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RESEARCH PAPER

S-nitrosoglutathione inhibits cerebrovascular angiotensin II-dependent and -independent AT_1 receptor responses: A possible role of *S*-nitrosation

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Background and Purpose: Angiotensin II (AngII) and NO regulate the cerebral circulation. AngII AT_1 receptors exert ligand-dependent and ligand-independent (myogenic tone [MT]) vasoconstriction of cerebral vessels. NO induces post-translational modifications of proteins such as *S*-nitrosation (redox modification of cysteine residues). In cultured cells, *S*-nitrosation decreases AngII's affinity for the AT_1 receptor. The present work evaluated the functional consequences of *S*-nitrosation on both AngII-dependent and AngII-independent cerebrovascular responses.

Experimental Approach: *S*-Nitrosation was induced in rat isolated middle cerebral arteries by pretreatment with the NO donors, *S*-nitrosoglutathione (GSNO) or sodium nitroprusside (SNP). Agonist-dependent activation of AT_1 receptors was evaluated by obtaining concentration–response curves to AngII. Ligand-independent activation of AT_1 receptors was evaluated by calculating MT (active vs. passive diameter) at pressures ranging from 20 to 200 mmHg in the presence or not of a selective AT_1 receptor inverse agonist.

Key Results: GSNO or SNP completely abolished the AngII-dependent AT_1 receptor-mediated vasoconstriction of cerebral arteries. GSNO had no impact on responses to other vasoconstrictors sharing (phenylephrine, U46619) or not (5-HT) the same signalling pathway. MT was reduced by GSNO, and the addition of losartan did not further decrease MT, suggesting that GSNO blocks AT_1 receptor-dependent MT. Ascorbate (which reduces *S*-nitrosated compounds) restored the response to AngII but not the soluble GC inhibitor ODQ, suggesting that these effects are mediated by *S*-nitrosation rather than by *S*-nitrosylation.

Conclusions and Implications: In rat middle cerebral arteries, GSNO pretreatment specifically affects the AT_1 receptor and reduces both AngII-dependent and AngII-independent activation, most likely through AT_1 receptor *S*-nitrosation.

Abbreviations: AngII, angiotensin II; AT_1 receptor, angiotensin II type 1 receptor; SNO, *S*-nitrosoglutathione; ID, internal diameter; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MCA, middle cerebral artery; MT, myogenic tone; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PE, phycoerythrin; Phe, phenylephrine; PSS, physiological salt solution; SNP, sodium nitroprusside; sGC, soluble GC

1 | INTRODUCTION

NO is a gaseous radical well known for its vascular dilator effect through the nitrosylation (coordination of NO to the ferrous haem prosthetic group) of **soluble GC (sGC)** and cGMP production (Rapoport, Draznin, & Murad, 1983). Besides, NO also induces the *S*-nitrosation of proteins (Stamler et al., 1992). The *S*-nitrosation is a redox-based modification of proteins resulting from the covalent bond between NO and the sulfhydryl function of cysteine residues. This post-translational modification of proteins is involved in the regulation of the activity of many proteins (see the SNObase database [Zhang et al., 2012] for an exhaustive review).

In the large field of NO-donors, *S*-nitrosothiols, in which the NO moiety is grafted on a sulfur atom, display low toxicity and no tolerance phenomenon (Munzel, Daiber, & Gori, 2011). In particular, *S*-nitrosoglutathione (GSNO), a low MW *S*-nitrosothiol formed by nitrosation of reduced **GSH**, mediates protein *S*-nitrosation and plays an important role in vascular signalling (Marozkina & Gaston, 2012). Several studies, including ours, have shown the therapeutic potential of exogenous GSNO in a preclinical model of ischaemic stroke (Khan et al., 2005; Parent et al., 2015). *S*-Nitrosation of target proteins seems to contribute to these cerebroprotective effects (Khan & Singh, 2016).

In 2006, Leclerc et al. showed that short exposure to NO donors of cells overexpressing the **angiotensin II** (AngII) type 1 receptor (**AT_1 receptor**) led to *S*-nitrosation of this receptor on cysteine 289, leading to a decrease in its affinity for AngII (Leclerc et al., 2006). The AT_1 receptor is involved in cerebral blood flow autoregulation in cerebrovascular structure and functions (Dupuis, Atkinson, Limiñana, & Chillon, 2005). At the cerebrovascular level, two of the main functions of the AT_1 receptor are (a) an AngII-dependent vasoconstriction and (b) an AngII-independent involvement in myogenic tone (MT, i.e., contractile response of arteries consecutive to an increase in arterial pressure; Mederos y Schnitzler et al., 2008). Both functions are inhibited by AT_1 receptor blockers. Following stroke and the consecutive increase in brain levels of AngII (Kagiyama, Kagiyama, & Phillips, 2003; Li et al., 2014), these contractile functions of AT_1 receptors might be deleterious if they reduce blood flow and result in enlarged infarct size. Reducing AT_1 receptor activity with AT_1 receptor blockers has protective effects against stroke in preclinical (Omura-Matsuoka et al., 2009) and clinical studies (Diener, 2009).

Based on the above, we hypothesized that *S*-nitrosation of the AT_1 receptor, by reducing its activity, might be an important mechanism that contributes to the cerebroprotective effects of GSNO.

The aim of this study was thus to determine the impact of exogenous GSNO administration on AT_1 receptor functions: AngII-dependent vasoconstriction and AngII-independent MT. We exposed isolated rat middle cerebral arteries (MCAs) to GSNO (Alencar et al., 2003) and tried to decipher the mechanisms involved in the altered AT_1 receptor functions.

First, concentration–response curves to AngII were built using isolated and perfused rat MCAs and compared to other vasoconstrictors. Specificity towards the AT_1 receptor was assessed using several vasoconstrictors that do or do not share the AT_1 receptor signalling pathway. **Phenylephrine** (Phe) and the TxA2 agonist **U46619** were chosen since both mediate vasoconstriction through receptors activating heterotrimeric $G_{q/11}$, like AT_1 receptors (Schleifenbaum et al., 2014). **5-HT** served as a positive control as its effects mainly involve the **5-HT_{1B} receptor** (Watts, Morrison, Davis, & Barman, 2012), which is coupled to G_i/G_o (Lin, Setya, Johnson-Farley, & Cowen, 2002).

Second, we evaluated the effect of GSNO on AT_1 receptor-dependent MT in arteries using the AT_1 receptor inverse agonist **losartan**.

Third, as GSNO can release both NO and GSH, we verified that the observed effect was only linked to the exogenous release of NO. Moreover, the role of endogenous NO production was assessed. As NO acts through two distinct mechanisms: nitrosylation and *S*-nitrosation, both of these pathways were investigated in order to determine GSNO's mode of action.

Finally, as receptor internalization is important in receptor resensitization and activation of downstream effectors (Hunyady, Catt, Clark, & Gaborik, 2000), we evaluated whether GSNO could modify basal or activated AT_1 receptor cell surface localization in a model of heterologous expression in transfected HEK293 cells.

What is already known about this subject

- Exposure of transfected cells to NO-donors reduces AT_1 receptor affinity for angiotensin II through *S*-nitrosation.

What this study adds

- *S*-nitrosation specifically abolishes ligand-dependent and -independent contractile responses mediated by AT_1 in cerebral vessels.

What is the clinical significance

- This AT_1 regulation is of potential interest in cerebrovascular diseases involving excessive AT_1 receptor-mediated responses.

2 | METHODS

2.1 | Ethics statement and animals

The experiments were conducted on 4- to 5-month-old (500 ± 50 g) male normotensive outbred Wistar rats (HanRj:WI, Janvier, Le Genest-Saint-Isles, France, RGD Cat# 13792727, RRID: RGD_13792727), a commonly used physiological model. Only male rats were used to avoid any gender parameters that could influence AngII response (De Silva, Broughton, Drummond, Sobey, & Miller, 2009). Animals were housed in a standard animal unit at 23°C (three animals per cage, carton tunnels as enrichment), exposed to

12 hr of light (lights on at 06:00 and off at 18:00 hr) and allowed free access to food and fluid. Animals were randomly allocated to the various pretreatments and protocols.

They ate standard rat chow (A04, Safe, Villemoison-Epinay sur Orge, France) and drank water (Aqua-clear®, Culligan, Northbrook, USA) ad libitum. All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals in the respect of the 3Rs' requirements for Animal Welfare. The project untitled "Nitro-Vivo" was positively evaluated by the regional ethical committee for animal experiments and approved by the French Ministry of Research (No. APAFIS#1614-2015090216575422v2). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Vasoactive experiments

2.2.1 | Preparation of MCAs

Rats were anaesthetized with sodium pentobarbitone (100 mg·kg^{-1}, i.p.), injected with heparin (1000 IU·kg^{-1}, i.v.), and then killed by decapitation. The brain was placed in a physiological salt solution (PSS) at 4°C (NaCl 119, $NaHCO_3$ 24, KCl 4.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $CaCl_2$ 1.6, and glucose 5.10^{-3} mol·L^{-1}).

MCAs were chosen as a classical model of isolated cerebral vessels. The first branch-free segment of the MCA immediately proximal to the circle of Willis was dissected out and mounted on two glass micropipettes (diameter, 125–150 μm) in a small vessel arteriograph (model CH/1/AU/SH, Living Systems Instrumentation, Burlington, VT, USA) and secured with silk thread (diameter, 20 μm, 2–0, 48 mm, 2540, Ethicon, Issy-les-Moulineaux, France). The vessel was then pressurized (intraluminal pressure of 80 mmHg) and perfused (intraluminal flow rate of 100 μl·min^{-1}) with PSS at 37°C. Arteries were placed in an organ bath with PSS at 37°C gassed with 5% CO_2/95% O_2 (extraluminal flow rate of 10 ml·min^{-1}) for measurement of isotonic vasoconstriction and assessment of viability.

Following initial pressurization, arteries were allowed to recover their basal MT for 60 min. Baseline internal diameter (ID) was measured using a video dimension analyser (V94, Living Systems Instrumentation, Burlington, VT, USA); signals were digitized and stored with WinDataq DI 720 data acquisition software (Dataq Instruments, Akron, OH, USA). Viability of the MCA was then assessed with potassium chloride (KCl, 4 x10^{-2} mol·L^{-1}) added to the extraluminal bath. The contractile response rapidly reached a steady state and was measured after a 5-min exposure. Responses were expressed as percentage change in ID from baseline ID (arteries showing less than 20% of contraction were excluded). Perfusion with KCl was followed by a 15-min washout period so that the ID returns to baseline.

2.2.2 | Pretreatment with GSNO

After assessment of viability, MCA were exposed for 30 min to GSNO (2 x10^{-6} mol·L^{-1}), which increased the ID. GSNO was then removed and the vessels submitted to a 1-hr washout period (PSS changed every 20 min, adapted from Alencar et al. (2003) that allowed MCA ID to return to baseline (data not shown). According to Alencar et al., *S*-nitrosation of cysteine residues is maintained after the washout (Alencar et al., 2003).

2.2.3 | AngII-dependent response and G_{q11}, G_i/G_o pathways

Concentration–response curves to AngII (5 min per concentration, from 10^{-12} to 10^{-6} mol·L^{-1}) were compared following PSS or GSNO pretreatment. Phe (10^{-12} to 10^{-4} mol·L^{-1}), U46619 (10^{-12} to 10^{-5} mol·L^{-1}), and 5-HT (10^{-12} to 10^{-5} mol·L^{-1}) concentration–response curves were performed in the same conditions. Then, for the following series of experiments, in order to shorten the duration of the protocols, MCA were successively exposed to two equiactive concentrations of AngII (10^{-10} and 10^{-9} mol·L^{-1}) and 5-HT (3.10^{-9} and 10^{-8} mol·L^{-1}, concentrations determined from the previous concentration–responses curves). In order to rule out any time-dependent effect, we reversed the order of administration of AngII and 5-HT (n = 2). Similar results were obtained regardless of the order AngII or 5-HT perfusion (Figure S1). Given the lack of available selective AT_1 agonists, the specific impact of GSNO on AT_1 receptor-mediated vasoactive responses was assessed using AngII in the presence of the **AT_2** receptor antagonist **PD123319** (10^{-6} mol·L^{-1}; Näveri, Strömberg, & Saavedra, 1994).

2.2.4 | AT_1 receptor-dependent MT experiment

MT was evaluated comparing the evolution of ID at pressures ranging from 20 to 200 mmHg under active or passive (20 mmol·L^{-1} EDTA) conditions in order to build MT pressure curves (Foulquier, Lartaud, & Dupuis, 2014). MCAs were exposed or not to the AT_1 inverse agonist losartan (10^{-5} mol·L^{-1}) to confirm the involvement of AT_1 receptors in MT (Schleifenbaum et al., 2014). MCA were pretreated with GSNO before exposure or not to losartan. The AUC (arbitrary unit) of MT as a function of pressure was obtained for each MCA using the following formula: $MT = (ID - ID_{EDTA}) \times 100/ID_{EDTA}$.

2.2.5 | NO involvement in AT_1 receptor-attenuated response

In order to rule out any antioxidant effect of GSNO (via GSH release), MCA were exposed to GSH (30 min, 2 x 10^{-6} mol·L^{-1}) instead of GSNO. To confirm the involvement of NO, MCA were exposed to another NO donor (**sodium nitroprusside, SNP**, 2.10^{-6} mol·L^{-1}) in the same conditions. Control MCA followed the same protocol with PSS instead of NO donors. Implication of basal level of NO was assessed using N^{ω}-nitro-L-arginine methyl ester (L-NAME) an **endothelial NOS** inhibitor. The concentration of L-NAME was fixed at 10^{-4} mol·L^{-1} (Briones, Alonso, Marin, & Salaices, 1999).

2.2.6 | Role of *S*-nitrosation

As most effects of NO are mediated by sGC nitrosylation, its implication was assessed using the sGC inhibitor **1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)**. The concentration of ODQ was fixed at 10^{-5} mol·L^{-1} (Yu, Sun, Maier, Harder, & Roman, 2002). A series a MCA underwent DMSO (i.e., ODQ solvent) treatment as control (final concentration 0.001%).

To confirm the involvement of *S*-nitrosation in the effect of GSNO, MCA were exposed to sodium ascorbate (20 min, 5.10^{-6} mol·L^{-1}, based on Figueiredo-Freitas et al., 2015) immediately after GSNO pretreatment to specifically reduce S–NO link. This was followed by 40 min PSS before exposure to AngII and 5-HT.

2.3 | Stable expression of fluorescently labelled EGFP-AT$_1$ receptor in HEK293 cells

HEK293 cells (ATCC Cat# CRL-1573, RRID:CVCL_0045) were cultured to ~80% confluence in T-75-cm^2 flasks in MEM with Earle's salt supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% antibiotics (penicillin/streptomycin) and re-plated twice a week.

The expression vector pIRES (Clontech) encoding for the fluorescent EGFP-AT$_1$ human receptor was a gift from Prof. Haiech and Prof. Kilhoffer (University of Strasbourg) and PCBIS UMS3286 CNRS-University of Strasbourg. The vector was constructed using a ligation-independent cloning strategy. Briefly, the receptor is expressed with, at its N-terminus, the fluorophore preceded by a signal peptide for translocation of the fusion protein to the membrane of the endoplasmic reticulum (Vollmer, Alix, Chollet, Takeda, & Galzi, 1999). The construct was verified by DNA sequencing before transfection.

For stable transfection of the EGFP-AT$_1$ receptor, cells were plated 1 day before on 10-cm dishes and transfected with 18 μg of plasmids using the calcium phosphate precipitation method (described in Vollmer et al., 1999). Selection with antibiotic started 2 days later with 600 μg·ml^{-1} G418 (Invitrogen). Clones were collected after 2 weeks of selection.

2.4 | Internalization experiments

Internalization were carried out according to Gicquiaux et al. (2002). Briefly, cells expressing stably EGFP-AT$_1$ receptors were suspended in HEPES-BSA buffer at a concentration of 10^6 cells·ml^{-1}, and the suspension was separated into tubes of 1 ml. Cells were either untreated (control) or treated with AngII (10^{-9} M) or GSNO (2.10^{-6} M) for 0, 5, 15, and 30 min at 37°C. A series of cells were pretreated for 30 min with GSNO at 37°C before activation with AngII (same times and concentrations). At the end of the incubations, cells were fixed with PBS containing 1% paraformaldehyde and 0.1% sodium azide then pelleted by centrifugation (at 4°C and suspended in PBS 0.2% BSA on ice to block nonspecific antibody labelling). Immunolabelling of EGFP-AT$_1$ receptors expressed at the cell surface was done on intact cells using a monoclonal mouse anti-GFP (Thermo Fisher Scientific Cat# A-11120, RRID:AB_221568; 1/100 dilution) for 30 min at room temperature and secondary labelling done using a R-phycoerythrin-conjugated AffiniPure F (ab')2 fragment goat anti-mouse IgG (Thermo Fisher Scientific Cat# 12-4010-82, RRID:AB_11063706; 1/100 dilution). Phycoerythrin (PE) staining was quantified by flow cytometric analysis (20,000 cells per sample) on a FACSCalibur cytometer (BD FACSCalibur Flow Cytometry System, RRID:SCR_000401). Using FACStar data software, mean PE fluorescence intensity was calculated after subtraction of the mean PE fluorescence of nonspecific staining by the two antibodies measured on non-transfected HEK cells. Results are expressed as percent variation of mean PE fluorescence between activated versus EGFP-AT$_1$ cells not exposed to agonists (100%) control for unwanted sources of variation such as non-specific staining of non-transfected cells or a slight drop in the number of cell surface receptors of the cell population as a function of culture time.

2.5 | Biotin switch technique

S-Nitrosated proteins were purified by the biotin switch technique as previously described with some adaptations (Belcastro et al., 2017; Forrester, Foster, Benhar, & Stamler, 2009).

EGFP-AT$_1$ receptor expressing HEK293 cells or untransfected HEK293 cells (as negative controls) were cultured for 2 days in T10 plates. Then, cells were lysed in 1 ml of cold 50 mM Tris (pH 6.8) buffer containing 0.15 M NaCl, 1% (v/v) Triton ×100, 0.1% (v/v) SDS, 1 mM EDTA, 0.1 mM neocuproine and protease inhibitor cocktail (EDTA-free Roche). Briefly, *S*-nitrosation was performed at 37°C on a wheel for 1 hr by 1 mM GSNO (or PBS as control). After a 10,000× *g* centrifugation for 15 min at 4°C, the free thiols in cell lysate supernatants were blocked with 50 mM of *N*-ethylmaleimide for 50 min at 40°C. Samples were precipitated by acetone. Pellets were then incubated with 10 mM sodium ascorbate to liberate thiols from the S–NO bonds, and free new thiols were immediately biotinylated. As negative controls, sodium ascorbate was omitted in one sample treated or not with GSNO. Under such conditions, S–NO bonds are not reduced and *S*-nitrosated proteins cannot be labelled by the biotin-HPDP. Excess biotin-HPDP was removed by acetone precipitation. Biotin-HPDP-labelled proteins were purified with High Capacity NeutrAvidin beads overnight on a wheel at 4°C. The following day, NeutrAvidinAgarose beads were washed three times in a high-salt containing buffer (50 mM Tris pH 7.5, 0.6 M NaCl, 1% [v/v] Triton ×100, 0.1% [v/v] SDS, 1 mM EDTA, 0.1 mM neocuproine). Beads were suspended in a 2× Laemmli buffer (25 mM Tris pH 6, 8, 3 M urea, 1.5% SDS, 3% [v/v] 2-mercaptoethanol) vortexed for 15 min at room temperature, heated for 20 min at 37°C, centrifuged for 3 min at 10,000× *g*. Biotin-HPDP-labelled proteins eluted from the beads were separated in 10% acrylamide gels then transferred onto PVDF membrane for 2 hr. The detection of the EGFP-AT$_1$ receptor proteins among the proteins that had been precipitated by streptavidin beads, indicating that the receptor was biotinylated during the biotin switch, was performed by immunoblotting with anti-GFP-HRP antibodies

(Abcam Cat# ab6663, RRID:AB_305636): incubation at 1/25,000 overnight revelation with Immobilon Western HRP and image acquisition on a LAS4000 camera (GE-Healthcare). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.6 | Statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology. Blinding of the operator could not be done as the various pretreatments could be easily distinguishable (colour of the solution for GSNO, impact of the pretreatment on basal MCA diameter such as dilatation induced by GSNO or SNP, or constriction induced by L-NAME). The values of ID were measured automatically with no intervention by the operator (except to set the start of the recording), ensuring reliable and objective measurements. Statistical analysis was performed using Graphpad Prism version 6.00 (RRID: SCR_002798).

Vasoactive responses of the MCA are expressed as percentage changes in ID from the value measured during the washout preceding administration of the drug. This normalization enables control for unwanted sources of variation such as anatomical, differences in MCA diameter. For each MCA experiment, the initial sample size was n = 8 to take into account experimental losses based on the predetermined viability criteria described above and a final sample size of at least n = 5 was obtained. The only exception is the group receiving DMSO, for which the sample size was reduced to n = 3 (given the low variability in this control group and to avoid unnecessary animal deaths) and was thus excluded from the statistical analyses. For MT determination, the sample size is greater due to a higher variability and the subsequent inclusion of partial preliminary data for the control group (n = 18). Concentration–response curves to vasoactive agents were built for each MCA and half maximal effective concentration (EC_{50}) was calculated using Hill's logistic equation:

$$\%\text{ID change} = E_{\min} + (E_{\max} + E_{\min}) / \left(1 + 10^{(\log EC_{50} - \log(\text{concentration}) \times \text{Hill slope})}\right),$$

where E_{min} and E_{max} are minimal and maximal response reached in each concentration–response curve. The value of the Hill slope for all experiments was 1.0 ± 0.1. Then $-\log EC_{50}$ values (pD_2 values) were calculated.

For functional studies after pretreatments (PSS, GSH, SNP GSNO ± ascorbate, L-NAME, or ODQ), differences in the responses to AngII ± PD123319, 5-HT, PHE, or U46619 (mean ± SEM) were determined by two-way ANOVAs (drug concentration, pretreatment) followed by a Bonferroni post-test ($P < 0.05$). For AT_1 receptor-dependent MT, IDs (active or passive) of MT or AUC were compared using one-way ANOVA (treatment) followed by a Bonferroni post-test ($P < 0.05$).

For internalization studies, differences in the responses to GSNO, AngII ± GSNO were determined by two-way ANOVAs (time, treatment) followed by a Bonferroni post-test ($P < 0.05$).

2.7 | Materials

All reagents were purchased from Sigma Chemical Company (St Louis, USA), U46619 from Enzo life Sciences (Farmingdale, USA), ODQ from Alfa Aesar (Haverhill, USA), sodium pentobarbital from Sanofi-Aventis (Libourne, France), High Capacity NeutrAvidin beads and biotin-HPDP from Fisher Scientific (Illkirch, France), anti-GFP-HRP antibodies from Abcam (Paris, France), Immobilon Western HRP from Promega (Charbonnières-les-Bains, France), primary monoclonal anti-GFP and secondary goat anti-mouse PE antibodies from Thermofisher (Waltham, USA). GSNO was prepared extemporaneously as previously described (Parent et al., 2013). All manipulations and assays involving GSH, ascorbate, and GSNO were performed under conditions of subdued light, in order to minimize light-induced degradation.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | NO acts specifically on AT_1 receptor signalling rather than on other G_{q11} or G_i/G_o signalling pathways

The data obtained on AngII-mediated vasoconstriction are in accordance with the literature (De Silva et al., 2009; Vincent et al., 2005). GSNO pretreatment specifically reduced the AngII-mediated vasoconstriction of the MCA (Figure 1). This was not linked to an increase in EC_{50} as GSNO completely abolished the response to AngII even at high concentrations. GSNO did not change the responses to other vasoconstrictors sharing the same G_{q11} pathway (U46619, Phe) or other pathways (5-HT; Figure 1). Moreover, the abolition of AngII vasoconstriction obtained in the presence of the AT_2-antagonist PD123319 confirmed a decrease of AT_1 receptor-mediated vasoconstriction rather than an increase of AT_2-mediated vasodilation (Figure 2).

3.2 | GSNO abolishes AT_1 receptor-mediated agonist-independent MT

As previously observed by others (Schleifenbaum et al., 2014), losartan, an inverse agonist of the AT_1 receptor, shifted the MT-pressure curve towards lower values (Figure 3a) and significantly reduced the AUC of the MT–pressure relationships versus controls (Figure 3b). This confirms the implication of AT_1 receptor on MT. GSNO significantly reduced MT to a lower extent than that

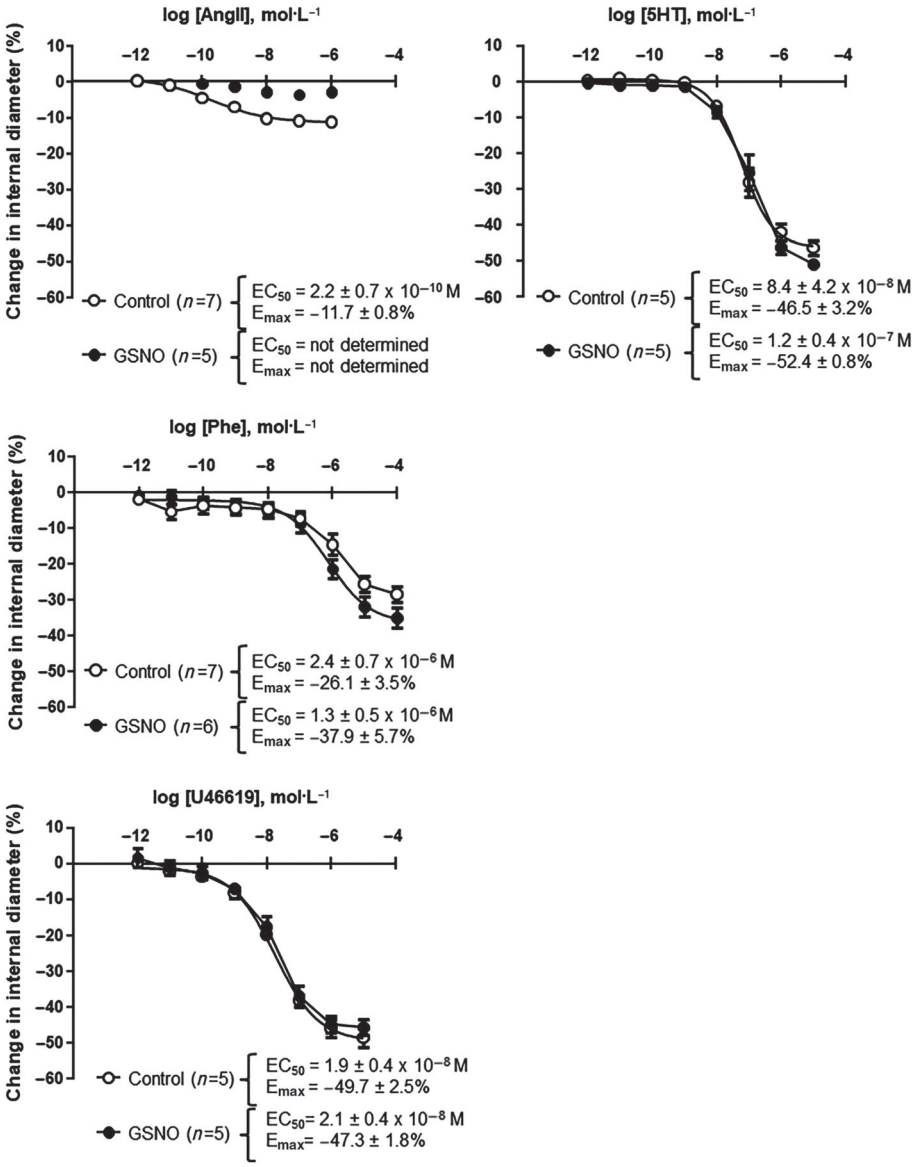

FIGURE 1 *S*-nitrosoglutathione (GSNO) effect lies specifically on AT_1 receptor signalling rather than on other G_{q11} or G_i/G_o signalling pathways. Contractile responses of isolated and perfused rat middle cerebral arteries to different vasoconstrictor agonists were measured. Concentration–response curves, EC_{50}, and maximal effect (E_{max}) values in response to (a) angiotensin II (AngII), α_1-adrenoceptor agonist phenylephrine (Phe), TxA2 agonist (U46619), and (b) 5-HT)were obtained after exposure (30 min) to either physiological salt solution (control) or GSNO (2×10^{-6} mol·L^{-1}), followed by a 1-hr washout. Fitting to Hill's logistic equation was used. Values for EC_{50} (mol·L^{-1}) and E_{max} (%) are expressed as means ± SEM

obtained with losartan alone (Figure 3a,b). The reduction of the AUC induced by GSNO + losartan was equivalent to that obtained by GSNO alone (Figure 3a,b) suggesting that GSNO decreased AT_1 receptor-dependent MT.

3.3 | GSNO abolishes AT_1 receptor functions through NO release

We ruled out any antioxidant properties of GSNO (via GSH release) as exposure to GSH did not change vasoconstriction induced by AngII (Figure 4a). Involvement of NO was confirmed as SNP (another NO donor) induced similar results to those produced by GSNO (Figure 4b). Involvement of endogenous NO was excluded as treatment with L-NAME (endothelial NOS inhibitor) did not restore vasoconstriction to AngII following GSNO pretreatment (Figure 4c).

3.4 | NO effects are mediated by a *S*-nitrosation mechanism

As most effects of NO are mediated by sGC nitrosylation, its implication was assessed using ODQ, a sGC inhibitor. ODQ did not prevent the loss of AngII vasoconstriction following GSNO pretreatment thus excluding involvement of sGC nitrosylation (Figure 5a). ODQ specifically decreased the response to 5-HT. However, this response was

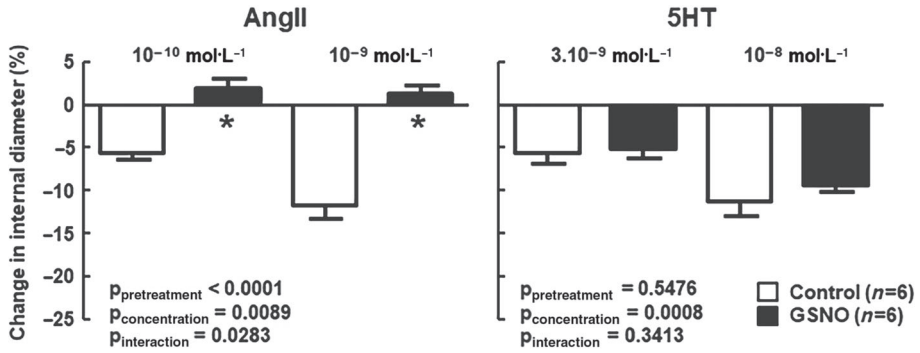

FIGURE 2 *S*-nitrosoglutathione (GSNO) specifically abolishes AT_1 receptor-induced vasoconstriction. Specific AT_1 receptor-induced vasoconstriction was assessed in response to angiotensin II (AngII) in the presence of PD123319 (a selective AT_2 receptor antagonist, 10^{-6} mol·L^{-1}) and compared to 5-HT after exposure (30 min) to either physiological salt solution (control) or GSNO (2 x 10^{-6} mol·L^{-1}), followed by a 1-hr washout. Changes in internal diameter (%) are expressed as means ± SEM. Two-way ANOVAs (pretreatment, concentration) were used to calculate *P* values; **P* < 0.05 versus control (Bonferroni post-test)

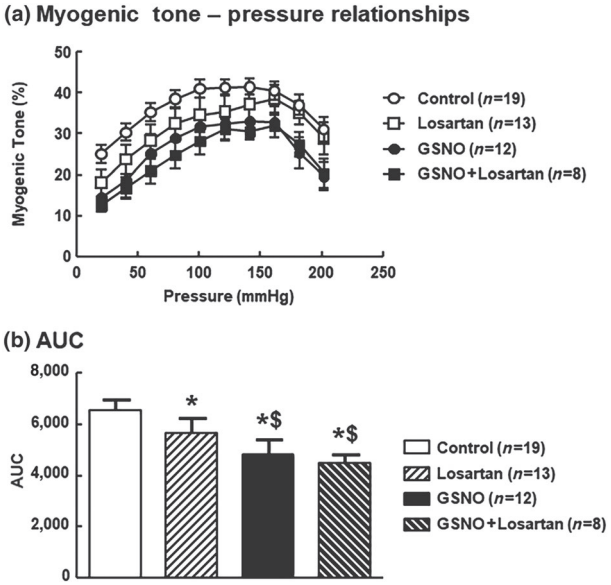

FIGURE 3 *S*-nitrosoglutathione (GSNO) decreases AT_1 receptor-dependant myogenic tone. Impact of an exposure to GSNO (2 x 10^{-6} mol·L^{-1}, 30 min followed by a 1-hr washout), losartan (10^{-5} mol·L^{-1}), or the combination GSNO + losartan on (a) myogenic tone and (b) AUC of myogenic tone of middle cerebral arteries isolated from Wistar rats. Values are expressed as means ± SEM. One-way ANOVA (treatment) was used to calculate *P* values; **P* < 0.05 versus control; $^{\$}P$ < 0.05 versus losartan; **P* < 0.05 versus control (Bonferroni post-test)

restored at higher concentration (10^{-6} mol·L^{-1}; Figure S2). No effect of DMSO (i.e., ODQ solvent) was observed on either AngII or 5-HT responses.

The response of MCA to AngII was restored when ascorbate (used to reduce S–NO bond) was added during the washout after GSNO exposure (Figure 5b) indicating that *S*-nitrosation is involved in the decrease of the AngII response following GSNO exposure. No change was observed for 5-HT.

3.5 | AT_1 receptor cell surface localization is not modified by NO

Due to the lack of specific antibodies directed against AT_1 receptor (Benicky, Hafko, Sanchez-Lemus, Aguilera, & Saavedra, 2012; Bouressam, Lartaud, Dupuis, & Lecat, 2018) precluding experiments on isolated MCA, the impact of GSNO on cell surface localization of AT_1 receptor was assessed in a heterologous expression system: EGFP-tagged AT_1 receptors expressed in HEK293 cells. Using fluorescence cell sorting analysis, the cell population was controlled to have a homogenous expression of the receptors as detected by a single Gaussian peak of GFP fluorescence as compared with non-transfected HEK cells (Figure 6a green line vs. black line, respectively). When exposed to GSNO alone (2.10^{-6} mol·L^{-1}) at 37°C, no variation of cell surface receptors was observed as detected by monoclonal anti-GFP antibody labelling followed by secondary anti-mouse antibody coupled to PE (Figure 6b, orange line and Figure 6c). The treatment of EGFP-AT_1 receptor transfected cells with 10^{-9} mol·L^{-1} of AngII induced a rapid decrease in the amount of receptors detected at the cell surface of intact cells (measurement of PE fluorescence intensities of the cell population, Figure 6b) while the total amount of receptors remained constant (measurement of GFP fluorescence intensities of the cell population, Figure 6a). The decrease of cell surface receptors upon activation is due to internalization of the receptor. Activated receptors are internalized with a maximal amplitude of 55–60% that is reached almost after 5 min of activation (Figure 6b, blue line and Figure 6c). When cells were treated with GSNO followed by incubation with AngII, the same decrease of PE fluorescence was observed as compared with AngII alone (Figure 6b red line vs. blue line, respectively) indicating that GSNO did not alter AT_1 receptor internalization amplitudes (55 vs. 53%, ns; Figure 6b,c). Similar results were obtained with cells exposed to AngII for 5, 15, or 30 min indicating that AT_1 receptor internalization kinetics are unaltered as well by GSNO pretreatment (Figure 6c).

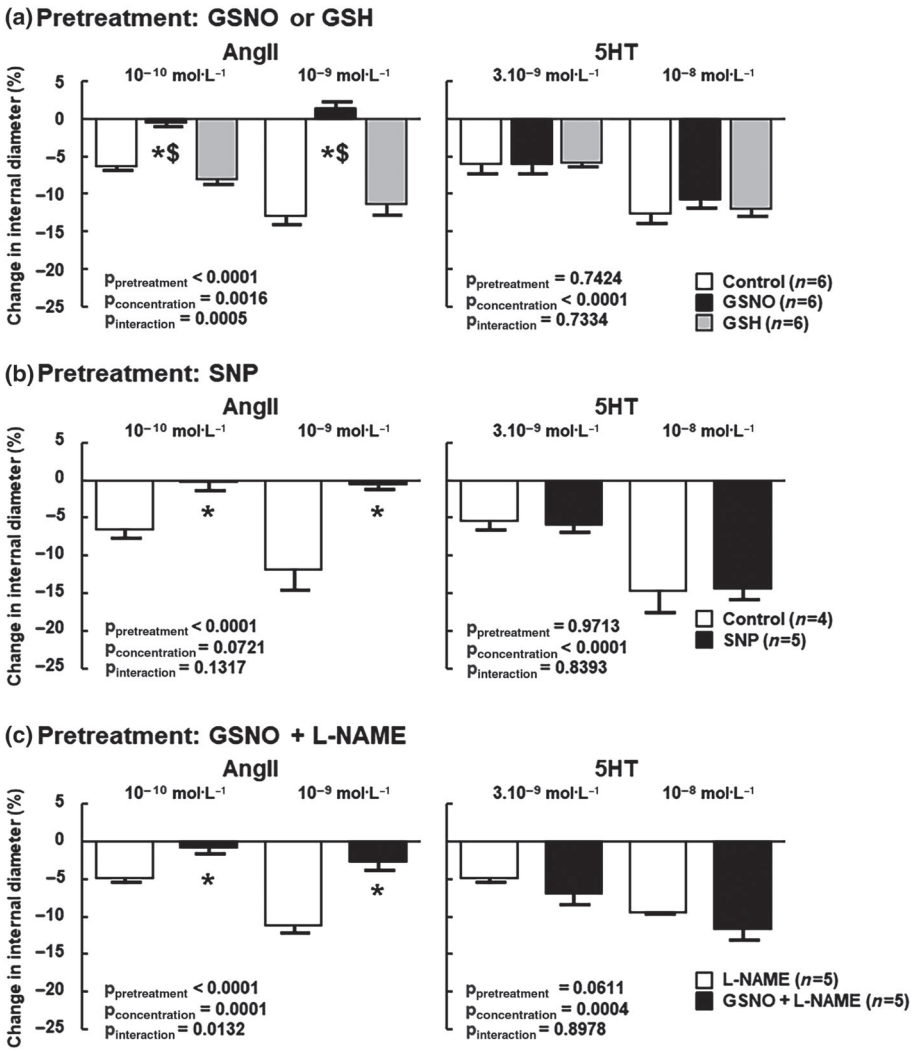

FIGURE 4 Exogenous NO induced by either *S*-nitrosoglutathione (GSNO) or sodium nitroprusside (SNP) is responsible for the loss of AngII response rather than GSH or endogenous NO. In each panel, 5-HT served as a positive control. (a) Middle cerebral arteries (MCA) were pretreated (30 min) with physiological salt solution (control), GSNO (2.10^{-6} mol·L^{-1}), or GSH (2.10^{-6} mol·L^{-1}), followed by a 1-hr washout. (b) MCA were pretreated (30 min) with physiological salt solution (control) or SNP (2×10^{-6} mol·L^{-1}), followed by a 1-hr washout. (c) MCA were pretreated (30 min) with physiological salt solution (control) or GSNO, followed by a 1-hr washout in the presence of L-NAME (10^{-4} mol·L^{-1}) throughout the experiment. Changes in internal diameter (%) are expressed as means ± SEM. Two-way ANOVAs (pretreatment, concentration) were used to calculate *P* values; $^{*}P < 0.05$ versus unexposed; $^{\$}P < 0.05$ versus GSH (Bonferroni post-test)

3.6 | GSNO induces *S*-nitrosation of AT_1 receptor

To directly test if GSNO is able to *S*-nitrosate the AT_1 receptor, we used the biotin switch technique on EGFP-tagged AT_1 receptor expressing HEK293 cells. This three-step procedure relies on the ability of ascorbate to selectively reduce *S*-nitrosothiol to a free sulfhydryl group, which is subsequently conjugated with a biotin adduct. The biotinylated proteins can then be enriched with avidin-affinity chromatography and analysed by conventional western blotting with antibodies against the target protein, here using antibodies targeting EGFP because of the lack of specific anti-AT_1 receptor antibodies (Benicky et al., 2012; Bouressam et al., 2018). After the biotin switch, EGFP-AT_1 receptor was specifically detected on western blot among the biotinylated proteins precipitated on streptavidin beads (Figure 7, Lane 7). When lysates were not treated with GSNO (Figure 7, Lanes 2 and 3) or not incubated with ascorbate after GSNO treatment (thus preventing SNO bonds to be labelled by biotin, Figure 7, Lane 5), there was no EGFP-AT_1 receptor detected on the western blot. These results indicate that EGFP-AT_1 receptor is *S*-nitrosated by GSNO.

4 | DISCUSSION

In the present work, we demonstrate that GSNO pretreatment of cerebral arteries specifically abolishes both ligand-dependent (AngII) and ligand-independent (MT) AT_1 receptor-mediated functions. Reduced AT_1 receptor signalling after GSNO pretreatment may depend

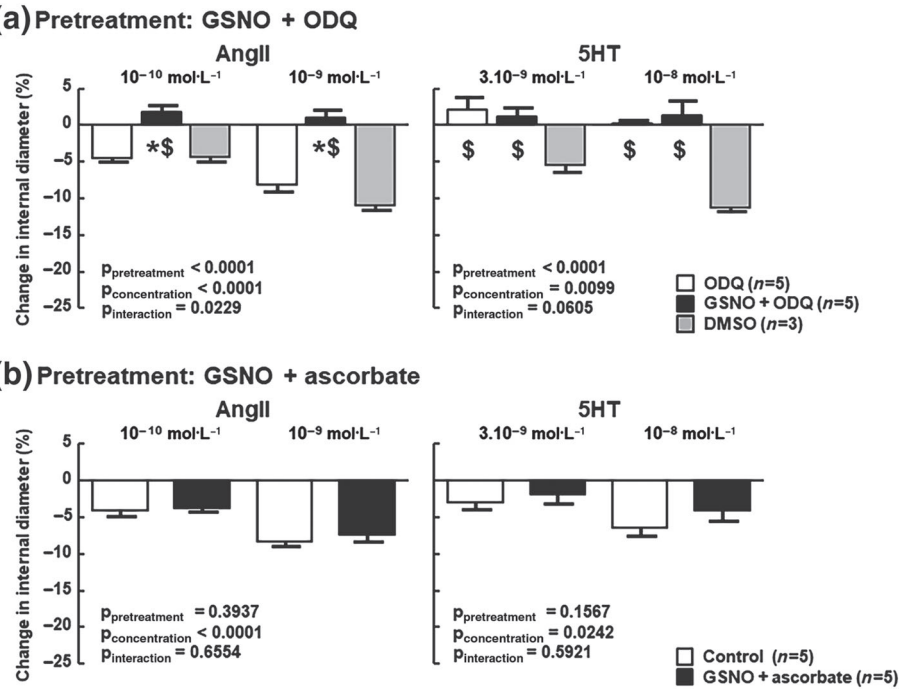

FIGURE 5 The effect of GSNO is independent of sGC activity as it is mediated by *S*-nitrosation. (a) MCA were pretreated (30 min) with physiological salt solution or with *S*-nitrosoglutathione (GSNO, 2 x 10^{-6} mol·L^{-1}) followed by a 1-hr washout, then ODQ (10^{-5} mol·L^{-1}) was added throughout the experiment. DMSO (i.e., ODQ solvent was used as control). (b) After pretreatment (30 min) with physiological salt solution (control) or with GSNO, MCA were exposed to sodium ascorbate (5 x 10^{-6} mol·L^{-1}, 20 min) to specifically reduce the *S*–NO bonds, followed by a 40-min washout. Control MCA underwent the same ascorbate washout as ascorbate alters the vasoactive responses to both AngII (Leclerc et al., 2008) and 5-HT (Muakkassah-Kelly, Andresen, Shih, & Hochstein, 1982). Changes in internal diameter (%) are expressed as means ± SEM. Two-way ANOVAs (pretreatment, concentration) were used to calculate *P* values; $^{*}P < 0.05$ versus unexposed; $^{\$}P < 0.05$ versus DMSO (Bonferroni post-test)

on *S*-nitrosation, as such a pretreatment of vessels has been reported to induce *S*-nitrosation of vascular proteins (Alencar et al., 2003).

We decipher the mechanisms involved using pharmacological tools. First, we argue that the GSNO effect is linked to NO (as SNP, another NO donor, produces similar results) rather than to its antioxidant properties. Indeed, ROS production is an important part of AngII signalling in cerebral arteries contributing to vasoconstriction (De Silva & Faraci, 2013). The effect of GSNO on AngII response could thus be related to a reduced ROS availability after AngII stimulation. The antioxidant properties of GSNO can be related to the release of NO and/or GSH. NO has been shown to reduce ROS production, mainly by inducing the *S*-nitrosation of **Nox5** (Qian et al., 2012). As ROS production induced by AngII is rather related to activation of **Nox1**, **Nox2**, and **Nox4** (De Silva & Faraci, 2013), the impact of NO on AngII-induced ROS production remains unlikely. The lack of effect of GSH pretreatment also rules out any reduction in ROS availability as the main mechanism responsible for the reduced AngII response. Then, we ruled out any involvement of endogenous NO production using the eNOS inhibitor L-NAME. The abolition of AngII-induced vasoconstriction of MCA was obtained using 2.10^{-6} mol·L^{-1} of GSNO, a concentration very close to its vasodilating EC_{50} (2.8 ± 0.4 10^{-6} mol·L^{-1}, Figure S3). Vasodilation involves the activation of the sGC/GMPc pathway through sGC nitrosylation. In our experiments, the 1-hr washout enables diameters to return to baseline, suggesting that nitrosylation-dependent activation of sGC disappears and could therefore not account for the reduced AngII constrictive response (Figure S4). This was further confirmed by the use of the sGC inhibitor, ODQ, which does not prevent the abolition of AngII responses, thus suggesting the involvement of other mechanisms.

As Alencar et al. previously showed, using a similar protocol (Alencar et al., 2003), that *S*-nitrosation of proteins remains despite a 1-hr washout, we tested if *S*-nitrosation could be involved in the reduced AngII response. Sodium ascorbate treatment, known to specifically reduce the S–NO bond (Forrester et al., 2009; Holmes & Williams, 2000), prevents the abolition of AngII vasoconstriction, thus suggesting that *S*-nitrosation contributes to this effect.

The abolition of AngII response following GSNO pretreatment is consecutive to a decreased AT_1-constriction rather than to an increased AT_2-related dilatation, as shown in the presence of AT_2 antagonist. This reduced vasoconstriction is not consecutive to a GSNO-induced alteration of the contractile apparatus, as 5-HT-induced vasoconstriction remained unaffected. While the $5\text{-}HT_{1B}$ receptor involved in cerebrovascular constriction (Watts et al., 2012) is coupled to a G_i/G_o G-protein (Lin et al., 2002), the AT_1 receptor, as well as the TxA2 and the α1-adrenergic receptors are coupled to the G_{q11} G-protein (Schleifenbaum et al., 2014). As GSNO specifically abolished AngII, but not U46619 nor Phe-induced vasoconstriction, this demonstrates that GSNO produces an alteration upstream of

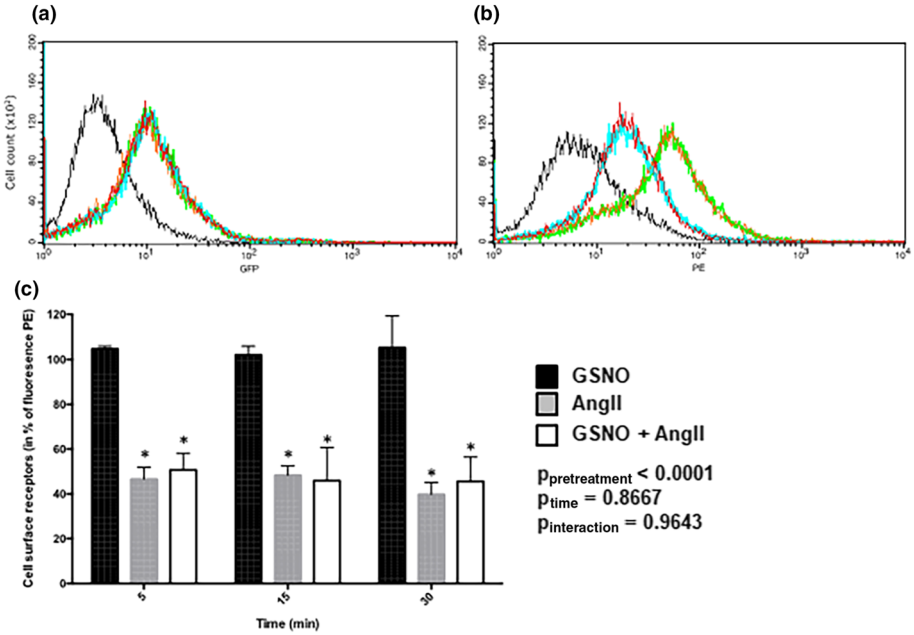

FIGURE 6 GSNO does not modify AT_1 receptor-internalization kinetics or amplitudes. Cell surface EGFP-AT_1 receptor receptors were evaluated by FACS using anti-GFP antibodies and secondary phycoerythrin-labelled antibodies (PE). (a) Cellular GFP or (b) cellular PE fluorescence intensities of non-transfected HEK cells (black line) and EGFP-AT_1 receptor cells (green line) or EGFP-AT_1 receptor cells activated with either AngII (30 min, 10^{-9} M—blue line), GSNO (30 min, 2 x 10^{-6} M—orange line), or GSNO (30 min, 2 x 10^{-6} M) together with AngII (30 min,10^{-9} M—red line). (c) Variation of EGFP-AT_1 receptor cell surface receptors normalized versus non activated EGFP-AT_1 receptor cells. Variations in cell surface receptors are expressed as means ± SEM of the mean fluorescence of PE. Two-way ANOVA (time, treatment) were used to calculate *P* values; **P* < 0.05 versus GSNO (Bonferroni post-test). *n* = 2 separate determinations, four points per condition

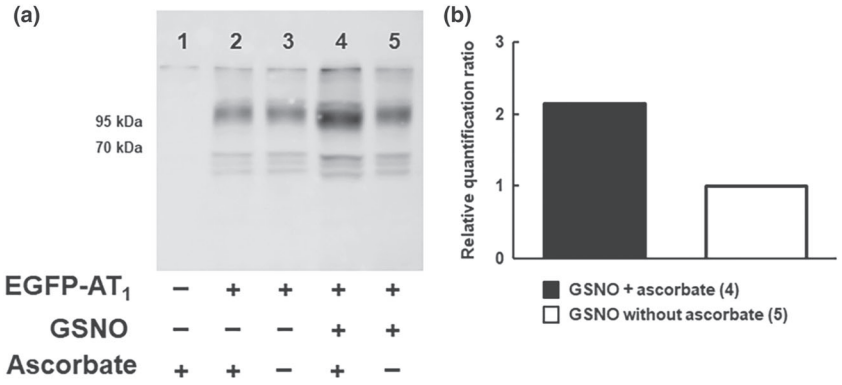

FIGURE 7 GSNO induces *S*-nitrosation of the AT_1 receptor. (a) Representative Western blot with anti-GFP-HRP antibodies. Cell lysates exposed to *S*-nitrosoglutathione (GSNO, 1 mM, 60 min, 37°C) underwent purification of *S*-nitrosated proteins with the biotin switch method. Lane 1 is a negative control conducted with untransfected HEK cells. Lanes 2 and 3 are negative controls without GSNO exposure. In Lane 5, the removal of ascorbate prevents SNO bonds being labelled by biotin and thus ensures the specificity of the biotin switch towards *S*-nitrosated proteins. (b) Relative quantification of *S*-nitrosated EGFP-AT_1 receptor (Lane 4 vs. Lane 5)

the G-protein G_{q11} and specifically on the AT_1 receptor signalling pathway.

Three hypotheses can explain our results. Decrease of AngII affinity for AT_1 receptor (Leclerc et al., 2006), uncoupling of G-protein, and/or changes in AT_1 receptor membrane expression. We ruled out the first hypothesis as AngII-independent response, MT, was also reduced by GSNO pretreatment.

Several studies have previously demonstrated that *S*-nitrosation can lead to uncoupling of G-proteins from their receptors, thus decreasing their activity (Hess, Lin, Freeman, & Norden, 1994; Miyamoto, Laufs, Pardo, & Liao, 1997). Activity of GPCRs could be either up-regulated (ACh receptor **M_2/M_4**) or down-regulated (purine receptor **$P2Y_{12}$**, lysophosphatidic acid receptor **LPA_1**, or cannabinoid receptor **CB_1**) by NO donors such as GSNO or CysNO (Kokkola, Savinainen, Monkkonen, Retamal, & Laitinen, 2005).

Several of our results point towards a probable *S*-nitrosation of the AT_1 receptor itself that would affect its conformational dynamics inhibiting its stabilization in the active state.

First, a decrease in AngII affinity for AT_1 receptor was observed on transfected HEK cells overexpressing AT_1 receptor in relation to a probable *S*-nitrosation on its Cys289 (Leclerc et al., 2006). Inhibition of isomerization into an active form with a better affinity for agonist

could also lead to a decrease in apparent affinity of the receptor, a phenomenon that has been previously described for a mutant of the tachykinin NK2 receptor (Lecat, Bucher, Mely, & Galzi, 2002). This would explain why, in our study, both ligand-dependent and independent functions are impaired.

Effectively, we report that GSNO also abolished the AngII-independent response of the AT_1 receptor, that is, its involvement in the setting of the cerebrovascular MT. It has been proposed that the molecular mechanism of AT_1 receptor activation upon mechanical stress is mediated by a change in the conformation of the receptor in which the transmembrane domain 7 of the receptor shifts into the agonist-binding pocket and triggers activation independently of agonist (Yasuda et al., 2008). This is why losartan, an inverse agonist that stabilizes the receptor in its inactive form, was shown to block the AT_1 receptor's involvement in the setting of the cerebrovascular MT. We observed that pretreatment with GSNO induced a stronger decrease in MT than treatment with losartan alone. When losartan was added to GSNO pretreatment, no further decrease was observed. This cannot be explained by a complete blockade of MT by GSNO (MT remaining relatively high—29% at 80 mmHg—after GSNO pretreatment) and thus suggests that AT_1 receptor-mediated MT was already abolished by GSNO. Thus, these results point towards the hypothesis that GSNO alters the conformational dynamics of the AT_1 receptor itself. The stronger decrease in MT compared to losartan alone is most likely related to the impact of GSNO on other mechanosensors, such as other GPCRs (Kauffenstein, Laher, Matrougui, Guerineau, & Henrion, 2012).

The third hypothesis to explain our results relies on changes in the amount of AT_1 receptors at the cell surface due to variation in expression, exocytosis, or internalization upon GSNO treatment. It seems unlikely that 30 min exposure to GSNO might affect AT_1 receptor transcription or gene expression as these require a much longer treatment (18–24 hr; that could not be detected within the time schedule of our experiments) or higher concentrations (100-fold more; Cahill, Redmond, Foster, & Sitzmann, 1995; Ichiki et al., 1998). However, general actors of receptor endocytosis, such as β-arrestins, clathrin, and/or dynamin (Gaborik et al., 2001), were shown to be regulated by *S*-nitrosation (Daaka, 2012).

Because there are no antibodies specifically targeting AT_1 receptor on the market (Bouressam et al., 2018), we could not test in MCA the two remaining hypotheses, that is, *S*-nitrosation of the receptor itself that alters its isomerization properties or the impact of GSNO on the amount of receptor at the cell surface. To directly prove that the AT_1 receptor can be *S*-nitrosated by GSNO, we had to rely on a heterologous expression system. We chose HEK293 cells, as Leclerc et al. did (2006), and used the biotin switch technique coupled to western blot to purify and identify *S*-nitrosated proetins (Belcastro et al., 2017). We were able to detect *S*-nitrosated EGFP-tagged AT_1 receptors after GSNO treatment of cell lysates. More sensitive methods, such as the DAN-Hg^{2+} method, enable the quantification of intracellular *S*-nitrosothiols directly on cells but do not allow the identification of a specific target protein. It would be interesting in the future to test *S*-nitrosation of the tagged-receptor expressed in a cellular context closer to its physiological environment.

Our results using EGFP-AT_1 receptor transfected HEK293 cells show that AngII induces AT_1 receptor internalization whereas GSNO alone has no effect. Pretreatment of the cells with GSNO did not affect the amplitude or kinetics of activated AT_1 receptor internalization. Whether GSNO pretreatment could affect proteins such as ATRAP (Tanaka et al., 2005) or ARAP1 (Guo, Chenier, Tardif, Orlov, & Inagami, 2003), which respectively promote internalization or membrane expression of AT_1 receptors, is thus unlikely.

Interestingly, the fact that GSNO alters AT_1 receptor-induced vasoconstriction (i.e., inhibits the G_q signalling pathway) without affecting AT_1 receptor internalization (i.e., the β-arrestin pathway) could also be explained by an alteration in the receptor conformational dynamics. Molecular dynamics simulation of the basal and the active states of AT_1 receptor suggests that activation of the G_{q11} pathway requires a specific conformational transition (i.e., a movement of the 7th transmembrane domain) to reach an active state, which is stabilized by the agonist. However, surprisingly, the activation of the β-arrestin pathway, leading to internalization, requires the receptor to be at a basal state (Cabana et al., 2015). This new concept that internalization and activation of AT_1 receptors are independently related to different conformational states has been confirmed by the recently developed biased agonist, TRV027, which activates the β-arrestin pathway while inhibiting the G-protein pathway (Boerrigter et al., 2011; Boerrigter, Soergel, Violin, Lark, & Burnett, 2012). Further studies are required to demonstrate whether *S*-nitrosation of the AT_1 receptor, which involves Cys289 (Leclerc et al., 2006) located within the 7th transmembrane domain, impairs these conformational dynamics.

Whether this blockade of the AngII-induced vasoconstriction occurs in physiological conditions and/or in vivo remains to be established. For example, one could speculate that increases in endogenous NO synthesis following ACh stimulation could reach the AT_1 receptor and thus decrease its activity. However, to the best of our knowledge, a reduction in AngII-induced vasoconstriction following ACh stimulation (a commonly used procedure in pharmacological studies to assess the viability of the endothelium) has never been reported. However, this lack of effect of ACh on AT_1 receptor function could be related to a compartmentalization of ACh-induced NO production targeting directly sGC and vascular smooth muscle cells. Indeed, several reports highlight the pivotal role of subcellular localization of eNOS (Biwer et al., 2016) and of transporters such as Hbα (Straub et al., 2012) or connexin-formed channels (Figueroa, Lillo, Gaete, Riquelme, & Saez, 2013) for NO to exert its dilating action. This well-regulated NO compartmentation could preclude ACh-induced NO synthesis to diffuse and reach proteins such as the AT_1 receptor.

In conclusion, we demonstrate a new and unexplored regulation pathway of the cerebrovascular renin angiotensin system: At the cerebrovascular level, GSNO pretreatment induces a decrease in AT_1 agonist-dependent or agonist-independent vasoconstriction which may depend on AT_1 receptor *S*-nitrosation. These results were obtained on the MCA, the artery most frequently involved in

ischaemic stroke (Hedna et al., 2013), and thus suggest that the redox regulation via *S*-nitrosation of the renin angiotensin system components might become an important therapeutic target.

Our preliminary data also indicate that the decrease in AngII-mediated vasoconstriction produced by GSNO is equivalent in arteries from spontaneously hypertensive rats (SHR) and in those from their normotensive controls (Figure S5). Therefore, besides its neuroprotective properties (Khan et al., 2005), GSNO, if administered after stroke, a situation in which AT_1 receptor-dependent vasoconstriction is exacerbated (Stenman & Edvinsson, 2004) and NO availability is reduced (Chrissobolis, Miller, Drummond, Kemp-Harper, & Sobey, 2011), could be beneficial by reducing AT_1 receptor signalling. We recently demonstrated that the microparticles loaded with GSNO protected the brain from the consequences of ischaemia (Parent et al., 2015). Further experiments are needed to link this cerebroprotection to the *S*-nitrosation in AT_1 receptor signalling pathway.

4.1 | Innovation

AngII is a key regulator of the cerebral circulation. The fine tuning of its AT_1 receptor (constrictor) is of critical importance in many pathophysiological processes, such as stroke. The present study is the first to identify *S*-nitrosation (the covalent bond between NO and cysteine residues) as a potential mechanism involved in the control of the AT_1 receptor-dependent cerebrovascular functions. GSNO exposure leads to reduced AngII vasoconstriction and MT and might thus represent a new therapeutic approach in cerebrovascular diseases, in which excessive vasoconstriction may lead to deleterious consequences.

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AUTHOR CONTRIBUTIONS

M.L.B., C.P.S., I.L., and F.D. conceived the ex vivo experiment and interpreted the results. M.L.B. and S.L. conceived the in vitro experiment. M.L.B. carried out all the experiment. S.L. and A.R. respectively conducted internalization and myogenic tone experiment. C.G. helped for the biotin switch experiments and analysis. All authors equally contributed to the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

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