role for H_2S as a coronary signaling molecule in this study.

Acknowledgments

This work was supported by a National Institutes of Health grant, HL092245. Dr. Goodwill was supported by National Institutes of Health T32HL079995. Dr. Owen was supported by National Institutes of Health T32DK064466. Dr. Moberly was supported by the IU Medical Scientist Training Program and Dr. Berwick was supported by the American Heart Association 10PRE4230035.

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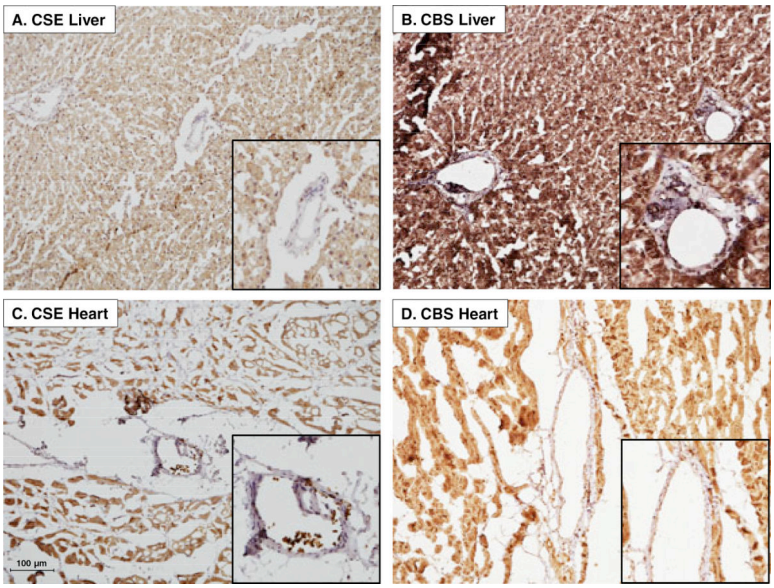

Figure 1.

Representative immunohistochemistry showing expression of the H_2S producing enzyme cystathionine γ-lyase (CSE) CSE in liver **(A)** and cardiac **(C)** tissue samples. Positive staining for the H_2S producing enzyme cystathionine β-synthase (CBS) in liver **(B)** and heart **(D). Insets:** Magnification of microvessels show modest staining for both CSE and CBS in liver and cardiac tissue samples.

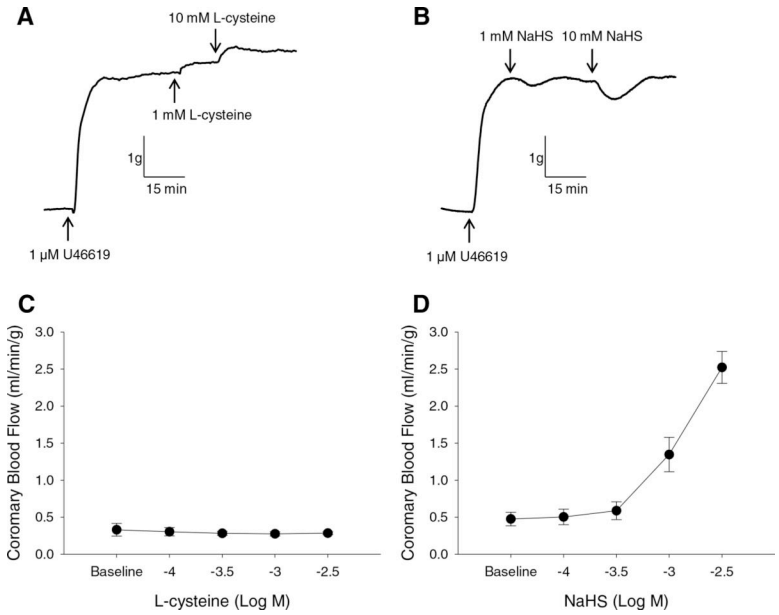

Figure 2.

Representative isometric tension recordings show modest effects of L-cysteine (n=3) **(A)** and NaHS (n=3) **(B)** on tension of isolated coronary artery rings pre-constricted with U46619. In-vivo intracoronary infusion of L-cysteine had no effect on basal coronary blood flow **(C)** while intracoronary infusion of NaHS significantly augmented coronary flow at plasma concentrations >300 μM **(D)**.

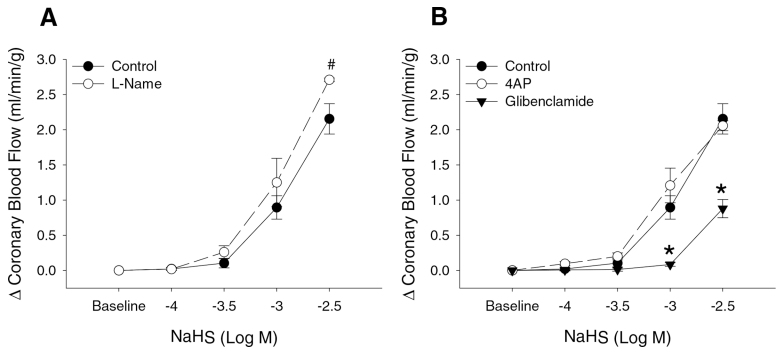

Figure 3.

Inhibition of NO synthase with L-Name produced a modest increase the coronary blood flow response to intracoronary NaHS (n=3) **(A)**. Coronary vasodilation to NaHS was unaffected by the inhibition of voltage-dependent K^+ channels with 4-AP (n=3) while blockade of K_{ATP} channels with glibenclamide (n=5) markedly reduced NaHS-induced dilation in the coronary circulation. # $P < 0.05$; * $P < 0.001$.

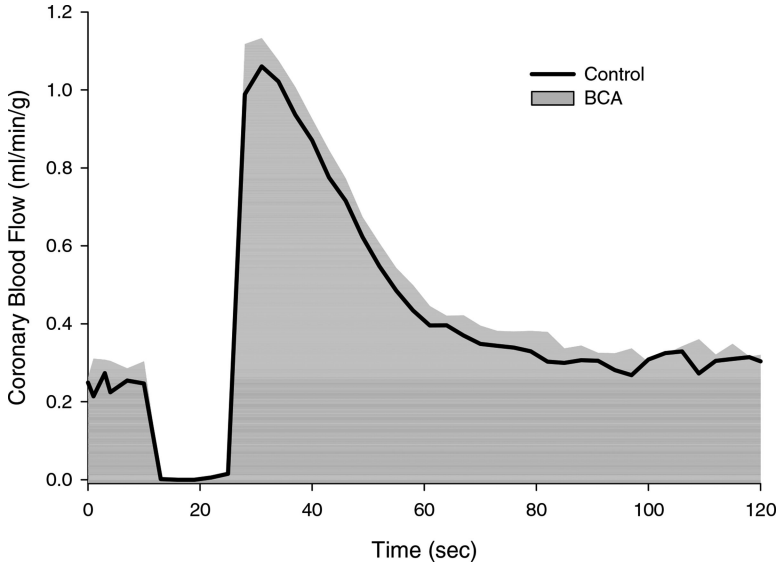

Figure 4.

Inhibition of the H_2S producing enzyme cystathionine γ-lyase (CSE) with β-cyano-L-alanine (BCA) (n=3) did not significantly alter the coronary blood flow response to a 15 sec coronary artery occlusion.

Table 1

Effects of inhibition of selected signaling pathways on baseline hemodynamic variables in anesthetized, open-chest dogs.

Heart Rate (beats/min)	Systolic Pressure Coronary Blood Flow (mmHg) (ml/min)	Diastolic Pressure (mmHg)	Mean Pressure (mmHg)	
Baseline	114 ± 6	74 ± 15	93 ± 8	99
± 26	0.47 ± 0.09			
L-NAME	114 ± 6	79 ± 10	96 ± 7	95
± 16	0.50 ± 0.11			
Baseline	112 ± 7	81 ± 15	96 ± 8	98
± 12	0.55 ± 0.15			
4AP	107 ± 14	76 ± 13	90 ± 14	92 ± 10
0.34 ± 0.07				
Baseline	107 ± 7	74 ± 8	89 ± 8	86
± 12	0.40 ± 0.04			
Glibenclamide	122 ± 4*	90 ± 6*	103 ± 5*	78 ± 10
0.25 ± 0.03				
Baseline	132 ± 15	90 ± 14	106 ± 14	79
± 24	0.27 ± 0.04			
BCA	125 ± 17	83 ± 15	100 ± 16	90 ± 31
0.31 ± 0.04				

Values are mean ± SE.

* indicates $P < 0.05$ vs. respective baseline. (L-NAME n = 3, 4AP n = 3, Glibenclamide n =5 , BCA n = 3).

Table 2

Effect of CSE inhibition on the coronary blood flow response to a 15 sec coronary artery occlusion.

	Control	BCA
Peak Flow (ml/min/g)	1.33 ± 0.13	1.36 ± 0.11
Debt Area (ml/g)	0.09 ± 0.01	0.10 ± 0.02
Repayment Area (ml/g)	18.1 ± 1.8	18.2 ± 1.6
Repayment/debt Ratio (%)	359 ± 35	323 ± 48

Values are mean ± SE. (Control n = 3, BCA n = 3).