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RESEARCH PAPER

Protective effects of exogenous and endogenous hydrogen sulfide in mast cell-mediated pruritus and cutaneous acute inflammation in mice

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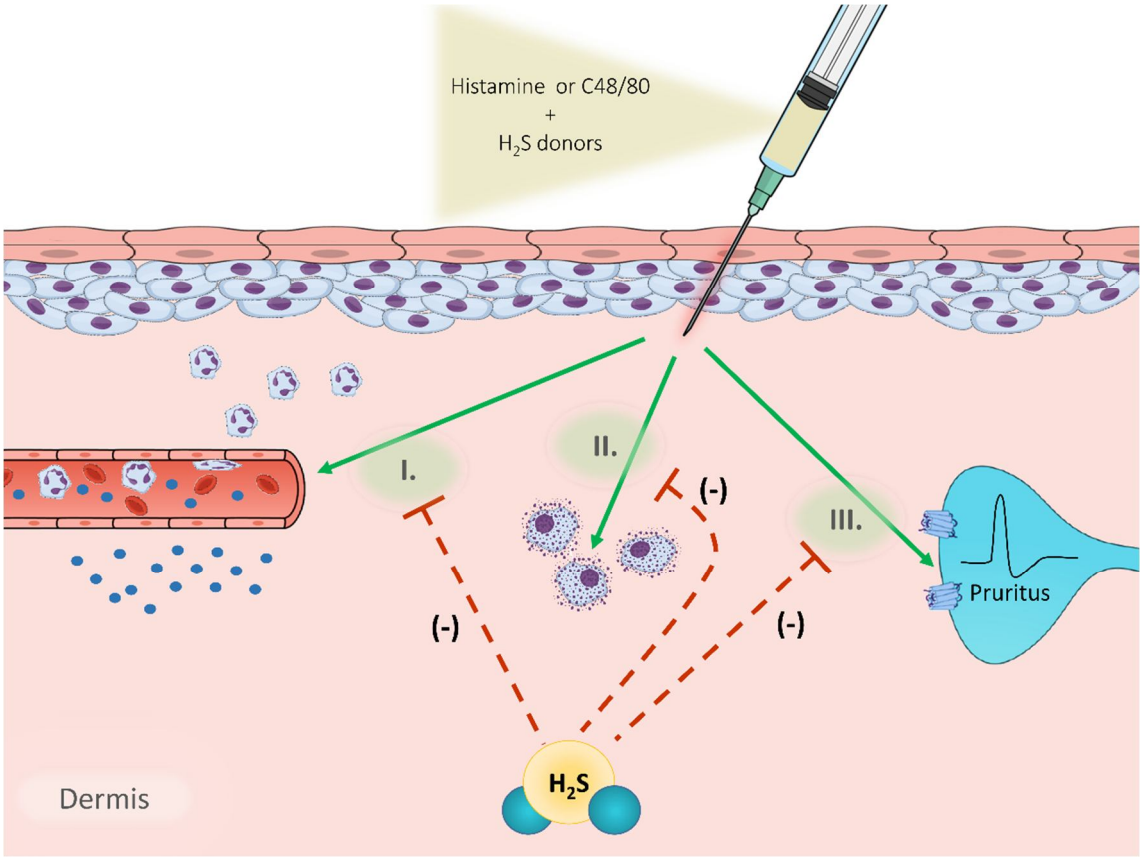
Graphical abstract



ABSTRACT

The recently described 'gasomediator' hydrogen sulfide (H_2S) has been involved in pain mechanisms, but its effect on pruritus, a sensory modality that similarly to pain acts as a protective mechanism, is poorly known and controversial. The effects of the slow-releasing (GYY4137) and spontaneous H_2S donors (Na_2S and Lawesson's reagent, LR) were evaluated in histamine and compound 48/80 (C48/80)-dependent dorsal skin pruritus and inflammation in male BALB/c mice. Animals were intradermally (i.d.) injected with C48/80 (3 µg/site) or histamine (1 µmol/site) alone or co-injected with Na_2S, LR or GYY4137 (within the 0.3 - 100 nmol range). The involvement of endogenous H_2S and K_{ATP} channel-dependent mechanism were also evaluated. Pruritus was assessed by the number of scratching bouts, whilst skin inflammation was evaluated by the

extravascular accumulation of intravenously injected ^{125}I-albumin (plasma extravasation) and myeloperoxidase (MPO) activity (neutrophil recruitment). Histamine or C48/80 significantly evoked itching behavior paralleled by plasma extravasation and increased MPO activity. Na_2S and LR significantly ameliorated histamine or C48/80-induced pruritus and inflammation, although these effects were less pronounced or absent with GYY4137. Inhibition of endogenous H_2S synthesis exacerbated C48/80-induced responses, whereas the blockade of K_{ATP} channels by glibenclamide did not. High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) revealed that Na_2S and LR, but not GYY4137, significantly attenuated C48/80-induced histamine release from rat peritoneal mast cell in vitro. We provide first evidences that H_2S exerted protective effect against acute pruritus mediated via histaminergic pathways in murine skin, thus making of H_2S donors a potential alternative/complementary therapy for treatment of acute pruritus.

Keywords: hydrogen sulfide; pruritus; skin; Na_2S; Lawesson's reagent; GYY4137

1. Introduction

Pruritus (itch) is an autonomous pain-independent sensation that, similarly to pain, acts as a distressing physiological self-protective mechanism in both humans and animals. This response greatly affects life quality and can be triggered by inflammatory skin diseases, systemic diseases, neuropathic conditions and psychogenic disorders. According to the etiology, it may be acute or chronic (duration longer than 6 weeks), localized or generalized [1], [2]. A range of mediators, such as histamine [3], prostaglandins [4], serotonin [5], bradykinin [6], cytokines [7], endothelin-1 [8], leukotrienes [9], proteases [10,11], neuropeptides [12] and opioids [13] orchestrate this response by acting on their receptors located on the nerve terminals. Pruritus (scratching behavior) is also a common symptom that results from insect bites, and can be experimentally induced in animals by the intradermal (i.d.) injection of insect saliva or venom toxins [12,14,15].

The management of pruritus, either dependent or independent of histaminergic pathways, is always recommended when removal of the trigger factors does not control the itch or perhaps unknown. Topical (e.g. anaesthetics, antihistamines, steroids, calcineurin inhibitors and capsaicin cream) or systemic approach (e.g. antihistamines, antidepressants and immunossupressors) are normally prescribed according to the etiology [2].

Interestingly, over the last ten years, hydrogen sulfide (H_2S) a new mediator that belongs to the class of endogenous gases such as nitric oxide (NO) and carbon monoxide (CO), has emerged and brought about divergent findings regarding its role in acute / chronic inflammatory responses and nociception [16–19]. However, the use of slow-releasing H_2S donors (such as SG-1002, diallyl trisulfide and GYY4137) and hybrid H_2S-releasing non steroidal anti-inflammatory compounds (such as the naproxen derivative ATB-346)

strengthens the beneficial therapeutical effects of H_2S in articular inflammation [20], colorectal cancer [21], periodontitis [22] and pain [23] with additional gastrointestinal safety [24]. More recently, low serum levels of H_2S has been associated with psoriasis [25], a disease often associated with pruritus [26]. In contrast, Wang and co-workers [27] showed that the i.d. injection of high doses of NaHS or Na_2S, but not GYY4137, evoked a dose-dependent scratching behavior in mice, which is possibly related to the H_2S releasing rate. Considering that the results on the effects of H_2S on itch behavior are rather limited and controversial, this study was carried out to evaluate whether slow and spontaneous-H_2S releasing donors, used at low doses, are able to reduce acute pruritus and the related cutaneous inflammation mediated by histamine.

2. Material and methods

2.1. Animals

Male BALB/c mice (20 - 30 g) and Wistar rats (180 - 200 g) were obtained from the local animal care facilities and housed in groups (up to five animals per cage) under standard controlled conditions (22°C; 12/12 h light/dark cycle) with free access to commercial rodent chow and water. All the experimental protocols were approved by the local ethics committee (CEUA–ICB; protocol n°. 33, pgs. 85, book no. 02/2010), in accordance with the guidelines from the Brazilian Council for Control of Animal Experimentation (CONCEA) and the Directive 2010/63/EU, comprising with the Animal Welfare Act.

2.2. Induction of pruritus (itching) in the mouse dorsal skin

Mice were transitorily anaesthetized with inhaled isoflurane (3% v/v in O_2) and the rostral part of the back (≅ 2 cm) near to the neck was shaved. Histamine (1 µmol/site), C48/80 (3 µg/site) or its corresponding vehicle Tyrode were i.d. injected, in a volume of 50 µl, alone or in combination with 0.3-10 nmol/site of Na_2S, Lawesson's reagent (LR; both, spontaneous H_2S donors) or the slow-release H_2S donor GYY4137. Mice were individually placed into a perspex transparent box (12x20x17 cm; Insight, Brazil) in a quiet room adapted with video camera (Sony HDR-PJ230), where the mice were daily acclimatized for 40 min during the two days previous to the experiments. A maximum of four mice were simultaneously recorded during the same period and the number of scratching bouts were counted as detailed in [12]. The number of scratching bouts was expressed either as absolute countings or as percentage values determined in 40 min. In all the experiments, the investigator who quantified the scratching behavior was unaware of the experimental group identities.

2.3. Assessment of dorsal cutaneous plasma extravasation

Mice were anesthetized with urethane (2.5 g/kg; i.p.), the rostral back shaved, and 100 µl of ^{125}I–bovine serum albumin (^{125}I–BSA, 0.037 MBq) was intravenously (i.v.) injected via the tail vein. Histamine (30 nmol/site), C48/80 (3 µg/site) or Tyrode were i.d. injected alone or co-injected with Na_2S, LR or GYY4137 (1–100 nmol/site) throughout six randomized skin sites as previously described [28]. The result were expressed as µl of plasma per g of tissue or percentage based on the control values (obtained with either histamine or C48/80 alone).

2.4. Pharmacological treatments

To investigate the involvement of K_{ATP} channel in H_2S donors-mediated protective effects, a set of mice was pretreated (- 30 min), via intraperitoneal (i.p.), with the K_{ATP} channel blocker, glibenclamide (10 or 30 mg/kg, i.p. [29]) or its corresponding vehicle carboxymethylcellulose (CMC; 0.1 ml, i.p.). In order to establish the effective dose of glibenclamide, another group of mice was pretreated (- 30 min) with glibenclamide 10 or 30 mg/kg and then i.d. injected with the K_{ATP} channel opener, pinacidil (10 - 30 nmol/site; i.d.). In order to assess the role of endogenous H_2S in histamine-induced skin pruritus and skin inflammation, two independent groups of mice were pretreated (- 60 min; i.p.) with the CSE and CBS inhibitors β-cyanoalanine (BCA, 50 mg/kg) and aminooxyacetic acid (AOAA, 20 mg/kg), a CSE and CBS inhibitors, respectively.

2.5. Biochemical analysis

2.5.1. Measurement of myeloperoxidase (MPO) activity

Mice were anaesthetized with isoflurane and i.d. injected with the test agents, as described above (item 2.3), and four hour later they were killed via an overdose of urethane followed by cervical dislocation. The injected skin sites were removed, and the myeloperoxidase (MPO) activity was measured as previously described [30]. The results were expressed as units of MPO per mg of protein (or percentage).

2.5.2. Production of H_2S by mouse dorsal skin

The endogenous H_2S production in the naïve mouse dorsal skin was carried out based on the formation of lead sulfide, according to [31]. Briefly, skin, brain and liver were excised and homogenized. After centrifugation (10,000 g, 10 min, 4°C), the

obtained supernatants (400 µg protein) was incubated with substrates (L-cysteine 10 mM and pyridoxal-5'-phosphate 2 mM) for 2h 30min at 37 ºC. The dark dots densities on the lead acetate white paper strips (12x8 cm) placed over the 96-wells microplate were analyzed from the digitalized images using the software ImageJ (NIH, USA). Hydrogen sulfide concentrations were extrapolated from a calibration curve generated with NaHS (1.95 - 2000 µM).

2.6. Rat mast cell isolation and quantification of in vitro histamine release by HPLC-MS/MS

Rats (n=6) were exsanguinated under deep isoflurane anaesthesia (5% v/v in O_2), mast cells were isolated from the peritoneal cavity and purified (95%) by Percoll gradients (as determined by Cytospin® preparations stained with May-Grünwald Giemsa, and trypan blue dye exclusion). Briefly, to 0.5 ml mast cell aliquots ($4x10^5$ cells/ml) were simultaneously added compound 48/80 (1 µg/ml) and the test agents (Na_2S, LR or GYY4137 at 100–1000 µM) and incubated at 37°C during 15 min. The amount of histamine released was quantified by high performance liquid chromatography coupled to an electrospray tandem mass spectrometry (HPLC-MS/MS), as described previously [32].

2.7. Drugs and reagents

Lawesson's reagent (2,4-bis[4-methoxyphenyl]-1,3,2,4-dithiadiphosphatane 2,4-disulphide), histamine (2-[1H-imidazol-4-yl]ethanamine), pyridoxal 5′-phosphate, L-cysteine, glibencamide (5-Chloro-N-[4-(cyclohexylureidosulfonyl) phenethyl]-2-methoxybenzamide), phenylmethanesulfonyl fluoride (PMSF), o-dianisidine dihydrochloride (3,3′-dimethoxybenzidine dihydrochloride), aminooxyacetic acid

(AOAA), urethane (carbamic acid ethyl ester), HTAB (hexadecyl trimethylammonium bromide), trypan blue and compound 48/80 (N-methyl-p-methoxyphenethylamine) were purchased from Sigma Chemical Co. (St Louis, MO, USA). CMC (carboxymethylcellulose) was obtained from Cromoline Química Fina Ltda (Diadema, São Paulo, Brazil). Percoll and $Na_2S.9H_2O$ were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden) and Dinâmica Química Contemporânea Ltda (Diadema, São Paulo, Brazil), respectively. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether), hydrogen peroxide and lead acetate were purchased from Cristália (Itapira, São Paulo, Brazil) and Labsynth® (Diadema, São Paulo, Brazil), respectively. BCA (β-cyanoalanine) was obtained from Cayman Chemical Company (USA) and GYY4137 was synthesized in house, as described in [33].

2.8. Statistical analysis

Data are expressed as mean ± SEM for n animals. Differences among the groups were analyzed by one-way ANOVA followed by Bonferroni or Dunnett's test for multiple comparisons, using the software GraphPad Prism (version 4.0, San Diego, CA, USA). Values of P lower than 0.05 were taken as significant.

3. Results

3.1. Histamine or C48/80-induced pruritus is reduced by H_2S donors

As shown in Figure 1, the i.d. injection of histamine (1 µmol/site) resulted in significant increase of pruritus in the dorsal skin compared with the Tyrode injected group. The co-injection of histamine with Na_2S (1 and 3 nmol/site, $P < 0.05$; Fig. 1A), LR (3 and 10 nmol/site, $P < 0.05$; Fig. 1B) and GYY4137 (1 nmol/site, $P < 0.05$; Fig. 1C)

reduced the number of scratching bouts in a dose-dependent manner compared to histamine alone, except that at a higher doses both Na_2S and GYY4137 failed to evoke this effect. The i.d. injection of higher dose of Na_2S (10 nmol/site), LR (10 nmol/site) or GYY4137 (10 nmol/site) by itself did not evoke scratching behavior (Fig. 1A-C).

Similarly, the i.d. injection of C48/80 (3 µg/site), a mast cells degranulator, also evoked a marked number of scratching bouts in comparison with Tyrode ($P < 0.05$ - $P < 0.001$; Fig. 2A-C). This response was significantly inhibited, but not in a dose-dependent fashion, by the co-injection with increasing doses of Na_2S (1, 3 and 10 nmol/site, $P < 0.05$; Fig 2A) or LR (0.3 - 10 nmol/site, $P < 0.05$ - $P < 0.001$; Fig 2B). GYY4137 i.d. injected in all tested doses (0.3 - 10 nmol/site) failed to significantly inhibit C48/80-induced pruritus (Fig 2C). None of the H_2S donors produced a significant scratching behavior compared to Tyrode injected group.

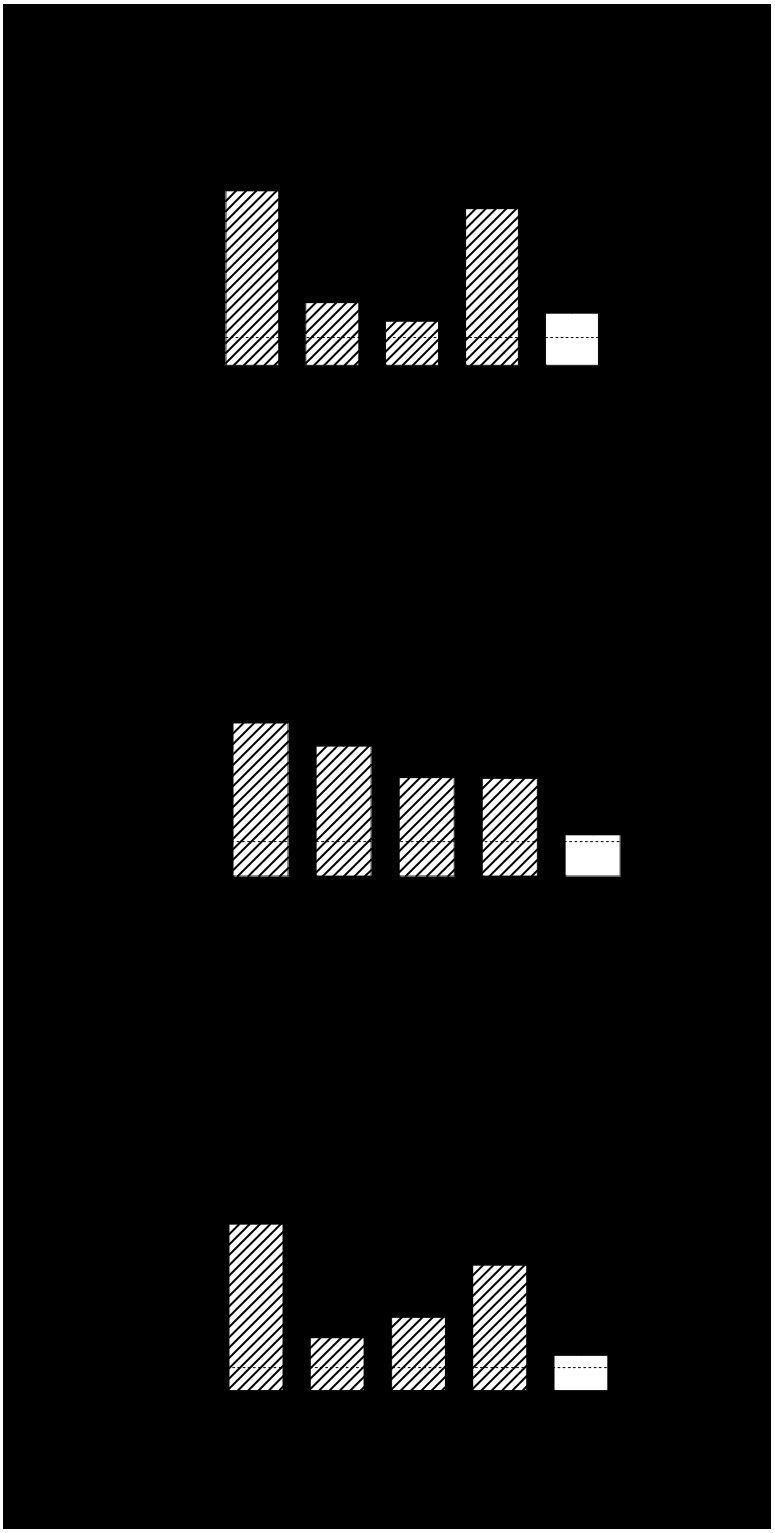


Fig. 1. Dose response relationship for H_2S-releasing donors on histamine-induced scratching bouts. The scratching bouts evoked by i.d. injection of histamine alone or co-injected with Na_2S (0.3 - 10 nmol/site., *n* = 5 - 10), LR (0.3 - 10 nmol/site., *n* = 5 - 8) and GYY4137 (0.3 - 10 nmol/site., *n* =5 - 6) are illustrated on panels A, B and C, respectively.

Independent groups of mice were i.d. injected only with H_2S donors at higher doses. Dashed line represents the pruritus induced by i.d. injection of vehicle, the Tyrode solution. Data are expressed as mean ± SEM. $^{**}P < 0.01$ –$^{***}P < 0.001$ *vs*. Tyrode, $^{\#}P < 0.05$ *vs*. histamine (One-way ANOVA followed by the Dunnett's test).

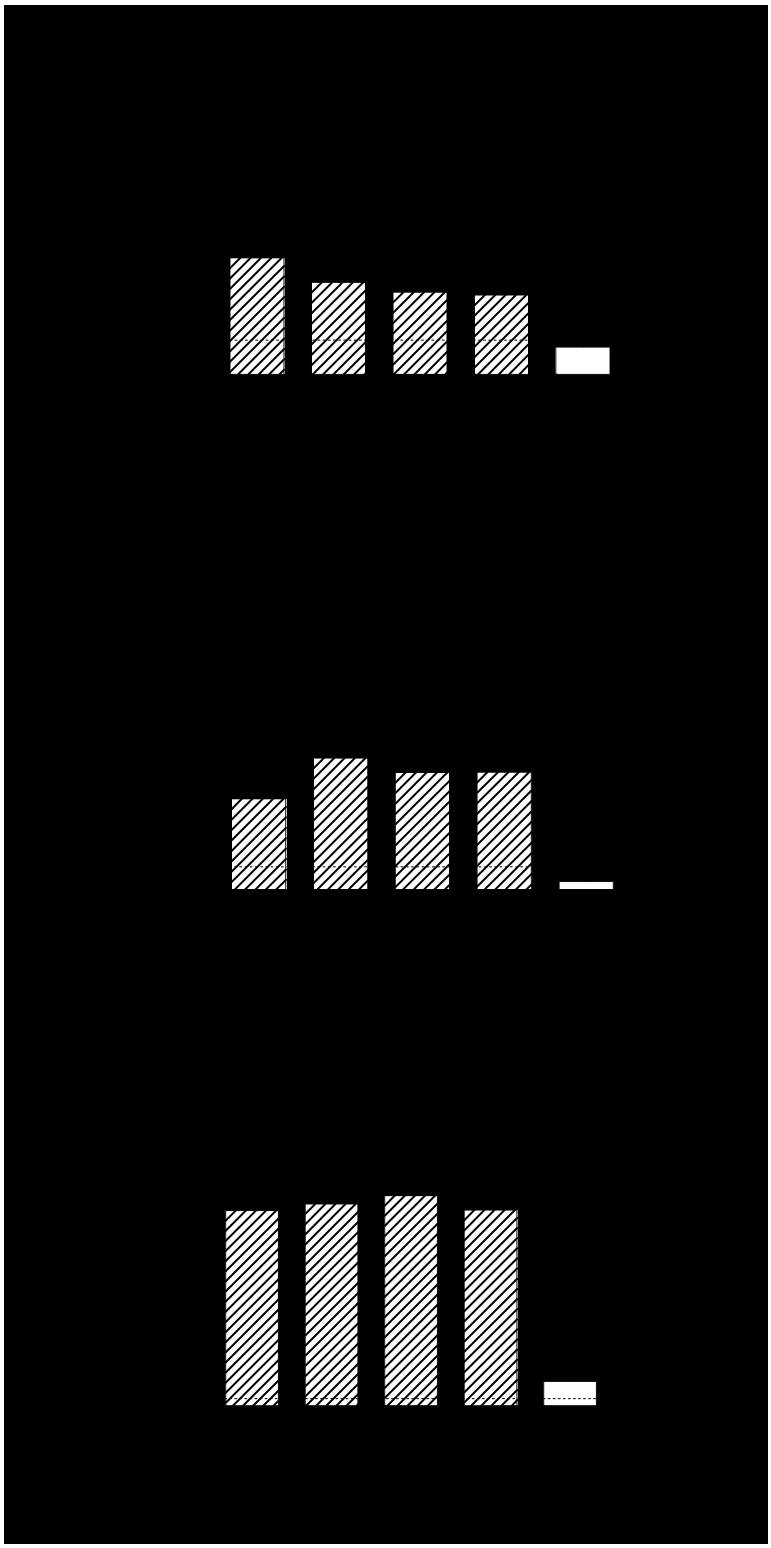


Fig. 2. Dose-response curves for H_2S-releasing donors on C48/80-induced scratching bouts. Panels (A), (B) and (C) show the percentage of scratching bouts evoked by C48/80 alone (3 µg/site; i.d) and co-injected with Na_2S (0.3 - 10 nmol/site, n = 5–8), LR (0.3 - 10 nmol/site, n = 5–7) and GYY4137 (0.3 - 10 nmol/site, n = 7), respectively. A set of mice received only i.d. injection of the H_2S donors at higher dose. Dashed line represents the pruritus evoked by i.d. injection of Tyrode. Data are expressed as mean ± SEM. $^{*}P < 0.05$ – $^{***}P < 0.001$ *vs*. Tyrode, $^{\#}P < 0.05$ – $^{\#\#\#}P < 0.001$ *vs*. C48/80 alone (One-way ANOVA followed by the Dunnett's test).

3.2. Effects of H_2S-releasing donors on dorsal skin plasma extravasation and neutrophil influx induced by amines (increased MPO activity)

The i.d. injection of histamine (30 nmol/site) induced a significant ($P < 0.001$) plasma protein extravasation in the mouse dorsal skin in comparison with Tyrode (Fig. 3). This response was dose-dependently reduced by the co-injection of Na_2S (3 - 100 nmol/site, $P < 0.05$; Fig. 3A) and LR (1 - 10 nmol/site, $P < 0.05$; Fig. 3B), but unaffected by GYY4137 (Fig. 3C). Neither Tyrode nor H_2S donors injected i.d. produced a significant increase in plasma extravasation.

The i.d. injection of C48/80 (3 µg/site) also resulted in significant ($P < 0.001$) amount of plasma extravasation in comparison with Tyrode (Fig. 4). Co-injected with H_2S donors Na_2S and LR, the plasma extravasation induced by C48/80 was significantly reduced ($P < 0.05$) at doses of 30 and 100 nmol/site (Fig. 4A and B). The simultaneous injection of GYY4137 with histamine failed to significantly affect the plasma extravasation induced by C48/80 (Fig. 4C). None of H_2S donors at a higher dose (100

nmol/site) increased significantly the microvascular permeability when i.d. injected alone in the mouse dorsal skin.

After 4 hours i.d injection of C48/80 in the mouse dorsal skin (3 µg/site), a marked ($P < 0.001$) increase in MPO activity was noticed compared with Tyrode (Fig. 5). The i.d. co-injection of Na_2S (3, 10 and 30 nmol/site; Fig. 5A), LR (10, 30 and 100 nmol/site; Fig. 5B) or GYY4137 (100 nmol/site; Fig. 5C) led to a significant ($P < 0.05$ - $P < 0.001$) inhibitory effect on C48/80-induced increased MPO activity. At a higher dose, Na_2S (100 nmol/site) failed to inhibit C48/80-induced increased MPO activity compared to this compound alone (Fig. 5A). H_2S donors had no effects when i.d. injected alone.

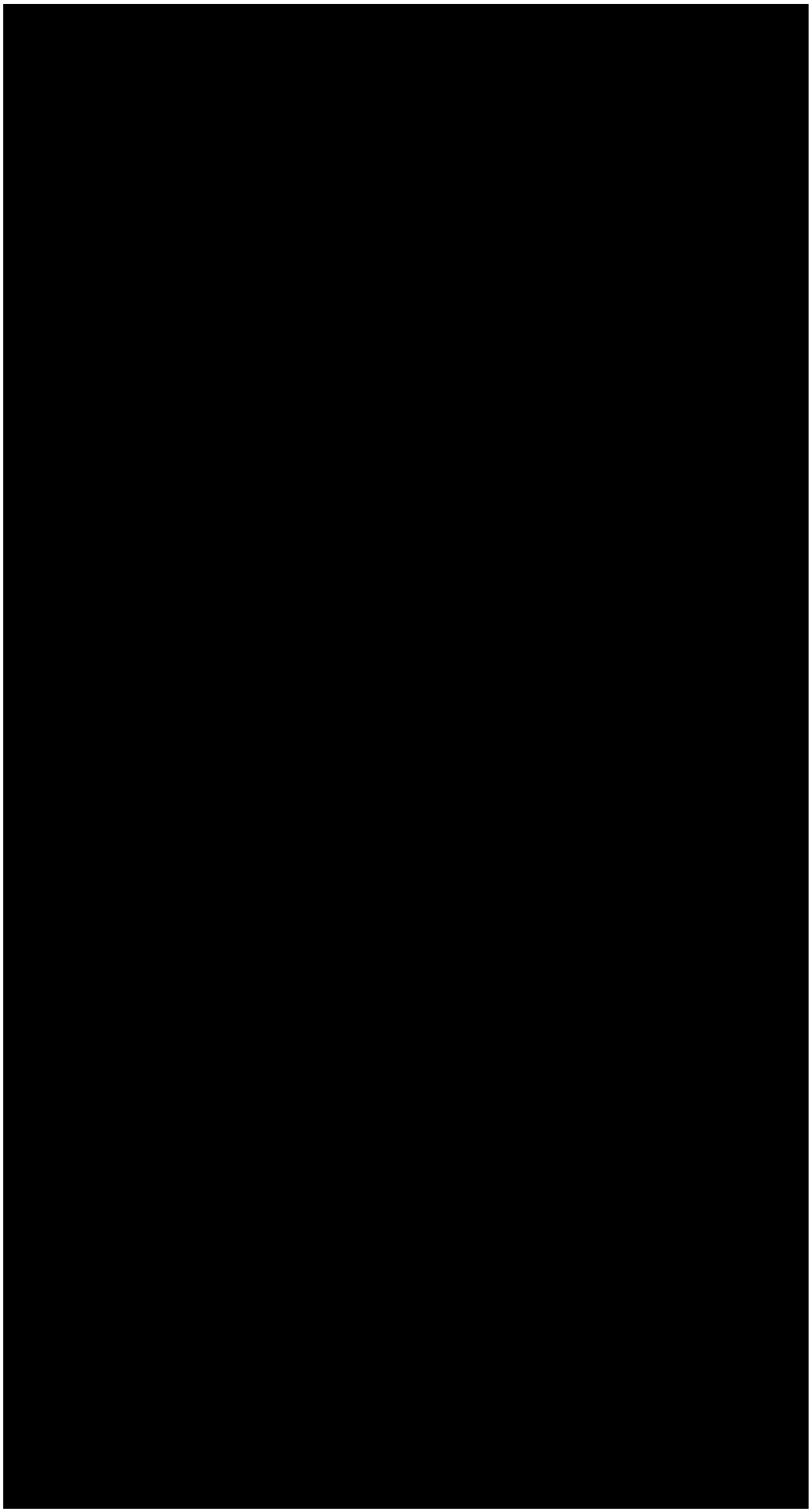


Fig. 3. Dose-response curves of the H_2S donors on plasma extravasation induced by i.d. injection of histamine. Panels (A), (B) and (C) show the co-injection effects of Na_2S (1 - 100 nmol/site, *n* = 5–7), LR (1 - 100 nmol/site, *n* = 5) and GYY4137 (1 - 100 nmol/site, *n* =7-10) on histamine (30 nmol/site)-induced plasma extravasation. At higher dose, H_2S donors i.d. injected induced similar plasma extravasation to that produced by its vehicle Tyrode (dashed line). Data are expressed as mean ± SEM. ***$P < 0.001$ *vs.* Tyrode, $^{\#}P < 0.05$ *vs*. Histamine alone (One-way ANOVA followed by the Dunnett's test).

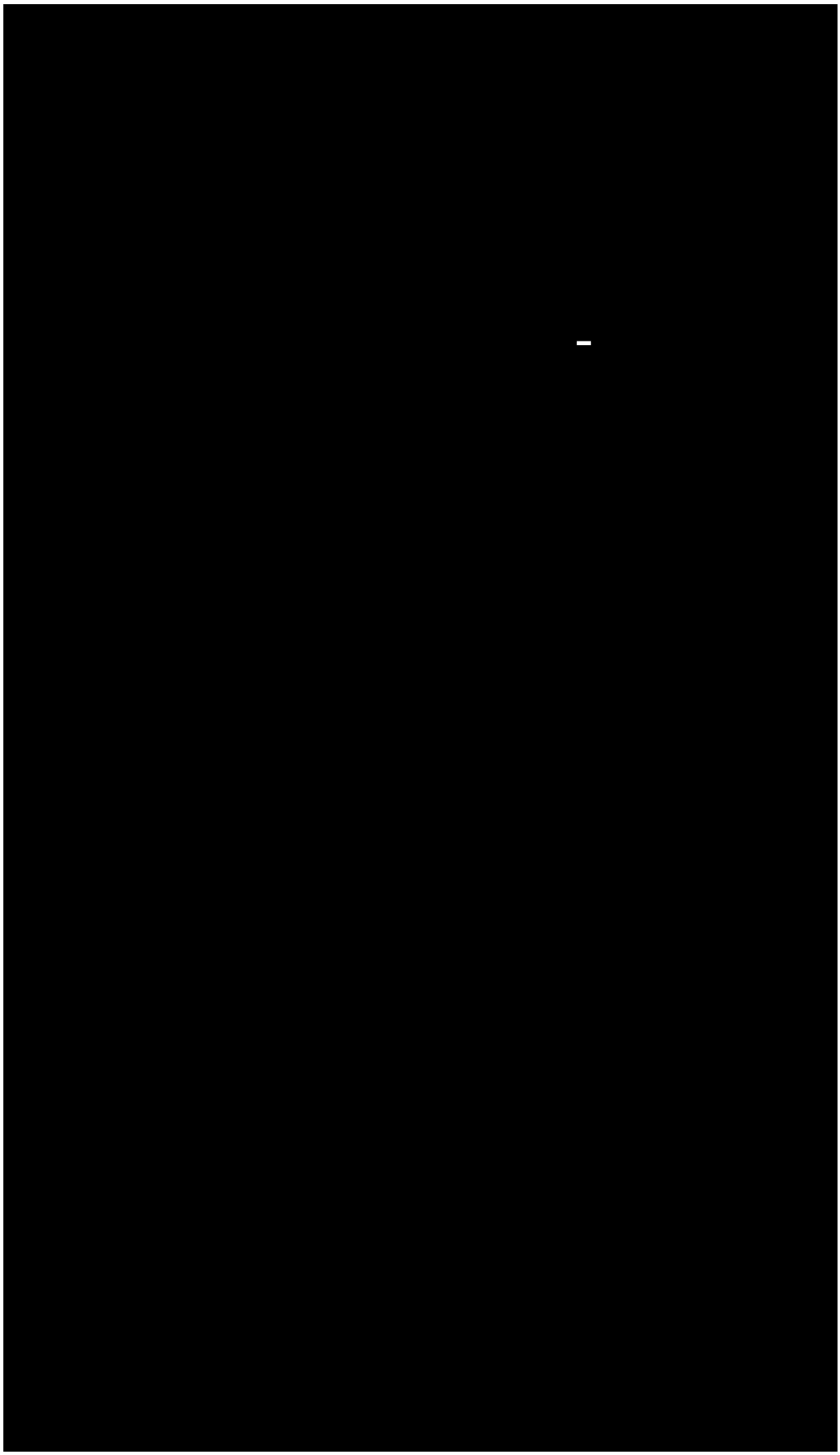


Fig. 4. Dose-response relationship between H_2S-releasing donors on C48/80-induced cutaneous plasma extravasation. Panels (A), (B) and (C) illustrate response evoked by i.d. injection of C48/80 (3 µg/site) alone and co-injected with Na_2S (1 - 100 nmol/site., *n* = 6), LR (1 - 100 nmol/site., *n* = 8) and GYY4137 (1 - 100 nmol/site., *n* =10), respectively. At higher dose, the i.d. of H_2S donors induced similar plasma extravasation to that produced by its vehicle Tyrode (dashed line). Data are expressed as mean ± SEM. ***P < 0.001 *vs*. Tyrode, $^{\#}P$ < 0.05 *vs*. C48/80 alone (One-way ANOVA followed by the Dunnett's test).

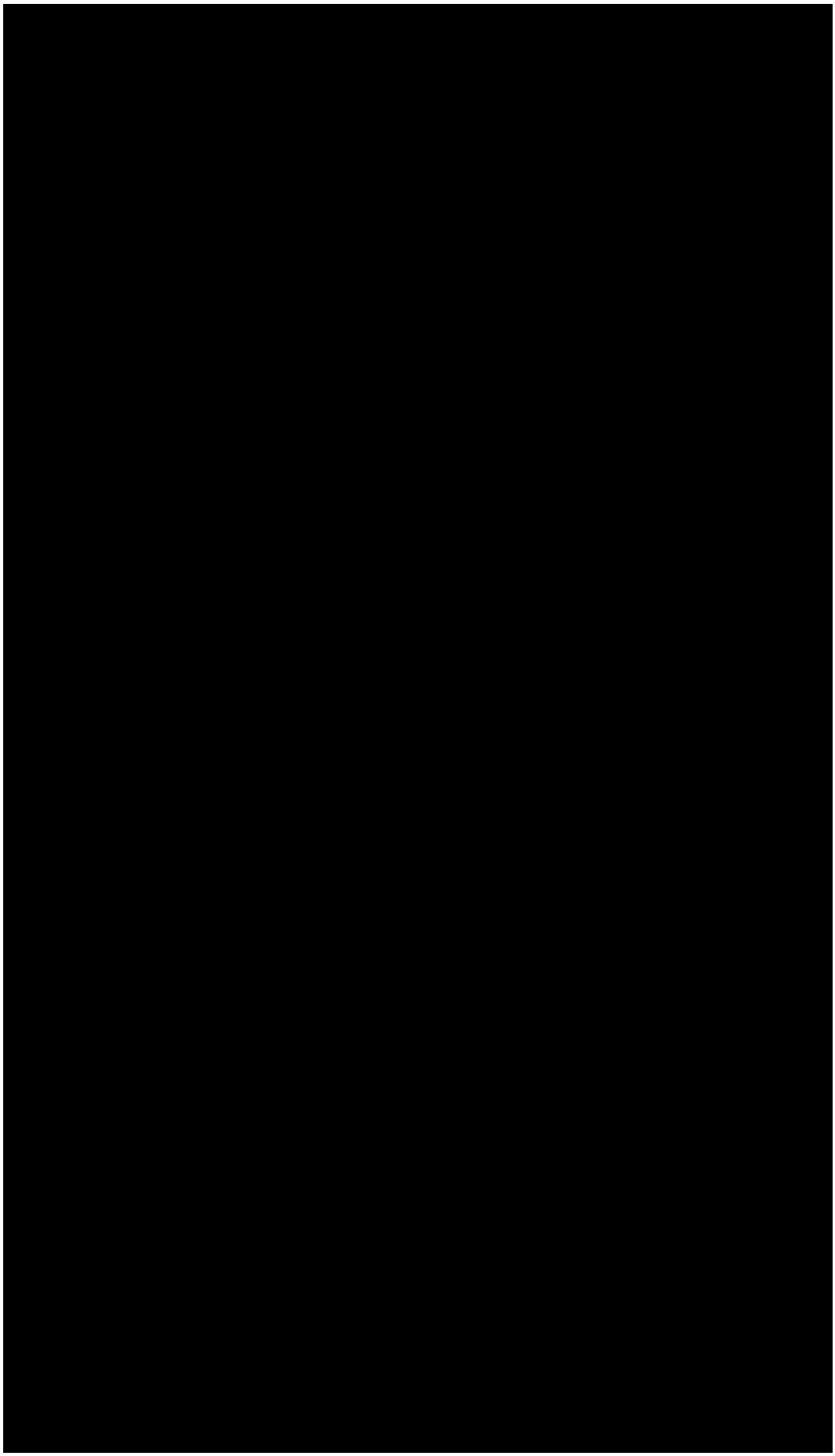


Fig. 5. Effects of H_2S donors on C48/80-induced increased MPO activity. Panels (A), (b) and (C) show the MPO activity evoked by i.d. injection of C48/80 (3 µg/site) alone and co-injected with Na_2S (1-100 nmol/site., *n* = 6), LR (1-100 nmol/site., *n* = 10) and GYY4137 (1-100 nmol/site ., *n* =8), respectively, 4 hours later. At higher doses, H_2S donors evoked similar response to that evoked by its vehicle Tyrode (dashed line). Data are expressed as mean ± SEM. ***$P < 0.001$ *vs.* Tyrode, $^{\#}P < 0.05$ - $^{\#\#\#}P < 0.001$ *vs.* C48/80 alone (One-way ANOVA followed by the Dunnett's test).

3.3. Role of endogenous H_2S in C48/80-induced both pruritus and neutrophil influx

As expected, the i.d. injection of C48/80 (3 µg/site) induced a significant increase on the number of scratching bouts (Fig. 6A and B) and MPO activity (Fig. 6C and D). The pretreatment of mice with the CSE inhibitor BCA (50 mg/kg; i.p., - 60 min) significantly exacerbated ($P<0.05$) C48/80-induced pruritus and MPO activity compared to control group pretreated with saline (Fig.6A and 6C), whilst the pretreatment of animals with the nonselective CBS inhibitor AOAA (20 mg/kg; i.p., - 60 min) did not. The i.d. injection of Tyrode in mice dorsal skin did not evoke a significant increase in MPO activity; however, the pretreatment of mice with BCA or AOAA resulted in a significant increase of MPO activity compared to Tyrode response in saline-pretreated mice (Fig. 6C and D).

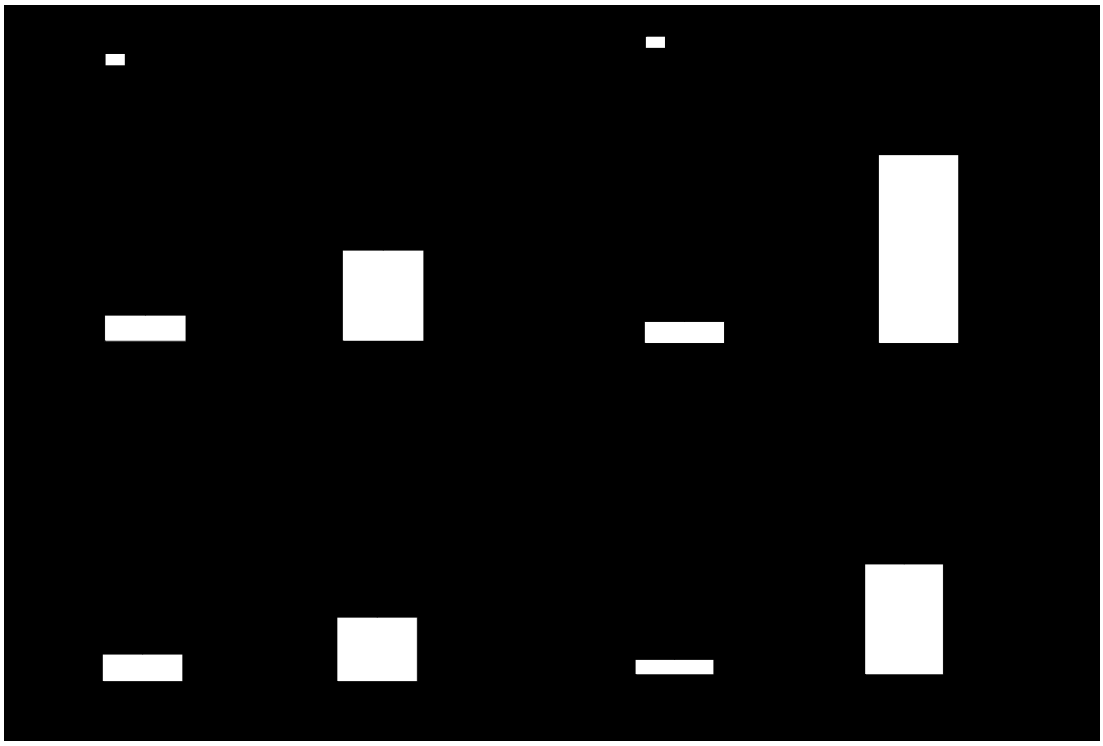


Fig. 6. Endogenous blockade of H_2S synthesis potentiated C48/80-induced pruritus and MPO activity in mouse dorsal skin. Panels (A) and (B) show the effects of pretreatment with either the CSE inhibitor, β-cyanoalanine (BCA, 50 mg/kg., -60 min) or the nonselective CBS inhibitor aminooxyacetic acid (AOAA, 20 mg/kg., -60 min) in the pruritus evoked by C48/80 (n=5-8). Panels (C) and (D) show the same effects of the same treatments in C48/80-induced MPO activity (n=5-8). Data are expressed as mean ± S.E.M. for n animals. * P <0.05 and *** P <0.001 *vs.* Tyrode. $^{\#\#}P$ < 0.01 and $^{\#\#\#}P$ < 0.001 *vs.* saline pretreated mice. (One-way ANOVA followed by Bonferroni's multiple comparison test).

3.4 Determination of H_2S in mouse dorsal skin

The naïve mouse skin, similarly to the brain, produce a significant and equivalent amount of H_2S (≅ 0.3 nmol / mg of protein/min), whereas a marked H_2S generation was generated in the liver (≅ 1.2 nmol / mg of protein/min) of naïve mice (Fig. 7).

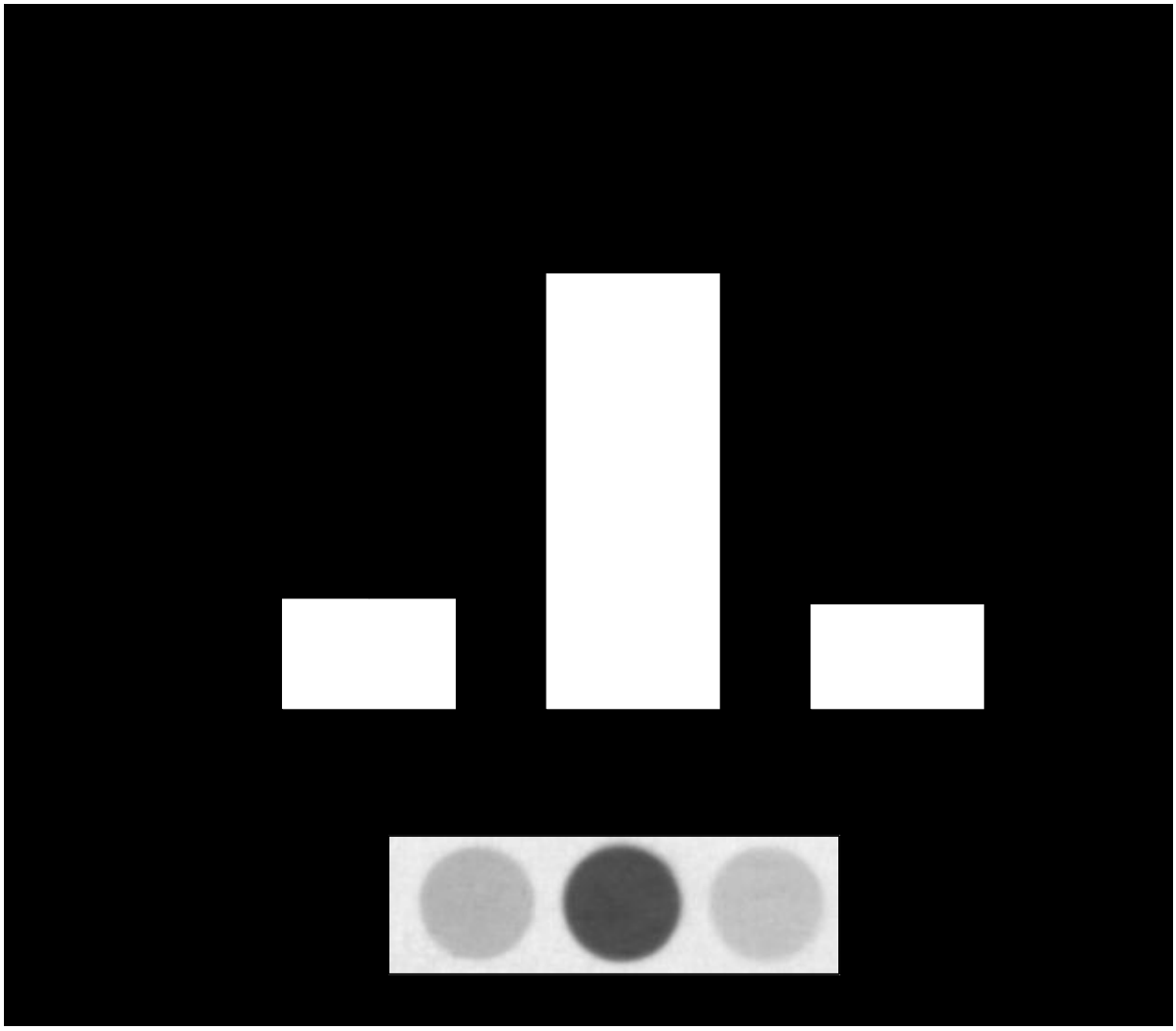


Fig. 7. Constitutive H_2S generation in the naïve skin, liver and brain of mice. Panel A shows the H_2S generation in the skin, liver and brain of naïve animals, expressed as nmol of H_2S per mg of protein/min, and panel B illustrates the representative corresponding skin, liver and brain dark dots densities on the lead acetate white paper strip. Data are expressed as mean ± S.E.M. for n = 4 animals.

3.5. Histamine release from mast cells activation is reduced by H_2S-releasing donors

C48/80 (1 µg/ml) markedly stimulated the release of histamine ($P < 0.001$) from rat peritoneal mast cells compared with histamine spontaneously released from mast cells

treated only with buffer (KRP; Fig. 8). The concomitant incubation of mast cells with C48/80 and Na_2S (100 and 1000 µM; $P < 0.05$ - $P < 0.01$) or LR (500 - 1000 µM; $P < 0.05$ - $P < 0.001$) resulted in significant decrease of histamine release from these cells. In all tested concentrations, GYY4137 did not prevent histamine release from C48/80-induced mast cell degranulation (Fig. 8). The % of histamine released from mast cells incubated with H_2S donors alone matched with KRP value (Fig. 8).

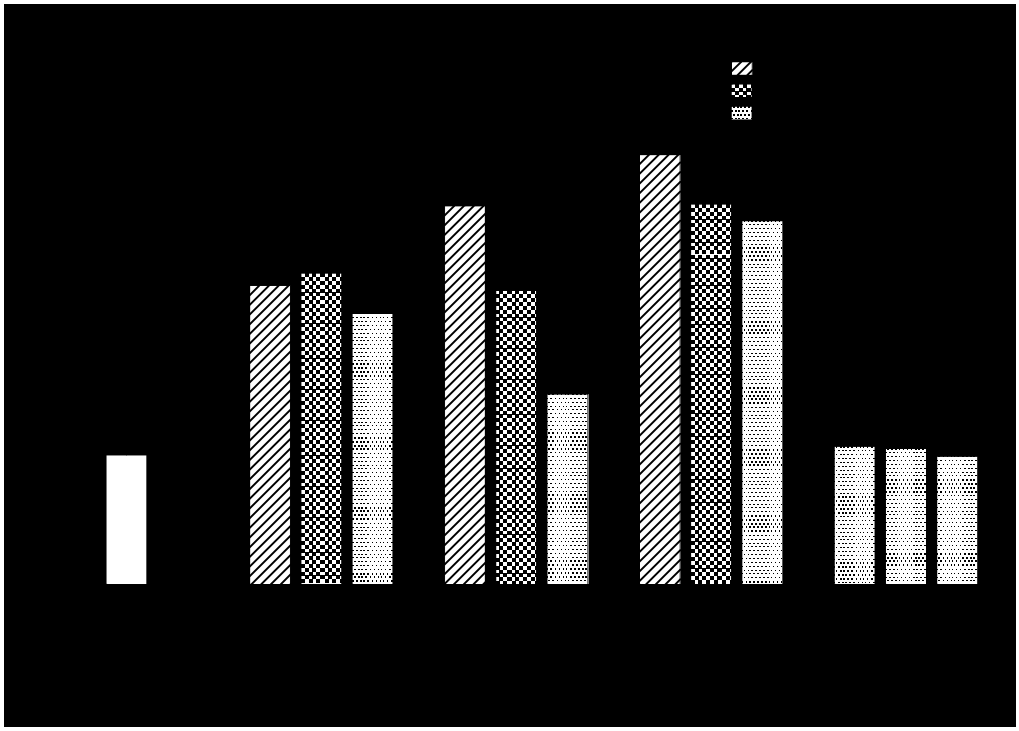


Fig. 8. Spontaneous**,** but not slow, H_2S donors reduce C48/80-induced mast cell degranulation. Black bar illustrates the % of histamine released from mast cells treated with C48/80 (1 µg/ml) in krebs ringer phosphate solution (KRP). Cross-hatched bars show the % of histamine release from mast cells in response to C48/80 plus Na_2S, LR or GYY4137 (100 - 1000 µM). Data are expressed as % of histamine release. Values are presented as mean ± SEM of three independent experiments. ***$P < 0.001$ *vs.* KRP (basal release), $^{\#}P < 0.05$- $^{\#\#}P < 0.01$-$^{\#\#\#}P < 0.001$ *vs.* C48/80 alone (One-way ANOVA followed by Bonferroni's multiple comparison test).

3.6. Lack of involvement of K_{ATP} channels in the antipruritic effect of H_2S

The pretreatment of mice with glibenclamide (10 or 30 mg/kg; i.p., - 30 min) significantly inhibited, at both doses, the K_{ATP} channel opener pinacidil (10 and 30 nmol/site)-induced exacerbation of plasma extravasation evoked by histamine (Fig. 9A), thus suggesting an effective blockade of this channel. The pretreatment of animals with 10 mg/kg of glibenclamide did not significantly affect histamine-induced plasma extravasation (Fig. 9B) or pruritus (Fig. 9C) compared to histamine responses in animals pretreated with vehicle CMC. Likewise, glibenclamide treatment did not alter the protective effect of Na_2S (3 or 30 nmol/site) against histamine-induced plasma extravasation or pruritus (Fig. 9B-C).

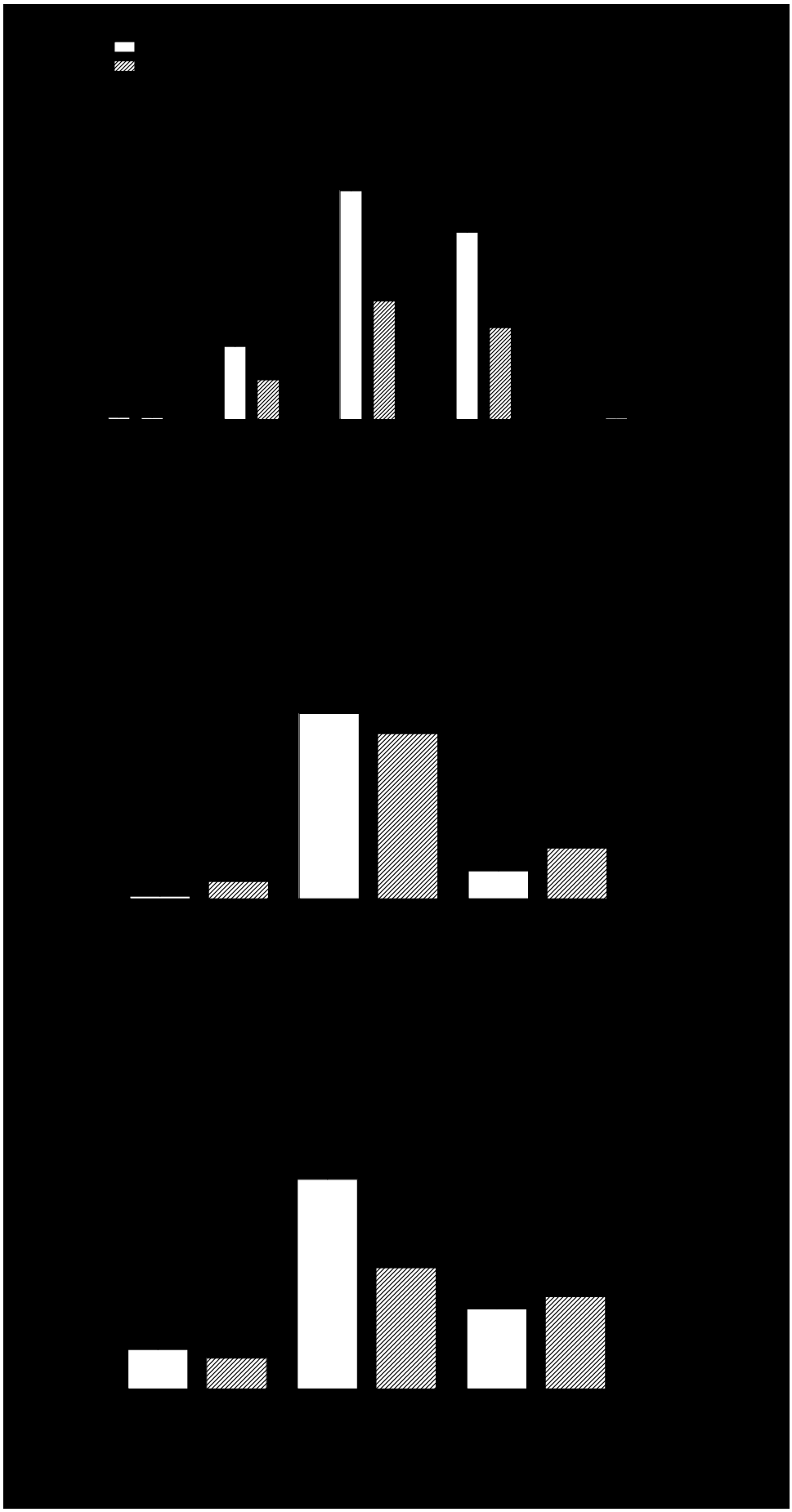


Fig. 9. Blockade of K_{ATP} channels failed to reverse Na_2S-induced protective effect against histamine-induced plasma extravasation and pruritus. Panel (A) shows the effect of glibenclamide (10 or 30 mg/kg., i.p., - 30 min) or its vehicle (CMC) on pinacidil (10 or 30 nmol/site)-induced potentiating of plasma extravasation in response to i.d. of histamine (30 nmol/site; *n*=5), whereas panels (B) and (C) show the effects of histamine in glibenclamide pretreated group (10 mg/kg., i.p., - 30 min) in addition to Na_2S (30 nmol/site., *n* = 5 - 6) and pruritus (1 µmol/site., *n* = 5 - 10). Data are presented as mean ± S.E.M. for n animals. ** *P* <0.01 - *** *P* <0.001 *vs*. Tyrode + CMC, $^{\#\#}$ *P* <0.01-$^{\#\#\#}$ *P* <0.001 *vs*. histamine + CMC, $^{+}$*P* <0.05 - $^{+++}$*P* <0.001 *vs* histamine + pinacidil. (One-way ANOVA followed by Bonferroni's test).

Discussion

The major finding in this study is to show that the spontaenous H_2S donors Na_2S and LR significantly ameliorated pruritus and the acute cutaneous inflammation induced by histamine and C48/80 in the mouse dorsal skin, being this effect less pronounced (or absent) in mice treated with the slow H_2S-releasing donor GYY4137, whilst the endogenous blockade of H_2S biosynthesis aggravates skin inflammation and pruritus.

Histamine-induced plasma extravasation (oedema) is mainly processed by activation of H1-histaminergic receptors and, to a lesser extent, H2 receptors expressed in the skin vessels of both rodents [34,35] and humans [36,37]. Herein, the simultaneous i.d. injection of H_2S donors Na_2S or LR within the 0.3-10 nmoles/site dose range, significantly inhibited pruritus and plasma extravasation produced by histamine, even though it did not follow a clear dose-dependent response. Instead, a trend to biphasic

pattern in the scratching behavior (pruritus) and plasma protein extravasation was observed with these H_2S donors, in particular with Na_2S.

Even though we have used smaller doses of Na_2S compared to previous study [27], it is possible that as this compound instantaneously delivers H_2S, the higher tested dose (100 nmol/site) led to an immediate non-physiologically concentration of H_2S in the local microcirculation, which may be distinct from that seen in the dorsal skin. In line with this, we provide the first evidence that the mouse dorsal healthy skin constitutively produces low amounts of H_2S (≅ 0.3 nmol of H_2S per mg of protein per min), similarly to that produced by the brain but less than that produced by the liver. Thus, these results point to the same suggestion made by previous studies, in terms that the application of high doses of a fast H_2S-releasing donors can rapidly lead to elevated concentrations of this mediator, which in turn may result in toxic effects, rather than the beneficial ones seen with low amounts of H_2S [33,38–41].

Histamine exerts its inflammatory effects, in part, through NO generation *in situ* [35], since the blockade of NOS significantly inhibited histamine-induced plasma extravasation in human nasal airway [42]. Interestingly, Ali and co-workers [43] demonstrated that low concentration of H_2S or spontaneous H_2S donors reversed histamine, but not isoprenaline-induced vasodilatation, possibly via a direct chemical reaction between H_2S and NO, which may lead to the formation of a nitrosothiol [44] or even nitroxyl species (NO-/HNO) [45] of poor vasorelaxant activities.

Similarly to histamine, the cationic secretagogue C48/80 also induces a marked pruritus behavior and skin inflammation characterized by plasma extravasation and neutrophil influx, except for the more pronounced response observed with the latter when applied at similar doses mainly due to the exocytosis of multiple preformed mediators from mast cells (e.g. histamine, 5-HT, etc.) on both blood vessels and neurons, via

interactions with their respective receptors. In fact, C48/80 effects can be significantly inhibited by antagonists of histamine H1 receptors, 5-HT or substance P (NK1) receptors [36,46,47].

C48/80-induced plasma extravasation and pruritus was partially, but significantly, inhibited by the simultaneous co-injection of the spontaenous H_2S donors Na_2S or LR, whereas neither histamine nor C48/80-induced plasma leakage was significantly affected by the slow H_2S-releasing donor GYY4137. It is thus possible that a significant amount of H_2S immediately available in the microvascular bed during the initial phases of the vascular response is necessary to interact with NO generated by histamine (or the histamine releaser C48/80), and consequently inhibit its potentiating action on microvascular permeability. Indeed, it is well established that plasma extravasation occurs immediately after the i.d. injection of chemical mediators (such as histamine), whereas the leukocyte recruitment into the cutaneous tissue takes longer periods of time (>3 h). In agreement, our results show that 4 hours after the i.d. injection of C48/80 a marked neutrophil influx ocurs (measured as MPO activity), and this response was effectively reduced by all the H_2S releasing donors. It is thus possible that the GYY4137 compound may have released enough H_2S along this time, which in turn can counteract with the dynamic of leukocyte influx in the microcirculation in response to C48/80.

A direct stabilizing effect of H_2S on mast cells degranulation could explain the reduced leukocyte influx in response to C48/80. In order to test this hypothesis we performed *in vitro* experiments using rat peritoneal mast cells stimulated with C48/80 and measured the release of histamine. In fact, we observed that Na_2S and LR, but not GYY4137 prevented mast cell degranulation. In agreement with our data, Zanardo and co-workers [48] have shown that spontaneous H_2S donors, such as Na_2S, NaHS and LR, markedly inhibited carrageenan-induced leukocyte adhesion and infiltration in a rodent

air pouch model. Similar results were obtained by Ekundi-Valentim and co-workers [16,20] in the model of carrageenan-induced synovitis in rats, and by Fiorucci and co-workers [49] in the acetyl salycilic acid-induced gastric injury in rats. Reduced expression of the adhesion molecules ICAM-1, VCAM-1, LFA-1, P-selectin, E-selectin in both endothelium and leukocytes has been suggested as the underlying mechanism of H_2S-mediated inhibition of leukocyte influx [40,48–50].

The activation of K_{ATP} channels by H_2S has been shown as the mechanism underlying the inhibitory effects of this mediator in a variety of experimental approaches, including aspirin-induced leukocyte adherence in mesenteric venules [48] and high glucose-induced cardiac cells injury [51]. However, this not seem to be the cause beyond the protective effects of the spontaneous H_2S donors against histamine-induced pruritus and skin inflammation, as pretreatment of mice with glibenclamide, a selective K_{ATP} channels blocker, did not antagonize the protective effects of H_2S, even when used at doses that abolish the vasodilatation induced in the mouse dorsal skin by the K_{ATP} channel opener pinacidil.

Compound 48/80 has been largely used as an IgE independent activator of mast cells, which mainly due to histamine release (and activation of peripheral H1 and H4 receptors), results in sensitization of afferent nerves [52,53]. Similarly to the spontaneous H_2S donor Na_2S, GYY4137 inhibited histamine-induced pruritus; however, this was not the case with C48/80-induced pruritus. Thus, either the intensity of response evoked by histamine released from C48/80 was higher than exogenous histamine when paralleled by the low availability of H_2S released from GYY4137 *in situ* on the peripheral afferent neurons or it is possibly that C48/80 produces scratching behavior that somehow depends on functional changes evoked by direct activation of pruriceptors. While direct excitatory effects of C48/80 on dorsal root ganglion (DRG), enteric neurons and visceral afferents

can occur independently of mast cell activation [54], other works show that the mechanism involved in C48/80-induced nociception is mediated by a cascade activation that starts after mast cell activation, including the release of mediators that can activate these nociceptors and promote pain [55].

Curiously, and in contrast to our findings, a recent work by Wang and co-workers [27] has shown that the i.d. injection of mice with the spontaneous H_2S donors NaHS and Na_2S, at mM range (7 – 280 and 1.7 – 67, respectively), led to a marked itching behavior and touch-evoked itching (allokinesis), while inhibition of endogenous H_2S synthesis by pretreatment of the mice with CSE and CBS inhibitors reduced C48/80-induced pruritus. Previously, Elies and co-workers [56] showed that NaHS at low concentrations (100-300 μM) markedly inhibits $Ca^{2+}{}_v3.2$ T-type calcium channel in HEK293 cells, while NaHS at high concentrations (3-10 mM) led to a marked activation of these channels, possibly due to the resultant high Na^+ concentrations. In line with these findings, we speculated that the presence of these high Na^+ concentrations may explain the discrepancies between our results and those shown in Wang's paper. We also show that inhibition of endogenous H_2S biosynthesis by BCA, but not by AOAA, resulted in enhanced C48/80-induced scratching behavior in addition to increased MPO activity. Again, the C48/80-induced pruritus results are in contrast with those produced by Wang and co-workers [27], but the increament in MPO activity are agreement with previous work by Zanardo and co-workers [48], in which they showed that animals treated with BCA exhibited enhanced leukocyte adherence and infiltration in an air pouch model [47]. Thus, it is possible that the administration route used by these authors (i.d.) may account for the discrepancies considering that local desensitization effects may be produced by these drugs independently of H_2S generation.

In addition to potentiating the scratching behavior, H_2S synthesis blockade by BCA or AOAA has also enhanced neutrophil influx into the dorsal skin, under basal conditions. Furthermore, the non-selective inhibition of CBS/CSE by AOAA increased the basal neutrophil influx to an extent similar to that induced by C48/80, thus reflecting the protective role of endogenous H_2S in the normal skin physiology.

Conclusions

Altogether the results shown in this study provide the first evidence that endogenous H_2S, as well as the administration of low doses of spontaneous H_2S donors, such as LR and Na_2S, exert protective roles against histamine/mast cell-mediated acute pruritus and inflammation of the dorsal mouse skin, independently of H_2S action on K_{ATP} channels.

Conflicts of interest

We have no conflict of interest to declare.

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