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Hydrogen sulfide dilates the isolated retinal artery mainly *via* the activation of myosin phosphatase

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ABSTRACT

Aims: Hydrogen sulfide (H_2S) is shown in ocular tissues and suggested to involve in the regulation of retinal circulation. However, the mechanism of H_2S-induced relaxation on retinal artery is not clarified yet. Herein, we aimed to evaluate the role of several calcium (Ca^{2+}) signaling and Ca^{2+} sensitization mechanisms in the relaxing effect of H_2S donor, NaHS, on retinal arteries.

Materials and methods: Relaxing effects of NaHS (10^{-5}–3×10^{-3}M) were determined on precontracted retinal arteries in Ca^{2+} free medium as well as in the presence of the inhibitors of Ca^{2+} signaling and Ca^{2+} sensitization mechanisms. Additively, Ca^{2+} sensitivity of the contractile apparatus were evaluated by $CaCl_2$-induced contractions in the presence of NaHS (3×10^{-3}M). Functional experiments were furtherly assessed by protein and/or mRNA expressions, as appropriate.

Key findings: The relaxations to NaHS were preserved in Ca^{2+} free medium while NaHS pretreatment decreased the responsiveness to $CaCl_2$. The inhibitors of plasmalemmal Ca^{2+}-ATPase, sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase, Na^{+}-Ca^{2+} ion-exchanger and myosin light chain kinase (MLCK) unchanged the relaxations to NaHS. Likewise, Ca^{2+} sensitization mechanisms including, rho kinase, protein kinase C and tyrosine kinase were unlikely to mediate the relaxation to NaHS in retinal artery. Whereas, a marked reduction was determined in NaHS-induced relaxations in the presence of MLCP inhibitor, calyculin A. Supportively, NaHS pretreatment significantly reduced phosphorylation of MYPT1-subunit of MLCP.

Significance: The relaxing effect of NaHS in retinal artery is likely to be related to the activation of MLCP and partly, to decrement in Ca^{2+} sensitivity of contractile apparatus.

1. Introduction

Hydrogen sulfide (H_2S) is a gasotransmitter known to regulate vascular tonus. With respect to its concentration, it was shown to induce generally vasodilatation in different vascular beds [1]. In several studies, the opening of potassium (K^+) channels, the activation of transient receptor potential A1 (TRPA1) channel [2–6] and the stimulation of Cl^-/HCO_3^- transporter [7,8] were reported to be responsible for the vasorelaxing mechanisms of H_2S possibly by inducing hyperpolarization which results in the reduction of intracellular Ca^{2+} concentration in vascular smooth muscle cells. In the past decade, H_2S was also shown to be a regulatory substance in the ocular vasculature [9]. GYY4137, a slow releasing H_2S donor, was notified to induce vasodilatation partially through the activation of K_{ATP} channels on isolated bovine ciliary artery [10]. Similarly, AP67 and AP72, the other slow releasing H_2S donors, were also reported to dilate bovine posterior ciliary artery through the mechanisms related to nitric oxide release and the opening of K_{ATP} channels [11]. In addition, NaHS, a rapid releasing H_2S donor, was reported to dilate bovine retinal artery partly *via* K_v and K_{ir} channels [12]. Moreover, H_2S released from the vascular wall and the perivascular retina was suggested to counteract spasmogen induced contraction in porcine retinal arteries in normoxic and hypoxic conditions [13]. Despite of the increasing evidence reporting its presence in ocular tissues and vasodilator effect on ocular vascular tone, the functional role of H_2S and the underlying mechanism of relaxation remain to be elucidated.

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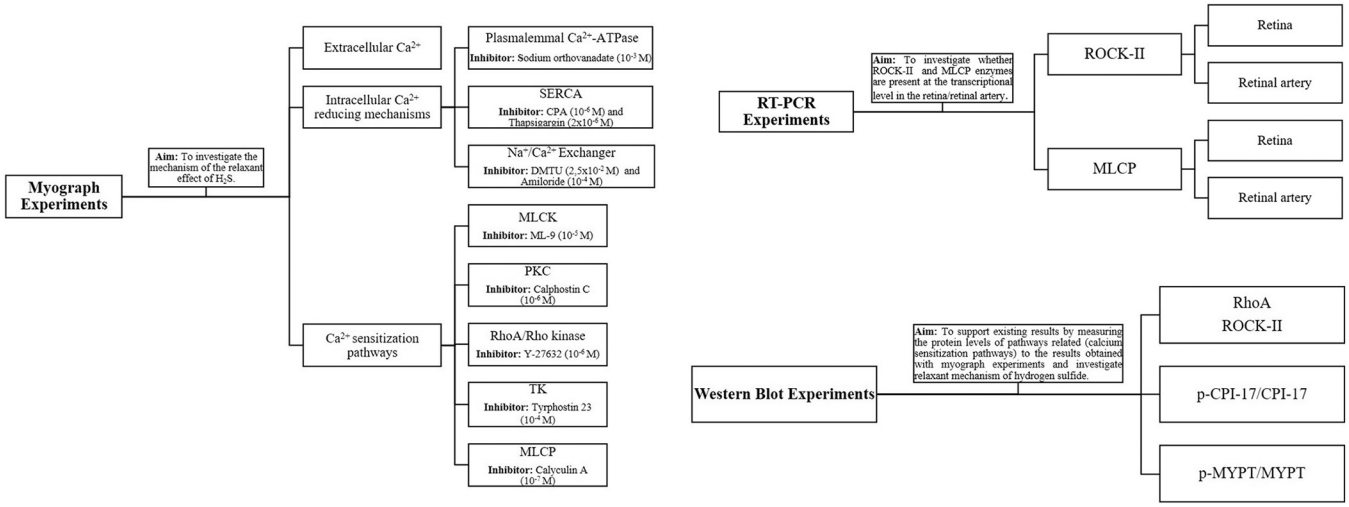


Fig. 1. The schematic representation of the experiments.

It is well known that the vascular contractile reactivity is regulated by Ca^{2+}-dependent and Ca^{2+}-independent mechanisms which related to the elevation of intracellular Ca^{2+} concentration and Ca^{2+} sensitization of the contractile apparatus, respectively. A decrease in intracellular Ca^{2+} concentration through mechanisms such as the inhibition of L-type voltage dependent Ca^{2+} channels, Na^{+}/Ca^{2+} exchanger or the activation of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATP_{ase} (SERCA) lead to dephosphorylation of 20 kDa myosin light chain (MLC_{20}) and induce vasodilatation [14]. On the other hand, the phosphorylation of MLC_{20} is regulated by Ca^{2+} independent mechanisms, as well. As known, the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), which are the enzymes phosphorylating and dephosphorylating MLC_{20}, respectively, importantly ascertain the degree of vascular smooth muscle tone [15]. Hence, the inhibition of MLCP can lead to phosphorylation of MLC_{20} and induce contraction without changing the intracellular Ca^{2+} concentration, which referred as Ca^{2+} sensitization [16]. Several kinase pathways have been linked to the inhibition of MLCP and induction of Ca^{2+} sensitization of the vascular contractile apparatus, such as RhoA/Rho kinase, protein kinase C (PKC) or tyrosine kinase [17,18].

Our previous study revealed that the H_2S donor, NaHS acts independently from L-type voltage dependent Ca^{2+} channels in bovine retinal artery [12]. Considering this finding, we suggest that the substantial relaxing effect of H_2S on retinal artery may involve an alteration in Ca^{2+} signaling and/or Ca^{2+} sensitization mechanisms of the contractile apparatus. Thus, in the present study, we aimed to characterize the possible role of intracellular Ca^{2+} reducing mechanisms, such as plasmalemmal Ca^{2+}-ATPase, sarcoplasmic/endoplasmic ATPase (SERCA) and Na^{+}-Ca^{2+} exchanger as well as Ca^{2+} sensitization mechanisms, such as RhoA/Rho kinase, protein kinase C and tyrosine kinase, in the relaxant effect of NaHS on isolated bovine retinal arteries. The role of RhoA/Rho kinase, CPI-17 and MLCP were furtherly assessed by determining related protein and/or mRNA expressions.

2. Materials and methods

2.1. Preparation of retinal arteries

Retinal arteries were prepared as previously described [12,19,20]. Briefly, bovine eyes (Brown Swiss [Montafoner], either sex, 2–4 years of age) were obtained from a local slaughterhouse and transported to the laboratory in ice-cold Krebs-Ringer bicarbonate solution with the following composition in mM: NaCl 135, KCl 5, $NaHCO_3$ 20, Glucose 10, $CaCl_2$ 2.5, $MgSO_4{\cdot}7H_2O$ 1.3, KH_2PO_4 1.2, ethylene diamine tetraacetic acid (EDTA) 0.026. For the experiments performed in Ca^{2+} free medium, $CaCl_2$ (2.5 mM) was removed from Krebs-Ringer bicarbonate solution and 0.5 mM ethylene glycol tetraacetic acid (EGTA) was added [20]. Extraocular muscles and connective tissues were dissected from the globe, vitreous was removed and cold Krebs-Ringer bicarbonate solution was poured into the eyecup for proceeding the dissection of the retinal artery under a stereomicroscope (Zeiss 2000, Oberkochen, Germany). A segment of central retinal artery located between the optic disc and the first branch was isolated and detached from the adhering retinal tissue. Four preparations were mounted parallelly in a multichamber wire myograph (Model 610M, DMT, Aarhus, Denmark). Two stainless steel wires in 40 µm diameter were treaded into the lumen of the retinal arteries and then fixed to the mounting devices of a force transducer and a micrometer. The retinal arteries were equilibrated for 1 h in Krebs-Ringer bicarbonate solution at 37 °C and aerated with 5% CO_2 + 95% O_2 (pH = 7.4). At the end of equilibration period, retinal arteries were set to a normalized internal circumference L_1 (0.9 L_{100}) in accordance to passive wall tension–internal circumference relationship under a passive transmural pressure of 100 mmHg [21]. Normalized retinal arteries were contracted twice with potassium chloride (K^{+}, 120 mM) plus noradrenaline (100 µM) in order to check the viability and provide standardization of the preparations. The preparations, which developed a tension < 0.5 mN/mm were discarded from the experiment. The isometric tension recordings of wire myograph system were obtained by using LabChart software programme (ADInstruments Ltd.). Functional endothelium and smooth muscle relaxation capacities of the retinal arteries were checked by acetylcholine (Ach, 10^{-8}–10^{-4} M) and sodium nitroprusside (SNP, 10^{-8}–10^{-4} M), respectively.

NaHS was used as the prominent donor of H_2S in the experiments [2,12,22]. Concentration-dependent effects of NaHS (10^{-5}–3×10^{-3}M) were investigated in a cumulative manner on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$: 3×10^{-5}M) precontracted retinal arteries as described previously [12]. In time-match control experiments, it is defined that the contraction induced by $PGF_{2\alpha}$ was stable enough for the period required to complete NaHS induced relaxant responses (data not shown).

2.2. Experimental protocol

The experimental procedures detailed below are summarized in flow chart (Fig. 1).

2.2.1. Investigation of the role of extracellular Ca^{2+}, Ca^{2+} sensitivity and intracellular Ca^{2+} reducing mechanisms

To evaluate the role of extracellular Ca^{2+} on NaHS induced

relaxations, isolated bovine retinal arteries were incubated in Ca^{2+} free medium for 30 min. [20] and increasing concentrations of NaHS (10^{-5}–3×10^{-3}M) were administered on retinal arteries precontracted with $PGF_{2\alpha}$ (3×10^{-5}M).

To assess the influence of NaHS on vascular smooth muscle Ca^{2+} sensitivity, the concentration-dependent contractions to $CaCl_2$ (10^{-5}–10^{-2} M) [20] were obtained in retinal arteries that incubated in Ca^{2+} free medium (30 min), depolarized with KCl (K^+; 20 mM; 20 min.) and pretreated with NaHS (3×10^{-3}M, 20 min). In addition, NaHS (10^{-5}–3×10^{-3}M) induced relaxations were also determined in the arteries precontracted with $CaCl_2$ (10^{-2} M) in Ca^{2+} free medium.

In order to verify the possible role of intracellular Ca^{2+} reducing mechanisms, the relaxant responses to NaHS (10^{-5}–3×10^{-3}M) were obtained in retinal arteries both in the absence (control) and presence of several related inhibitors, namely, plasmalemmal Ca^{2+}-ATPase inhibitor, sodium orthovanadate (10^{-3} M, 30 min) [23], sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA; 10^{-6} M, 30 min) [20] and thapsigargin (2×10^{-6}M, 45 min) [23] and Na^+-Ca^{2+} ion exchanger inhibitor, 1,3-dimethyl-2-thiourea (DMTU; 2.5×10^{-2} M, 20 min) and amiloride (10^{-4} M, 20 min) [23]. In these experiments, the relaxations to NaHS were compared with that of the corresponding responses obtained in the absence of these inhibitors (control).

2.2.2. Investigation of the role of MLCK and Ca^{2+} sensitization mechanisms

In order to assess the role of MLCK, the relaxation responses to NaHS (10^{-5}–3×10^{-3}M) were determined in the absence (control) and presence of MLCK inhibitor, ML-9 (10^{-5} M, 20 min) [24]. In order to identify the role of Ca^{2+} sensitization mechanisms, the relaxation responses to NaHS (10^{-5}–3×10^{-3}M) were determined in the absence (control) and the presence of protein kinase C (PKC) inhibitor, calphostin C (10^{-6} M, 20 min) [25], Rho kinase inhibitor, Y-27632 (10^{-6} M, 20 min) [26,27], tyrosine kinase (TK) inhibitor, tyrphostin 23 (10^{-4} M, 20 min) [28] and MLCP inhibitor, calyculin A (10^{-7} M, 20 min) [20,29].

2.2.3. RT-qPCR analysis

To determine whether the enzymes, Rho kinase (ROCK-II) and MLCP are expressed or not in bovine retina and/or retinal arteries, RT-qPCR analysis were performed. We also aimed to evaluate whether $PGF_{2\alpha}$ (10^{-4} M, 20 min) or $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) treatments influenced mRNA expression levels of these enzymes. Thus, mRNA expression levels of Rho kinase (ROCK-II) and MLCP were quantified in the retina and retinal arteries of the control, $PGF_{2\alpha}$ (10^{-4} M, 20 min) treated and $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) treated groups by RT-qPCR analysis. Total RNA from bovine retinas and retinal arteries were isolated with RNAzol® (MRC-Molecular Research Center, Cincinnati, USA). Isolated RNA (1 μg) was used to synthesize cDNA by using Fermentas, RevertAid First Strand cDNA Synthesis Kit (Roche). Reversibly transcribed cDNA was amplified by Real-time quantitative polymerase chain reaction (qPCR) under standard conditions using SYBR Green system (Bio-Rad). All data of threshold Cycle (Ct) were normalized by Ct of house-keeping gene β-actin and expressed as fold change. To determine the level of expression, the differences (Δ) between the threshold cycles (Ct) were measured. Fold change was calculated as $2^{-(\Delta\Delta Ct)}$. After the $2^{-(\Delta\Delta Ct)}$ values were calculated, mRNA expression changes (as increase or decrease) between the experimental groups were compared for significance in control, $PGF_{2\alpha}$ and $PGF_{2\alpha}$ + NaHS groups. All values given were representative of at least three duplicated independent experiments. The samples were collected from different 50 retinal arteries and 30 retinas isolated from the bovine eyes. The samples were harvested in pool. The following primers were used: bovine ROCK-II: forward; 5′-GCTCGAGAGAAGGCTGAAAA-3′, reverse; 5′-CGCTTGTTTGATTCCT GCT-3′; bovine myosine phosphatase: forward; 5′-CCAGTCCCAGACTT TCCTCC-3′, reverse; 5′-TCAGATGTACTGGCAAGCCG-3′; bovine β-actin: forward; 5′-TAGCACAGGCCTCTCGCCTTCGT-3′, reverse; 5′-GCACATG CCGGAGCCGTTGT-3′.

2.2.4. Western blot analysis

To evaluate the role of Ca^{2+} sensitization mechanisms in the relaxant effects of NaHS, protein expression levels of possible signaling pathways were measured in retinal arteries. By using Western blot analysis, the role of RhoA/Rho kinase and CPI-17 pathways as well as MLCP were determined in the control, $PGF_{2\alpha}$ (10^{-4} M, 20 min) treated and $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) treated retinal arteries. For the evaluation of RhoA/Rho kinase pathway, RhoA and Rho kinase (ROCK II) protein levels were determined whereas, for CPI-17 pathway, the phosphorylation levels of CPI-17 and total CPI-17 and, for MLCP activity, the phosphorylation levels of myosin phosphatase target subunit-1 (MYPT-1) at Thr696 and total MYPT1 were determined in isolated bovine retinal arteries. Retinal arteries were homogenized with RIPA buffer and protease inhibitory cocktail (Santa Cruz, Dallas, Texas, USA) and then centrifuged. Following centrifugation, protein concentrations of supernatants were determined and equal amount of proteins were seperated by 7–10% SDS-PAGE and blotted to PVDF membranes. Protein loaded membranes were blocked with 5% non-fat dried milk and then, incubated overnight with primary antibodies (RhoA, ROCK-II, p-CPI-17, total CPI-17, p-MYPT, total MYPT1, β-actin, 1/1000 dilution rate) followed by incubation for 1 h with appropriate secondary antibody (1/10,000 dilution rate) conjugated with horseradish peroxidase. β-Actin was used as an internal loading control. Enhanced chemiluminescence was used to visualize protein bands on the membrane. The quantification of protein band intensity was analyzed using ImageJ 1.50i software program. The measured optical density value of the protein of interest was normalized by dividing by the beta-actin density value. Thus, the relative density of each protein was calculated. After the relative densities were calculated, protein expression changes (as increase or decrease) between the experimental groups were compared for significance in control, $PGF_{2\alpha}$ and $PGF_{2\alpha}$ + NaHS treated groups. All values given were representative of three independent experiments.

2.3. Chemicals

The chemicals used in myograph experiments were purchased from Sigma Chemical Co. (Taufkirchen, Germany) except for ML-9 hydrochloride (Tocris, Bristol, United Kingdom), calyculin A (MP Biomedicals, California, USA) and tyrphostin 23 (TRC-Toronto Research Chemicals, Toronto, Canada). RIPA buffer and protease inhibitory cocktail, secondary antibodies and primary antibodies for RhoA (Santa Cruz Biotechnology Cat# sc-179-G, RRID: AB_632346), ROCK-II (Santa Cruz Biotechnology Cat# sc-365275, RRID: AB_10844331), β-actin (Santa Cruz Biotechnology Cat# sc-47778 HRP, RRID: AB_2714189)) used in Western Blot analysis were purchased from Santa Cruz (USA) except for p-MYPT (Millipore Cat# ABS45, RRID: AB_11212365), p-CPI-17 (Millipore Cat# 36-006, RRID: AB_310815), CPI-17 (Millipore Cat# 07-344, RRID: AB_310537) and MYPT (Thermo Fisher Scientific Cat# PA5-17164, RRID: AB_10978517).

ML-9, cyclopiazonic acid, thapsigargin were dissolved in dimethyl sulfoxide (DMSO), calphostin C and calyculin A were dissolved in ethanol and, tyrphostin 23 was dissolved in acetone. Whereas, NaHS and all other drugs were dissolved in distilled water. Dilutions of NaHS were prepared freshly on the day of each experiment and protected from light. Noradrenaline was dissolved in 0.001 N HCl and ascorbic acid (1 mg/ml) was added to avoid oxidation. The final concentrations of DMSO, ethanol and acetone in the organ bath did not exceed 0.1% and all these solvents were determined to have no direct influence on the contractile tone as well as on the relaxant responses.

2.4. Statistical analyses

The data are shown as "mean ± S.E.M." and "n" is the number of bovine retinas and the retinal arteries used in the experiments. The contraction responses to contractile agents are expressed as "mN/mm", and the relaxation responses to NaHS, Ach and SNP are indicated as percent decreases of the precontraction evoked by the spasmogen ($PGF_{2\alpha}$ or $CaCl_2$). The maximal relaxation or contraction responses to vasoactive agents are expressed as E_{max}. The sensitivities of the retinal arteries to contractile and relaxant agents were calculated as the effective concentration that elicit 50% of the maximal response (EC_{50}) by using non-linear regression curve fit and expressed as -log M (pEC_{50}). Statistical analyses were determined by Student's paired and unpaired *t*-tests as well as by repeated measures or one-way analysis of variance (ANOVA) followed by Tukey Kramer *post hoc* analysis, as appropriate. A "*p*" value less than "0.05" was considered statistically significant.

3. Results

The pre-contraction levels with $PGF_{2\alpha}$ was comparable between control and inhibitor groups for all vessels and they started from the same baseline (+Inhibitors: 1.14 ± 0.09 mN/mm *vs* control: 1.36 ± 0.09 mN/mm, n = 51; $p < 0.05$).

Endothelium-dependent vasodilator, Ach (10^{-8} M–10^{-4} M) and endothelium-independent vasodilator, SNP (10^{-8} M–10^{-4} M) produced moderate relaxations (E_{max} for Ach: 34.45 ± 4.72% and E_{max} for SNP: 44.48 ± 2.80%, n = 10) on isolated bovine retinal arteries precontracted with $PGF_{2\alpha}$ (3×10^{-5}M). The moderate relaxation responses to Ach and SNP suggested that NO cannot be considered as potent relaxant in retinal artery.

3.1. The influence of extracellular Ca^{2+} removal, Ca^{2+} sensitivity and Ca^{2+} reducing mechanisms on NaHS induced relaxation

The relaxant responses of NaHS (10^{-5}-3×10^{-3}M) on $PGF_{2\alpha}$ (3×10^{-5}M) precontracted retinal arteries were found comparable either in the presence or absence of Ca^{2+} in Krebs-Ringer bicarbonate solution (Table 1). In Ca^{2+} free medium, cumulative administration of $CaCl_2$ (10^{-5}–10^{-2} M) elicited concentration-dependent contractions on isolated bovine retinal arteries depolarized with K^+ (Fig. 2). Pretreatment of the retinal arteries with NaHS (3×10^{-3}M; 20 min) significantly reduced the contractions to $CaCl_2$ (+NaHS E_{max}: 0.20 ± 0.05 mN/mm *vs* control E_{max}: 0.45 ± 0.05 mN/mm, n = 11; $p < 0.05$) but did not produce a significant difference in EC_{50} values (+NaHS pEC_{50}: 3.59 ± 0.26 *vs* control pEC_{50}: 3.46 ± 0.16; n = 11, $p > 0.05$). Moreover, NaHS (10^{-5}–3×10^{-3}M) elicited prominent relaxations on retinal arteries precontracted with $CaCl_2$ (10^{-2} M) in Ca^{2+} free medium (E_{max}: 71.41 ± 4.24%; pEC_{50}: 4.02 ± 0.13, n = 9) (Fig. 2).

In relation to intracellular Ca^{2+} reducing mechanisms, incubation of isolated bovine retinal arteries with Na^+-ortovanadate (10^{-3} M, 30 min) did not produce a statistically significant difference in NaHS induced relaxation (+Na^+-ortovanadate; E_{max}: 42.41 ± 5.30%, pEC_{50}: 2.89 *vs* corresponding control, $p > 0.05$, n = 10) (Fig. 3). Similar results were obtained in the presence of sarcoplasmic reticulum Ca^{2+}-ATPase inhibitors; CPA (10^{-6} M, 30 min) or thapsigargin (2×10^{-6}M, 45 min) (+CPA E_{max}: 45.41 ± 5.30%, pEC_{50}: 2.92 n = 8; +thapsigargin E_{max}: 47.39 ± 7.15%, n = 9, pEC_{50}: 3.01 *vs* corresponding control, $p > 0.05$). Likewise, Na^+-Ca^{2+} ion exchanger inhibitors DMTU (2.5×10^{-2}M, 20 min) or amiloride (10^{-4} M, 20 min) did not significantly modify the relaxing response to NaHS (+DMTU E_{max}: 48.41 ± 6.24%, n = 8; +amiloride E_{max}: 46.39 ± 4.45%, pEC_{50}: 2.84 and 2.90, n = 7 *vs* corresponding control, $p > 0.05$) (Fig. 3).

These results thought that the relaxation responses to NaHS, were unrelated to extracellular and intracellular Ca^{2+} levels.

Table 1
The maximum relaxation responses (E_{max}) and pEC_{50} values of NaHS (10^{-5}–3×10^{-3}M) obtained in $PGF_{2\alpha}$ (3×10^{-5}M) precontracted retinal arteries that incubated either in Ca^{2+} containing (+) or in Ca^{2+} free (−) medium.

NaHS induced relaxation			
	E_{max} (%)	pEC_{50} (−log M)	n
Ca^{2+}(+)	64.38 ± 5.74	4.16 ± 0.16	6
Ca^{2+}(−)	73.73 ± 10.13	4.17 ± 0.08	6

$p > 0.05$, when compared the values obtained in Ca^{2+} containing (+) and Ca^{2+} free (−) conditions.

3.2. The effects of the inhibitors of MLCK, Ca^{2+} sensitization mechanisms and MLCP

The relaxation response to NaHS did not change in the presence of the MLCK inhibitor, ML-9 (+ML-9; E_{max}: 62.75 ± 9.05%, pEC_{50}: 4.55 ± 0.13 *vs* corresponding control, $p > 0.05$, n = 10) (Fig. 4). In relation to Ca^{2+} sensitization mechanisms, the maximum relaxations to NaHS obtained either in the presence of Rho kinase inhibitor, Y-27632, protein kinase C inhibitor, calphostin C or tyrosine kinase inhibitor, tryphostin 23 were found comparable to their corresponding controls (+Y-27632, E_{max}: 70.56 ± 2.86%, pEC_{50}: 4.07 ± 0.10 n = 7; +Calphostin C, E_{max}: 44.72 ± 6.88%, pEC_{50}: 2.40 ± 0.05, n = 8; and +Tyrphostin 23, E_{max}: 63.01 ± 10.23%, pEC_{50}: 3.01 ± 0.11 n = 8 *vs* corresponding controls, $p > 0.05$). While, in the presence of the MLCP inhibitor, calyculin A, the maximum relaxation response to NaHS on bovine retinal arteries was considerably decreased (+Calyculin A, E_{max}: 12.45 ± 5.92% *vs* corresponding control, n = 7, $p < 0.05$) (Fig. 4).

The decrease in relaxation responses to H_2S in the presence of MLCP inhibitor and the uneffectivenes of Rho kinase, PKC or TK inhibitors to H_2S relaxation were thought that H_2S induces relaxation directly by dephosphorylation of MLC_{20} without using Rho kinase, PKC or TK pathways.

3.3. mRNA expression levels of ROCK-II and MLCP

ROCK-II enzyme was found to be expressed in both bovine retina and the retinal artery (Table 2). Stimulation of the retinal artery either with $PGF_{2\alpha}$ (10^{-4} M, 20 min) or $PGF_{2\alpha}$ (10^{-4} M) + NaHS (10^{-3} M, 20 min) did not significantly modify ($p > 0.05$, n = 3) ROCK-II mRNA expression levels compared to control ($2^{-(\Delta\Delta Ct}$, Table 2).

Similar findings were also observed in the mRNA expression levels of MLCP enzyme. It was found to be expressed in both bovine retina and the retinal artery. In retinal artery, mRNA expression levels of MLCP ($p > 0.05$, $n = 3$) in between $PGF_{2\alpha}$ (10^{-4} M, 20 min) and $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) pretreated groups were similar and both were found comparable to control ($2^{-(\Delta\Delta Ct)}$, Table 2).

Based on myograph data, relaxation response was related to MLCP activitiy and Rho kinase was also an important pathway of calcium sensitivity, Rho kinase and MLCP transcription levels were investigated with RT-PCR. The results indicated that these enzymes expressed in both retina and retinal artery. However, neither contraction with $PGF_{2\alpha}$ nor relaxation response to NaHS change the expression levels of the enzymes.

3.4. Protein expressions of Rho A, Rho kinase, p-CPI-17 and p-MYPT-1

3.4.1. Rho A protein levels

Rho A protein levels in retinal arteries pretreated with $PGF_{2\alpha}$ (10^{-4} M, 20 min) were found significantly higher than the control arteries (+$PGF_{2\alpha}$: 0.68 ± 0.09 *vs* corresponding control: 0.56 ± 0.07,

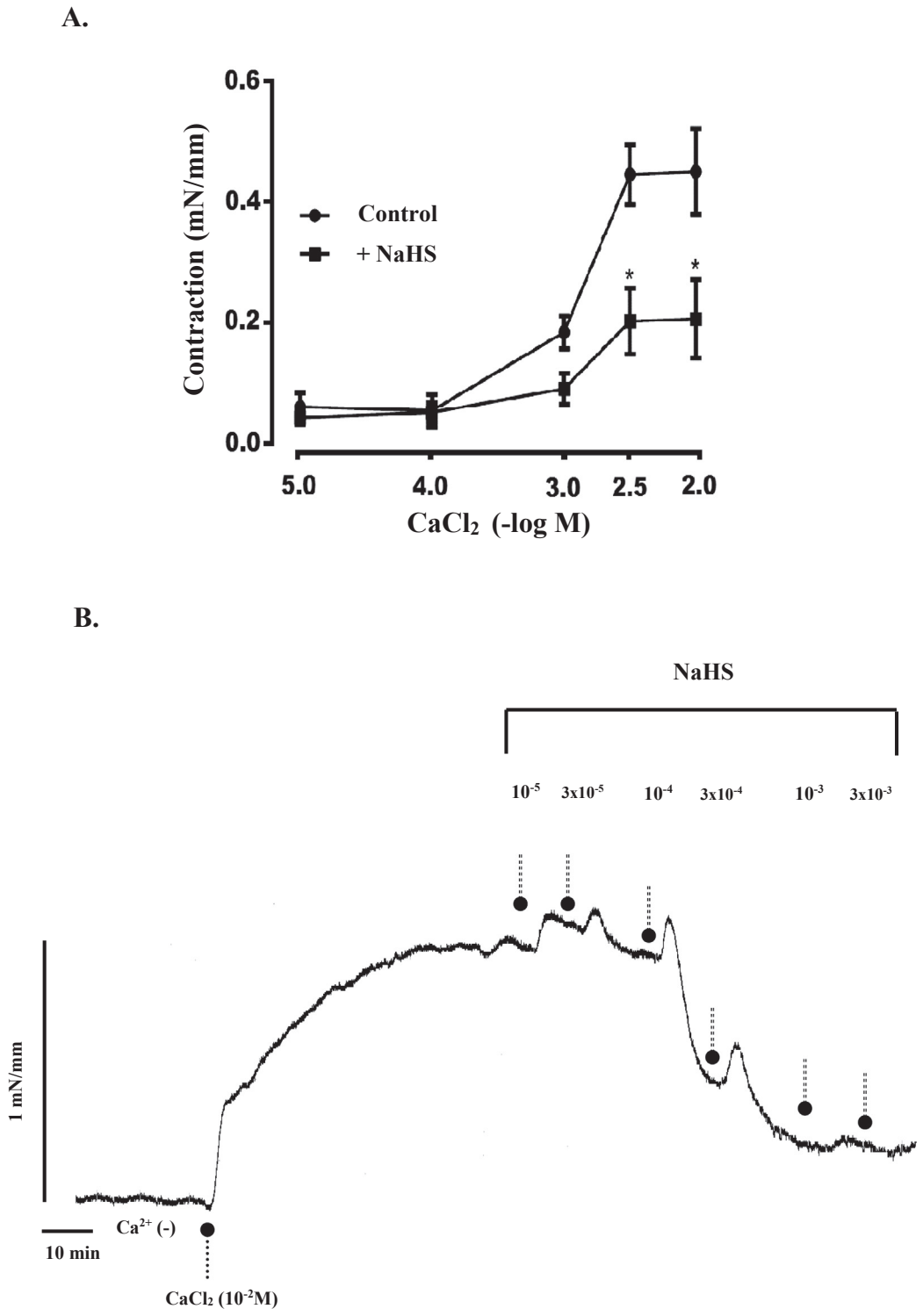


Fig. 2. (A) The effect of NaHS (3×10^{-3}M) pretreatment on the concentration-dependent contraction responses of $CaCl_2$ (10^{-5}–10^{-2} M) obtained in Ca^{2+} free medium, n = 11, $^*p < 0.05$ compared to the corresponding control. (B) An original tracing that demonstrates the substantial relaxations to NaHS (10^{-5}–3×10^{-3}M) on retinal arteries precontracted with $CaCl_2$ (10^{-2} M) in Ca^{2+} free medium.

$n = 3$, $p < 0.05$). However, no statistically significant difference was observed in Rho A protein levels of $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) pretreated group compared to either $PGF_{2\alpha}$ pretreated or the control group ($PGF_{2\alpha}$ + NaHS: 0.60 ± 0.06 *vs* + $PGF_{2\alpha}$ or control groups, $p > 0.05$) (Fig. 5).

3.4.2. ROCK-II protein levels

Rho kinase (ROCK-II) protein levels of the retinal arteries in $PGF_{2\alpha}$ (10^{-4} M, 20 min) and $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) pretreated groups were found statistically significantly higher than the control group (+$PGF_{2\alpha}$: 0.64 ± 0.11; +$PGF_{2\alpha}$ + NaHS: 0.64 ± 0.08 *vs* control: 0.30 ± 0.05, $n = 3$, $p < 0.05$). Whereas, ROCK-II protein levels between $PGF_{2\alpha}$ and $PGF_{2\alpha}$ + NaHS groups were

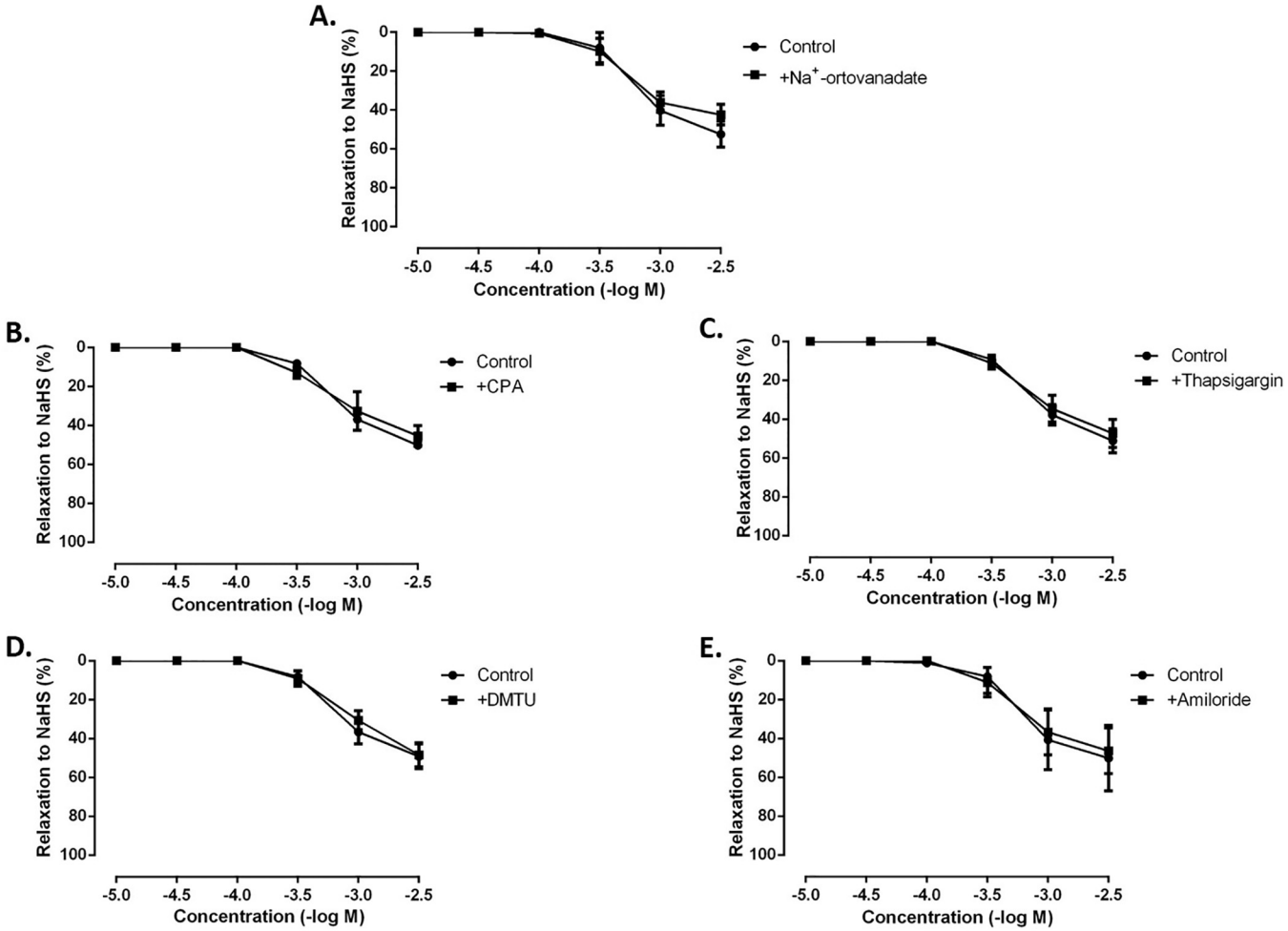


Fig. 3. Concentration-dependent relaxation responses of NaHS (10^{-5}–3×10^{-3}M) obtained in $PGF_{2\alpha}$ (3×10^{-5} M) precontracted bovine retinal arteries either in the presence of (A) plasmalemmal Ca^{2+}-ATPase inhibitor, sodium orthovanadate (Na_3VO_4; 10^{-3} M, n = 10), (B) sarco/endoplasmic reticulum ATPase inhibitors cyclopiazonic acid (CPA; 10^{-6} M, n = 8) and thapsigargin (2×10^{-6}M, n = 9), or (C) Na^+-Ca^{2+} ion exchanger pump inhibitors, 1,3-dimethyl-2-thiourea (DMTU; 2.5×10^{-2}M, n = 8) and amiloride (10^{-4} M, n = 7). Statistically significance level is $p > 0.05$ compared to corresponding controls.

found similar ($p > 0.05$) (Fig. 5).

3.4.3. p-CPI-17 protein levels

The phosphorylated protein levels of CPI-17 in bovine retinal arteries pretreated with $PGF_{2\alpha}$ (10^{-4} M, 20 min) or $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) were not found statistically significantly different from the control group (+$PGF_{2\alpha}$: 1.23 ± 0,81; +$PGF_{2\alpha}$ + NaHS: 1.27 ± 0.76 *vs* control: 0.61 ± 0.31, n = 3 $p > 0.05$). Likewise, p-CPI-17 protein levels between $PGF_{2\alpha}$ and $PGF_{2\alpha}$ + NaHS groups were found similar ($p > 0.05$) (Fig. 5).

3.4.4. p-MYPT protein levels

The phosphorylated protein levels of MYPT-1 (p-MYPT) in $PGF_{2\alpha}$ (10^{-4} M, 20 min) pretreated retinal arteries were found comparable to the control group (+$PGF_{2\alpha}$: 0.60 ± 0.09 *vs* control: 0.60 ± 0.15, n = 3 $p > 0.05$). However, p-MYPT protein levels in $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) pretreated retinal arteries were found significantly decreased compared to $PGF_{2\alpha}$ treated and the control group (+$PGF_{2\alpha}$ + NaHS: 0.32 ± 0.10 *vs* + $PGF_{2\alpha}$: 0.60 ± 0.09 or control group: 0.60 ± 0.15, n = 3 $p < 0.05$) (Fig. 5).

Western blot analysis showed that Rho A, Rho kinase, CPI-17 and MYPT are expressed in retinal artery at protein levels. The decrement, observed in phosphorylation levels of MYPT-1 and activation of MLCP which dephosphorylate MLC_{20} suggested to be responsible from H_2S induced relaxation responses. Moreover, Rho A/Rho kinase have role in $PGF_{2\alpha}$ induced contraction and phosphorylation of MYPT-1 subunit which seems to be responsible for maintaining the basal tone of retinal artery.

4. Discussion

Vascular tone is known to be regulated by Ca^{2+}-dependent and -independent mechanisms [14]. Previous studies suggested that H_2S could modulate Ca^{2+} signals in the vascular system. Stimulation of Ca^{2+} influx by Na^+-Ca^{2+} exchanger [30] and L-type Ca^{2+} channels [31,32] and activation of SERCA [5] have been reported to mediate the effects of H_2S in different vessels. While, in retinal arteries the substantial relaxation to NaHS, a H_2S donor, was insensitive to inhibition by L-type voltage-dependent Ca^{2+} channels [12]. Herein, we evaluated other effective mechanisms regulating intracellular Ca^{2+} levels including, plasmalemmal Ca^{2+}-ATPase, SERCA and Na^+-Ca^{2+} exchanger by using their specific inhibitors. However, none of them changed the relaxation profile to NaHS suggesting that H_2S induced relaxation is insensitive to modulation of intracellular Ca^{2+} levels in retinal artery. As is known, increased intracellular Ca^{2+} concentrations trigger Ca^{2+}-calmodulin binding and activation of MLCK to initiate smooth muscle contraction *via* phosphorylation of MLC_{20} [14,16]. Herein, the relaxation response to NaHS was unmodified by MLCK inhibition which further confirms that changes in intracellular Ca^{2+} concentration do not mediate this relaxation and suggests the involvement of Ca^{2+}

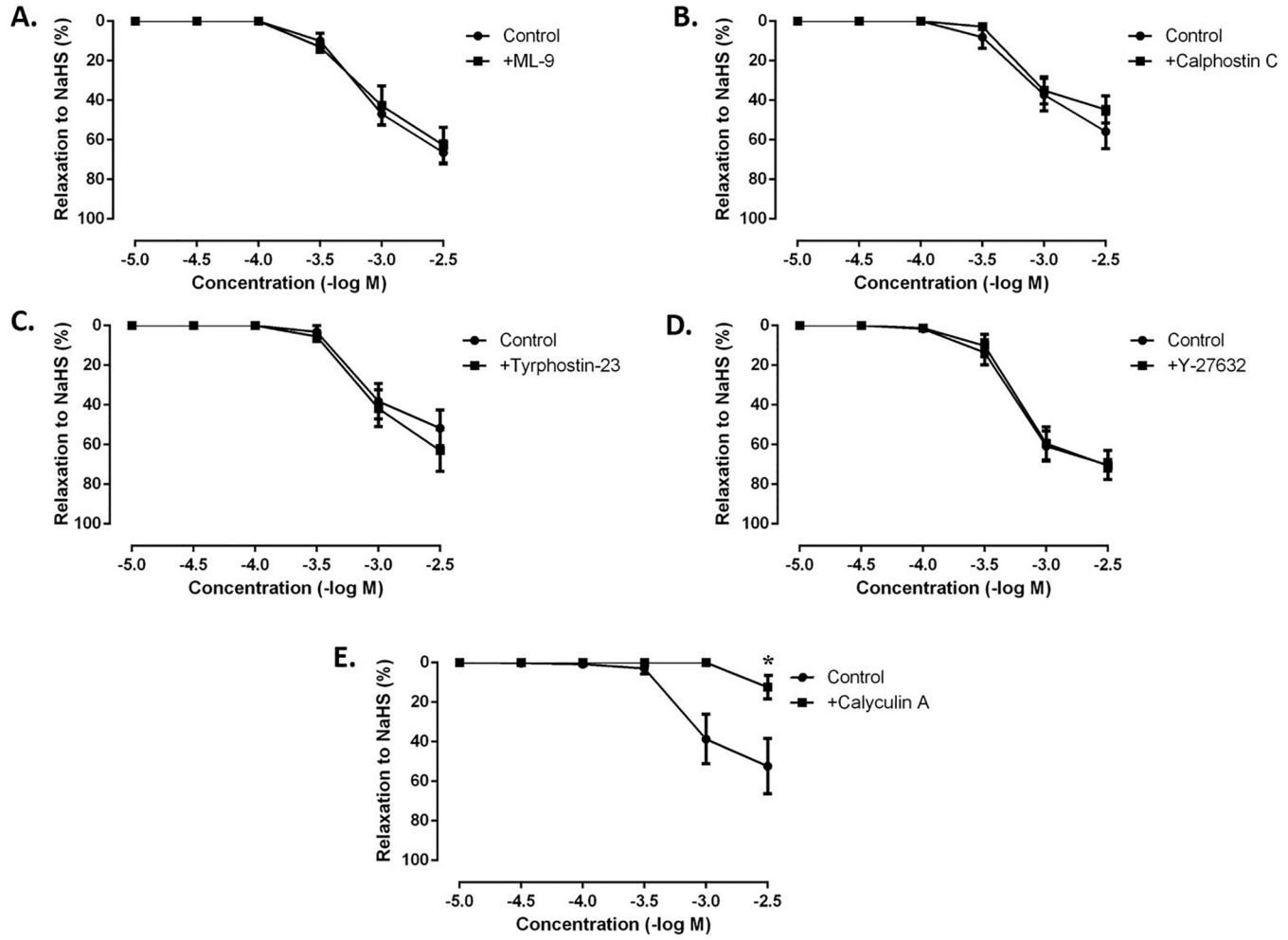


Fig. 4. Concentration-dependent relaxation responses of NaHS (10^{-5}–3×10^{-3}M) obtained in $PGF_{2\alpha}$ (3×10^{-5} M) precontracted bovine retinal arteries that pretreated with either of the following inhibitors, (A) MLCK inhibitor, ML-9 (10^{-5} M, n = 10), (B) protein kinase C inhibitor, Calphostin C (10^{-6} M, n = 8), (C) Rho kinase inhibitor, Y-27632 (10^{-6} M, n = 7), (D) tyrosine kinase inhibitor, tyrphostin-23 (10^{-4} M, n = 8), $p > 0.05$, (E) MLCP inhibitor, Calyculin A (10^{-7} M, n = 7), $^{*}p < 0.0001$ compared to the corresponding control.

Table 2
mRNA expression levels of Rho kinase (ROCK-II) and myosin light chain phosphatase (MLCP) in the control, and + $PGF_{2\alpha}$ (10^{-4} M) pretreated retinas as well as control, + $PGF_{2\alpha}$ (10^{-4} M, 20 min) and PGF_{2a} + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) pretreated retinal arteries.

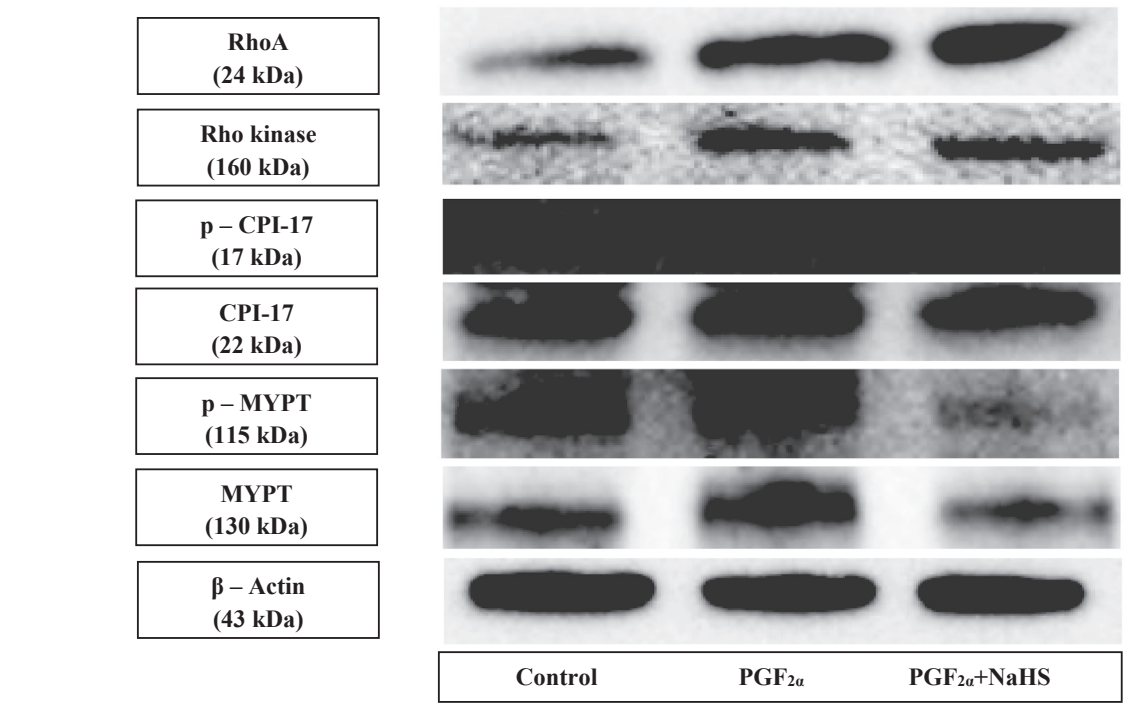
mRNA expression level		
	ROCK-II	MLCP
Retina		
Control	1	1
+ PGF_{2a}	1.41	1.13
Retinal artery		
Control	1	1
+ PGF_{2a}	0.62	2.16
+ PGF_{2a} + NaHS	0.88	1.23

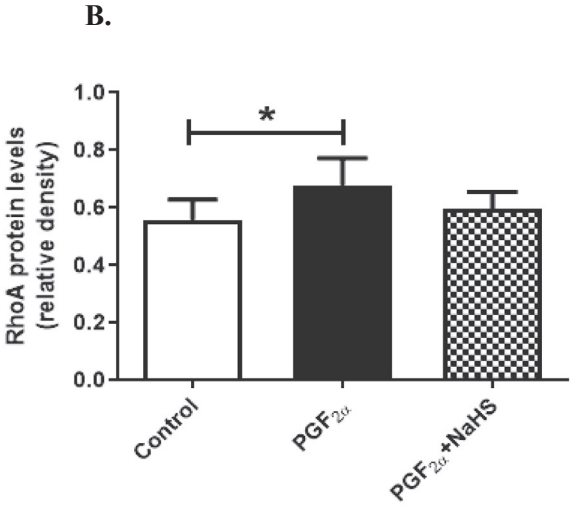
ROCK-II $p = 0.2089$, MLCP $p = 0.2290$, one-way ANOVA, n = 3. mRNA expression levels of ROCK-II and MLCP were compared by using $2^{-(\Delta\Delta Ct)}$ values of the groups.

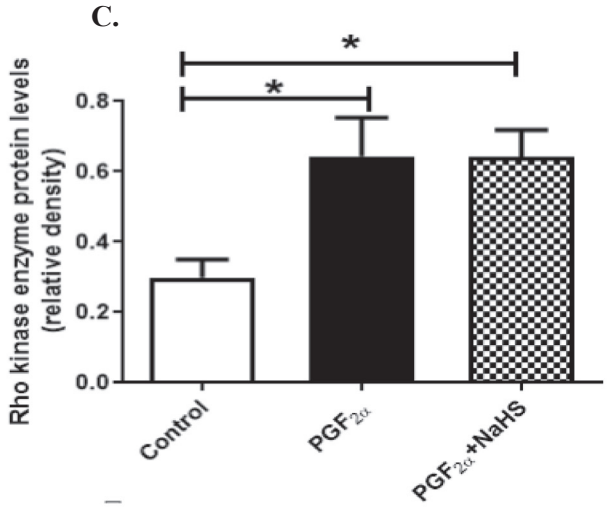
sensitization mechanisms. Of note, NaHS induced relaxations were fully apparent in Ca^{2+}-free medium and pretreatment of retinal arteries with NaHS markedly reduced contractions to $CaCl_2$. In parallel, NaHS elicited substantial relaxations in $CaCl_2$ contracted arteries. These findings reveal that, NaHS induced relaxation is partly associated with a decrement in Ca^{2+} sensitivity of the contractile apparatus in bovine retinal arteries.

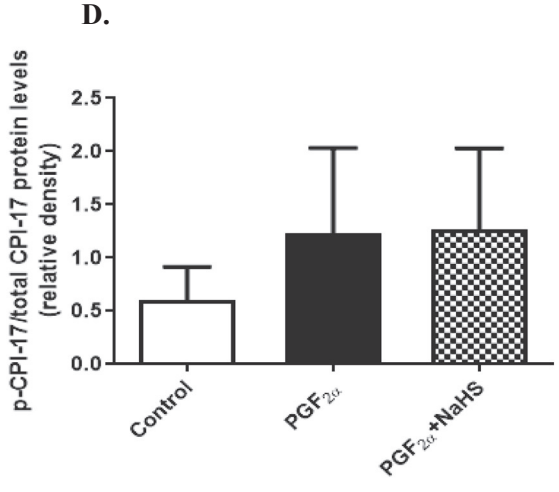
Ca^{2+} sensitization refers to smooth muscle contraction without changing of intracellular Ca^{2+} concentrations, but through the inhibition of MLCP to cause MLC_{20} phosphorylation and activation of myosin adenosine triphosphatase [15,16]. Several kinase pathways are linked to inhibition of MLCP and initiation of Ca^{2+} sensitization in the contractile apparatus [17,18]. MLCP inhibition is initiated mainly *via* three mechanisms, *i.e.*, phosphorylation of myosin phospatase target subunit (MYPT1) [33], phosphorylation of CPI-17 (type 1 protein phosphatase inhibitory protein) which in turn phosphorylates PP1c, the catalytic subunit of MLCP [34] and modification of the heterotrimeric structure of MLCP [35].

Protein kinase C (PKC) is activated by several vasoconstrictor substances which phosphorylate CPI-17 by acting on their receptors and in turn, inhibit MLCP and enhance myofilament force sensitivity to Ca^{2+} [36]. The role of PKC pathway in NaHS induced relaxation was investigated by using its selective inhibitor, Calpostin C as well as by determining the phosphorylated CPI-17 protein levels in $PGF_{2\alpha}$ + NaHS pretreated retinal arteries. No change was apparent in NaHS induced relaxation in the presence of Calpostin C, and p-CPI-17 protein levels were found similar in $PGF_{2\alpha}$ + NaHS pretreated arteries compared to $PGF_{2\alpha}$ pretreated and control arteries. These findings exclude the contribution of PKC pathway and CPI-17 dephosphorylation in the relaxing mechanism of NaHS in bovine retinal artery. Opposing results were









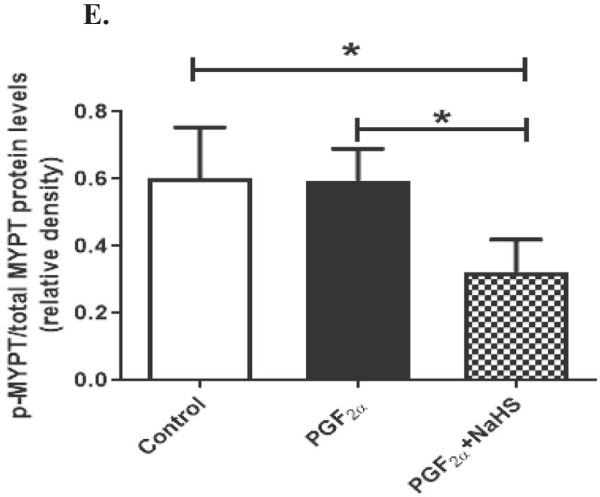


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Fig. 5. (A) Representative protein bands of RhoA, rho kinase (ROCK-II), phospho CPI-17, total CPI-17, phospho MYPT (p-MYPT), total MYPT and internal control β-actin obtained from protein extracts of retinal arteries in control, $PGF_{2\alpha}$ and $PGF_{2\alpha}$ + NaHS groups by using Western blot analysis. The comparison of the relative densitometric values of (B) RhoA, (C) Rho kinase (ROCK-II) (D) p-CPI-17/total CPI-17, and (E) p-MYPT/total MYPT in the control, $PGF_{2\alpha}$ (10^{-4} M, 20 min) and $PGF_{2\alpha}$ + NaHS (10^{-4} M, 20 min + 10^{-3} M, 20 min) groups. A prominent decrease was observed in the staining of p-MYPT bands in $PGF_{2\alpha}$ + NaHS pretreated group compared to $PGF_{2\alpha}$ treated and the control group, $^{*}p < 0.05$, n = 3.

noticed in NaHS and endogenous H_2S induced relaxations in rabbit stomach smooth muscles, which displayed the stimulation of MLCP activity partly through the inhibition of PKC and phosphorylation of CPI-17. This difference possibly relies on the type of smooth muscle and the precontractile agent used, *i.e.*, carbachol, which increased PKC-sensitive CPI-17 phosphorylation [37] unlike to $PGF_{2\alpha}$ determined here in retinal artery.

RhoA/Rho kinase is other substantial pathway to inhibit MLCP and provide Ca^{2+} sensitization [18]. In this study, RhoA and Rho kinase protein levels were found increased in retinal arteries pretreated with $PGF_{2\alpha}$. This indicates that Ca^{2+} sensitization *via* RhoA/Rho kinase activation is involved in $PGF_{2\alpha}$ induced contraction which correlates with a previous report in rabbit aorta [38]. While, RhoA and Rho kinase protein levels in $PGF_{2\alpha}$ + NaHS pretreated retinal arteries were found similar to that of pretreated with $PGF_{2\alpha}$. Moreover, mRNA expressions of Rho kinase isoform ROCK-II were defined in both retina and the retinal artery, in regard to previous documentations in other vascular and nonvascular smooth muscles [36,39]. This is the first time expression of ROCK-II has been demonstrated in these tissues. This expression provides support of a role for ROCK-II in the contractile mechanism of retinal artery, while, we did not observe a difference in expression levels detected in $PGF_{2\alpha}$ + NaHS and $PGF_{2\alpha}$ pretreated arteries. Notably, the relaxation response to NaHS was also unchanged in the presence of Rho kinase inhibitor, Y-27632. Considering these data, we suggest that inhibition of RhoA/Rho kinase do not play a role in the relaxing mechanism of NaHS in retinal artery. Of note, the majority of previous studies evaluating the relaxing effects of endogenous (L-cystein) or exogenous H_2S (NaHS) were on nonvascular tissues and presented divergent results on the role of Rho kinase pathway [37,40–43]. One vascular study was noticed reporting Rho kinase activation but was related to contraction induced by NaHS [32].

Tyrosine kinase contributes to Ca^{2+} sensitization of smooth muscle by phosphorylating and activating GEFs (guanine nucleotide exchange factor) which are required for the activation of RhoA and Rho kinase [14,18]. Unchanged relaxations in the presence of protein tyrosine kinase inhibitor, Tryphostin 23 are likely to eliminate the inhibitory influence of NaHS on tyrosine kinase pathway in retinal artery.

MLCP inhibition is recognized as the major mechanism of Ca^{2+} sensitization [18]. Considerable reduction in NaHS induced relaxation in the presence of MLCP inhibitor, Calyculin A, as well as the significant decrease in p-MYPT protein levels in arteries pretreated with $PGF_{2\alpha}$ + NaHS indicated for the first time that NaHS, *i.e.*, H_2S, dilates retinal artery mainly *via* the activation of MLCP which refers to Ca^{2+} desensitization. Despite of one contrary evidence on MYPT1 dephosphorylation in rat mesenteric artery [44], no data is available in literature regarding to the inhibitory influence of NaHS or H_2S on Ca^{2+} sensitization of vascular smooth muscle contraction. Whereas, our findings are consistent with various studies on nonvascular smooth muscles such as mouse gastric fundus [40], rabbit gastric smooth muscle [37] and rat jejunum circular muscle [45] which indicated MLCP activation and Ca^{2+} desentitization in NaHS induced relaxation.

MYPT1 phosphorylation at Thr^{696} is the basic site for the inhibition of MLCP activity and Ca^{2+} sensitization. While, phosphorylation of Thr^{853} site may also be responsible, in conjunction with Thr^{696}, as documented for Rho kinase activation [17,46]. In this study, Thr^{696} phosphorylation was evident in retinal arteries at resting (control) conditions which raises the possibility that MLCP activity is constitutively inhibited at Thr^{696} phosphrylation, at least in part, regardless of the contractile state. This inhibition may have an impact in the regulation of basal tone of retinal artery. Thereby, the significant reduction in Thr^{696} phosphorylation in $PGF_{2\alpha}$ + NaHS pretreated arteries compared to control may imply a regulatory role for H_2S in the maintenance of basal tone in retinal artery. Moreover, we noticed that MYPT1 phosphorylation at Thr^{696} in $PGF_{2\alpha}$ pretreated retinal arteries were similar to basal (control) levels. Considering increased RhoA and Rho kinase protein expressions in $PGF_{2\alpha}$ pretreated arteries, it is reasonable to suggest that $PGF_{2\alpha}$ induced contraction and RhoA/Rho kinase related MLCP inhibition may be associated with the phosphorylation of Thr^{853} site, rather than Thr^{696}, in retinal artery. Supportively, previous studies reported an increased phosphorylation at Thr^{853} site or an unchanged Thr^{696} phosphorylation over basal levels after stimulation with contractile agonists in vascular and nonvascular tissues [47,48]. In this regard, phosphorylation of Thr^{853} in $PGF_{2\alpha}$ induced contraction and whether dephosphorylation at Thr^{853} regulates MLCP activity and mediates the relaxation to NaHS, in conjunction with Thr^{696}, remains to be determined in retinal artery.

5. Conclusion

Ca^{2+} signaling mechanisms including plasmalemmal Ca^{2+}-ATPase, SERCA, Na^{+}-Ca^{2+} exchanger and MLCK do not seem to play a role in the substantial relaxations to NaHS in bovine retinal artery, while, the inhibition of Ca^{2+} sensitivity of contractile proteins was notable. NaHS induced relaxations were considerably decreased by the inhibition of MLCP. Additively, the significant reduction in MYPT1 phophorylation at Thr^{696} reinforced the role of MLCP activation and Ca^{2+} desensitization in NaHS induced relaxation in bovine retinal artery. However, inhibition of RhoA/Rho kinase, PKC or tyrosine kinase pathways does not seem to play a fundamental role in MLCP activation stimulated by NaHS. Further studies are needed to elucidate the contribution of other candidate pathways such as integrin linked kinase, p21-activated protein kinase, zip kinase or zip like kinase [15] in NaHS induced relaxation. Moreover, the investigation of MYPT1 phophorylation at Thr^{853} will be an intriguing approach as well. Considering the potential impact of H_2S in the regulation of retinal vascular tone, characterization of relaxing mechanisms might be useful to reveal its therapeutic value in some ocular diseases.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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