Nitroxyl (HNO) is a redox sibling of nitric oxide (NO) that targets distinct signalling pathways with pharmacological endpoints of high significance in the treatment of heart failure. Beneficial HNO effects depend, in part, on its ability to release calcitonin gene-related peptide (CGRP) through an unidentified mechanism. Here we propose that HNO is generated as a result of the reaction of the two gasotransmitters NO and H$_2$S. We show that H$_2$S and NO production colocalizes with transient receptor potential channel A1 (TRPA1), and that HNO activates the sensory chemoreceptor channel TRPA1 via formation of amino-terminal disulphide bonds, which results in sustained calcium influx. As a consequence, CGRP is released, which induces local and systemic vasodilation. H$_2$S-evoked vasodilatory effects largely depend on NO production and activation of HNO–TRPA1–CGRP pathway. We propose that this neuroendocrine HNO–TRPA1–CGRP signalling pathway constitutes an essential element for the control of vascular tone throughout the cardiovascular system.
HNO-induced disulphide formation on TRPA1. hTRPA1 is rich in cysteine residues and C621, C641 and C665 of the N terminus are responsible for electrophilic activation. To test whether these specific cysteine residues play a role, experiments were performed on HEK293 cells expressing hTRPA1 and cysteine- and lysine-neutralized mutants, hTRPA1-C621S/C641S/C665S (hTRPA1-3C) and hTRPA1-C621S/C641S/C665S/K710R (hTRPA1-3CK). AS had no effect on non-transfected HEK cells but induced large responses in hTRPA1-transfected cells. Cells expressing hTRPA1-3CK or hTRPA1-3C did not respond to AS at all (Fig. 2a). Although many TRPA1 agonists at higher concentrations also activate TRPV1 (ref. 26), no responses could be observed in experiments on hTRPV1-transfected HEK cells (Supplementary Fig. 2c).

Providing that the observed effects originate from disulphide formation, the reducing agent dithiothreitol (DTT) should interfere with the outlasting TRPA1 responses. Indeed, the decay of the AS responses was considerably accelerated when 5 mM DTT was externally applied, and the downward inflection upon DTT onset almost restored intracellular Ca\(^{2+}\) to baseline level within 10 min (Supplementary Fig. 3). HC030031 applied after AS caused similar effects, however, after its removal, a rebound rise in intracellular Ca\(^{2+}\) occurred, confirming a temporary block in presence of a sustained TRPA1 modification by HNO.

The outlasting AS-induced inward currents were instantly reversed by the administration of 5 mM DTT and did not recur upon its washout (Fig. 2b). When the cells were subsequently re-exposed to AS, the responses were smaller (62.5% of first response). These currents could be blocked by HC030031 only for the duration of its application, and DTT again deactivated the recurred inward current completely.

Formation of disulphides upon HNO stimulation of TRPA1 was further supported using a modified biotin-switch assay (Fig. 2c) on V5-poly-His-tagged mTRPA1 expressed and purified from HEK cells (Supplementary Fig. 4a–d). The protein that was further supported using a modified biotin-switch assay (Fig. 2c) on V5-poly-His-tagged mTRPA1 expressed and purified from HEK cells (Supplementary Fig. 4a–d). The protein that was exposed to HNO provided positive staining in a concentration-dependent manner while the control or the iodoacetamide (IA)-pretreated protein exposed to HNO showed no disulphide bond formation (Fig. 2c).
To identify the reaction site, a custom-made synthetic peptide consisting of 64 amino acids of the hTRPA1 N terminus, including the three critical and three neighbouring cysteine residues, was analysed by MALDI-TOF mass spectrometry (MS) (Fig. 2d,e). MS analysis revealed disulphide formation between the critical Cys 621 and the neighbouring Cys 633 as well as between Cys 651 and the critical Cys 665 (Fig. 2f).

Formation of disulphides by HNO would go step-wise, with initial formation of a (hydroxyamino)sulfinyl derivative at critical cysteine residues and then fast subsequent reaction with another cysteine in vicinity, leading to substantial allosteric deformation and channel opening (Fig. 2g). Such disulphide bonds may account for the observed slow deactivation of TRPA1 after AS treatment. *Ab initio* structure prediction of a 200 amino acid long N-terminal sequence covering important cysteine residues (Fig. 2h) revealed that the cysteine residues 665 and 651 are connected by a flexible loop and could easily get into each other’s vicinity, a condition that would facilitate disulphide bond formation. In addition, disulphide bond formation is facilitated between the Cys 633 and 621, where the atom distance is estimated to be 4.4 Å.

**TRPA1 is responsible for HNO-induced release of CGRP.** Next we tested how HNO-induced TRPA1 activation affects CGRP release from isolated tissues. CGRP is typically released from polymodal A-delta and C-fiber afferents. At first, the release of CGRP from rat dura mater exposed in a hemisected skull preparation was measured in 5-min samples of incubation fluid using enzyme immunoassays (Fig. 3a–c). Exposure to AS induced an increase in CGRP release (by 46.4 ± 5.3 pg ml⁻¹) while DEA NONOate was ineffective (Fig. 3b,c) confirming previous observations of distinct biochemical pathways that these two congeners enter downstream of nitric oxide synthase (NOS)¹⁻⁴. HC030031 significantly inhibited the AS-evoked responses (18.1 ± 4.6 pg ml⁻¹, Fig. 3a,c).

We also added evidence from another species and innervation territory measuring CGRP release from sciatic nerve (Fig. 3d) and cranial dura mater (Fig. 3e) of wild-type and TRPA1−/− mice. In both preparations, HC030031 had the same inhibitory effects (Fig. 3f) as observed in rat (Fig. 3a,c) but preparations taken from congenic TRPA1−/− mice did not respond to AS stimulation at all (Fig. 3f). These results confirm that HNO-induced CGRP release is selectively mediated via TRPA1 receptor channels.

CGRP is a potent vasodilator contributing to overall cardiovascular homeostasis. It is also released from trigeminal nerve fibres accompanying meningeal blood vessels and plays an established role in migraine. In anaesthetized rats, topically applied AS (60 nmol) induced an increase in meningeal blood flow (recorded by laser Doppler flowmetry) by 24 %, while co-application of HC030031 (50 μM) reduced this response by 64% (Fig. 4a). Co-application of CGRP receptor antagonist, CGRP₈⁻₃₇, also inhibited the AS-evoked dilatation, although not as effectively as TRPA1
Figure 2 | HNO activates TRPA1 via disulphide bond formation. (a) AS (300 μM, 45 s) increases intracellular Ca\(^{2+}\) in hTRPA1-transfected HEK cells but not in cells expressing hTRPA1-3C; mean ± s.e.m., n = 250 cells for each group. Representative pseudocolor images (scale bar, 100 μm). (b) AS-evoked inward currents (n = 7) can be reversed by application of DTT (5 mM, 60 s), or temporarily blocked by HC030031 (50 μM, 10 s). Inset: AS (400 μM)-evoked peak inward currents are significantly reduced by HC030031 or DTT (analysis of variance following honestly significant difference post hoc test, P<0.001 each; n = 7; error bars represent s.e.m.). (c) Detection of disulphide bond formation on purified mTRPA1 channel protein (100 mM) treated with AS, using modified biotin-switch assay. Lane 1: TRPA1 treated with 1.5 mM AS, lane 2: TRPA1 treated with 0.5 mM AS, lane 3: untreated protein, lane 4: TRPA1 pretreated with IA and then treated with 1.5 mM AS. (d,e) MALDI-TOF MS of AS-treated TRPA1 synthetic peptide. Peptide fragments containing cysteine residues differ in AS-treated (black, d) and control (red, e) samples. The same fragmenting pattern is observed in both cases but with m/z of the AS-treated fragments being shifted towards lower masses (by m/z 116 = 2IA + 2H), indicating formation of disulphides. (f) Amino-acid sequence of synthetic peptide used in the study to mimic the part of hTRPA1 N terminus with critical, that is, activating cysteines and a rationale for deciphering disulphide bond positions based on observed fragments. Yellow marked cysteine residues form disulphide bonds and red cysteine residues are found to be modified by IA even after exposure to AS. (g) Schematic model of TRPA1 with cysteine-rich region (red dots) and formation of disulphide bonds causing major conformational changes. Chemical structure (bottom) of two cysteine-SH residues reacting with HNO to form hydroxylamine (NH₂OH) and a disulphide bond and causing conformational change. (h) Ab initio model of the 200 amino acid long polypeptide chain of the N terminus of hTRPA1 displaying five essential cysteine residues and two indicated disulphides (dotted lines).
channel blockade, most likely due to modification of the inhibitory peptide by HNO (Supplementary Fig. 5).

To assess to what extent the systemic hypotensive effect of HNO depends on TRPA1, the mean arterial blood pressure (MAP) of anesthetized mice was measured. Mice were injected intravenously with freshly dissolved AS (61 μg kg⁻¹ body weight) in saline solution (pH 7.0). AS induced a drop of MAP by 18.8 ± 0.9 mm Hg in wild type, but only 8.8 ± 0.9 mm Hg in TRPA1⁻/⁻ mice (Fig. 4b), indicating that a significant proportion of the vasodilatory effect of HNO is mediated by TRPA1.

CGRP release from sensory nerve fibres does not necessarily require action potential discharge; subliminal depolarization is sufficient. Nonetheless, strong activation of TRPA1-expressing peptidergic nerve fibres should evoke pain. This was tested in human volunteers by double-blinded intracutaneous injection of AS (0.7 μmol, Fig. 4c). Injections of AS caused an immediate burning pain declining over ~7.5 min with a mean maximum rating of 3.6 ± 0.4 (on a numerical rating scale 0–10, n = 6). Decomposed AS and DEA NONOate were neither rated as painful nor as itching (Fig. 4d). Although TRPA1 is not activated by noxious heat in heterologous expression systems, its activation by cinnamaldehyde causes heat hyperalgesia in humans. In our experiments, injection of AS but not of DEA NONOate or decomposed AS increased the pain ratings (from 2.6 ± 0.6 to 4.3 ± 0.7, P < 0.001, analysis of variance and large significant difference) in response to noxious heat (5 s, 47°C). In addition, injection of AS into the skin of the volar forearm induced a large axon-reflex erythema (Fig. 4c) visualized by laser Doppler scanning of superficial blood flow (Fig. 4e, Supplementary Fig. 6a), which was not affected by the pre-administration of Clemastine, an H1 histaminic receptor blocker, indicating that the AS-induced vasodilation is not due to mast cell degranulation (Supplementary Fig. 6b,c).

Generation of HNO from NO and H₂S. The cross-talk of H₂S and NO has been suggested but the actual direct reaction of NO with H₂S has not been studied before. The reaction of NO and H₂S solutions was initially probed amperometrically using H₂S and NO selective electrodes. The drop of the H₂S electrode response upon addition of gaseous NO suggested immediate H₂S consumption under aerobic conditions (Fig. 5a) and the apparent rate of this reaction remained unchanged even in the presence of 20-fold excess of glutathione in anoxic conditions (Supplementary Fig. 7a). In parallel, NO release from DEA NONOate was prevented when H₂S was present in the solution (Fig. 5b). Both, the addition of NO to degassed H₂S solution, or H₂S to NO solution led to immediate sulphur/polysulphide formation suggesting that H₂S was oxidized and consequently NO was reduced.

The ultimate proof for the in vitro HNO formation came from the recently developed HNO-selective electrode. The results show that a rate of HNO production through an anaerobic reaction between NO and H₂S is orders of magnitude faster than for any known HNO donor (Fig. 5c, Supplementary Fig. 7b). For example, 2 μM combination of each NO and H₂S yields a peak HNO concentration of ca. 0.5 μM, similar to effects of almost three orders of magnitude higher AS concentration (1 mM, Fig. 5c).

Finally, HNO formation was also tested on cellular level using a HNO-selective fluorescent sensor. Only the combination of applied NO and H₂S provided strong fluorescence signals characteristic of HNO (Fig. 5d). Interestingly, a basal...
fluorescence of the untreated cultured neurons was observed, suggesting that some basal formation of HNO was constitutively present. Thus, we inhibited enzymatic NO and/or H2S production to see the effect on basal intracellular HNO level. In neurons, treatment for 2 h with either L-NMMA (inhibitor of NOS), oxamic acid (inhibitor of cystathionine beta synthase, CBS) or with the combination of both resulted in a dramatic decrease of the basal intracellular HNO signal (Supplementary Fig. 7c). DRG cells deprived of arginine, cysteine or both for 2 h (Fig. 5e) provided the same results, suggesting that the majority of intracellularly produced HNO originated from the interaction of NO and H2S that are constitutively produced.

Both NO and H2S are gasotransmitters and as such, could freely diffuse into nerve fibres to deliver paracrine signals. In addition, upon adequate stimulation including Ca2+ influx, neurons may be able to produce HNO by themselves, modifying TRPA1 to become more sensitive or activated in a sustained way. Neuronal NOS has already been shown to colocalize with TRPA1 (ref. 32), but CBS has not been examined before. We thus focused on detecting CBS and TRPA1 in sensory neurons and fibres. TRPA1 immunoreactivity was preferably found in small and middle-sized trigeminal neurons, producing a yellow colour in the merged image where both were colocalized (Fig. 5f). High magnification shows the immune products in discrete, well-organized structures confirming a strong co-expression of the H2S-producing enzyme with TRPA1. In confocal images of cross-sections through the rat spinal trigeminal nucleus caudalis (Supplementary Fig. 7d), bundles of immunostained afferent nerve fibres were seen running through the trigeminal tract and into the superficial laminae of the nucleus. Most of the nerve fibres show signals for both TRPA1 and CBS, producing the yellow colour in the merged image.

HNO from NO and H2S activates TRPA1 to cause CGRP release. While we show that NO provided by a pure NO donor does not directly activate TRPA1 (Supplementary Fig. 2a), we also tested H2S on cultured DRG neurons and could not see any significant change in intracellular Ca2+ levels when using 100–500 μM (Supplementary Fig. 8a). Prolonged exposures to

Figure 4 | Vasodilation by HNO-induced CGRP release involves TRPA1 in vivo. (a) AS-evoked increase in meningeal blood flow measured in anaesthetized rats was significantly reduced by topical application of HCO30031 (50 μM) (mean ± s.e.m., repeated measures ANOVA and HSD post hoc tests, *P < 0.05, n given in the figure). (b) AS-induced drop in MAP under anaesthesia was smaller in TRPA1−/− than in C57Bl/6 mice (P < 0.002; U-test; n = 7). (c) Photograph of a subject’s volar forearm with noticeable axon-reflex erythema upon intradermal injection of AS (0.7 μmol). (d) Magnitude and time-course of AS (0.7 μmol)-evoked pain in human volunteers (n = 6, 3 male and 3 female) on a numerical rating scale (NRS 0–10). Decomposed Angeli’s salt and DEA NONOate (0.23 μmol) were used as controls. (e) Areas of increased superficial blood flow following injections of AS and decomposed AS as a control. QST indicates time point of quantitative sensory testing. Pseudocolor representations of laser Doppler scanned images of superficial blood flow evoked by injection of AS, DEA NONOate and decomposed AS (repeated measures analysis of variance, least significant difference post hoc tests; *P ≤ 0.05; n = 6; all error bars represent s.e.m.).
H$_2$S did, however, induce channel activation in a cysteine-dependent manner (Supplementary Fig. 8b), but this treatment inevitably leads to inhibition of cell respiration and transient ROS formation both of which could activate TRPA1 (refs 35,36) and cannot account for the physiological effects of H$_2$S in low concentration. However, when combined, H$_2$S and NO did induce dramatic effects. The effects of combined H$_2$S and NO (10–75 µM) were scrutinized in the same way as previously for AS, using Ca$^{2+}$ imaging and patch clamp, TRPA1 agonists and antagonists, knockouts and mutants and the outcomes were strikingly identical to what was observed with HNO stimulation, a clear specific activation of TRPA1 in sensory neurons through interaction with the critical N-terminal cysteines (Fig. 6a–d and Supplementary Fig. 9). Combination of 10 µM of each NO and H$_2$S induced a similar maximal response as that observed with 75 µM concentrations (Fig. 6a,c).

H$_2$S has been suggested to modify cysteine residues inducing formation of persulphides, however, a direct reaction of H$_2$S with cysteine residues is chemically impossible unless in the presence of an oxidant. Nonetheless, we used the purified N terminus of TRPA1 channels (amino acids 1–719) and treated it with DEA NONOate, H$_2$S or both under hypoxic (2–5% O$_2$) conditions to minimize the artifactual oxidation of cysteine residues. Following this, the protein was treated with IA to block all free cysteine modifications and finally treated with Ellman’s reagent. If any product was observed, confirming that neither NO nor H$_2$S directly modify the channel (Supplementary Fig. 10a). Furthermore, a purified N terminus of TRPA1 mutant hTRPA1-C621S/C641/C665S (amino acids 1–719) did not show a positive Ellman’s reaction when exposed to the same treatments (Supplementary Fig. 10b). Using the synthetic model peptide as in Fig. 2, we confirmed this finding (Fig. 6f; Supplementary Fig. 10c) and could show that, when exposed to NO/H$_2$S, the peptide gets modified, because the m/z shift by 4 implies that two disulphide bonds are formed as expected from our results with AS (Fig. 6f).

Finally, we purified the TRPA1 channel by immunoprecipitation from dorsal root ganglia and compared the levels of endogenously present disulphides, S-nitrosothiols and persulphides in controls and ganglia treated with combination of NOS and CBS inhibitors. Only endogenously present disulphides were found and their level
Figure 6 | HNO generated from NO and H2S activates TRPA1 to release CGRP. (a) Combination of NO and H2S (10 \( \mu \)M each) induces increases of intracellular Ca\(^{2+}\) in DRG neurons of C57Bl/6 mice. AITC (100 \( \mu \)M, 20 s) and capsaicin (0.3 \( \mu \)M, 10 s at 4-min intervals) were used as controls. Data represent mean \( \pm \) s.e.m. (b) Similar to the currents evoked by AS (Fig. 1b), in mTRPA1-expressing Chinese hamster ovary cells, inward currents are induced as soon as DEA NONOate (75 \( \mu \)M) is added to H2S (75 \( \mu \)M). Currents (mean \( \pm \) s.e.m., \( n = 7 \)) can reversibly be blocked by HC030031 (50 \( \mu \)M, 10 s). (c) Combination of NO and H2S (75 \( \mu \)M each) induces reversible increases of intracellular calcium in Fura-2-stained DRG neurons of C57Bl/6 mice (AITC and capsaicin were used as controls as above). Responses were absent when DEA NONOate was decomposed before combined application with H2S and abolished following treatment with HC030031 (50 \( \mu \)M) or in TRPA1\(^{-/-}\) mice (mean \( \pm \) s.e.m.; \( n = 75 \)). (d) DTT (5 mM, 60 s) reversed NO + H2S-evoked inward currents (mean \( \pm \) s.e.m., \( n = 7 \)), which could temporarily be blocked by HC030031. (e) Combination of NO and H2S (75 \( \mu \)M each) activates hTRPA1 increasing intracellular Ca\(^{2+}\) in transfected HEK cells but not in cells expressing hTRPA1-3CK; mean \( \pm \) s.e.m., \( n = 250 \) cells each. (f) \( m/z = (-4) \) spectral shift of 64 amino acid long peptide treated with the combination of NO and H2S (red) compared with untreated peptide (black). (g) Detection of intramolecular disulphides (1–2), S-nitrosothiols (3–4) and S-sulfhydration (5–6) in TRPA1 isolated from DRG neurons (lanes 1, 3, 5) or DRG neurons treated with 2 mM combination of oxamic acid and L-NMMA for 12 h (lanes 2, 4, 6). Lower picture: total protein load. (h) CGRP release from hearts of mice induced by superfusion with 250 nmol NO and/or H2S. (ANOVA least significant difference post hoc test, \( P < 0.003 \) for C57Bl/6 compared with either NO, H2S or TRPA1\(^{-/-}\), \( n > 11 \) for TRPA1\(^{-/-}\) and C57Bl/6, \( n = 6 \) for NO and H2S, error bars represent s.e.m.).
was significantly reduced by the treatment with L-NMMA and oxamic acid, suggesting that HNO is a constitutive endogenous regulator of TRPA1 activity (Fig. 6g).

Finally, we tested the ability of the NO/H2S combination to induce CGRP release from the isolated mouse heart (Fig. 6h, Supplementary Fig. 11a,b), because the heart is an important target organ of circulating CGRP. TRPA1, CGRP is released from chemosensory primary afferent nerve fibres located in the epicardial surface of the heart, and human cardiomyocytes do express the receptor for CGRP (Supplementary Fig. 11c,d). We show that neither 250 nmol H2S nor NO have any significant effects on CGRP release. However, the combination of both proved to be a robust stimulant with CGRP peaking at 38 ± 4 pg ml⁻¹ in the effluvate (Fig. 6h). While HC030031 reduced this effect, the failure of the TRPV1 blocker BCTC, as well as the use of TRPV1⁻/⁻ mice proved that TRPV1 is not involved in this process (Supplementary Fig. 11b). If hearts from TRPA1⁻/⁻ mice were used, the peak of the CGRP release was reduced and the stimulant effect lasted much shorter compared with congeneric C57Bl/6 mice (Fig. 6h).

**H2S vasodilatory effects are NO and TRPA1 and CGRP dependent.** To assess to what extent the H2S vasodilatory effects are related to reduction of endogenous NO to HNO and activation of the HNO–TRPA1–CGRP pathway, we first performed laser Doppler recordings of meningeal blood flow in the rat. Topical application of H2S induced a clear increase in blood flow (Fig. 7a,b). This effect was significantly inhibited by topical application of HC030031 as well as by intravenous (i.v.) injection of the NOS inhibitor L-NMMA (Fig. 7a,b). Some H2S response was retained even 90 min after L-NMMA injection, and this was completely inhibited by topical application of glibenclamide (Fig. 7a,b), the K_ATP channel antagonist, confirming the additional role of K_ATP channel activation in the action of H2S (refs 8,38).

I.v. injection of 0.9 µg kg⁻¹ Na2S led to an increase of blood flow in the rat medullary brainstem, which was followed by an increase of CGRP levels in the cerebrospinal fluid (Fig. 7c,d). CGRP release was completely abolished in the presence of L-NAME and TRPA1 antagonists (Fig. 7c,d) confirming that H2S has to react with NO to stimulate the TRPA1–CGRP pathway.

To strengthen our hypothesis that the observed effects of H2S could originate from the reaction with endogenous NO, we applied H2S on cultured DRG neurons loaded with the specific NO fluorescent indicator DAF–FM–DA. Cells that were exposed to 100 µM H2S showed lower intensity of fluorescence, implying that by H2S treatment NO is depleted from the cells (Supplementary Fig. 11a). This effect was not restricted to DRG neurons, but also evident in aorta rings, where the lower fluorescence intensity was again observed in H2S treated tissue (Supplementary Fig. 11b).

In addition to local vasodilation, the effect of H2S on arterial blood pressure was monitored. We first assessed whether the TRPA1–CGRP part of the pathway is important for the regulation of systemic blood pressure. Application of HC030031 and CGRPₓ₈–₃₇ led to a rise of blood pressure in rats, similar to that observed with L-NAME application (Supplementary Fig. 11c), suggesting that both CGRP release and TRPA1 activation play a constitutive role in the regulation of blood pressure. I.v. injection of H2S caused a transient drop of blood pressure by 45 ± 1 mm Hg in wild-type mice, in a similar manner and comparable amplitude as reported previously. However, significant reductions of the H2S-induced blood pressure decrease were observed in TRPA1⁻/⁻ mice (by 28 mm Hg) and CGRP⁻/⁻ mice (by 25 mm Hg), as well as in wild types.

---

**Figure 7 | Local neurovascular effects of H2S depend on NO and TRPA1 and CGRP.** (a) Original recordings of meningeal blood flow in anaesthetized rats using laser Doppler flowmetry; H2S (60 nmol), HC030031 (50 µM), i.v. L-NMMA (10 mg kg⁻¹) and 1 mM glibenclamide with i.v. L-NMMA. (b) Mean values of flow increase (normalized to baseline) within 5 min after topical administration of H2S (ANOVA least significant difference (LSD) post hoc tests; *P < 0.05, n = 6). (c) Changes in brainstem blood flow upon i.v. injection of 70.2 µg kg⁻¹ Na2S. The TRPA1 antagonist (HC030031), CGRP receptor antagonists (BIBN4096BS and CGRPₓ₈–₃₇) and NOS inhibitor (L-NAME) were applied i.v. (d) Changes of CGRP levels in cerebrospinal fluid following the treatment shown in c (repeated measures ANOVA, LSD post hoc test; n given in the figure; *P < 0.05 compared with Na2S, all error bars represent s.e.m.).
receiving L-NMMA for 7 days through the drinking water (by 24 mm Hg; Fig. 8a).

If our hypotheses were valid, part of the NOS-generated NO would react with H$_2$S to give HNO and activate the TRPA1–CGRP pathway. Inhibition of H$_2$S production should thus affect the MAP changes induced by NOS inhibitors. Indeed, the administration of L-NMMA led to an increase of blood pressure (12.5 ± 1.2 mm Hg), but this change was significantly inhibited by pre-administration of H$_2$S inhibitors (6.5 ± 0.5 mm Hg) confirming that part of the vasodilation induced by NOS activity is H$_2$S dependent (Supplementary Fig. 12c).

We further addressed the above question using the rat isolated mesenteric artery, which is densely innervated with CGRP-containing sensory nerve fibres and potently relaxed by exogenous CGRP as well as TRPA1 agonists$^{20,39,40}$. The treatment with H$_2$S of mouse mesenteric vessels, in which TRPA1 activators cause CGRP-mediated relaxation$^{40}$, led to a robust CGRP release, an effect that was completely abolished by pretreatment with L-NMMA (Fig. 8b). Furthermore, using only 10 μM H$_2$S, we induced almost complete relaxation of the preconstricted rat blood vessels, an effect that was completely reversed by subsequent addition of the CGRP receptor antagonist (Fig. 8c). Pretreatment with the CGRP receptor antagonist or the TRPA1 channel blocker inhibited the vasodilatory effect of H$_2$S, as did pretreatment with capsaicin, which served to deplete the neurogenic pools of CGRP (Fig. 8d,f,g). Most importantly, treatment with L-NMMA completely blocked the relaxant effect of H$_2$S (Fig. 8e). L-NMMA treatment had no effect, however, on ring segments of rat isolated thoracic aortas, which display a minor CGRP innervation and negligible CGRP vasodilator responses$^{39}$, and an effect of H$_2$S was observed only at toxic concentrations higher than 1 mM (not shown). This strongly suggests a systemic relevance of the interaction between H$_2$S and endogenous NO to activate the HNO–TRPA1–CGRP pathway and thus contributes to the control of vascular tone.

To add human translational evidence six adult volunteers (three male, three female) received intradermal injections of DEA NONOate, Na$_2$S and the combination of both, in a double-blinded study. NO induced a small, circumscribed vasodilation, suggestive for a localized activation of the classical cGMP pathway (Fig. 9a,b). H$_2$S induced a minute and transient spot dilatation in conduit arteries$^{45–47}$ and also in mediating non-nitric oxide-dependent (Supplementary Fig. 12c).

Discussion

In the past decade both chemical and physiological research on nitroxy1 (HNO) has shown that this congener of nitric oxide has distinct ways of action$^{3,4,6}$. Studies have reported that HNO may be a co-product of NOS activity being converted back to NO by Cu/Zn superoxide dismutase and other suitable electron acceptors$^{32,43}$. It has also been reported that the NOS intermediate HO-Arg can be oxidized by catalase and hydrogen peroxide or cytochrome P450 enzymes to produce HNO$^{44}$ suggesting that HNO can not only be produced directly by NO but also from precursors such as HO-Arg. HNO operates together with NO to mediate the classical EDRF-induced dilatation in conduit arteries$^{45–47}$ and also in mediating nitricergic neurotransmission$^{48}$. Still, the main pathways for physiological generation of HNO and its way of action remained elusive.

Here we provide translational evidence for a direct, HNO-induced activation of neuronal TRPA1 channels, which is followed by Ca$^{2+}$ influx and, consequently, by the release of CGRP from ubiquitous, polymodal, sensory nerve fibres and endings. These effects are completely absent in mice lacking TRPA1, blocked by a selective TRPA1 inhibitor, and not induced by a pure NO donor, leading to the conclusion that the
chemosensory TRPA1 channel is the prime target for HNO-induced CGRP release and resulting cardiovascular effects.

Recent emergence of another gasotransmitter, H₂S, raised the possibility for its interaction with NO. Several groups have considered that H₂S effects could be linked to NO, including the early studies that showed physiological effects of H₂S (ref. 13). In addition, positive inotropic effects of these combined gasotransmitters on the heart have been demonstrated in vivo, which is one of the hallmarks of HNO physiology. However, these studies mainly used sodium nitroprusside (SNP) as a source of NO. Although it has ‘NO-like’ physiological effects, such as cGMP-dependent vasodilation, SNP does not release NO unless exposed to light. We have recently published that the above mentioned observations were in fact artefacts due to the fact that SNP reacts with H₂S directly to generate HNO and thiocyanates, without the actual involvement of NO.

Some of us were the first to identify that H₂S could be involved in TRPA1 receptor activation with several follow-up studies suggesting the same. Furthermore, previous studies have also linked H₂S to TRPA1 and microvascular blood flow mediated by CGRP and H₂S and TRPA1/TRPV1 intestinal pro-secretory actions. However, only very high concentrations of H₂S activated TRPA1 (as we also observed, Supplementary Fig. 10). These effects are, however, most likely outside the physiological concentration range and should stand for inhibition of cell respiration and/or generation of ROS both of which can induce TRPA1 activation. In addition, polysulphides, inevitable contaminants of NaHS solutions used in these studies, could be the reason for the activation of the TRPA1, as shown recently. H₂S, just like NO, cannot react directly with thiols due to thermodynamic constraints.

Using selective methods for in vitro and intracellular HNO detection, our data suggest that the gasotransmitter H₂S may transform endogenous NO to HNO, which activates the HNO-TRPA1-CGRP cascade, suggesting broad physiological relevance of the findings. Mechanistic details of direct reaction between NO and H₂S will be subject of further studies. As we previously demonstrated, it is plausible that H₂S-mediated generation of HNO in vivo could be additionally related to its reaction with S-nitrosothiols, as well as with metal nitrosyls.

Data supporting the notion that H₂S may react with NO to give HNO, and our demonstration that H₂S effects could be diminished by either blocking NOS activity or deleting TRPA1 or CGRP are in favour of a new signalling pathway for cardiovascular control. In addition, co-expression of TRPA1 and CBS in small to medium-sized sensory neurons and axons together with the recent demonstration of co-expression of TRPA1 and nNOS suggest a structural and functional organization for constitutive HNO generation and subsequent activation of TRPA1-dependent CGRP release. This functional unit is of importance in the regulation of peripheral blood flow (as demonstrated in dura mater and brainstem) and even of systemic blood pressure. In addition, positive inotropic and lusitropic cardiac effects of circulating and/or paracrine CGRP have been reported. The positive inotropic and lusitropic effects of HNO were originally ascribed to CGRP release and completely blocked by the use of CGRP receptor antagonist.

**Figure 9 | Combination of NO and H₂S induces axon-reflex erythema in humans.** (a) Laser Doppler scanned pseudocolor images of volar forearm of human volunteers (3 male, 3 female) after intracutaneous injection of DEA NONOate (0.23 μmol), Na₂S (0.35 μmol) or both. (b) Flare size after intracutaneous injection of Na₂S, DEA NONOate or combination of both. When H₂S and NO were intracutaneously injected to the volar forearm of human volunteers, blood flow increased locally following DEA NONOate (0.23 μmol), while H₂S (0.35 μmol) hardly evoked any response apart from slight irritation due to the injection needle. Injection of H₂S and NO in combination however led to widespread vasodilatation in agreement with formation of an axon-reflex erythema due to activation of nerve fibers, antidromically propagated action potentials and concomitant CGRP release (repeated measures analysis of variance (ANOVA) honestly significant difference (HSD) post hoc tests; *P < 0.01 H₂S and NO versus H₂S; *P < 0.01 H₂S and NO versus H₂S and NO; n = 6; all error bars represent s.e.m.). (c) Pain and (d) itch ratings from the experiments shown in a (repeated measures ANOVA HSD post hoc tests; *P < 0.01 H₂S and NO versus H₂S; *P < 0.01 H₂S and NO versus H₂S and NO; n = 6; all error bars represent s.e.m.).
known to produce NO and H2S, both of which freely diffuse and activate guanylyl cyclase and KATP channels, respectively, to induce vasodilatation. In neurons, there are several possibilities of their reaction leading to formation of HNO that targets the cysteines of TRPA1 in close vicinity. (i) NO and H2S produced in endothelium react to give HNO that could reach paravascular TRPA1-expressing sensory nerve fibres, inducing Ca2+ influx and CGRP release. (ii) Production of H2S and NO from colocalized CBS and neuronal NOS leads to intracellular HNO formation and TRPA1 activation; (iii) Taking into account that constitutive levels of NO in neurons are very low, it is also plausible that, for instance in the CNS, astrocyte-derived NO, as a paracrine signal, meets with endothelial or neuronal H2S, forming HNO, which activates TRPA1 in primary afferent peptidergic terminals. Recent work showed that the NO vasodilatory effect on aorta rings is partially blocked by inhibiting H2S production, vice versa. H2S effects are diminished by inhibiting NO production, further strengthening the link between these gasotransmitters. Our data on isolated blood vessels suggest that most of H2S-induced vasodilation is directly dependent on its reaction with NO to form HNO, as well as on functional presence of TRPA1 and CGRP.

Formation of disulphides as a mode of channel activation, as shown in this study, may have a broader impact on understanding the multiple mechanisms for TRPA1 activation, as other activators could use the same mechanism. We and others have recently demonstrated the formation of disulphides in the N terminus of TRPA1 by other endogenous (methyglyoxal) and exogenous (N-methylmaleimide) activators. Although the initial reaction with C621, C641 and/or C665 is unquestionable, the extent to which neighbouring cysteines interact, as well as the extent to which the size, hydrophilicity and charge of the activator could determine the half-life of the disulphide-induced conformational change, remain to be elucidated in further studies. In case of HNO, the activation is particularly long lasting and could be reversed by a reducing agent. Thus, if reducing equivalents are scarce, as in conditions of oxidative stress, and/or when H2S and NO are produced in excess, pathophysiological effects of the HNO–TRPA1–CGRP (plus substance P) pathway are well conceivable. Being a nociceptive transduction channel in the first place, TRPA1 contributes to pain/itch sensations and possibly excessive CGRP release into the jugular venous blood, for example, where also plenty of metabolic NO products are found during migraine attacks.

The deciphering of the HNO–TRPA1–CGRP pathway provides several new targets for future drug design. A century after

**Figure 10 | H2S-NO-HNO–TRPA1–CGRP pathway in neurovascular regulation and synaptic transmission.** (a) The trigeminal system as an example of CGRP-containing nerve fibres and its neuronal and vascular interaction sites. By means of diffusion of H2S or NO, produced either in the endothelium or in neurons, there are several possibilities of their reaction leading to formation of HNO that targets the cysteines of TRPA1 in close vicinity. (b) TRPA1/CGRP expressing nerve endings in the periphery communicate with the smooth muscle cells surrounding the endothelium of blood vessels. Endothelial cells are known to produce NO and H2S, both of which freely diffuse and activate guanylyl cyclase and KATP channels, respectively, to induce vasodilatation. However, H2S and NO also react with each other to give HNO, which could reach paravascular TRPA1-expressing sensory nerve fibres, inducing Ca2+ influx and CGRP release. (c) Other potential sites of NO-H2S interaction in neurons: (i) TRPA1 channels are co-expressed with nNOS and CBS in primary afferents forming a functional signalling complex that leads to confined HNO generation and TRPA1 gating upon activation of the gasotransmitter-generating enzymes. In addition, NO (ii) or H2S (iii) could originate from either side of a synaptic cleft (or from nearby axons of passage) and freely diffuse into adjacent neurons (or nerve fibres). There, they react with their counterpart producing HNO in vicinity of TRPA1, which leads to its activation, Ca2+ influx and release of CGRP. Apart from its vascular functions, CGRP acts as a co-transmitter, facilitating synaptic transmission, which may play a role in migraine headaches. Glu, glutamate; SC, satellite cells.
the demonstration that the classical TRPA1 agonist mustard oil acts as a strong vasodilator, the ion channel responsible for this action could be considered as a co-mediator of endogenous ligand effects that lower the blood pressure and strengthen the heart.

Methods

Chemicals. Stock solutions of DEA NONOate and AS were prepared in 10 mM KOH. KOH was titrated tests as stated above by biological buffer (pH 7) immediately before use to minimize the loss of activity due to spontaneous decomposition of these salts at neutral pH. 300 mM phosphate buffer (KPi) was prepared with nanopure water, stirred with Chelex-100 resins to remove traces of heavy metals (and when appropriate supplemented with 100 mM neocuproine) and kept above the resins until used. Sodium sulphide (Na2S) was purchased as anhydrous, opened and stored in a glove box (<1 p.p.m. O2 and <1 p.p.m. H2O). 100 mM and 10 mM stock solutions of sodium sulphide were prepared in the glove box using argon-bubbled nanopure water and stored in glass vials with PTFE septa at +4°C, for a maximum of one week. The concentration of H2S was determined using a H2S selective electrode (World Precision Instruments, USA). Gas-tight Hamilton syringes were used for handling Na2S solution.

Cell culture. DRGs of C57Bl/6 or TRPA1 /− /− mice were excised and transferred to Dulbecco’s modified Eagle’s medium containing 50 mg ml−1 gentamicin. Following treatment with 1 mg ml−1 collagenase and 0.1 mg ml−1 protease for 30 min ganglia were dissociated by trituration with a fire-polished silicone-coated Pasteur pipette. For plating, cover slips were coated with poly-L-lysine (200 μg ml−1 Sigma-Aldrich, Germany) and cells were cultured in TNB 100 cell culture medium supplemented with TNB 100 lipid-protein complex, 100 μg ml−1 streptomycin and penicillin (all from Biochrom, Berlin, Germany) and mouse NGF (100 ng ml−1, Alomone Labs, Tel Aviv, Israel) at 37°C and 5% CO2. All experiments were performed within 24 h of dissociation.

Animals. TRPA1 knockout mice were a gift from Drs. David Corey and Kelvin Kwan (Harvard University, Boston, USA). TRPV1+/− mice came from Dr John Davis (former chief, Harlow, UK) and GCP (Calca) /− /− from Dr Jean-Pierre Chan-geux (Institut Pasteur, Paris, France). For measuring CGRP release and culturing neurons experiments the same day. Human and mouse TRPA1 cDNA and cDNA of a mutant hTRPA1 lacking lysine and/or cysteine residues in the intracellular domain (C621S, C641S, C665S/−/−, K710R) were a kind gift from Dr John Davis (former chief, Harlow, UK) and GCP (Calca) /− /− from Dr Jean-Pierre Chan-geux (Institut Pasteur, Paris, France). For measuring CGRP release and culturing neurons experiments the same day.

Human tissue samples. The retrospective examination of pseudonymized human (cardiac) tissue samples (from archival tissues or obtained from autopsies with permission by relatives and institutional approval for collection of research biomaterials), was performed in compliance with university ethics committee guidelines and the German federal law for correct usage of archival tissue for clinical research (Reference Dtsch. Arzteblatt 2003:100: A1632).

Statistics. Comparison of two groups was performed by the respective t-test and with a non-parametric test for n < 10. More than two groups were compared by analysis of variance following least significant difference or honestly different difference post hoc tests as stated using StatSoft, Inc. (Tulsa, USA). Differences were considered significant at *P < 0.05.

HNO fluorescent imaging. For nitroxy fluorescent imaging, DRG neurons were plated in poly-L-lysine coated 35 mm high μ-dishes siliTreat (Ibidi, Germany). Cells were incubated with 10 μM CuBOT1 in Hank’s buffer for 20 min. After washout, cells were treated with AS, DEA NONOate, Na2S (100 μM each) or medium as a control for 15 min. In addition, cells were preincubated with 1 mM L-NAME, 1 mM NO synthase inhibitor or combination of both for 2 h, or without arginine, cysteine or without both for partial depletion of NO and/or H2S, before staining with CuBOT1, to quantify the effects on basal HNO production. Fluorescence microscopy was carried out using Carl Zeiss Axiovert 40 CLF inverted microscope, equipped with green fluorescent filters and AxioCam ICm1. Images were post-processed in ImageJ software where semi-quantitative fluorescence intensity was determined.

Analysis of NO and H2S. Two microlitres of 50 mM KPi buffer pH 7.4 was added into the chamber and the electrodes were immersed into it. Depending on the type of measurement, different concentrations of Na2S solution were injected followed by the addition of either NO or DEA NONOate solutions. The studies were performed with NO or DEA NONOate being injected first and then followed by injection of H2S. The fate of H2S and NO during the course of the reaction was confirmed by a Free Radical Analyser (WPI, USA) connected to a computer equipped with DataTrax software for the signal processing. Experiments were performed in a four-channel chamber (WPI) with both electrodes at the same time or each of them separately. When needed the solutions were degassed with argon and kept closed in measuring chamber securing anaerobic conditions, which were constantly monitored by additional oxygen electrode.

High-resolution ESI–TOF–MS. Being previously degassed with argon for 5 min, 20 μM model peptide was exposed to 1 mM H2S, 1 mM DEA NONOate or the combination of both. The samples were analysed on maXis, a high-resolution mass spectrometer with an electrospray source (Bruker, Bremen, Germany). The samples were injected using a syringe pump at a flow rate of 240 μl−1. Nitrogen was used as the nebulizing gas at a pressure of 10 psi and as the drying gas at a temperature of 180°C and a flow rate of 5 min−1. All experiments were carried out in the positive ion mode and obtained spectra deconvoluted and further processed in Data Analysis software provided by Bruker Daltonics. Analytical samples were prepared by mixing peptide samples in water with a mixture of 0.1% formic acid in water/acetonitrile (1:1, v/v).

Modified biotin-switch assay for disulfide bond detection. For disulfide bond detection, purified mTRPA1 channel (50 μM) was first treated with 1 mM

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms5381 | ARTICLE
DTT and subsequently purified using Micro Bio-Spin Columns with Bio-Gel P-6 (Bio-Rad). The protein was then exposed to either 500 µM or 1.5 mM AS for 15 min. After a wash, the sample was further modified with IA to block all the available SH groups before application of 1.5 mM AS was used as negative control. The samples were desalted on Micro Bio-Spin Columns with Bio-Gel P-6 (Bio-Rad) and processed as follows: (i) 5 mM IA (45 min at 37°C), (ii) 1 mM DTT (1 at 37°C) and (iii) 5 mM biotin-maleimide (45 min at 37°C). The presence of biotinylated protein was confirmed with mouse monoclonal anti-biotin peroxidase labelled antibodies (Clone BN-34, A0184, Sigma-Aldrich).

MALDI-TOF characterization of AS-induced modification of hTRPA1. A model peptide of the intracellular N-terminal part of hTRPA1 containing amino acids 607–670 (UniProt database, O75762, Therm Fischer Scientific) was used to evaluate AS effects on cysteines. A quantity of 50 µM peptide in 20 mM ammonium bicarbonate buffer pH 7.4 was treated with 500 µM Angeli’s salt for 15 min and then incubated with 1 mM IA for 45 min. Hydrolysis by MALDI-TOF was pre-crystallized with sinapinic acid matrix, a supersaturated solution of 4-hydroxy-3,5-dimethoxycinnamic acid in acetonitrile, 0.1% TFA (50:50, v/v). Samples were mixed with sinapinic acid in a 1:3 ratio and spotted onto the plate.

Ab initio peptide structure prediction. A model structure of the peptide containing critical cysteine residues was obtained by ab initio protein folding and protein structure prediction using QUARK software.

Detection of disulfides, S-nitrosohistidine and persulphides. DRGs were prepared as described above. In addition, cells were treated with 2 mM combination of oxamic acid and L-NAME for 12 h. After that cells were lysed in TNE buffer (0.15 M NaCl, 20 mM Tris–HCl, 1 mM EDTA pH 7.4) supplemented with 1% NP-40, 10% proteinase inhibitor cocktail (Sigma-Aldrich) and 50 mM MSBT-A27 using ultrasonicator. TRPA1 was immunoprecipitated with anti-TRPA1 antibodies (rabbit polyclonal IgG, ab58844, Abcam) and the samples split on three to be analysed for: (i) disulfide formation by reducing the proteins with DTT, removing DTT on Micro Bio-Spin Columns with Bio-Gel P-6 (Bio-Rad) and labelling with biotin-maleimide; (ii) S-nitrosotrinoid content, by treating the extracts with 1 mM ascorbate, 5 µM copper sulphate and biotin-maleimide; and (iii) persulphide formation, by treating the cells with CN-biotin27. The presence of biotinylated proteins was confirmed with mouse monoclonal anti-biotin peroxidase-labelled antibodies (Clone BN-34, A0184, Sigma-Aldrich).

Patch clamp. Membrane currents were acquired with an EPC10 USB HEKA amplifier (HEKA Elektronik, Lampertheim, Germany), low pass at 1 kHz, and sampled at 2 kHz. Electrodes were pulled from borosilicate glass tubes (TW150F-3; World Precision Instruments, Berlin, Germany) and heat polished to give a tip resistance of 5–5 MΩ. The standard external solution contained (in mM) NaCl 140, KCl 5, MgCl2 2, NaHCO3 15, NaH2PO4 1.2 and D-glucose 10; pH 7.4 was adjusted with Angeli’s salt, currents were measured during 500 ms long voltage ramps from −100 to +100 mV. All experiments were performed at room temperature. Solutions were applied with a gravity-driven PTFE/glass multi-barrel perfusion system. The PatchMaster/FitMaster software (HEKA Elektronik) was used for acquisition and off-line analysis.

Ratios of [Ca2+]c measurements. Cells were stained by 3 µM fura-2-AM and 0.02% pluronic (both from Invitrogen) for about 30 min. Coverslips were mounted on an Olympus IX71 inverse microscope with a ×10 objective and constantly superfused with extracellular solution (in mM: NaCl 145, KCl 5, MgCl2 2, EGTA 5, HEPES 10 and glucose 10 (pH 7.4) was adjusted with tetramethylammonium hydroxide). The internal solution contained (in mM) KCl 145, MgCl2 5, EGTA 2, HEPES 5, KCl 100 and HEPES 10 (pH 7.4 was adjusted with KOH). If not otherwise noted, cells were held at −60 mV. For IV curves with and without Angeli’s salt, currents were measured during 500 ms long voltage ramps from −100 to +100 mV. All experiments were performed at room temperature. Solutions were applied with a gravity-driven PTFE/glass multi-barrel perfusion system. The PatchMaster/FitMaster software (HEKA Elektronik) was used for acquisition and off-line analysis.

Assessment of systemic blood pressure in mice. Male mice weighing up to 25 g were anaesthetized with isoflurane supplied with a mask and the right carotid arteries were catheterized to measure intraarterial blood pressure via a transducer connected to a polygraph (Hellige, Freiburg, Germany) while the substances were...
injected through the catheterized left jugular vein. AS was injected at a dose of 61 μg kg−1 freshly dissolved in saline pH 7.0, which also served as a control. In addition, anaesthetized C57Bl/6 mice were injected with 39 nmol kg−1 NaS. One group of C57Bl/6 mice was also treated with 10 mg l−1 of L-NMMA in drinking water ad lib for 7 days prior the injection of NaS. C57Bl/6 mice were also treated with 6 μg kg−1 of oxamic acid and propargylglycine each, and after 10 min 6 μg kg−1 of L-NMMA was injected. A reverse perfusion was performed with first injecting the L-NMMA, and then cervical dislocation of OXA and PG. At the end of experiments, animals were killed by cervical dislocation in deep anaesthia.

Psychophysics. Studies on human volunteers were limited to some authors of the present study and experimental procedures were approved to fulfill the requirements of the Declaration of Helsinki by the ethics committee of the Friedrich-Alexander University of Erlangen-Nuremberg. AS (10 nmol kg−1, DEA NONOate (3.33 nmol kg−1/C0) was injected through the catheterized left jugular vein. AS was injected at a dose of 61 μg kg−1. Area of superficial vasodilation was analysed with a fine brush. A laser Doppler imager (LDI, Moore, London, UK) was used for recording microvascular blood flow. Two baseline scans of 0.5 mm spatial resolution were taken, following scans every 2.5 min starting right after the injections and a final one after 20 min. Area of superficial vasodilation was analysed with ImageJ software.

Immunohistochemistry of aortas. For immunohistochemistry of aorta Wistar rats subjected to autopsies and from archival tissues or obtained from autopsies with permission by relatives and institutional approval for collection of research biomaterials, was performed in compliance with Universitätsklinikum Ulm ethics committee guidelines and the German federal law for correct usage of archival tissue for clinical research (Reference Druch. Ärzteblatt 2003:100, A1632). For immunofluorescence staining paraaffin-embedded tissue was washed for 5 min three times with xylene, twice with 100% ETOH, once with 95% ETOH and once with PBS. Sections were boiled in Tris pH.60 for antigen retrieval, rinsed in deionized water for 15 min and washed with PBS. Sections were blocked in 1% BSA, 0.3% Triton X-100 PBS and incubated with a 1:1,000 dilution of rabbit-anti-CRLR antibody (kindly provided by Nigel Bunnett/ Eileen Grady). The specificity of the antibody has previously been determined and was additionally confirmed with preabsorption controls and omission of the primary antibody. Images were taken using an Olympus BX51 Fluorescent Microscope (Olympus, Melville, NY) with appropriate filter settings and software packages (MetaSystems, Allhusheim, Germany).

References

1. Fukuto, J. M., Switzer, C. H., Miranda, K. M. & Wink, D. A. Nitroxyl (HNO): chemistry, biochemistry, and pharmacology. Annu. Rev. Pharmacol. Toxicol. 45, 335–355 (2005).

2. Fukuto, J. M. & Carrington, S. J. HNO signalling mechanisms. Antioxid. Redox Signal. 14, 1649–1657 (2011).

3. Paolocci, N. et al. Positive inotropic and lusitropic effects of HNO/NO− in failing hearts: independence from beta-adrenergic signaling. Proc. Natl Acad. Sci. USA 100, 5537–5542 (2003).

4. Paolocci, N. et al. Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by catalysis of gene-related peptide signaling. Proc. Natl Acad. Sci. USA 98, 10463–10468 (2001).

5. Favaloro, J. L. & Kemp-Harper, B. K. The nitroxyl anion (HNO) is a potent physiological vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322, 587–590 (2008).

6. Mustafa, A. K. et al. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulphhydrates potassium channels. Circ. Res. 107, 1259–U1169 (2011).

7. Brain, S. D. & Grant, A. D. Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol. Rev. 84, 903–934 (2004).

8. Dux, M., Santha, P. & Jancso, G. Cystathionine-β-synthase knockout mice: new insights into cystathionine metabolism. Arch. Biochem. Biophys. 475, 212–220 (2008).

9. Filipovic, M. R. et al. Cystathionine beta synthase gene silencing results in cystathionine accumulation in rat brain. Neurosci. Lett. 452, 245–249 (2009).

10. Yang, G. et al. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine γ-lyase. Science 322, 587–590 (2008).

11. Mustafa, A. K. et al. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulphhydrates potassium channels. Circ. Res. 107, 1259–U1169 (2011).

12. Li, L., Rose, P. & Moore, P. K. Hydrogen sulfide and cell signaling. Annu. Rev. Pharmacol. Toxicol. 51, 169–187 (2011).

13. Abe, K. & Kimura, H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J. Neurosci. 16, 1066–1071 (1996).

14. Coletta, C. et al. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. Proc. Natl Acad. Sci. USA 109, 9161–9166 (2012).

15. Yong, Q. C. et al. Regulation of heart function by endogenous gaseous mediators: crosstalk between nitric oxide and hydrogen sulfide. Antioxid. Redox Signal. 14, 2081–2091 (2011).

16. Filipovic, M. R. et al. Chemical characterization of the smallest S-Nitrosothiol, HSNO; cellular cross-talk of H2S and S-Nitrosolites. J. Am. Chem. Soc. 134, 12016–12027 (2012).

17. Lancaster, J. R. Nitrosative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. Chem. Rev. 109, 1160–1174 (2009).

18. Wink, D. A. et al. Reaction kinetics for nitrosation of cytochrome and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O2 reaction. Chem. Res. Toxicol. 7, 519–525 (1994).

19. Streng, T. et al. Distribution and function of the hydrogen sulfide-sensitive TRPA1 ion channel in rat urinary bladder. Eur. J. Pharmacol. 53, 391–399 (2008).

20. Bautista, D. M. et al. Pungent products from garlic activate the sensory ion channel TRPA1. Proc. Natl Acad. Sci. USA 102, 12248–12252 (2005).
...tory, 45(5), 541–545 (2007).
21. M. J. Eberhardt, M. J.
22. T. N. Asan, A. D. A., Gentry, C., Moss, S. & Bevan, S. Transient receptor potential channel A1 (TRPA1) a possible mechanism of metabolic neuropathies. J. Biol. Chem. 287, 28291–28306 (2012).
23. H. H. Min, H. H., Bautista, D. M. & D. I. J. D. Julius, D. TRP channel activation by reversible covalent modification. Proc. Natl Acad. Sci. USA 103, 19564–19568 (2006).
24. P. J. Macpherson, L. J.
25. K. M. Miranda, K. M.
26. W. Everaerts, W.
27. S. A. Suarez, S. A., Bikiel, D. A., Wetzler, D., Marti, M. A. & Doctorovich, F. A1 is a sensory receptor for multiple products of oxidative stress. Angew. Chem. Int. Ed. 53, 10262–10269 (2013).
28. A. D. Andersson, D. A., Gentry, C., Moss, S. & Bevan, S. Transient receptor potential channel A1 (TRPA1) a possible mechanism of metabolic neuropathies. J. Biol. Chem. 287, 28291–28306 (2012).
29. S. A. Suarez, S. A., Bikiel, D. A., Wetzler, D., Marti, M. A. & Doctorovich, F. A1 is a sensory receptor for multiple products of oxidative stress. Angew. Chem. Int. Ed. 53, 10262–10269 (2013).
30. S. M. Koenitzer, J. R.
31. D. P. Poole, D. P.
32. D. P. Poole, D. P.
33. D. J. Koenitzer, J. R.
34. L. J. Macpherson, L. J.
35. L. J. Macpherson, L. J.
36. D. A. Andersson, D. A., Gentry, C. & Bevan, S. TRPA1 has a key role in the somatic pro-nociceptive actions of hydrogen sulfide. J. Physiol. 591, 137–142 (2012).
37. P. M. Zygga, P. M.
38. G. Tang, G. & B. N. E. D. H. and P. M. Z. are grateful for support from the Swedish Research council. This work was supported by intramural funds provided by the University of Erlangen and the Emerging Field Initiative for Metabolic Diseases of the University of Erlangen. Additional support came from DFG (KOFO30: LU728/3-1) to P.R. and from DFG (NA 970/1-1) to B.N. E.D.H. and P.M.Z. are grateful for support from the Swedish Research council (2010–3347 and 2010–5787) and Lund University. M.D. acknowledges the support from...
TAMOP 4.2.2.A-11/1/KONV-2012-0052 and K.M. and M.F. the support from FP7/EUROPHEADPAIN (no. 602633). J.K.L. was supported by Else Kröner-Fresenius-Foundation. TRPA1 knockout mice were a gift from Drs David Corey and Kelvin Kwan (Harvard University, Boston, USA); TRPV1 +/− came from Dr John Davis (formerly GSK, Harlow, UK) and CGRP (Calca) +/− from Dr Jean-Pierre Changeux (Institut Pasteur, Paris, France). CHO cells expressing mTRPA1 were a gift from Dr Ardem Patapoutian (The Scripps Research Institute, La Jolla, USA). HNO fluorescent sensor, CuBOT1, was a gift from Dr Stephen J. Lippard (MIT, USA). We also thank Dr Ardem Patapoutian for reading the manuscript and suggestions in the initial phase of this study. The authors are also grateful to Mrs Birgit Vogler, Mrs Jana Schramm and Mrs Iwona Izydorzyk (Physiology, Erlangen) for assistance and support. D.B., S.A.S., M.A.M. and F.D. are members of CONICET and acknowledge financial support from UBA (UBACYT W583 and 2010-12), ANPCyT (PICT 2010-2649 and 2010-416), CONICET (PIP1207 and 112-201001-00125) and the Bunge y Born Foundation.

Author contributions

M.R.F., P.R. and M.E. developed the concept, designed the study, analysed the data and wrote the paper. M.E. performed most of the experiments. M.R.F. provided substances and supervised all experiments. K.M. and M.D. planned and analyzed and K.M., M.D. and C.W. conducted in vivo blood flow experiments, CGRP release studies, immunohistochemistry of vessels and the trigeminovascular system. M.R.F. and J.M. conducted all biochemical studies. M.R.F., F.D., S.A.S., D.B., M.A.M. and I.I.B. designed and conducted chemical experiments. B.N. supervised and conducted human studies. M.F. provided tagged TRPA1 receptor DNA, purified TRPA1 protein and was involved in data interpretation. A.B. performed calcium imaging experiments on TRPV1. T.I.K. conducted CGRP release studies on mesenterium. A.Ls. and A.La. supervised patch clamp experiments. J.d.l.R. performed patch clamp experiments. J.K.L. and K.D. performed immunohistochemistry on human heart tissue. J.I. and N.C. conducted blood pressure experiments on anaesthetized mice. E.D.H., P.M.Z. conducted vasorelaxation studies and were involved in data interpretation. All authors discussed the results and implications and commented on the manuscript at all stages.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Eberhardt, M. et al. H2S and NO cooperatively regulate vascular tone by activating a neuroendocrine HNO–TRPA1–CGRP signalling pathway. Nat. Commun. 5:4381 doi: 10.1038/ncomms5381 (2014).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/