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

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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 increased both Tyrode and C48/80-induced responses in the skin, whereas the blockade of K_{ATP} channels by glibenclamide did not. H_2S-releasing donors significantly attenuate C48/80-induced mast cell degranulation either *in vivo* or *in vitro*. We provide first evidences that H_2S donors confer protective effect against histamine-mediated acute pruritus and cutaneous inflammation. These effects can be mediated, at least in part, by stabilizing mast cells, known to contain multiple mediators and to be primary initiators of allergic processes, thus making of H_2S donors a potential alternative/complementary therapy for treating inflammatory allergic skin diseases and related pruritus.

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 exper-

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imentally 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 no. 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 (12 × 20 × 17 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 anaesthetized 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 and i.d. injected (Tyrode and C48/80 3 μg/site) mouse dorsal skin was carried out based on the formation of lead sulfide after 30 min and 4 h post injection, accordingly [31]. Briefly, naïve and i.d. injected 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 2 h 30 min at 37 °C. The dark dots densities on the lead acetate white paper strips (12 × 8 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 (7.8–500 μM).

2.6. Assessment of rat mast cell degranulation

2.6.1. Intravital microscopy assay

Under a mixture of ketamine and xylazine anaesthesia (100 and 10 mg/kg, respectively, i.p.), the rat was placed on a homeothermic blanket system (37 °C), and the mesentery was exposed for microscopic observation as described previously [32]. The exteriorized mesentery was superfused with Ringer–Locke solution (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2{\cdot}2H_2O$, 6 mM $NaHCO_3$, 5 mM glucose, 0.025 mM ascorbic acid pH 7.2–7.4, 37 °C) and a volume of 30 μl of Tyrode or C48/80 (10 μg) was applied to the mesentery. Alternatively, 30 μl of the test agents Na_2S, LR or GYY4137 (30 nmol)

was applied to the mesentery field prior (−2 min) to C48/80. The transilluminated images of the mesentery were assessed via optical intravital microscopy equipped with the objective x40.0/0.80 (DMLFS, Leica, Wetzlar, Germany) and captured for 3 min by a video camera (DFC300 FX, Leica) adapted to a TV monitor and a PC. The number (or percentage) of intact and degranulated mast cells surrounding the mesentery was determined by topically applying 1% toluidine blue at the termination of each experiment.

2.6.2. Isolation of mast cells and quantification of histamine release by HPLC–MS/MS

Rats (n = 6) were exsanguinated under deep isoflurane anaesthesia (5% v/v in O_2), and 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 (4×10^5 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 [33].

2.6.3. Tissue preparation (dorsal skin) and staining for mast cell histological analysis

Mouse dorsal skin sites were i.d. injected with Tyrode, C48/80 (3 μg/site) alone and co-injected with Na_2S, LR or GYY4137 (30 nmol/site). After 30 min, skin sites (8 mm) were removed and fixed in 4% buffered formalin (pH 7.4) before embedding in paraffin. 5 μm-thick sections were cut, mounted onto slides and stained with acidified toluidine blue (1%). The number of intact and degranulated mast cells was assessed via optical microscopy (×400, Leica DM 2500; Switzerland).

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, toluidine 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 $Na_2S{\cdot}9H_2O$ 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. Ketamine and xylazine were obtained from Virbac do Brasil (São Paulo, Brazil) and Konig do Brasil (Mairinque, São Paulo, Brazil), respectively. BCA (β-cyanoalanine) was obtained from Cayman Chemical Company (USA) and GYY4137 was synthesized in house, as described in Ref. [34].

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 Fig. 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 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.

3.2. Effects of H_2S-releasing donors on dorsal skin plasma extravasation and neutrophil influx induced by amines

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.

Four hours after i.d injection of C48/80 in the mouse dorsal skin (3 μg/site) markedly ($P < 0.001$) increasead MPO activity compared with Tyrode (Fig. 5). The 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.

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

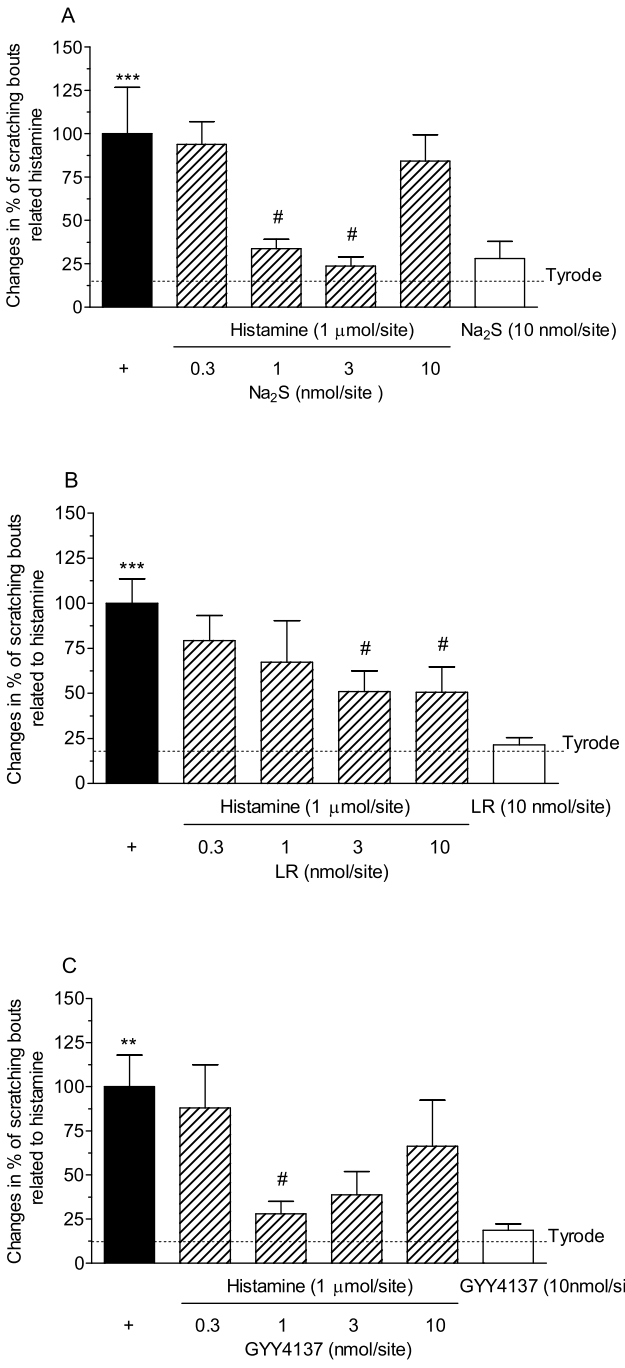

Fig. 1. Dose response relationship for H_2S-releasing donors on histamine-induced scratching bouts. The scratching bouts evoked by i.d. injection of histamine alone or co-injected with Na_2S (0.3–10 nmol/site., $n = 5$–10), LR (0.3–10 nmol/site., $n = 5$–8) and GYY4137 (0.3–10 nmol/site., $n = 5$–6)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

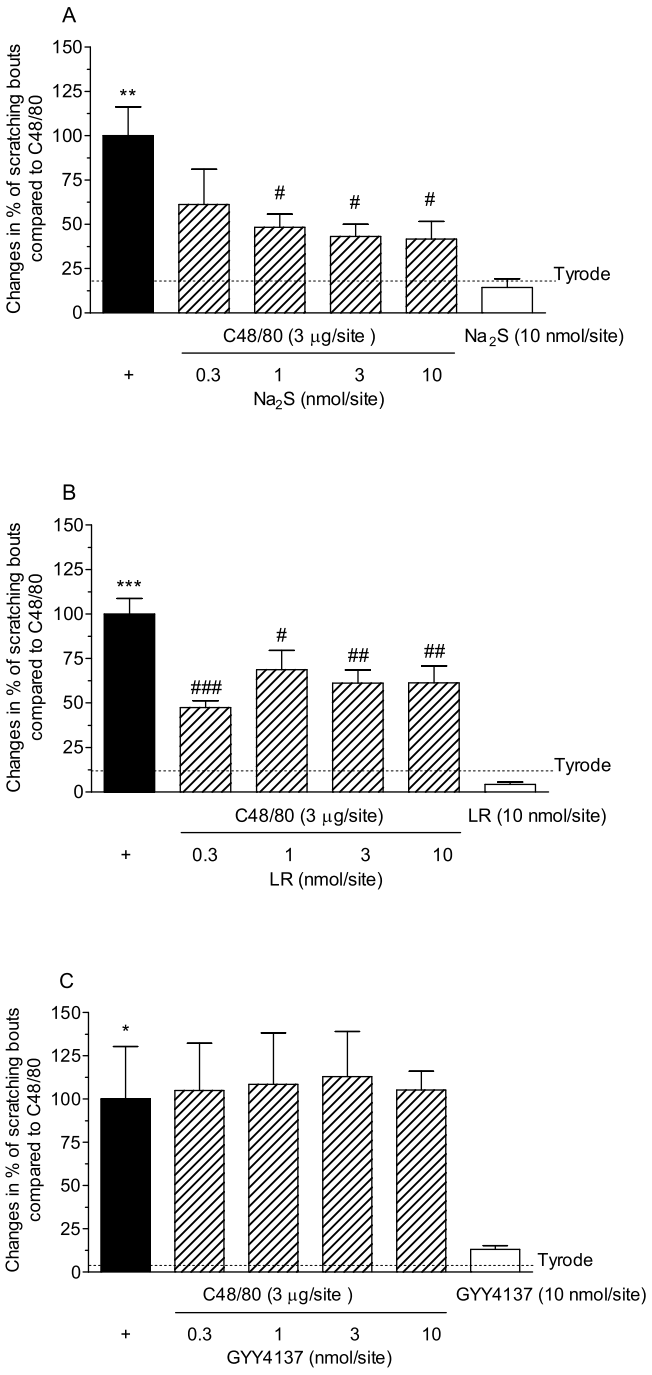

Fig. 2. Dose-response curves for H_2S-releasing donors on C48/80-induced scratching bouts. Panels (A–C) show the percentage of scratching bouts evoked by C48/80 alone (3 μg/site; i.d) and co-injected with Na_2S (0.3–10 nmol/site, $n = 5$–8), LR (0.3–10 nmol/site, $n = 5$–7) and GYY4137 (0.3–10 nmol/site, $n = 7$), 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).

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 C), 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).

3.4. Determination of H_2S in mouse dorsal skin

The naïve mouse skin, similarly to the brain, produced a significant and equivalent amount of H_2S, whereas a marked H_2S generation was measured in the liver of naïve mice (Fig. 7A). The i.d.

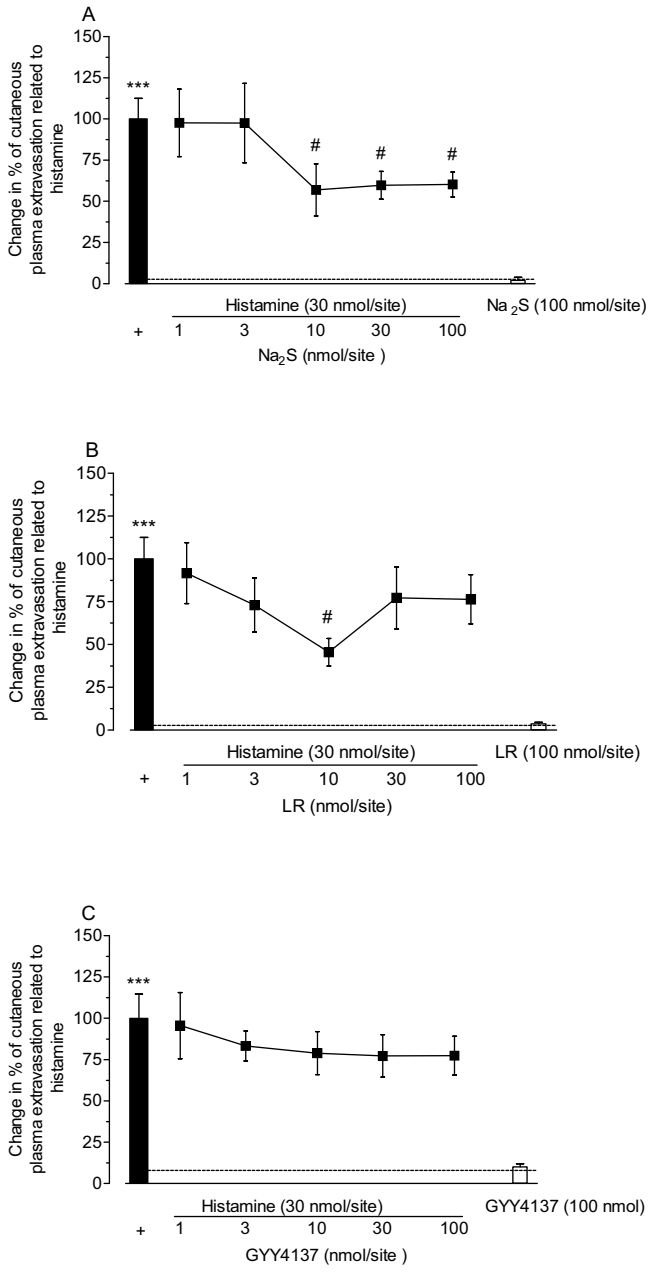

Fig. 3. Dose-response curves of the H_2S donors on plasma extravasation induced by i.d. injection of histamine. Panels (A–C) show the co-injection effects of Na_2S (1–100 nmol/site, $n = 5$–7), LR (1–100 nmol/site, $n = 5$) and GYY4137 (1–100 nmol/site, $n = 7$–10) on histamine (30 nmol/site)-induced 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).

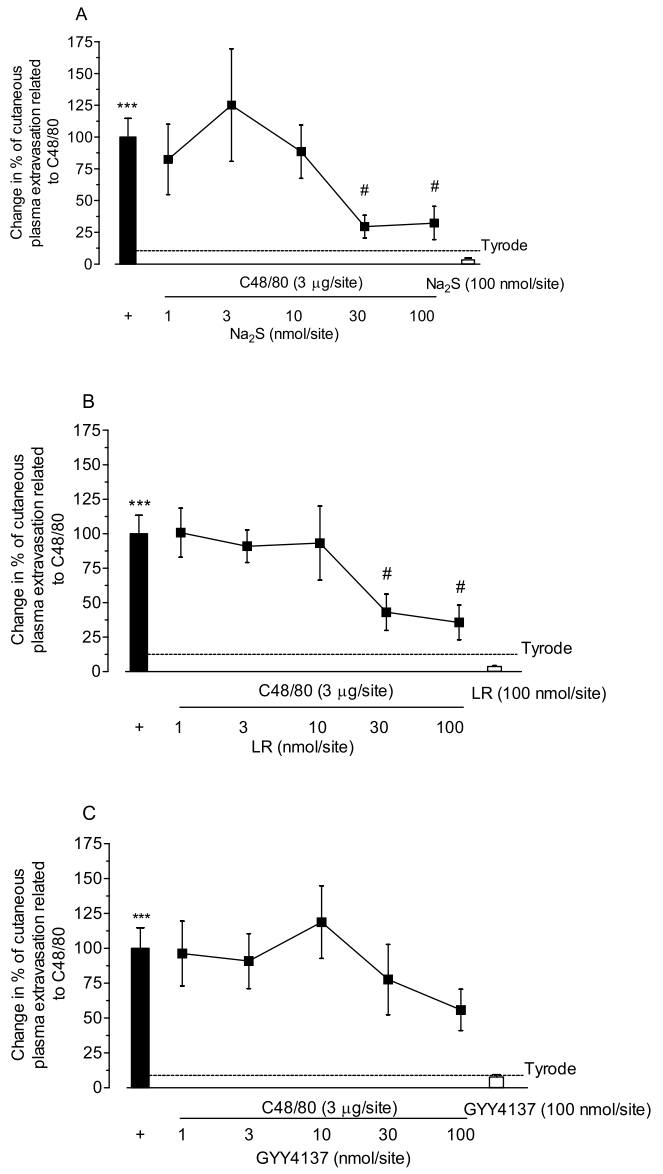

Fig. 4. Dose-response relationship between H_2S-releasing donors on C48/80-induced cutaneous plasma extravasation. Panels (A–C) illustrate response evoked by i.d. injection of C48/80 (3 μg/site) alone and co-injected with Na_2S (1–100 nmol/site., $n = 6$), LR (1–100 nmol/site., $n = 8$) and GYY4137 (1–100 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).

injection of C48/80 (3 μg/site) significantly increased the endogenous H_2S production after 30 min, but not 4 h post injection, as compared to the vehicle Tyrode (Fig. 7B and C).

3.5. *Effects of H_2S-releasing donors on C48/80-induced mast cell degranulation*

3.5.1. *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).

3.5.2. *H_2S-releasing donors attenuate C48/80-induced mast cell degranulation* in vivo

When applied to the mesentery conjunctive bed, the C48/80 (10 μg) also significantly degranulated mast cells (4.0 ± 0.4 DMC per field, n = 5) as compared to Tyrode (0.5 ± 0.2 DMC per field, n = 5,

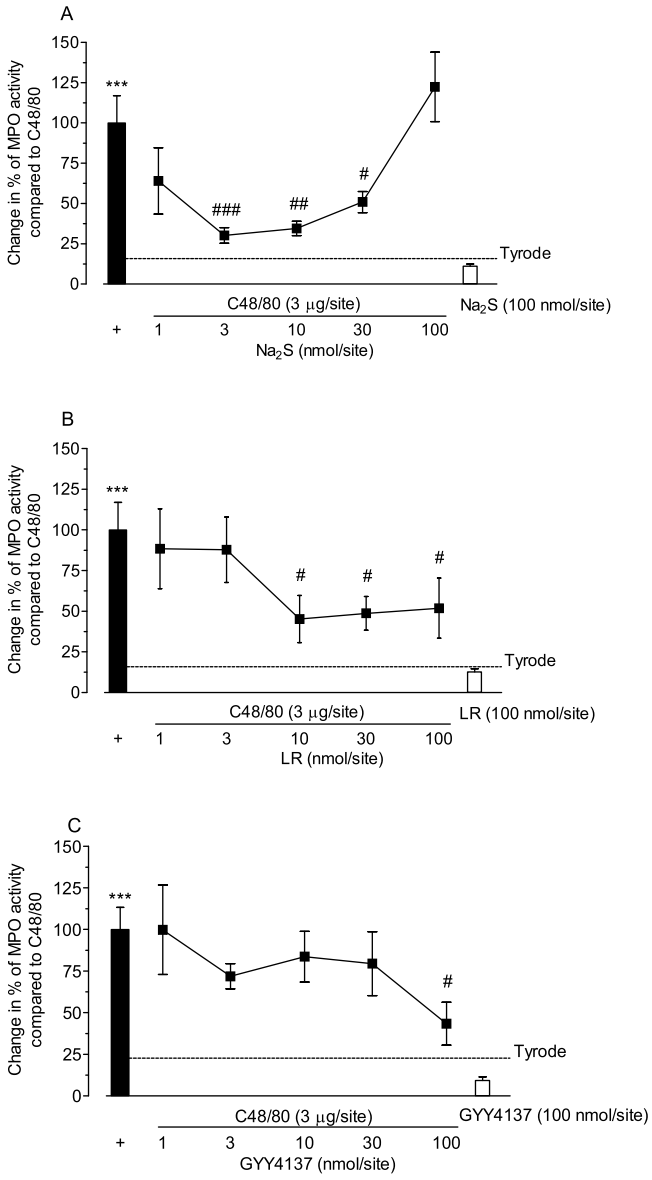

Fig. 5. Effects of H_2S donors on C48/80-induced increased MPO activity. Panels (A–C) show the MPO activity evoked by i.d. injection of C48/80 (3 μg/site) alone and co-injected with Na_2S (1–100 nmol/site., $n = 6$), LR (1–100 nmol/site., $n = 10$) and GYY4137 (1–100 nmol/site., $n = 8$), respectively, 4 h 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).

Fig. 9A and B). The pretreatment of the rat mesentery with the H_2S-releasing donors Na_2S, LR and GYY4137 (30 nmol, 30 μl) resulted in higher population of intact mast cells and a smaller number of activated/degranulated cells in response to C48/80 (1.8 ± 0.9, 0.9 ± 0.5 and 1.5 ± 0.6 DMC, respectively, n = 5 each group, Fig. 9A and B) as compared to the mesentery treated only with C48/80.

Likewise, the representative photomicrography of the mouse dorsal skin i.d. injected with C48/80 (3 μg/site) shows an increased population of degranulated mast cells together discrete intact cells as compared to naive or Tyrode-injected skin (Fig. 10A–C). The i.d. co-injection of H_2S-releasing donors Na_2S, LR or GYY4137 (30 nmol/site) with C48/80 reduced significantly the number of degranulated mast cells which is paralleld by a significant increase in intact cells (Fig. 10D–F).

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. 11A), 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. 11B) or pruritus (Fig. 11C) 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. 11B and C).

4. Discussion

The major finding in this study is to show that the spontaneous H_2S donors Na_2S and LR significantly ameliorated pruritus and the acute cutaneous inflammation induced by histamine and the mast cell degranulation in the mouse dorsal skin, this effect being 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. This suggests that low doses of spontaneous/fast-releasing H_2S donors (Na_2S and LR) might be preferable to the slow-releasing H_2S donor (GYY4137) to treat acute pruritus and cutaneous inflammation.

Several factors including lipid solubility, compartimentalization within cells, physico-chemical characteristics, local pH/environment, rate of cellular uptake and H_2S availability/rate of release within the microcirculation and the unmyelinated C-fibers may differ among the compounds. Furthermore, since mast cells contain several inflammatory/pruritogenic mediators in addition to histamine, this may also contribute to the weak effect of GYY4137 in the C48/80-induced responses.

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 [35,36] and humans [37,38]. 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 studies [27], it is possible that as this compound instantaneously delivers H_2S, the highest tested dose (100 nmol/site) led to an immediate non-physiological concentration of H_2S in the local microcirculation, which may be distinct from that seen in the dorsal skin. These results point to the same conclusion from previous studies, where high doses of 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 [34,39–42]. In line with this, we provide the first evidence that the mouse healthy dorsal skin constitutively produces low amounts of H_2S (≅0.3 nmol of H_2S per mg of protein per min), similar to that produced by the brain but less than that produced by the liver. Importantly, a significant increase in the endogenous production of H_2S in response to C48/80 was measured after 30 min post injection, but not 4 h post injection. Taking together with the results obtained under H_2S-synthesis inhibition, it is plausible to suggest that the early increase (30 min post i.d. injection of C48/80) in H_2S production in response to mast cell

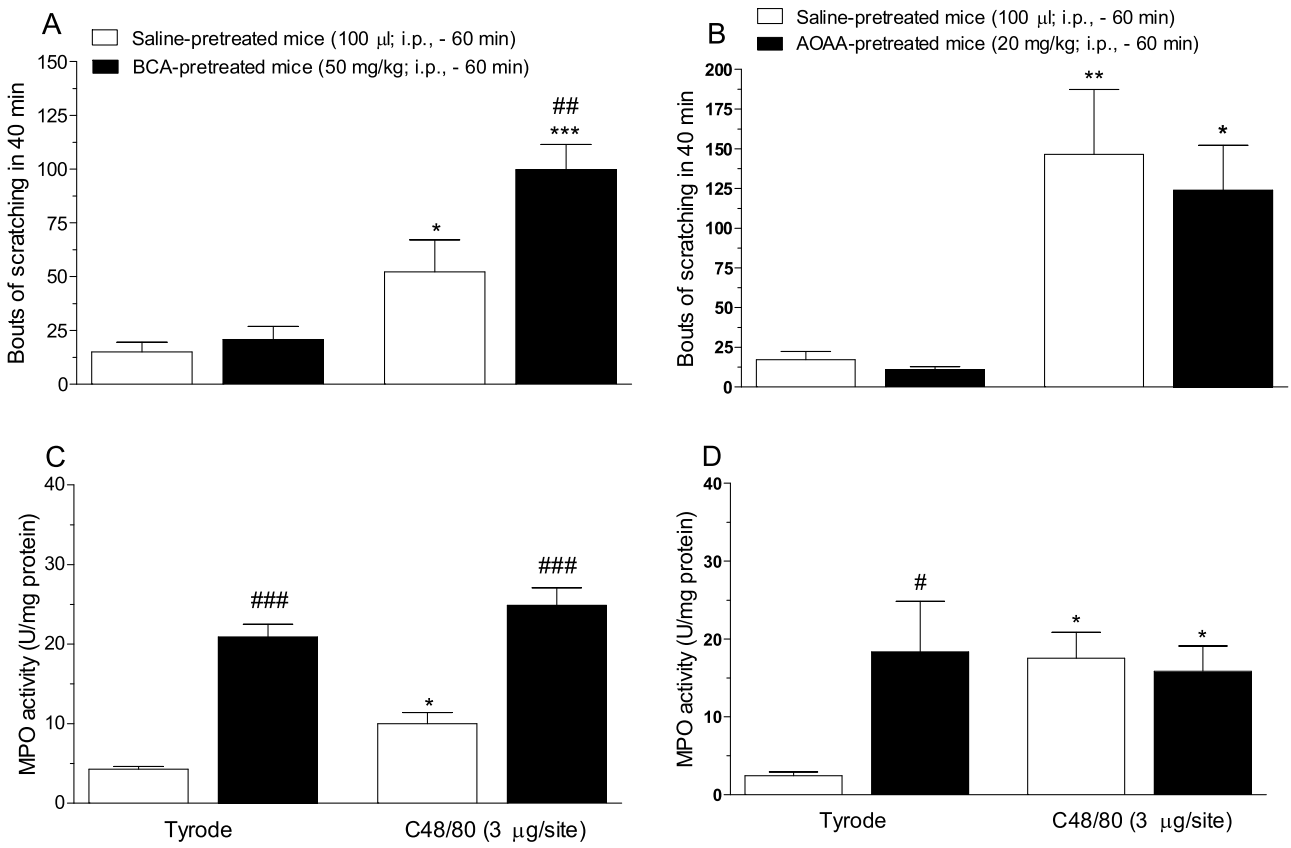

Fig. 6. Endogenous blockade of H_2S synthesis potentiated C48/80-induced pruritus and MPO activity in mouse dorsal skin. Panels (A) and (B) show the effects of pretreatment with either the CSE inhibitor, β-cyanoalanine (BCA, 50 mg/kg, −60 min) or the nonselective CBS inhibitor aminooxyacetic acid (AOAA, 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).

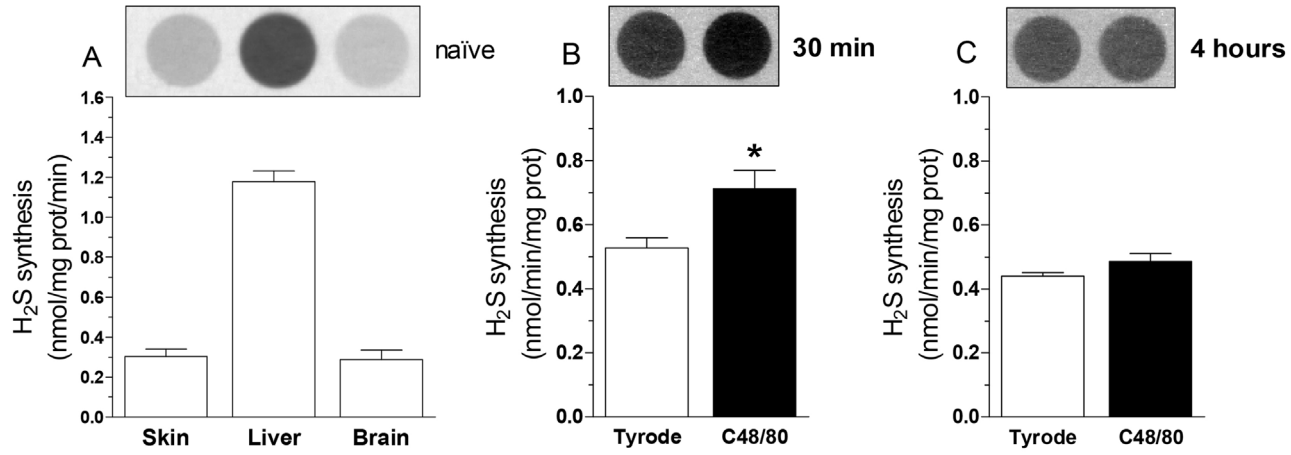

Fig. 7. Hydrogen sulfide generation in both the naïve and inflamed skin. Panel (A) shows the H_2S generation in the skin, liver and brain of naïve mice ($n = 4–5$), whereas panels B and C show this gas generation in response to i.d. injected Tyrode and C48/80 (3 μg/site) after 30 min and 4 h post injection, respectively. Data are mean ± SEM for n = 5. $^{*}P < 0.05$ *vs.* Tyrode (One-way ANOVA followed by the Dunnett's test).

degranulation is an attempt of the host to control the inflammatory response.

Histamine exerts its inflammatory effects, in part, through NO generation *in situ* [36], since the blockade of nitric oxide synthase (NOS) significantly inhibited histamine-induced plasma extravasation in human nasal airway [43]. 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. Indeed, C48/80-mediated responses can be significantly inhibited by antagonists of histamine H1 receptors, 5-HT or substance P (NK1) receptors [37,44,45].

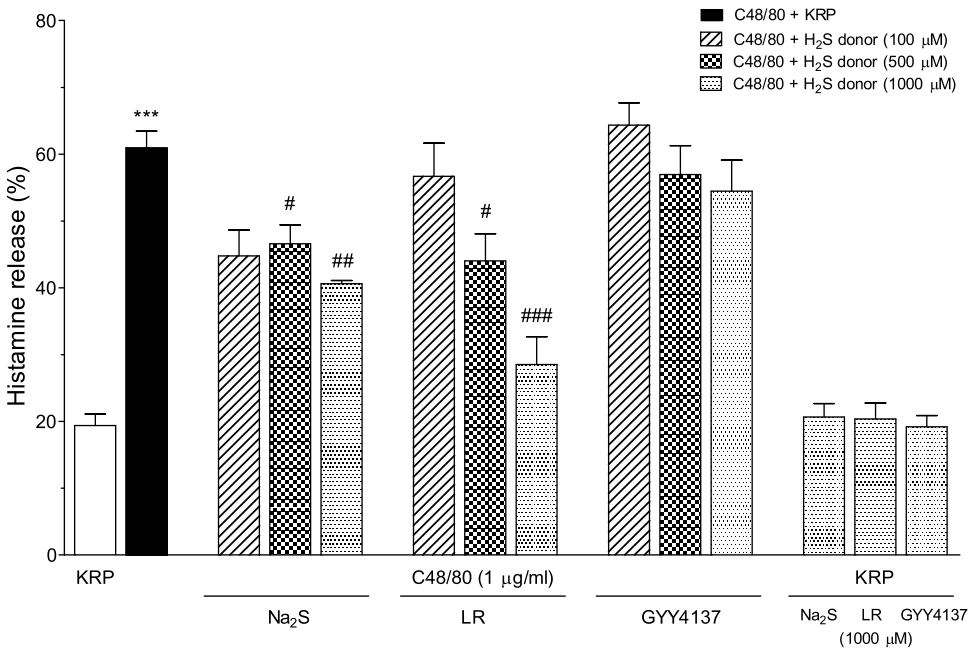

Fig. 8. Spontaneous, but not slow, H_2S donors reduce C48/80-induced mast cell degranulation. Black bar illustrates the % of histamine released from mast cells treated with C48/80 (1 μg/ml) in krebs ringer phosphate solution (KRP). Cross-hatched bars show the % of histamine release from mast cells in 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).

In this study, C48/80-induced plasma extravasation and pruritus was partially, but significantly, inhibited by the simultaneous co-injection of the spontaenous H_2S donor 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 h 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 during 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 both *in vivo* and *in vitro* experiments. By using isolated rat peritoneal mast cells stimulated with C48/80 *in vitro* in order to measure the histamine release, we observed that Na_2S and LR, but not GYY4137 prevented mast cell degranulation. Corroborating this *in vitro* data, the use of *in vivo* intravital microscopic approach to study the effects of exogenous H_2S on mast cells integrity clearly established a stabilizing effect for both spontaneous/fast and slow H_2S-releasing donors on mast cell degranulation index in response to C48/80 administration to the rat mesentery. Likewise, representative photomicrograph from mouse dorsal skin stained with toluidine reinforces this findings, as it clearly illustrates that either Na_2S or LR and at a lesser extent GYY4137 promoted direct antidegranulating effects in the mast cells of the mouse dorsal skin i.d. injected with C48/80.

In spite of the discrepancies between the *in vitro* and *in vivo* pharmacological data obtained with the slow H_2S-releasing donor GYY4137 on mast cells stability. It is worthwhile mentioning that *in vivo* organisms differ greatly from *in vitro* studies, mainly considering the variable drug fate and biotransformation pathways. For example, high concentration of H_2S produced apoptosis in intestinal epithelial cells *in vitro* [49], whereas the *in vivo* systemic treatment of rodents with high doses of H_2S donors did not evoke death or important side effects in the rat [46]

Importantly, Zanardo and co-workers [47] were one of the first to show by using intravital microscopy that spontaneous/fast H_2S-releasing donors (Na_2S, NaHS and LR) markedly inhibited carrageenan-induced leukocyte adhesion and infiltration in a rodent air pouch model, and that the endogenous production of H_2S acts as an important modulator of acute inflammation. 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 [48] 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 [41,47–49].

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 [46] and high glucose-induced cardiac cells injury [50]. However, this does 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} channel 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 [51–53]. Similarly to the spontaneous H_2S

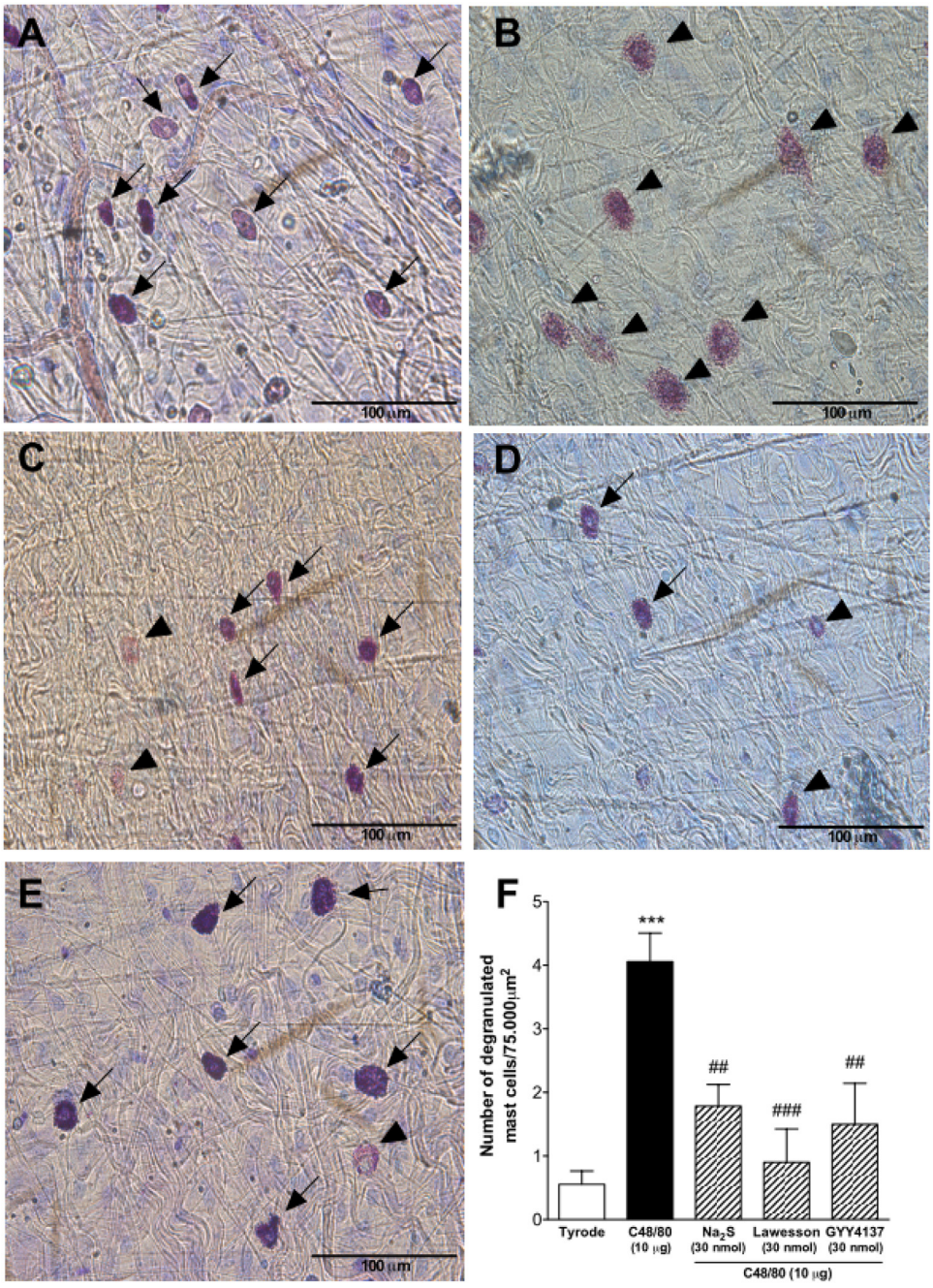

Fig. 9. Stabilizing effects of H_2S-releasing donors on mast cell degranulation index in the rat mesentery. The mesentery conjunctive bed was exposed to Tyrode or C48/80 (10 μg, panel A and B, respectively) alone or together with H_2S-releasing donors (30 nmol, −2 min; panels C–F). The exemplar images of the mesenteric bed stained with 1% toluidine blue show intact and or degranulated mast cells in the naïve mesentery bed (naïve, panel A) exposed to either Tyrode (control; panel A) or C48/80 alone (panel B) or C48/80 together with 30 nmol of H_2S-releasing donors: Na_2S, LR and GYY3147 (panels C–E, respectively). Black arrows and the arrows heads indicate intact and degranulated mast cells, respectively. Data are expressed as mean ± S.E.M. of degranulated cells per area for n = 6 animals/group. $^{***}P < 0.05$ vs Tyrode. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ *vs.* C48/80 alone (One-way ANOVA followed by Dunnett's test).

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, 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 [53–55].

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. Elies and co-workers [56] showed that NaHS at low concentrations

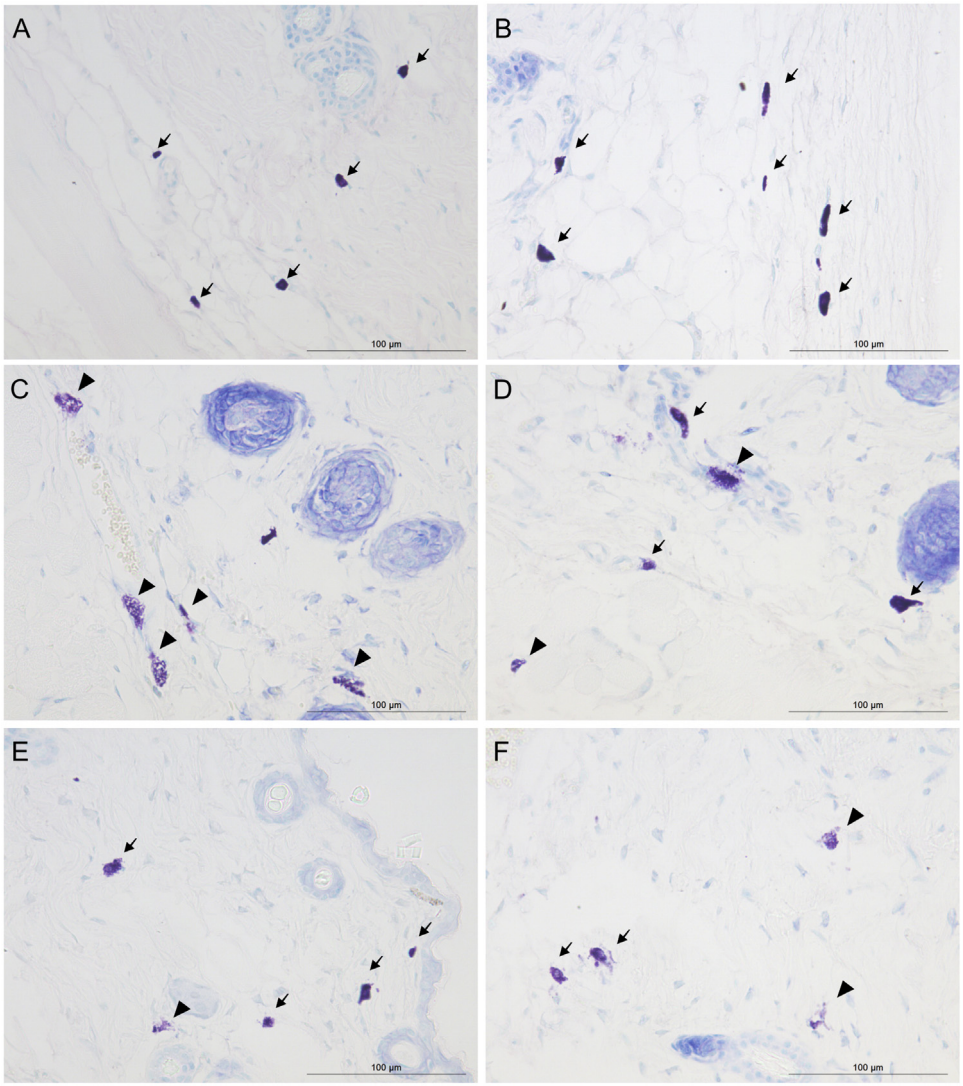

Fig. 10. Representative photomicrographs from mouse dorsal skin stained with 1% toluidine blue show the protective effects of H_2S-releasing donors on C48/80-induced mast cell degranulation. Panel A (naïve skin) shows intact mast cells features (black arrows) with no evidence of degranulated cells. Panel B (Tyrode) shows intact cells and scarce activated mast cells with no clear evidence of degranulated cells. Panel C (C48/80 3 µg/site, i.d.) illustrates several degranulated mast cells (arrows heads) and scarce intact cells (black arrows). Panels D–F (C48/80 + Na_2S or LR or GYY4137 30 nmol) illustrate that C48/80-induced mast cell degranulation is greatly reduced by spontaneous H_2S-releasing donors and at a lesser extent by GYY4137. Light microscopy with high magnification (400×) and scale bar = 100 µm.

(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 demonstrate that inhibition of endogenous H_2S biosynthesis by BCA, but not by AOAA, resulted in further enhancement of C48/80-induced scratching behavior in addition to increased MPO activity. There has been a discrepancy in the results among BCA and AOAA elesewhere. This should be strengthened by the fact that BCA more selectively inhibits CSE while AOAA can inhibit both CSE and CBS. As stated earlier, the mechanisms involved in both pruritus and inflammation evoked by C48/80 are complex and multimediated, since several mediators together with histamine are released from mast cells and in turn can also activate mast cells [54,55]. Altogether these findings contribute to the suggestion that endogenous H_2S synthesis inhibition seems to be detrimental, since it led to a marked increase of leukocyte influx in response to either non noxious (Tyrode) or noxious stimuli (C48/80).

Again, the C48/80-induced pruritus results are in contrast with those produced by Wang and co-workers [27], but the increase in MPO activity is in agreement with previous work by Zanardo and co-workers [46], in which they showed that animals treated with BCA exhibited enhanced leukocyte adherence and infiltration in an air pouch model [47]. 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.

5. 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 exogenous spontaneous/fast H_2S-releasing donors (*e.g.* LR and Na_2S), and at a lesser extent the slow H_2S-releasing donor GYY4137, exert protective effects against histamine/mast cell-mediated acute pruritus and inflammation in the dorsal skin, and this effect being independently of K_{ATP} channels modulation.

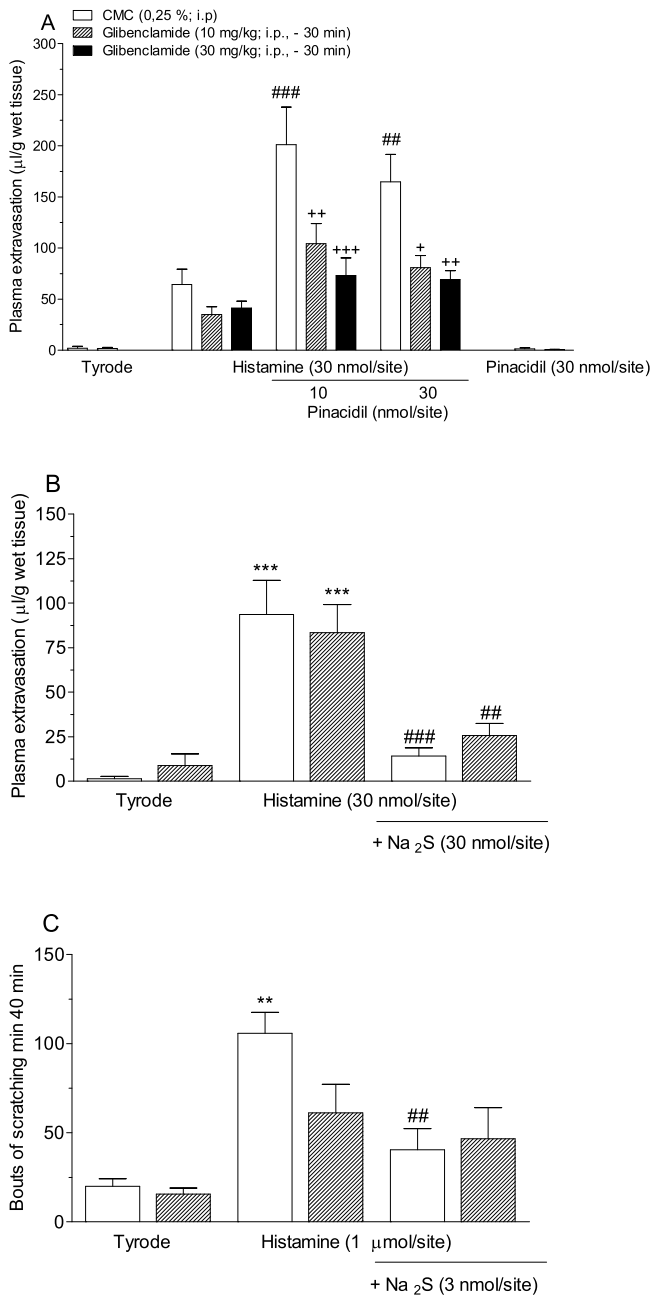

Fig. 11. Blockade of K_{ATP} channels failed to reverse Na_2S-induced protective effect against histamine-induced plasma extravasation and pruritus. Panel (A) shows the effect of glibenclamide (10 or 30 mg/kg., i.p., −30 min) or its vehicle (CMC) on pinacidil (10 or 30 nmol/site)-induced potentiating 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).

Conflicts of interest

We have no conflict of interest to declare.

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