Supplemental material to this article can be found at:
http://molpharm.aspetjournals.org/content/suppl/2013/11/07/mol.113.088864.DC1.html

1521-0111/85/1/175–185$25.00
MOLECULAR PHARMACOLOGY
Copyright © 2013 by The American Society for Pharmacology and Experimental Therapeutics
http://dx.doi.org/10.1124/mol.113.088864
Mol Pharmacol 85:175–185, January 2014

MOLECULAR PHARMACOLOGY

# Transnitrosylation Directs TRPA1 Selectivity in *N*-Nitrosamine Activators[S]

Daisuke Kozai, Yoji Kabasawa, Maximilian Ebert, Shigeki Kiyonaka, Firman, Yuko Otani, Tomohiro Numata, Nobuaki Takahashi, Yasuo Mori, and Tomohiko Ohwada

*Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering (D.K., M.E., S.K., T.N., N.T., Y.M.), Department of Technology and Ecology, Hall of Global Environmental Studies (S.K., T.N., Y.M.), and Advanced Biomedical Engineering Research Unit (N.T.), Kyoto University, Kyoto, Japan; Laboratory of Organic and Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan (Y.K., F., Y.O., T.O.); and Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Tokyo, Japan (S.K., Y.M.)*

Received August 1, 2013; accepted November 7, 2013

## ABSTRACT

*S*-Nitrosylation, the addition of a nitrosyl group to cysteine thiols, regulates various protein functions to mediate nitric oxide (NO) bioactivity. Recent studies have demonstrated that selectivity in protein *S*-nitrosylation signaling pathways is conferred through transnitrosylation, a transfer of the NO group, between proteins via interaction. We previously demonstrated that sensitivity to activation by synthetic NO-releasing agents via *S*-nitrosylation is a common feature of members of the transient receptor potential (TRP) family of $Ca^{2+}$-permeable cation channels. However, strategies to confer subtype selectivity to nitrosylating agents targeted to TRP channels are yet to be developed. Here, we show selective activation of TRPA1 channels by novel NO donors derived from the ABBH (7-azabenzobicyclo[2.2.1]heptane) *N*-nitrosamines, which exhibit transnitrosylation reactivity to thiols without releasing NO. The NNO-ABBH1 (*N*-nitroso-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane) elicits *S*-nitrosylation of TRPA1 proteins, and dose-dependently induces robust $Ca^{2+}$ influx via both recombinant and native TRPA1 channels, but not via other NO-activated TRP channels. TRPA1 activation by NNO-ABBH1 is suppressed by specific cysteine mutations but not by NO scavenging, suggesting that cysteine transnitrosylation underlies the activation of TRPA1 by NNO-ABBH1. This is supported by the correlation of N–NO bond reactivity and TRPA1-activating potency in a congeneric series of ABBH *N*-nitrosamines. Interestingly, nonelectrophilic derivatives of ABBH also activate TRPA1 selectively, but less potently, compared with NNO-ABBH1. Thus, ABBH *N*-nitrosamines confer subtype selectivity on *S*-nitrosylation in TRP channels through synergetic effects of two chemical processes: cysteine transnitrosylation and molecular recognition of the nonelectrophilic moiety.

## Introduction

Nitric oxide (NO) controls diverse biological processes such as vascular relaxation and neurotransmission (Moncada et al., 1991), and is produced in vivo by the family of NO synthase (NOS) isoforms (Alderton et al., 2001). In addition to the well characterized binding of NO to the heme iron of soluble guanylate cyclase, physiologic NO-based protein modification might also be mediated by *S*-nitrosylation, the covalent attachment of an NO moiety to the sulfur atom of cysteine residues to form *S*-nitrosothiol (Hess et al., 2005). A large number of *S*-nitrosylated proteins (>1000) have been identified (Seth and Stamler, 2011), supporting the notion that NO exerts its biological activity via *S*-nitrosylation.

NO is highly reactive and diffusible within cells, posing the question of how *S*-nitrosylation–dependent regulation of cellular signaling is achieved with any selectivity. Recent studies have identified protein–protein transnitrosylation, the transfer of the NO group between proteins in the absence of apparent NO release, as a potentially important targeting pathway (Stamler and Hess, 2010; Anand and Stamler, 2012; Nakamura and Lipton, 2013). Transnitrosylation has been reported for anion exchanger-1 by hemoglobin, caspase-3 by thioredoxin (and vice versa), X-linked inhibitor of apoptosis by caspase-3, and various nuclear proteins by glyceraldehyde-3-phosphate dehydrogenase

This work was supported by Grant-in-Aid for Scientific Research(A) [Grants 24249017 and 20249015]; and the University of Tokyo.
dx.doi.org/10.1124/mol.113.088864.
[S] This article has supplemental material available at molpharm.aspetjournals.org.

**ABBREVIATIONS:** ABBH, 7-azabenzobicyclo[2.2.1]heptane; AP-18, (*Z*)-4-(4-chlorophenyl)-3-methylbut-3-en-2-oxime; 2-APB, 2-aminoethyl diphenylborinate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; eNOS, endothelial NOS; GFP, green fluorescent protein; HEK, human embryonic kidney; HPDP-biotin, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; NCHO-ABBH, *N*-formyl-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane; NEM, *N*-ethylmaleimide; NH-ABBH, *N*-H-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane; NMe-ABBH, *N*-methyl-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane; NNO-ABBH1, *N*-nitroso-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane; NO, nitric oxide; NOR3, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide; NOS, NO synthase; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; TRP, transient receptor potential.



Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

(Pawloski et al., 2001; Mitchell and Marletta, 2005; Kornberg et al., 2010; Nakamura et al., 2010). The negative impact of binding-disruptive mutations on transnitrosylation demonstrates the importance of protein-protein binding to this reaction (Kornberg et al., 2010; Nakamura et al., 2010).

*Drosophila melanogaster* transient receptor potential (TRP) protein and its homologs are putative six-transmembrane polypeptide subunits that assemble into tetramers to form $Ca^{2+}$-permeable cation channels (Clapham, 2003; Clapham et al., 2005). Mammalian TRP channels comprise six related protein subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML. TRP channels are activated by diverse stimuli, including receptor stimulation, heat, osmotic pressure, mechanical stress, and environmental irritants from the extracellular and intracellular milieu. Some members of the TRPC, TRPV, TRPM, and TRPA subfamilies act as cell sensors for redox mediators (Hara et al., 2002; Aarts et al., 2003; Hinman et al., 2006; Yoshida et al., 2006; Macpherson et al., 2007; Andersson et al., 2008; Bessac et al., 2008; Salazar et al., 2008; Takahashi et al., 2008, 2011; Taylor-Clark et al., 2008; Xu et al., 2008). We and others have described the *S*-nitrosylation of TRP channels. Receptor-activated (TRPC5, TRPC1, and TRPC4) and thermosensor (TRPV1, TRPV3, TRPV4, and TRPA1) TRPs are activated by the exogenous NO-releasing donors SNAP (*S*-nitroso-*N*-acetyl-DL-penicillamine) and NOR3 ((±)-*(E)*-4-ethyl-2-[*(E)*-hydroxyimino]-5-nitro-3-hexenamide) through oxidative cysteine modification (Yoshida et al., 2006; Sawada et al., 2008; Takahashi et al., 2008; Miyamoto et al., 2009), but with very limited TRP selectivity. Endowing NO donors with TRP channel subtype specificity mediated by transnitrosylation is an attractive strategy for the improvement of these agents.

To begin the development of transnitrosylation-based subtype-selective activators of TRP channels, it is necessary to first identify a synthetic NO donor that has only transnitrosylative reactivity. *S*-Nitrosoglutathione is a biological transnitrosylating agent, but also releases NO (Foster et al., 2003; Makita et al., 2013). *N*-Nitroso derivatives of ABBH (7-azabenzobicyclo[2.2.1]heptanes) constitute a new class of NO donors that, at physiological pH and temperature, transnitrosylate thiols to generate *S*-nitrosothiols without releasing NO (Ohwada et al., 2001, 2011; Yanagimoto et al., 2007; Karaki et al., 2012). This article describes the development of ABBH *N*-nitrosamines into subtype-selective nitrosylation activators of TRP channels.

## Materials and Methods

**Synthesis of ABBH Derivatives.** The details of the synthesis of ABBH derivatives are provided in the Supplemental Materials and Methods.

**Reagents.** AP-18 [(*Z*)-4-(4-chlorophenyl)-3-methylbut-3-en-2-oxime] was synthesized as previously reported (Takahashi et al., 2011). Dithiothreitol (DTT) was purchased from Nacalai Tesque (Kyoto, Japan). Carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] was purchased from Dojindo (Kumamoto, Japan). SNAP, 2-APB (2-aminoethyl diphenylborinate), and NEM (*N*-ethylmaleimide) were purchased from Sigma-Aldrich (St. Louis, MO). Ascorbic acid was purchased from Wako Pure Chemical Industries (Osaka, Japan). HPDP-biotin (*N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide) was purchased from Thermo Scientific (Rockford, IL). Derivatives of ABBH were prepared as stock solutions in *N*-methyl pyrrolidone and were diluted at working concentrations in aqueous solutions containing 0.1% *N*-methyl pyrrolidone. These were protected from light. AP-18, SNAP, 2-APB, and carboxy-PTIO were prepared as stock solutions in dimethylsulfoxide and were diluted at working concentrations in aqueous solutions containing 0.01% or 0.1% dimethylsulfoxide. DTT was directly dissolved in aqueous solutions at working concentrations.

**Cell Culture.** Human embryonic kidney (HEK) 293 and HEK 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 30 units $ml^{-1}$ penicillin, and 30 $\mu g\ ml^{-1}$ streptomycin at 37°C under 5% $CO_2$. WI-38 cells were cultured in modified Eagle's medium containing 10% fetal bovine serum, 30 units $ml^{-1}$ penicillin, and 30 $\mu g\ ml^{-1}$ streptomycin at 37°C under 5% $CO_2$. WI-38 (RCB0702) was purchased from RIKEN BRC through the National Bio-Resource Project of the MEXT (Tsukuba, Japan).

**cDNA Cloning and Recombinant Plasmid Construction.** Plasmids carrying human TRPA1 cDNA were prepared as previously described (Takahashi et al., 2011). TRPA1 and TRPA1 cysteine mutants were subcloned into the expression vector pCI-neo (Promega Corporation, Madison, WI) or the enhanced green fluorescent protein (EGFP) expression vector, pEGFP-C (Clontech Laboratories, Mountain View, CA). Plasmids of pCI-neo vector carrying mouse TRPC5, mouse TRPC1$\alpha$, mouse TRPC4$\beta$, human TRPV1, human TRPV3, or human TRPV4 cDNA were used (Yoshida et al., 2006; Takahashi et al., 2011). Plasmid of pEGFP-N vector carrying TRPV1 was also used.

**cDNA Expression in Cells.** HEK 293 cells and HEK 293T cells were transfected with recombinant plasmids using SuperFect transfection reagent (Qiagen, Valencia, CA) and Lipofectamine 2000 transfection reagent (Invitrogen/Life Technologies Corporation, Grand Island, NY), respectively, according to the manufacturer's instructions. In intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) measurement and electrophysiological measurements, recombinant plasmids were cotransfected with pEGFP-F (Clontech Laboratories) and pEGFP-N1 (Clontech Laboratories), respectively, and HEK cells with green fluorescence were analyzed. Transfected cells were grown for 36–40 hours prior to $[Ca^{2+}]_i$ measurement and electrophysiological measurements, and were grown for 48 hours prior to *S*-nitrosylation assay.

**Small Interfering RNA.** The TRPA1 small interfering RNA (siRNA) sequence targeting the coding region of TRPA1 mRNA (5′-AAACTTTGTTGAGGTCTACAG-3′) was used. To construct siRNA oligomers, the Silencer siRNA construction kit (Ambion/Life Technologies Corporation) was used. The glyceraldehyde-3-phosphate dehydrogenase siRNA was used as the control provided with the kit. Transfection of siRNAs at 200 nM to WI-38 cells was carried out using Lipofectamine 2000. The cells treated with siRNAs were subjected to reverse-transcription polymerase chain reaction (RT-PCR) or $[Ca^{2+}]_i$ measurement 48 hours after transfection.

**RT-PCR.** Total RNA of siRNA-transfected WI-38 cells was extracted using ISOGEN (Wako Pure Chemical Industries). Reverse transcription of total RNA to cDNA was performed using the RNA LA PCR Kit (TaKaRa-Bio, Shiga, Japan). Suppression of RNA expression was confirmed by RT-PCR analyses. The primer pairs used for TRPA1 and $\beta$-actin were as follows: TRPA1, 5′-GACCACAATGGCTGGACAGCTT-3′ (forward) and 5′-GTACCATTGCGTTGAGGGCTGT-3′ (reverse); $\beta$-actin, 5′-CATCCGCAAAGACCTGTACGCCAACAC-3′ (forward) and 5′-CTCGTCATACTCCTGCTTGCTGATCCAC-3′ (reverse). RT-PCR was conducted under the following conditions: 94°C for 5 minutes followed by 33 cycles for TRPA1 or 25 cycles for $\beta$-actin at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds, and finally 72°C for 7 minutes. Predicted lengths of products were 540 and 228 base pairs for TRPA1 and $\beta$-actin, respectively.

**$[Ca^{2+}]_i$ Measurement.** Transfected HEK cells, WI-38 cells, and siRNA-transfected WI-38 cells were subjected to $[Ca^{2+}]_i$ measurement 3–16 hours after plating onto poly-L-lysine–coated glass coverslips. The fura-2 (Dojindo) fluorescence was measured in HEPES-buffered saline containing the following: 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, and 20 mM HEPES (pH adjusted to 7.4 with NaOH). Fluorescence images of the cells were recorded and analyzed with the video image analysis system AQUACOSMOS (Hamamatsu Photonics, Shizuoka, Japan) according

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

to the manufacturer's instructions. The 340:380-nm ratio images were obtained on a pixel-by-pixel basis. Fura-2 measurements were carried out at 21 ± 1°C in HEPES-buffered saline. The 340:380-nm ratio images were converted to $Ca^{2+}$ concentrations by in vivo calibration using 10 $\mu$M ionomycin (Calbiochem/EMD Chemicals, San Diego, CA) as described previously (Takahashi et al., 2008).

**Electrophysiology.** For electrophysiological measurements, coverslips with cells were placed in dishes containing bath solutions. Currents from cells were recorded at room temperature (22–25°C) using patch-clamp techniques in whole-cell mode and excised inside-out mode, with an EPC-10 (Heka Elektronik, Lambrecht/Pfalz, Germany) patch-clamp amplifier as previously described (Okada et al., 1999). The patch electrodes prepared from borosilicate glass capillaries had a resistance of 2–4 MΩ for whole-cell recordings and 5–7 MΩ for single-channel recordings. Current signals were filtered at 2.9 kHz with a 4-pole Bessel filter and digitized at 20 kHz. Patchmaster (Heka Elektronik) software was used for command pulse control, data acquisition, and analysis. For whole-cell recordings, series resistance was compensated (to 70–80%) to minimize voltage errors. Ramp pulses were applied every 5 seconds from +100 to –100 mV at a speed of 4 mV $ms^{-1}$ after a 50-millisecond step to +100 mV from a holding potential of 0 mV. The bath solution contained the following: 140 mM NaCl, 5 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 adjusted with NaOH, and osmolality adjusted to 320 mOsM with D-mannitol). The pipette solution contained the following: 140 mM Cs-aspartate, 5 mM BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1.374 mM Ca-gluconate, 2 mM $Na_2ATP$, 2 mM $MgSO_4$, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 adjusted with CsOH, and osmolality adjusted to 300 mOsM with D-mannitol). The free $Ca^{2+}$ concentration was 30 nM, calculated with CaBuf software (provided by Dr. Droogmans, G., Katholieke Universiteit Leuven, Leuven, Belgium). Single-channel recordings were performed in inside-out excised patches configuration. Single-channel events were detected using the 50% threshold detection method. From the single-channel events list, the $NP_O$ ($N$, number of channels; $P_O$, open probability) of single-channels was calculated by dividing the total time spent in the open state by the total time of continuous recording (30 seconds) in the patches containing active channels. The amplitude of single-channel currents was measured as the peak-to-peak distance in Gaussian fits of the all-point amplitude histogram. The pipette solution contained the following: 100 mM CsCl, 1 mM EGTA, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 adjusted with CsOH, and osmolality adjusted to 300 mOsM with D-mannitol). The intracellular side was exposed to the bath solution, which contained the following: 50 mM CsCl, 50 mM Cs-aspartate, 10 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $Na_5P_3O_{10}$, and 10 mM HEPES (pH 7.4 adjusted with CsOH, and osmolality adjusted to 300 mOsM with D-mannitol).

***S*-Nitrosylation Assay.** The *S*-nitrosylation assay was performed as described previously (Jaffrey et al., 2001; Yoshida et al., 2006) with a few modifications. Microsomal fractions were prepared from transfected HEK 293T cells. In brief, cells were washed with phosphate-buffered saline, collected with a cell scraper, and homogenized with HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, and 0.1 mM neocuproine). The resulting homogenate was centrifuged at 2000*g* for 10 minutes. The supernatant was subsequently centrifuged at 100,000*g* for 1 hour to pellet the microsomal fraction. The fraction was incubated with 1 mM ABBH derivatives or 1 mM SNAP in the dark at room temperature for 10 minutes. After the centrifuge, the pellet was solubilized in HEN buffer containing 1% Triton X-100 (ICN Biomedicals, Aurora, OH). The extracts were incubated with 20 mM NEM and 2.5% SDS at room temperature for 30 minutes. Excess NEM was removed by precipitation with –20°C acetone. After resuspending the proteins in HEN buffer containing 1% SDS, ascorbic acid (20 mM final concentration) and HPDP-biotin (1 mM final concentration) were added. The mixtures were incubated for 1 hour at room temperature in the dark with intermittent vortexing. Residual HPDP-biotin was removed by precipitation with an equal volume of –20°C acetone. The pellet was resuspended in 0.1 ml HEN buffer containing 1% SDS. Two volumes of neutralization buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) were added, and biotinylated proteins were incubated batch-wise with NeutrAvidin-Plus beads (Thermo Scientific) overnight at 4°C with constant shaking. Beads were rinsed three times with neutralization buffer containing 600 mM NaCl. The proteins were eluted in sample buffer containing DTT (50 mM) at room temperature for 30 minutes and analyzed by 7.5% SDS-PAGE and Western blotting with an antibody to green fluorescent protein (GFP) (Clontech Laboratories).

**Griess Assay.** The Griess assay was performed as described previously (Yanagimoto et al., 2007). The strength of the N–NO bond of ABBH *N*-nitrosamines, or the propensity to release $NO^+$ or NO in acidic solution, can be estimated with the Griess method. NO reacts with oxygen in water to generate nitrite ion $NO_2^-$, which yields $NO^+$ in an acidic medium (Griess solvents). The visible absorption at 570 nm of the red dye formed upon diazo coupling of the Griess reagents provides a measure of the amount of NO and $NO^+$ formed by cleavage of N–NO bonds of ABBH *N*-nitrosamines. The dye formation was estimated after 5 hours at 37°C.

**Statistical Analyses.** All data are expressed as means ± S.E.M. We accumulated the data for each condition from at least three independent experiments. The statistical analyses were performed using Student's $t$ test. A value of $P < 0.05$ was considered significant.

## Results

**Transnitrosylating ABBH *N*-Nitrosamine Shows High Subtype Selectivity for TRPA1 Channels.** It was shown previously that NNO-ABBH1 (*N*-nitroso-2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane) (Fig. 1A) does not generate free NO by using electron spin resonance spin trap experiments under neutral cell-free conditions, and that NNO-ABBH1 participates in transnitrosylation reactions with a model thiol, triphenylmethylthiol, to yield *S*-nitrosothiols (Yanagimoto et al., 2007). However, NNO-ABBH1 has yet to be tested for protein thiol transnitrosylation activity (Fig. 1B).

The activation of homomeric TRPV1, TRPV3, TRPV4, TRPC5, and TRPA1 channels and heteromultimeric TRPC1/TRPC5 and TRPC4/TRPC5 channels by the exogenous NO-releasing donor SNAP is well characterized (Yoshida et al., 2006; Takahashi et al., 2008). We therefore tested whether the transnitrosylating agent NNO-ABBH1 activates these SNAP-sensitive TRP channels. In $[Ca^{2+}]_i$ measurements, 300 $\mu$M NNO-ABBH1 stimulation of HEK 293T cells recombinantly expressing TRPA1 elicited robust elevation in $[Ca^{2+}]_i$ (Fig. 1C). In contrast, responses were completely absent in cells expressing TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, and TRPC4/TRPC5. As a positive control, the same cells were stimulated with 300 $\mu$M SNAP as previously reported (Yoshida et al., 2006; Takahashi et al., 2008), and all were found to be activated (Fig. 1D). These experiments revealed an amazing activation selectivity of the transnitrosylating agent NNO-ABBH1 to TRPA1 channels.

NNO-ABBH1–elicited $[Ca^{2+}]_i$ increases in HEK 293 cells expressing TRPA1 occurred in a concentration-dependent manner (Fig. 1E). NNO-ABBH1–induced $Ca^{2+}$ responses were attributable mainly to $Ca^{2+}$ entry through the TRPA1 channel, because NNO-ABBH1 did not evoke $[Ca^{2+}]_i$ elevation in TRPA1-expressing HEK 293 cells in the absence of extracellular $Ca^{2+}$ (Fig. 1F). Furthermore, $Ca^{2+}$ influx evoked by NNO-ABBH1 in HEK 293 cells expressing TRPA1 was significantly suppressed by AP-18, a selective blocker of TRPA1 (Petrus et al., 2007) (Fig. 1G).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

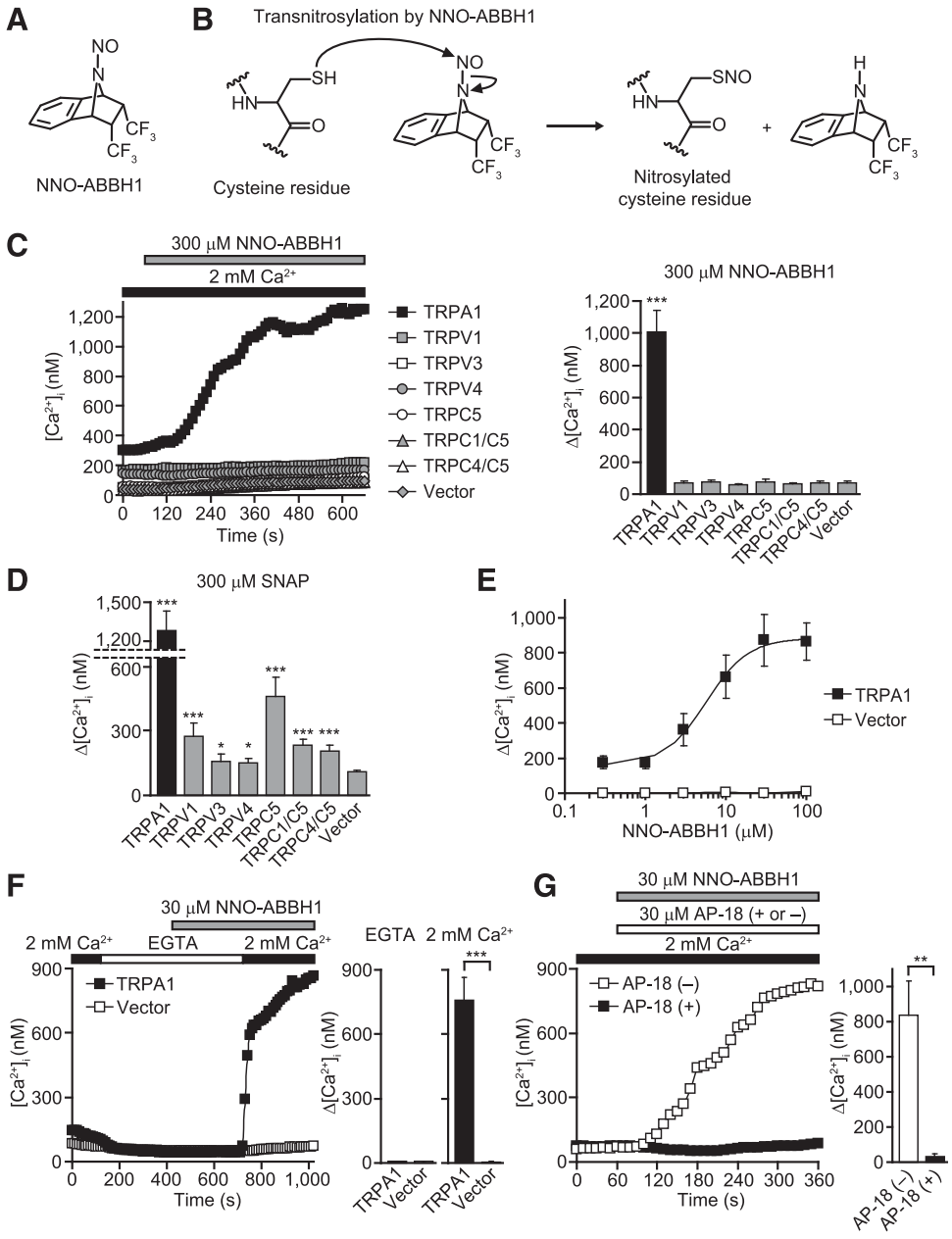


**Fig. 1.** Highly selective activation of TRPA1 channels by ABBH *N*-nitrosamine. (A) The chemical structure of NNO-ABBH1. (B) The chemical mechanism underlying the trans-nitrosylating action of NNO-ABBH1 on the protein thiol group. (C) $[Ca^{2+}]_i$ changes evoked by 300 $\mu$M NNO-ABBH1 in HEK 293T cells expressing TRPA1, TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, TRPC4/TRPC5, or vector. Averaged time courses and maximum increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) ($n$ = 47–107). ***$P < 0.001$ compared with vector. (D) $\Delta[Ca^{2+}]_i$ evoked by 300 $\mu$M SNAP in HEK 293T cells expressing TRPA1, TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, TRPC4/TRPC5, or vector ($n$ = 22–111). *$P < 0.05$; ***$P < 0.001$ compared with vector. (E) Dose-response relationships for NNO-ABBH1–induced $\Delta[Ca^{2+}]_i$ in HEK 293 cells expressing TRPA1 or vector during 5 minutes of stimulation ($n$ = 15–70). The plots were fitted to the Hill equation: $f(x) = A_0 + (A_{max} - A_0)/[1 + (EC_{50}/x)^n]$, where $A_0$ is the basal response, $A_{max}$ is the maximum response, $x$ is the NNO-ABBH1 concentration, and n is the Hill coefficient. The $EC_{50}$ value is 5.67 $\mu$M, and the Hill coefficient is 1.65. (F) $[Ca^{2+}]_i$ changes induced by 30 $\mu$M NNO-ABBH1 in $Ca^{2+}$-free, 0.5 mM EGTA-, or 2 mM $Ca^{2+}$-containing external solution in HEK 293 cells transfected with TRPA1 or vector. Averaged time courses and $\Delta[Ca^{2+}]_i$ ($n$ = 28–31). ***$P < 0.001$. (G) Effects of 30 $\mu$M AP-18 on 30 $\mu$M NNO-ABBH1–induced $[Ca^{2+}]_i$ increase in HEK 293 cells expressing TRPA1. Averaged time courses and $\Delta[Ca^{2+}]_i$ ($n$ = 15–27). **$P < 0.01$. Data points are the mean ± S.E.M.

In whole-cell mode of patch clamp recordings, we found that NNO-ABBH1 increased whole-cell currents in TRPA1-expressing HEK 293 cells (Fig. 2, A and B). NNO-ABBH1–activated whole-cell currents showed an outward-rectifying current-voltage (*I-V*) relationship and a reversal potential ($E_{rev}$) characteristic of nonselective cationic channels ($E_{rev}$ = 1.2 ± 0.63 mV, $n$ = 5). In cell-free excised inside-out patches, single-channel currents were prominently enhanced by NNO-ABBH1 (Fig. 2, C and D), and single-channel conductance at –60 mV was 97.9 ± 3.97 pS, as previously reported (Takahashi et al., 2011). These results suggest that NNO-ABBH1 activation of TRPA1 occurs by direct interaction rather than via intracellular components.

**Activation of TRPA1 by ABBH *N*-Nitrosamines Is by Transnitrosylation.** To biochemically identify *S*-nitrosylation of TRPA1 proteins, we treated TRPA1-expressing HEK 293T cells with NNO-ABBH1 and subjected them to an *S*-nitrosylation assay (Jaffrey et al., 2001; Yoshida et al., 2006). Cysteine *S*-nitrosylation of GFP-TRPA1 proteins was detected after the selective conversion of nitrosylated residues into biotinylated cysteines, and was induced by NNO-ABBH1 (Fig. 3A). *S*-Nitrosylation of TRPA1 was increased even more dramatically by SNAP, indicating the relative promiscuity of SNAP compared with NNO-ABBH1 in nitrosylating TRPA1 cysteine residues. Interestingly, *S*-nitrosylation of TRPV1-GFP proteins was induced by SNAP but not by NNO-ABBH1 (Fig. 3B), supporting the selective action of NNO-ABBH1 via *S*-nitrosylation on TRPA1.

The N–NO bond strength of the *N*-nitroso derivatives of ABBH can be controlled by changing the distal substituents (Yanagimoto et al., 2007) (NNO-ABBH2–NNO-ABBH5; Fig. 4A). The reactivity of the *N*-nitrosamine N–NO bonds can be estimated through their propensity to release $NO^+$ or NO by cleavage of the N–NO bond in acidic conditions (pH = 2.1) in

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

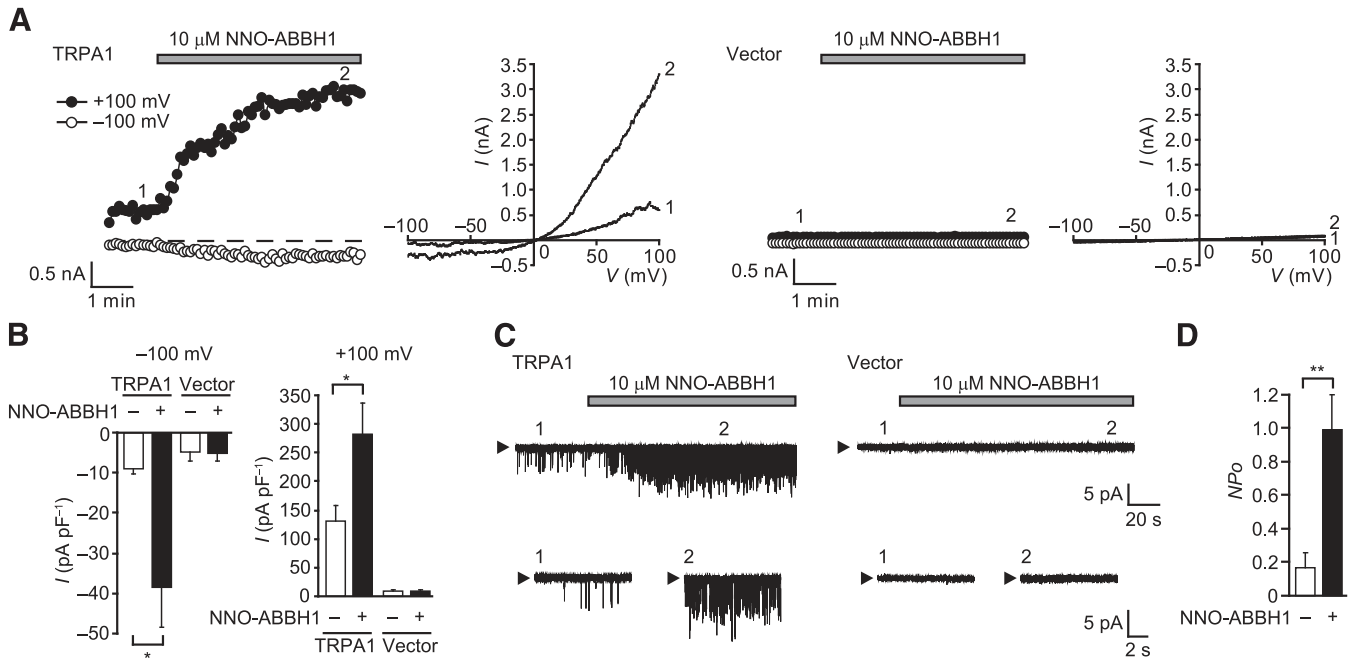


**Fig. 2.** Electrophysiological characterization of TRPA1 activation by NNO-ABBH1. (A) Whole-cell currents evoked by 10 $\mu$M NNO-ABBH1 in HEK 293 cells transfected with TRPA1 or vector. Representative time courses of outward and inward currents recorded at +100 and –100 mV, respectively, under ramp clamp. Corresponding *I-V* relationships at the time points 1 and 2. (B) Peak current densities at –100 and +100 mV before and during the application of 10 $\mu$M NNO-ABBH1 ($n$ = 5). $^*P < 0.05$. (C) Single-channel activities evoked by 10 $\mu$M NNO-ABBH1 at –60 mV in inside-out patches excised from TRPA1- or vector-transfected HEK 293 cells. Time-expanded current traces before and during the application of 10 $\mu$M NNO-ABBH1 are shown at time points 1 and 2. Arrowheads represent the closed state. (D) Averages of $NP_O$ representing single TRPA1 channel activity before and during the application of NNO-ABBH1 ($n$ = 5). $^{**}P < 0.01$. Data points are the mean ± S.E.M.

the Griess assay (Ohwada et al., 2001; Yanagimoto et al., 2007). An aromatic group (NNO-ABBH1–NNO-ABBH4) and electron-withdrawing groups, such as trifluoromethyl (NNO-ABBH1) and ester (NNO-ABBH2 and NNO-ABBH3) groups, enhanced N–NO bond cleavage (NNO-ABBH1 > NNO-ABBH2 > NNO-ABBH3 > NNO-ABBH4 > NNO-ABBH5; Fig. 4B). Using this series of ABBH *N*-nitrosamine compounds, we investigated the correlation between TRPA1-activating capacity and N–NO bond reactivity. N–NO bond reactivity was well correlated with the $[Ca^{2+}]_i$ elevation induced by these compounds in TRPA1-expressing HEK 293 cells (Fig. 4, C and D). We then measured the contribution of free NO to the NNO-ABBH1–induced activation of TRPA1 using the NO scavenger, carboxy-PTIO (Akaike et al., 1993). Incubation of TRPA1-expressing HEK 293 cells with 300 $\mu$M carboxy-PTIO had no significant effect on TRPA1 activation by NNO-ABBH1 (Fig. 4E). In contrast, carboxy-PTIO inhibited TRPA1 activation by SNAP (Fig. 4F). Thus, it is probable that ABBH *N*-nitrosamines

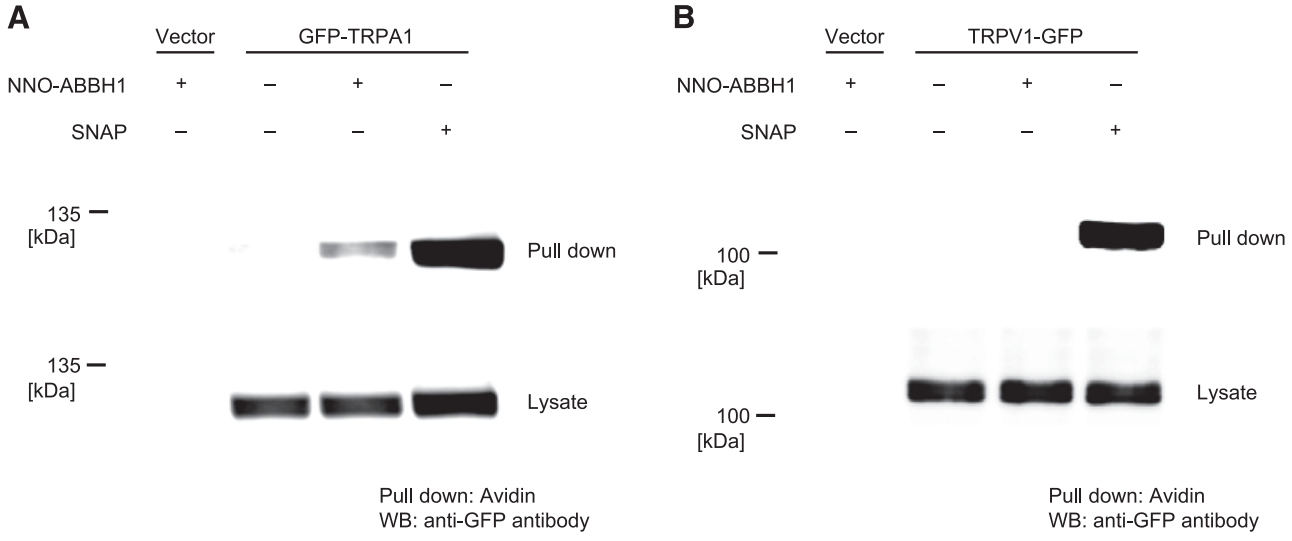


**Fig. 3.** *S*-Nitrosylation of TRPA1 protein by NNO-ABBH1. *S*-Nitrosylation labeling assay of TRPA1 (A) and TRPV1 (B). Microsomal fractions prepared from transfected HEK 293T cells with and without treatment of NNO-ABBH1 (1 mM) or SNAP (1 mM) were subjected to *S*-nitrosylation assay. Western blotting (WB) of total lysates indicates comparable protein expression (lysate).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

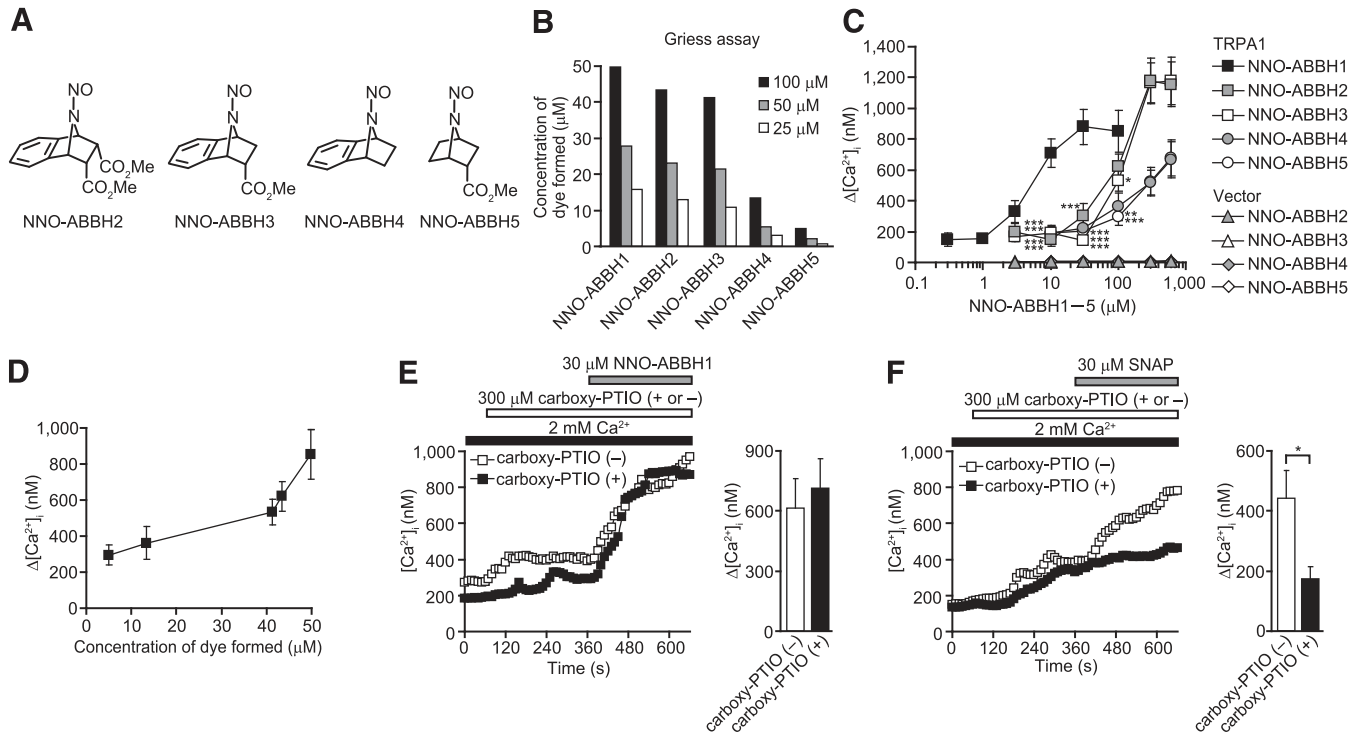


**Fig. 4.** Activation of TRPA1 by *N*-nitroso derivatives of ABBH through transnitrosylation. (A) The chemical structures of *N*-nitroso derivatives of ABBH (NNO-ABBH2–NNO-ABBH5). (B) Estimation of reactivity of the N–NO bond of *N*-nitroso derivatives of ABBH by the Griess assay. The dye formation of 25, 50, and 100 $\mu$M NNO-ABBH1–NNO-ABBH5 is examined. (C) Dose-response relationships for NNO-ABBH1–NNO-ABBH5–induced $\Delta[Ca^{2+}]_i$ in HEK 293 cells transfected with TRPA1 or vector during 5 minutes of stimulation ($n$ = 12–73). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with NNO-ABBH1. (D) Relationship between the dye formation by each *N*-nitroso derivative of ABBH in the Griess assay and $\Delta[Ca^{2+}]_i$ in response to 100 $\mu$M NNO-ABBH1–NNO-ABBH5. Data points are plotted using the data shown in B and C. (E) Effects of carboxy-PTIO on TRPA1 responses evoked by NNO-ABBH1 in HEK 293 cells. Five minutes prior to the treatment with 30 $\mu$M NNO-ABBH1 and continuing during the treatment, cells are incubated with or without 300 $\mu$M carboxy-PTIO. Averaged time courses and NNO-ABBH1–induced $\Delta[Ca^{2+}]_i$ ($n$ = 19–25). (F) Effects of carboxy-PTIO on TRPA1 responses evoked by 30 $\mu$M SNAP in HEK 293 cells. Averaged time courses and SNAP-induced $\Delta[Ca^{2+}]_i$ ($n$ = 20–24). $^*P < 0.05$. Data points are the mean ± S.E.M.

activate TRPA1 through transnitrosylation rather than through the release of NO.

**Identification of the TRPA1 Cysteine Residues Critical for the Activation by NNO-ABBH1.** The cell-permeable reducing agent DTT, which reverses disulfide formation and nitrosylation of cysteine sulfhydryls (Macpherson et al., 2007; Takahashi et al., 2008), was used to examine whether NNO-ABBH1 activates the TRPA1 channel via oxidative sulfhydryl modification. DTT significantly diminished NNO-ABBH1–induced TRPA1-mediated $[Ca^{2+}]_i$ responses (Fig. 5A). We next mutated 29 individual cysteine residues in human TRPA1 to serine (Takahashi et al., 2011) and tested the responsiveness of these TRPA1 mutants. Compared with wild type, mutants C414S, C421S, C540S, C641S, and C665S showed significantly suppressed responses to NNO-ABBH1 (Fig. 5B). C414S and C421S are likely to have deleterious effects on channel function because they also significantly suppressed responses to 100 $\mu$M 2-APB (Fig. 5C), which normally activates TRPA1 independently of cysteine modification (Hinman et al., 2006). TRPA1 nitrosylation by NNO-ABBH1 was prominently suppressed by the C540S mutation, and more moderately by the C641S and C665S mutations (Fig. 5D). These results suggest that Cys540, Cys641, and Cys665 are putative TRPA1 target sites of NNO-ABBH1.

**Modulation of TRPA1 by Nonelectrophilic Analogs of NNO-ABBH1.** To further explore the structure-function relationship of the profound selectivity of NNO-ABBH1 for TRPA1, we developed a new group of nonelectrophilic analogs of NNO-ABBH1, including *N*-H (NH-ABBH), *N*-formyl (NCHO-ABBH), and *N*-methyl (NMe-ABBH) derivatives of 2-exo,3-exo-ditrifluoromethyl-7-azabenzobicyclo[2.2.1]heptane (Fig. 6A). These analogs were less potent than NNO-ABBH1 in activating TRPA1, but maximum responses to the analogs were similar to those induced by NNO-ABBH1 (Fig. 6B).

Importantly, *S*-nitrosylation of GFP-TRPA1 proteins was induced by NNO-ABBH1, but not by NMe-ABBH (Fig. 6C). In $[Ca^{2+}]_i$ measurements, TRPA1 activity evoked by NMe-ABBH took a time course to decrease after the treatment by DTT similar to the washout of NMe-ABBH (Fig. 6D). Furthermore, compared with wild type, cysteine mutants C540S, C641S, and C665S showed similar $[Ca^{2+}]_i$ responses to NH-ABBH, NCHO-ABBH, and NMe-ABBH (Fig. 6E). These results suggest that oxidative modification of cysteine residues is not critical for the activation of TRPA1 by nonelectrophilic analogs of NNO-ABBH1.

Interestingly, the NMe-ABBH sensitivity of TRPA1 was significantly enhanced by SNAP at a subthreshold concentration (10 $\mu$M) (Fig. 6F), in support of the idea of positive synergistic effects between nitrosylation and molecular recognition on TRPA1 activation. It is important to note that TRPC5 and TRPV1 failed to respond to NMe-ABBH (Fig. 6G). Thus, molecular recognition of chemical groups other than NO contributes to the selective activation of TRPA1.

**Native TRPA1 Channels Are Activated by ABBH *N*-Nitrosamines.** The action of ABBH *N*-nitrosamines on endogenous TRPA1 channels was characterized in WI-38 human lung fibroblasts, which are known to express functional TRPA1 (Jaquemar et al., 1999; Hu et al., 2010). NNO-ABBH1 induced

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

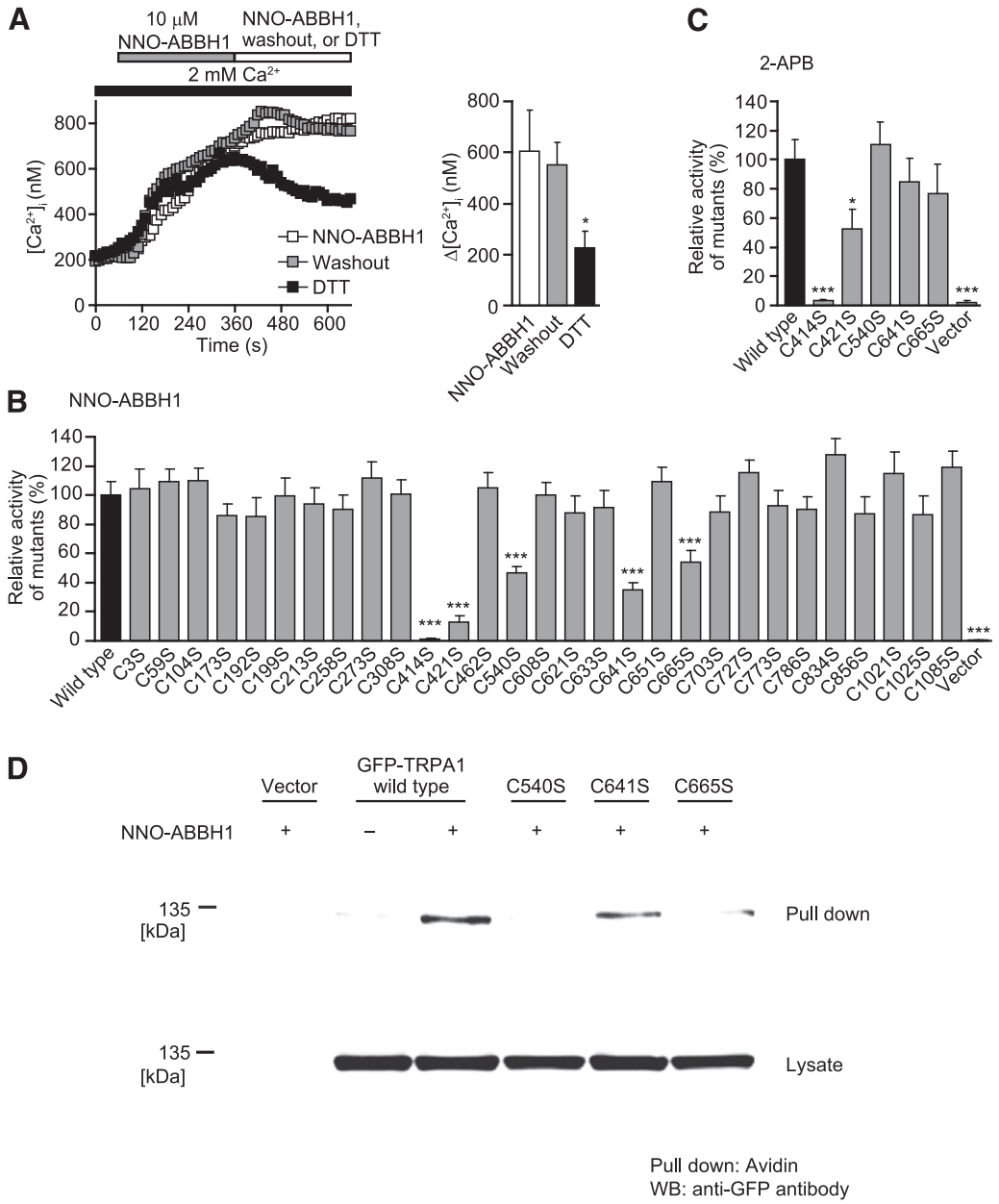


**Fig. 5.** Identification of cysteine residues contributing to NNO-ABBH1–induced activation of TRPA1. (A) Effects of 5 mM DTT on 10 $\mu$M NNO-ABBH1–induced $[Ca^{2+}]_i$ increase in HEK 293 cells expressing TRPA1. Averaged time courses and $[Ca^{2+}]_i$ increases at 660 seconds ($n$ = 20–38). *$P < 0.05$ compared with NNO-ABBH1. (B) Relative $[Ca^{2+}]_i$ responses evoked by 10 $\mu$M NNO-ABBH1 in HEK 293 cells expressing each of TRPA1 cysteine mutants during 5 minutes of stimulation ($n$ = 25–81). ***$P < 0.001$ compared with wild type. (C) Relative $[Ca^{2+}]_i$ responses induced by 100 $\mu$M 2-APB in HEK 293 cells expressing each of TRPA1 cysteine mutants during 5 minutes of stimulation ($n$ = 22–34). *$P < 0.05$; ***$P < 0.001$ compared with wild type. Data points are the mean ± S.E.M. (D) Effects of mutations C540S, C641S, or C665S on *S*-nitrosylation of TRPA1 proteins by 1 mM NNO-ABBH1. Western blotting (WB) of total lysates indicates comparable TRPA1 expression (lysate).

$[Ca^{2+}]_i$ increases in a concentration-dependent manner (Fig. 7A), and these were suppressed by the absence of extracellular $Ca^{2+}$, indicating the role of $Ca^{2+}$ influx in these responses (Fig. 7B). $Ca^{2+}$ influx evoked by NNO-ABBH1 was significantly suppressed by AP-18 (Fig. 7C) and by transfection of an siRNA for TRPA1, which suppressed the RNA expression of TRPA1 in WI-38 cells (Fig. 7, D and E). These results suggest that endogenous TRPA1 in WI-38 cells mediates $Ca^{2+}$ entry in response to NNO-ABBH1.

In agreement with the results from TRPA1-expressing HEK 293 cells, the N–NO bond reactivity of NNO-ABBH1–NNO-ABBH5 was correlated with the evoked $[Ca^{2+}]_i$ increases in WI-38 cells (Fig. 7F), and the reducing agent DTT significantly diminished NNO-ABBH1–induced $[Ca^{2+}]_i$ responses (Fig. 7G). The NO group was essential for the action of NNO-ABBH1, because NH-ABBH, NCHO-ABBH, and NMe-ABBH gave significantly smaller responses compared with NNO-ABBH1 in WI-38 cells (Fig. 7H). Thus, ABBH *N*-nitrosamines potently activate native TRPA1 channels in WI-38 cells.

## Discussion

In the present study, with the aim of establishing novel TRP subtype-selective nitrosylating agents, we have characterized

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

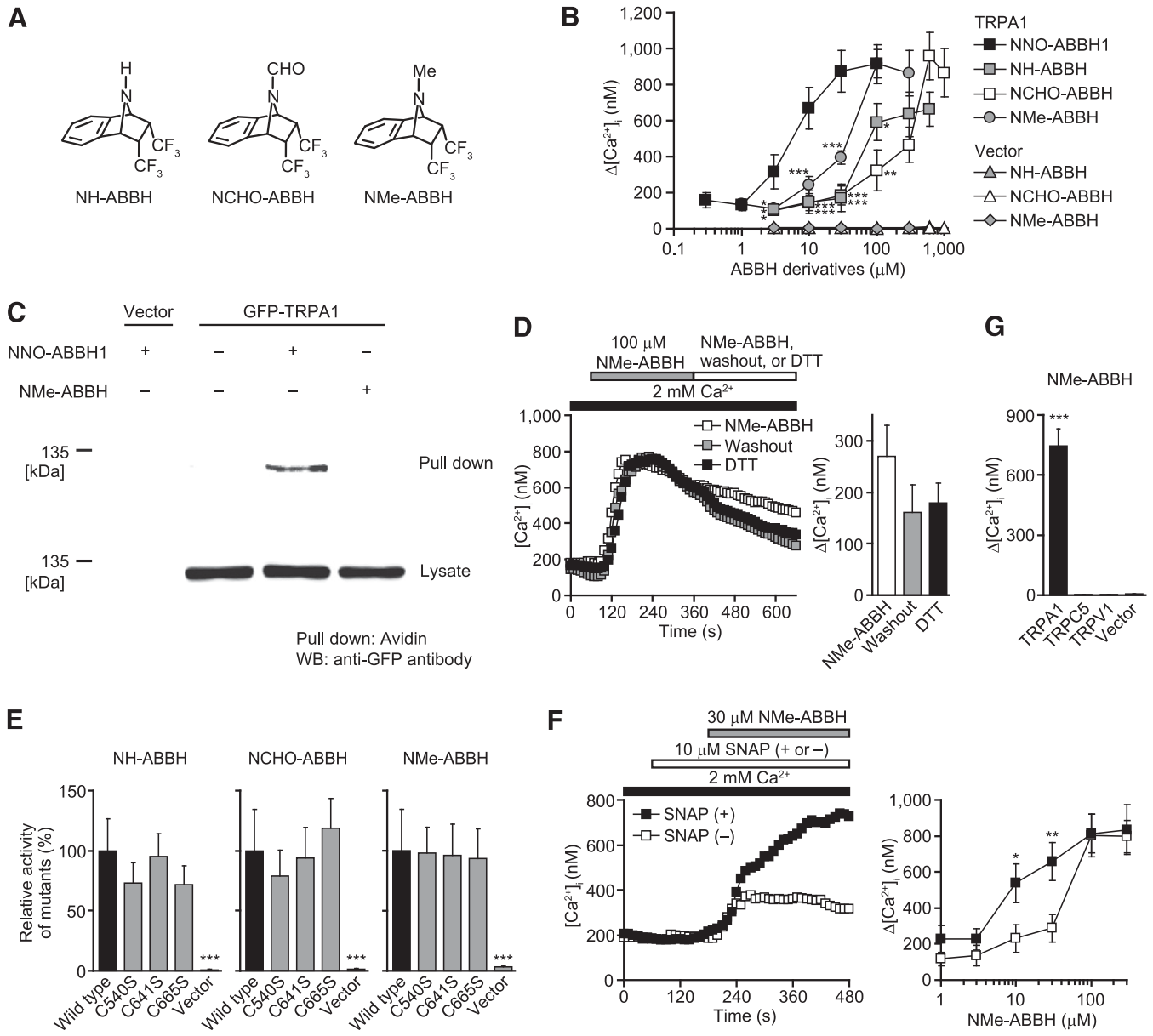


**Fig. 6.** Modulation of TRPA1 by nonelectrophilic derivatives of NNO-ABBH1. (A) The chemical structures of NH-ABBH, NCHO-ABBH, and NMe-ABBH which are nonelectrophilic derivatives of NNO-ABBH1. (B) Dose-response relationships for $\Delta[Ca^{2+}]_i$ induced by ABBH derivatives (NNO-ABBH1, NH-ABBH, NCHO-ABBH, and NMe-ABBH) in HEK 293 cells transfected with TRPA1 or vector during 5 minutes of stimulation ($n$ = 6–70). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with NNO-ABBH1. (C) *S*-Nitrosylation labeling assay of TRPA1 with treatment of 1 mM NMe-ABBH or 1 mM NNO-ABBH1. Western blotting (WB) of total lysates indicates comparable TRPA1 expression (lysate). (D) Effects of 5 mM DTT on 100 $\mu$M NMe-ABBH–induced $[Ca^{2+}]_i$ increase in HEK 293 cells expressing TRPA1. Averaged time courses and $[Ca^{2+}]_i$ increases at 660 seconds ($n$ = 22–27). (E) Relative $[Ca^{2+}]_i$ responses evoked by 100 $\mu$M NH-ABBH, 300 $\mu$M NCHO-ABBH, or 100 $\mu$M NMe-ABBH in HEK 293T cells expressing each of TRPA1 cysteine mutants during 5 minutes of stimulation ($n$ = 12–48). $^{***}P < 0.001$ compared with wild type. (F) Effects of SNAP on NMe-ABBH dependence of $[Ca^{2+}]_i$ responses of TRPA1 in HEK 293 cells. The treatment of 10 $\mu$M SNAP was started 2 minutes prior to the treatment with NMe-ABBH. Averaged time courses and dose-response relationships for $\Delta[Ca^{2+}]_i$ ($n$ = 25–65). $^*P < 0.05$; $^{**}P < 0.01$. (G) $\Delta[Ca^{2+}]_i$ evoked by 300 $\mu$M NMe-ABBH in HEK 293 cells transfected with TRPA1, TRPC5, TRPV1, or vector during 5 minutes of stimulation ($n$ = 29–77). $^{***}P < 0.001$ compared with vector. Data points are the mean ± S.E.M.

several ABBH *N*-nitrosamines, a new series of NO donors that transnitrosylate thiols without releasing NO. The TRPA1 selectivity of these ABBH *N*-nitrosamines is likely mediated through the synergetic effects of two chemical processes: cysteine transnitrosylation and molecular recognition of a nonelectrophilic moiety.

In $[Ca^{2+}]_i$ measurements and whole-cell mode of patch-clamp recordings, NNO-ABBH1 robustly activated TRPA1. There is a possibility that NNO-ABBH1 reacts with intracellular components to generate NO-bound intracellular components that modulate TRPA1 activity, because the cytoplasm contains high concentrations of glutathione, metals, and proteins that can bind to NO. However, in excised inside-out patches, single-channel currents were enhanced by NNO-ABBH1, suggesting that intracellular components are not required for the activation of TRPA1 by NNO-ABBH1. *S*-Nitrosylation assay strongly showed that TRPA1 proteins are *S*-nitrosylated by NNO-ABBH1. In a congeneric series of ABBH *N*-nitrosamines,

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

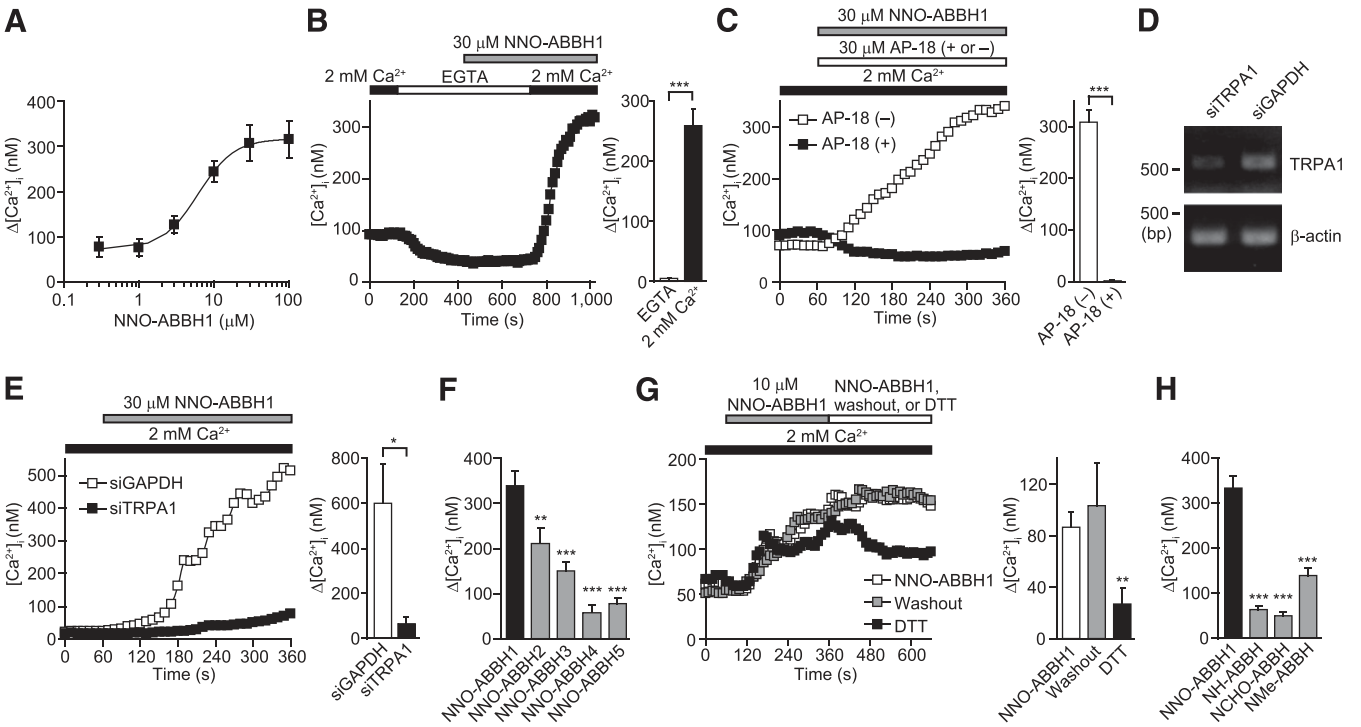


**Fig. 7.** Activation of native TRPA1 in WI-38 human fibroblasts by ABBH derivatives. (A) Dose-response relationships for NNO-ABBH1–induced $\Delta[Ca^{2+}]_i$ in WI-38 cells during 5 minutes of stimulation ($n$ = 37–138). $EC_{50}$ value obtained through Hill equation is 6.19 $\mu$M, and the Hill coefficient is 1.82. (B) Averaged time courses and $\Delta[Ca^{2+}]_i$ induced by 30 $\mu$M NNO-ABBH1 in $Ca^{2+}$-free, 0.5 mM EGTA-, or 2 mM $Ca^{2+}$-containing external solution in WI-38 cells ($n$ = 42). ***$P < 0.001$. (C) Effects of 30 $\mu$M AP-18 on 30 $\mu$M NNO-ABBH1–induced $[Ca^{2+}]_i$ increase in WI-38 cells. Averaged time courses and $\Delta[Ca^{2+}]_i$ ($n$ = 69–76). ***$P < 0.001$. (D) RT-PCR analysis of TRPA1 RNA expression in WI-38 cells treated with TRPA1-specific siRNA (siTRPA1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–specific siRNA (siGAPDH). $\beta$-Actin is used as control. (E) $[Ca^{2+}]_i$ changes induced by 30 $\mu$M NNO-ABBH1 in WI-38 cells treated with siTRPA1 or siGAPDH. Averaged time courses and $\Delta[Ca^{2+}]_i$ ($n$ = 11–15). *$P < 0.05$. (F) $\Delta[Ca^{2+}]_i$ induced by 30 $\mu$M ABBH $N$-nitrosamines (NNO-ABBH1–NNO-ABBH5) in WI-38 cells during 5 minutes of stimulation ($n$ = 69–92). **$P < 0.01$; ***$P < 0.001$ compared with NNO-ABBH1. (G) Effects of 5 mM DTT on 10 $\mu$M NNO-ABBH1–induced $[Ca^{2+}]_i$ increase in WI-38 cells. Averaged time courses and $[Ca^{2+}]_i$ increases at 660 seconds ($n$ = 58–104). **$P < 0.01$ compared with NNO-ABBH1. (H) $\Delta[Ca^{2+}]_i$ induced by 30 $\mu$M nonelectrophilic analogs of NNO-ABBH1 (NH-ABBH, NCHO-ABBH, and NMe-ABBH) in WI-38 cells during 5 minutes of stimulation ($n$ = 77–149). ***$P < 0.001$ compared with NNO-ABBH1. Data points are the mean ± S.E.M.

N–NO bond reactivity was correlated with $[Ca^{2+}]_i$ elevation via TRPA1. Activation of TRPA1 by NNO-ABBH1 was not inhibited by the NO scavenger carboxy-PTIO. Thus, these results clearly show that NNO-ABBH1 activates TRPA1 by transnitrosylation.

Multiple cysteine residues in TRPA1 have been identified as sensors of various electrophilic ligands (Hinman et al., 2006; Macpherson et al., 2007; Takahashi et al., 2008). The contribution of multiple cysteine residues to the oxidation sensitivity of protein function has also been reported for other proteins (Dinkova-Kostova et al., 2002; Voss et al., 2004). This is consistent with our site-directed mutagenesis and pharmacological experiments, which reveal that Cys540, Cys641, and Cys665 of human TRPA1 are involved in its modification by NNO-ABBH1. Cys641 and Cys665 are also required for responsiveness to SNAP (Takahashi et al., 2008; Miyamoto et al., 2009). Cys540 may be a unique target for NNO-ABBH1.

Within the predicted structure according to molecular modeling and docking into the mouse TRPA1 electron microscopy map, cysteine residues in the cytoplasmic N-terminal region of mouse TRPA1 (Cys415, Cys422, and Cys622), which are conserved in human TRPA1 (Cys414, Csy421, and Cys621), form a ligand binding pocket between subunits (Cvetkov et al., 2011). Cys414 and Cys421 in the ankyrin repeats from one subunit could interact with Cys621 located on a flexible region between ankyrin repeats and transmembrane domain on an adjacent subunit. It is possible that covalent modification of cysteine residues around this pocket could alter the interaction between subunits and promote conformational changes and disulfide formation, leading to activation (Cvetkov et al., 2011; Wang et al., 2012). Modification of Cys540 in ankyrin repeats and Cys641 and Cys665 on a flexible region by NNO-ABBH1 may induce conformational changes of ankyrin repeats and affect subunit–subunit interaction and disulfide formation, leading to activation of TRPA1.

The NO group of ABBH $N$-nitrosamine is essential, because NH-ABBH, NCHO-ABBH, and NMe-ABBH showed significantly suppressed activity in activating TRPA1. NH-ABBH, NCHO-ABBH, and NMe-ABBH did induce $[Ca^{2+}]_i$ increases in TRPA1-expressing HEK 293 cells, but only at higher concentrations. It has been shown that some nonelectrophilic compounds can activate TRPA1 by noncovalent ligand–receptor interaction (Karashima et al., 2007; Maher et al., 2008; Xiao et al., 2008; Hu et al., 2010; Liu et al., 2010; Nagatomo et al., 2010; Zhong et al., 2011a,b). However, the precise molecular mechanism by which noncovalent binding compounds activate TRPA1 remains controversial. Our results obtained regarding NH-ABBH, NCHO-ABBH, and NMe-ABBH support the concept of a synergetic mechanism of TRPA1 activation by NNO-ABBH1 comprising two main processes: transnitrosylation and

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

molecular recognition. However, it is unclear to what extent the transnitrosylation site and the nonelectrophilic group-activating site overlap in TRPA1. NNO-ABBH1 may be considered as a hybrid activator, apparently combining covalent binding to reactive thiols with noncovalent molecular recognition in the same binding pocket of TRPA1.

Our $[Ca^{2+}]_i$ measurements revealed that TRPV1, TRPV3, TRPV4, TRPC5, TRPC1/TRPC5, and TRPC4/TRPC5 failed to respond to 300 $\mu$M NNO-ABBH1. In addition, *S*-nitrosylation of TRPV1 proteins was not induced by NNO-ABBH1. This may be due to the absence in these TRP channels of structural features required for the binding of NNO-ABBH1. Considering the nature of transnitrosylation, the compounds must be able to reach the target site cysteine residues for transnitrosylation. Our findings suggest that molecular recognition is critical for this. Indeed, TRPC5 and TRPV1 failed to respond to the nonelectrophilic derivative NMe-ABBH, suggesting that these proteins may lack the molecular recognition site for the ABBH skeleton. However, we cannot exclude the possibility that the ABBH compound binds to, but does not activate, these channels. Importantly, the site for *S*-nitrosylation by SNAP varies among TRP channels, being on the N-terminal side of the pore region in TRPC5 and TRPV1, but in the cytoplasmic N-terminal region of TRPA1 (Yoshida et al., 2006; Takahashi et al., 2008; Miyamoto et al., 2009).

In addition to transnitrosylation via direct protein–protein interaction, selectivity of *S*-nitrosylation is provided by a proximity effect of the subcellular colocalization of NOS and the targets of the generated NO, exemplified by the *N*-methyl-D-aspartate receptor/NO signaling module (Hess et al., 2005). We have demonstrated that TRPC5 is activated by nitrosylation via colocalized endothelial NOS (eNOS) following ATP receptor stimulation in endothelial cells (Yoshida et al., 2006). It is known that eNOS localizes in caveolae, a specialized lipid raft domain, and interacts with caveolin-1, a major coat protein of caveolae (García-Cardeña et al., 1996a,b; Shaul et al., 1996). In addition, caveolin-1 is known to interact with TRPC1, which can form heteromers with TRPC5 (Lockwich et al., 2000; Strübing et al., 2001). Therefore, it is possible that eNOS and TRPC5 are in close proximity due to these interactions in caveolae. It has been reported that the activation of TRPV1 by ligands in endothelial cells may trigger $Ca^{2+}$-dependent signaling, leading to enhanced TRPV1 phosphorylation, the formation of a TRPV1-eNOS complex, activation of eNOS, and NO production (Ching et al., 2011). Production of NO is known to activate TRPA1 in dorsal root ganglion neurons (Miyamoto et al., 2009), although it is unknown whether NOS binds to TRPA1. Thus, TRPC5 and TRPV1 can achieve NO signaling specificity through subcellular colocalization with NOS, instead of through transnitrosylation.

Our studies using the WI-38 human fibroblast cell line revealed that endogenous TRPA1 is activated through transnitrosylation by ABBH *N*-nitrosamines. Recently, it has been shown that TRPA1 is natively expressed in endothelial cells as well, and allyl isothiocyanate–induced TRPA1 activation in endothelial cells elicits vasodilation of cerebral arteries (Earley et al., 2009). Thus, transnitrosylating agents ABBH *N*-nitrosamines will be useful for investigating the effect of transnitrosylation-induced TRPA1 activation on vasodilation.

In conclusion, our finding that *N*-nitroso derivatives of ABBH are selective activators of TRPA1 channels and act via transnitrosylation presents the opportunity for developing selective transnitrosylating modulators of proteins. Our results also suggest that an important concept in designing such pharmacological agents is that they should contain both a transnitrosylating moiety and a skeleton that imbues target protein selectivity via molecular recognition.

#### Acknowledgments

The authors thank F. Karaki for providing NNO-ABBH5 and performance of the Griess assay. The authors thank M. X. Mori, N. Ogawa, and T. Kuwamura for experimental advice and helpful discussions.

#### Authorship Contributions

*Participated in research design*: Kozai, Takahashi, Mori, Ohwada.

*Conducted experiments:* Kozai, Kabasawa, Ebert, Firman, Otani, Ohwada.

*Contributed new reagents or analytic tools:* Kabasawa, Firman, Otani, Ohwada.

*Performed data analysis:* Kozai, Kabasawa, Ohwada.

*Wrote or contributed to the writing of the manuscript:* Kozai, Ebert, Kiyonaka, Otani, Numata, Takahashi, Mori, Ohwada.

#### References

Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF, and Tymianski M (2003) A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**:863–877.

Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, and Maeda H (1993) Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. *Biochemistry* **32**:827–832.

Alderton WK, Cooper CE, and Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357**:593–615.

Anand P and Stamler JS (2012) Enzymatic mechanisms regulating protein S-nitrosylation: implications in health and disease. *J Mol Med (Berl)* **90**:233–244.

Andersson DA, Gentry C, Moss S, and Bevan S (2008) Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci* **28**: 2485–2494.

Bessac BF, Sivula M, von Hehn CA, Escalera J, Cohn L, and Jordt SE (2008) TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest* **118**: 1899–1910.

Ching LC, Kou YR, Shyue SK, Su KH, Wei J, Cheng LC, Yu YB, Pan CC, and Lee TS (2011) Molecular mechanisms of activation of endothelial nitric oxide synthase mediated by transient receptor potential vanilloid type 1. *Cardiovasc Res* **91**: 492–501.

Clapham DE (2003) TRP channels as cellular sensors. *Nature* **426**:517–524.

Clapham DE, Julius D, Montell C, and Schultz G (2005) International Union of Pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels. *Pharmacol Rev* **57**:427–450.

Cvetkov TL, Huynh KW, Cohen MR, and Moiseenkova-Bell VY (2011) Molecular architecture and subunit organization of TRPA1 ion channel revealed by electron microscopy. *J Biol Chem* **286**:38168–38176.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, and Talalay P (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* **99**:11908–11913.

Earley S, Gonzales AL, and Crnich R (2009) Endothelium-dependent cerebral artery dilation mediated by TRPA1 and $Ca^{2+}$-Activated $K^+$ channels. *Circ Res* **104**: 987–994.

Foster MW, McMahon TJ, and Stamler JS (2003) S-nitrosylation in health and disease. *Trends Mol Med* **9**:160–168.

García-Cardeña G, Fan R, Stern DF, Liu J, and Sessa WC (1996a) Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J Biol Chem* **271**:27237–27240.

García-Cardeña G, Oh P, Liu J, Schnitzer JE, and Sessa WC (1996b) Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. *Proc Natl Acad Sci USA* **93**:6448–6453.

Hara Y, Wakamori M, Ishii M, Maeno E, Nishida M, Yoshida T, Yamada H, Shimizu S, Mori E, and Kudoh J et al. (2002) LTRPC2 $Ca^{2+}$-permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol Cell* **9**:163–173.

Hess DT, Matsumoto A, Kim SO, Marshall HE, and Stamler JS (2005) Protein *S*-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol* **6**:150–166.

Hinman A, Chuang HH, Bautista DM, and Julius D (2006) TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci USA* **103**:19564–19568.

Hu H, Tian J, Zhu Y, Wang C, Xiao R, Herz JM, Wood JD, and Zhu MX (2010) Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. *Pflugers Arch* **459**:579–592.

Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, and Snyder SH (2001) Protein *S*-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* **3**:193–197.

Jaquemar D, Schenker T, and Trueb B (1999) An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *J Biol Chem* **274**:7325–7333.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015

Karaki F, Kabasawa Y, Yanagimoto T, Umeda N, Firman, Urano Y, Nagano T, Otani Y, and Ohwada T (2012) Visible-light-triggered release of nitric oxide from N-pyramidal nitrosamines. *Chemistry* **18**:1127–1141.
Karashima Y, Damann N, Prenen J, Talavera K, Segal A, Voets T, and Nilius B (2007) Bimodal action of menthol on the transient receptor potential channel TRPA1. *J Neurosci* **27**:9874–9884.
Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM, Law L, Hester LD, and Snyder SH (2010) GAPDH mediates nitrosylation of nuclear proteins. *Nat Cell Biol* **12**:1094–1100.
Liu K, Samuel M, Ho M, Harrison RK, and Paslay JW (2010) NPPB structure-specifically activates TRPA1 channels. *Biochem Pharmacol* **80**:113–121.
Lockwich TP, Liu X, Singh BB, Jadlowiec J, Weiland S, and Ambudkar IS (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J Biol Chem* **275**:11934–11942.
Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, and Patapoutian A (2007) Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445**:541–545.
Maher M, Ao H, Banke T, Nasser N, Wu NT, Breitenbucher JG, Chaplan SR, and Wickenden AD (2008) Activation of TRPA1 by farnesyl thiosalicylic acid. *Mol Pharmacol* **73**:1225–1234.
Makita N, Kabasawa Y, Otani Y, Firman, Sato J, Hashimoto M, Nakaya M, Nishihara H, Nangaku M, and Kurose H et al. (2013) Attenuated desensitization of β-adrenergic receptor by water-soluble N-nitrosamines that induce S-nitrosylation without NO release. *Circ Res* **112**:327–334.
Mitchell DA and Marletta MA (2005) Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. *Nat Chem Biol* **1**:154–158.
Miyamoto T, Dubin AE, Petrus MJ, and Patapoutian A (2009) TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS ONE* **4**:e7596.
Moncada S, Palmer RM, and Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43**:109–142.
Nagatomo K, Ishii H, Yamamoto T, Nakajo K, and Kubo Y (2010) The Met268Pro mutation of mouse TRPA1 changes the effect of caffeine from activation to suppression. *Biophys J* **99**:3609–3618.
Nakamura T and Lipton SA (2013) Emerging role of protein-protein transnitrosylation in cell signaling pathways. *Antioxid Redox Signal* **18**:239–249.
Nakamura T, Wang L, Wong CC, Scott FL, Eckelman BP, Han XM, Tzitzilonis C, Meng F, Gu Z, and Holland EA et al. (2010) Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Mol Cell* **39**:184–195.
Ohwada T, Ishikawa S, Mine Y, Inami K, Yanagimoto T, Karaki F, Kabasawa Y, Otani Y, and Mochizuki M (2011) 7-Azabicyclo[2.2.1]heptane as a structural motif to block mutagenicity of nitrosamines. *Bioorg Med Chem* **19**:2726–2741.
Ohwada T, Miura M, Tanaka H, Sakamoto S, Yamaguchi K, Ikeda H, and Inagaki S (2001) Structural features of aliphatic *N*-nitrosamines of 7-azabicyclo[2.2.1]heptanes that facilitate N–NO bond cleavage. *J Am Chem Soc* **123**:10164–10172.
Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, and Imoto K et al. (1999) Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. $Ca^{2+}$-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. *J Biol Chem* **274**: 27359–27370.
Pawloski JR, Hess DT, and Stamler JS (2001) Export by red blood cells of nitric oxide bioactivity. *Nature* **409**:622–626.
Petrus M, Peier AM, Bandell M, Hwang SW, Huynh T, Olney N, Jegla T, and Patapoutian A (2007) A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain* **3**:40.
Salazar H, Llorente I, Jara-Oseguera A, García-Villegas R, Munari M, Gordon SE, Islas LD, and Rosenbaum T (2008) A single N-terminal cysteine in TRPV1 determines activation by pungent compounds from onion and garlic. *Nat Neurosci* **11**:255–261.
Sawada Y, Hosokawa H, Matsumura K, and Kobayashi S (2008) Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. *Eur J Neurosci* **27**:1131–1142.
Seth D and Stamler JS (2011) The SNO-proteome: causation and classifications. *Curr Opin Chem Biol* **15**:129–136.
Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG, and Michel T (1996) Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J Biol Chem* **271**:6518–6522.
Stamler JS and Hess DT (2010) Nascent nitrosylases. *Nat Cell Biol* **12**:1024–1026.
Strübing C, Krapivinsky G, Krapivinsky L, and Clapham DE (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* **29**:645–655.
Takahashi N, Kuwaki T, Kiyonaka S, Numata T, Kozai D, Mizuno Y, Yamamoto S, Naito S, Knevels E, and Carmeliet P et al. (2011) TRPA1 underlies a sensing mechanism for $O_2$. *Nat Chem Biol* **7**:701–711.
Takahashi N, Mizuno Y, Kozai D, Yamamoto S, Kiyonaka S, Shibata T, Uchida K, and Mori Y (2008) Molecular characterization of TRPA1 channel activation by cysteine-reactive inflammatory mediators. *Channels (Austin)* **2**:287–298.
Taylor-Clark TE, Undem BJ, Macglashan DW, Jr, Ghatta S, Carr MJ, and McAlexander MA (2008) Prostaglandin-induced activation of nociceptive neurons via direct interaction with transient receptor potential A1 (TRPA1). *Mol Pharmacol* **73**:274–281.
Voss AA, Lango J, Ernst-Russell M, Morin D, and Pessah IN (2004) Identification of hyperreactive cysteines within ryanodine receptor type 1 by mass spectrometry. *J Biol Chem* **279**:34514–34520.
Wang L, Cvetkov TL, Chance MR, and Moiseenkova-Bell VY (2012) Identification of *in vivo* disulfide conformation of TRPA1 ion channel. *J Biol Chem* **287**:6169–6176.
Xiao B, Dubin AE, Bursulaya B, Viswanath V, Jegla TJ, and Patapoutian A (2008) Identification of transmembrane domain 5 as a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels. *J Neurosci* **28**:9640–9651.
Xu SZ, Sukumar P, Zeng F, Li J, Jairaman A, English A, Naylor J, Ciurtin C, Majeed Y, and Milligan CJ et al. (2008) TRPC channel activation by extracellular thioredoxin. *Nature* **451**:69–72.
Yanagimoto T, Toyota T, Matsuki N, Makino Y, Uchiyama S, and Ohwada T (2007) Transnitrosation of thiols from aliphatic *N*-nitrosamines: *S*-nitrosation and indirect generation of nitric oxide. *J Am Chem Soc* **129**:736–737.
Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, Tominaga M, Shimizu S, Sato Y, and Mori Y (2006) Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol* **2**:596–607.
Zhong J, Minassi A, Prenen J, Taglialatela-Scafati O, Appendino G, and Nilius B (2011a) Umbellulone modulates TRP channels. *Pflugers Arch* **462**:861–870.
Zhong J, Pollastro F, Prenen J, Zhu Z, Appendino G, and Nilius B (2011b) Ligustilide: a novel TRPA1 modulator. *Pflugers Arch* **462**:841–849.

**Address correspondence to:** Dr. Yasuo Mori, Laboratory of Molecular Biology, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura Campus, Nishikyo-ku, Kyoto 615-8510, Japan. E-mail: mori@sbchem.kyoto-u.ac.jp; or Dr. Tomohiko Ohwada, Laboratory of Organic and Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: ohwada@mol.f.u-tokyo.ac.jp

Downloaded from molpharm.aspetjournals.org at ASPET Journals on March 6, 2015