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NS-398, a selective cyclooxygenase-2 blocker, acutely inhibits receptor-mediated contractions of rat aorta: role of endothelium

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Abstract

NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)-methane sulfonamide) is a selective inhibitor of the cyclooxygenase-2 isozyme in vitro and in vivo. This study reports on acute inhibition of receptor-mediated contractions of isolated rat aorta by NS-398 and its modulation by endothelium-derived nitric oxide. NS-398 (1–10 μM) blocked norepinephrine, and 5-hydroxytryptamine (5-HT) evoked contractions and suppressed E_{max} responses for both agonists. E_{max} changes occurred in endothelium-intact vessel rings and in the absence, as well as in the presence of cycloheximide or dexamethasone in the physiological salt solution (PSS) bathing the tissues. NS-398 altered contractions to these receptor agonists in denuded rings only at 10 μM, and did not significantly alter contractions to KCl and sodium fluoride in all situations. NS-398 (3 and 10 μM) reduced aortic contractions initiated by cyclopiazonic acid (CPA), a sarcoplasmic reticulum Ca^{2+}-ATPase blocker, in endothelium intact rings bathed with PSS with/without nitro-D-arginine methyl ester (D-NAME;100 μM), but did not alter contractions to the compound in endothelium-denuded aortic rings and in vessel rings bathed with PSS + L-NAME (100 μM). Western blot analyses reveal significantly denser cyclooxygenase-2 protein expressions in freshly isolated endothelium-intact, compared to, denuded vessel segments. We conclude that: (1) cyclooxygenase-2 is constitutively expressed in rat aortic endothelial and smooth muscle cells, and (2) NS-398 modulates aortic contractions principally through an action on endothelial cyclooxygenase-2. Our data strongly suggest that cyclooxygenase-2 and/or its product(s), in concert with endothelium-derived nitric oxide, regulates the sarcoplasmic reticulum Ca^{2+} pump activity in rat aorta.

Keywords: NS-398; Cyclooxygenase-2; Aorta, rat; Endothelium derived; Nitric oxide; SERCA (Ca^{2+} pump); Sarcoplasmic reticulum

1. Introduction

Nonsteroidal antiinflammatory drugs inhibit the enzyme cyclooxygenase, which is responsible for prostaglandin synthesis in a variety of tissues (Vane, 1971). Cyclooxygenase exists in two isoforms. Cyclooxygenase-1 is constitutive to most tissues (Funk et al., 1991; Simmons et al., 1991; O'Neil et al., 1994; Smith et al., 1994) and elaborates prostanoids involved in physiological processes, for example, gastric mucosal cytoprotection (De Witt and Smith, 1988; Merlie et al., 1988). Cyclooxygenase-2, on the other hand, is normally undetectable in most tissues, but can be rapidly induced by proinflammatory cytokines and mitogens at sites of inflammation (Cryer and Dubois, 1998; Mitchell and Warner, 1999). Its expression is also increased by lipopolysaccharides and hormones (Cryer and Dubois, 1998) and also by YC-1, an activator of soluble guanylate cyclase (Chang et al., 2002). Although cyclooxygenase-1 and -2 share 60% homology (Cryer and Dubois, 1998), they differ with respect to susceptibility to inhibition by pharmacological agents (Cryer and Dubois, 1998; Mitchell and Warner, 1999) and in expression. Cyclooxygenase-2, but not -1, is found in colon and gastric forms of cancer (Eberhart et al., 1994; Ristimaki et al., 1997).

NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)-methane sulfonamide) is the first documented agent to have selective inhibition for cyclooxygenase-2 both in vitro and in vivo (Futaki et al., 1993, 1994; Gilroy et al., 1998; Copeland et al., 1994; Masferrer et al., 1994). Because the majority of nonsteroidal antiinflammatory drugs in current clinical use are either selective cyclooxgenase-1 or have dual cyclooxygenase-1/2 inhibitory properties (Meade et al., 1993) with varying degrees of gastric and renal side effects (Bate-

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man, 1994), the discovery of NS-398 and other such selective cyclooxygenase-2 blockers is believed to be a significant improvement to current therapy.

We have consistently observed that NS-398 inhibits receptor-mediated contractions of rat aorta in vitro. The observations led us to wonder whether cyclooxygenase-2-inducing factors prevailed during our experimental conditions. Thus, we performed experiments during the continuous presence of protein synthesis inhibition with cycloheximide or dexamethasone since the induced expression of cyclooxygenase-2 can be prevented by protein synthesis inhibitors and by glucocorticoids (Cryer and Dubois, 1998). We also determined whether the acute inhibition of aortic contraction by NS-398 is related to blockade of cyclooxygenase-2 that is native to aorta or, simply, a nonspecific blockade of a common event (Ca^{2+} availability) to receptor agonists' signal transduction process.

2. Materials and methods

2.1. Isolated thoracic aorta

Thoracic aortae were excised from male Sprague–Dawley (Harlan, Indianapolis; 250–300 g) rats, cleaned of connective tissues and cut into 4–5-mm ring segments. Each ring was mounted between stainless steel triangular hooks and suspended under 2-g passive tension in 20-ml organ baths containing physiological salt solution (PSS) maintained at 37 °C and bubbled with 95% O_2/5% CO_2. Isometric tension was recorded with Grass FT 0.03 force displacement transducers coupled to a Grass polygraph (model 7H). The composition (in mM) of our PSS is as follows: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1. High-K^+ (10, 30, 50 and 100 mM) depolarizing PSS were prepared by substituting equimolar amounts of K^+ for Na^+ in the respective solutions. The pH of the solutions after saturation with 95% O_2/5% CO_2 gas mixture was 7.4. As a routine, tissues were allowed to equilibrate for 1 h before the start of all experiments.

2.2. Experimental protocol

2.2.1. Series 1

Experiments in this series involved testing the effects of NS-398 on aortic contractions initiated by agonists acting through distinct processes: norepinephrine, 5-HT (specific receptor activation), KCl (a voltage-operated Ca^{2+} channel agonist) and sodium fluoride (a dual G-protein activator/phosphatase inhibitor). First, cumulative concentration–response curves were established to each agonist. This was followed by addition of increasing concentrations (1–10 μM) of NS-398 or its vehicle (dimethylsulfoxide) in appropriate volumes to the tissue bathing medium for 30 min. Concentration–response curves were thereafter reestablished. Agonist E_{max} and pD_2 values were obtained in the absence (control) and in the presence of different concentrations of NS-398. We also tested the effects of two other chemically distinct cyclooxygenase-2 inhibitors: celecoxib (1–10 μM) and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)-phenyl-2(5*H*)-furanone, or L-752860 (10–100 μM), on contractions initiated by norepinephrine.

2.2.2. Series 2

This series of experiments investigated the influence of protein synthesis inhibition and of endothelium on NS-398/agonist interactions. Two forms of investigations were carried out, and the protocol for the tests is as in Series 1 above. First, we studied the interaction of NS-398 with norepinephrine or 5-HT on aortic rings bathed with PSS containing cycloheximide (10 μM) or dexamethasone (1 μM) as protein synthesis inhibitors. Aortic rings used for these experiments were exposed continuously to cycloheximide or dexamethasone from equilibration time to the end of the experiments. Second, we investigated the influence of endothelium by comparing NS-398/agonist interactions in: (a) endothelium-intact or -denuded aortic rings bathed with normal PSS; (b) endothelium-intact vessel rings bathed with PSS containing 100 μM of either L- or D-NAME; and (c) endothelium-intact or -denuded rings incubated with PSS containing the nitric oxide donor, sodium nitrite in the absence (denuded rings) or presence (intact rings) of L-NAME. Endothelium was denuded mechanically by rubbing a piece of cotton swab

Table 1
Influence of NS-398 on rat aortic reactivity (pD_2) to norepinephrine in the absence and during treatment with cycloheximide or dexamethasone

Aortic vessel reactivity (pD_2)				
Experimental conditions	Control	+1 μM NS-398	+3 μM NS-398	+10 μM NS-398
+ Endo aorta				
PSS	7.65 ± 0.05	7.64 ± 0.10	7.42 ± 0.15[a]	7.01 ± 0.15[a]
PSS + cycloheximide (10 μM)	8.00 ± 0.03[b]	7.82 ± 0.13	7.39 ± 0.12[c]	7.01 ± 0.17[c]
PSS + dexamethasone (1 μM)	7.57 ± 0.21	6.58 ± 0.09[d]	5.87 ± 0.07[d]	5.62 ± 0.13[d]
PSS + L-NAME (100 μM)	8.13 ± 0.05[e]	7.81 ± 0.06[f]	7.57 ± 0.04[f]	7.34 ± 0.04[f]
− Endo aorta				
PSS	9.10 ± 0.06	8.67 ± 0.09[g]	8.23 ± 0.10[g]	7.96 ± 0.07[g]
PSS + cycloheximide (10 μM)	9.68 ± 0.65	8.33 ± 0.19[h]	7.63 ± 0.13[h]	7.21 ± 0.07[h]

Data represent the means ± S.E.M. ($n = 8$).
[a] Statistical difference ($P < 0.05$) from the corresponding control value.
[b] Statistical difference ($P < 0.05$) from value obtained with PSS only.
[c] Statistical difference ($P < 0.05$) from the corresponding control value.
[d] Statistical difference ($P < 0.05$) from the corresponding control value.
[e] Statistical difference ($P < 0.05$) from value obtained with PSS only.
[f] Statistical difference ($P < 0.05$) from the corresponding control value.
[g] Statistical difference ($P < 0.05$) from the corresponding control value.
[h] Statistical difference ($P < 0.05$) from the corresponding control value.

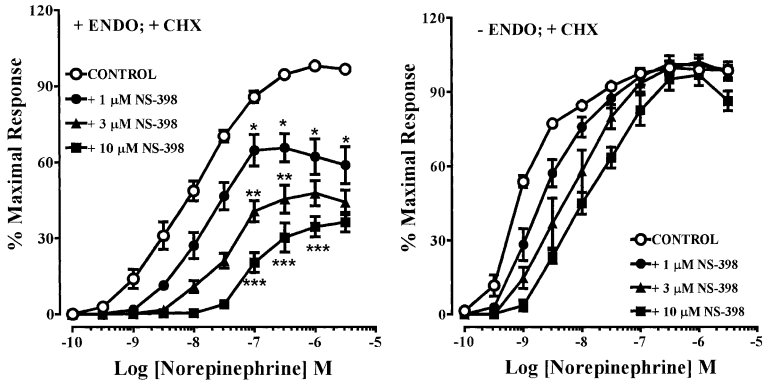

Fig. 1. Effect of NS-398 on responses initiated by norepinephrine in endothelium-intact (+ENDO; left panel) or endothelium-denuded (− ENDO; right panel) rat aortic rings. The PSS bathing the tissues contained cycloheximide (CHX, 10 μM). In both panels, (○) represents responses to norepinephrine alone, while (●), (▲) and (■) represent responses to norepinephrine in the presence of 1, 3 and 10 μM NS-398, respectively. Each data point on the graphs represents the mean ± S.E.M., $n=8$; *, ** and *** denote statistical differences ($P<0.05$) between E_{max} of control and treated tissues to norepinephrine.

against the intimal lining of aortic vessel rings and the degree of endothelial removal was established functionally with the response of precontracted vessel rings to 1 μM acetylcholine.

2.2.3. Series 3

Experiments in this series were conducted to determine the influence of NS-398 on contractions initiated by readmission of $CaCl_2$ in aortic rings bathed with external Ca^{2+}-free PSS and primed with norepinephrine (1 μM). The experiments were performed in two stages. First, aortic contractions initiated by 1 μM norepinephrine during nominal omission of extracellular Ca^{2+} were determined. This was followed 5 min later by addition of 1 mM Ca^{2+} to PSS in the baths. Thereafter, effects of different concentrations of NS-398 or its solvent, dimethylsulfoxide, on the contractile activity to readmission of Ca^{2+} were evaluated.

2.2.4. Series 4

Experiments in this series were designed to investigate the influence of NS-398 on contractions initiated by intracellular Ca^{2+} leak from the sarcoplasmic reticulum following blockade of Ca^{2+}-ATPase function with cyclopiazonic acid (CPA; 1 or 10 μM). As with norepinephrine, the experiments were performed on: (a) endothelium-intact and denuded rings bathed with normal PSS; (b) endothelium-intact vessel rings bathed with PSS containing 100 μM of either L- or D-NAME; and (c) endothelium-intact or denuded rings incubated with PSS containing sodium nitrite (50 μM) in the absence (denuded rings) or presence (intact rings) of L-NAME. In all cases, aortic contractions initiated by CPA were established in the presence of either NS-398 (3 or 10 μM), or its vehicle, dimethylsulfoxide.

2.3. Western blot analysis for cyclooxygenase-2

Western immunoblotting analyses were performed in order to determine the locus (endothelium and/or vascular smooth muscle cells) of cyclooxygenase-2 protein expression in rat aortic vessels. Total cellular proteins were obtained by glass–glass homogenization of freshly isolated, endothelium-intact or denuded aortic vessel samples. The homogenization was accomplished in a sample buffer con-

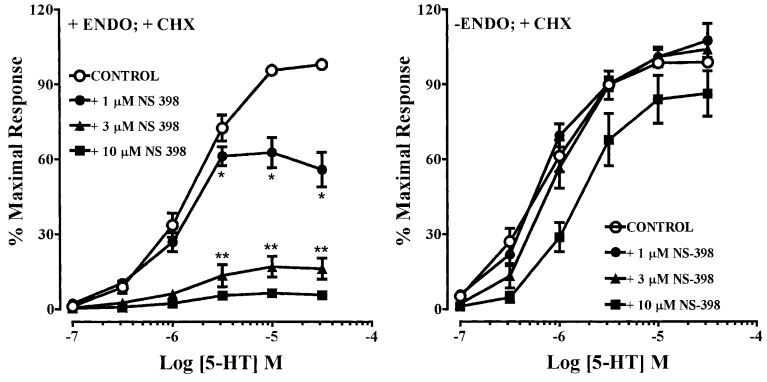

Fig. 2. Effect of NS-398 on responses initiated by 5-hydroxytryptamine (5-HT) in endothelium-intact (+ENDO; left panel) or endothelium-denuded (− ENDO; right panel) rat aortic rings. The tissue bathing PSS contained cycloheximide (CHX, 10 μM). In both panels, (○) represents responses to 5-HT alone, while (●), (▲) and (■) represent responses to 5-HT in the presence of 1, 3 and 10 μM NS-398, respectively. Each data point on the graphs represents the mean ± S.E.M., $n=8$; * and ** denote statistical differences ($P<0.05$) between 5-HT E_{max} of control and treated tissues.

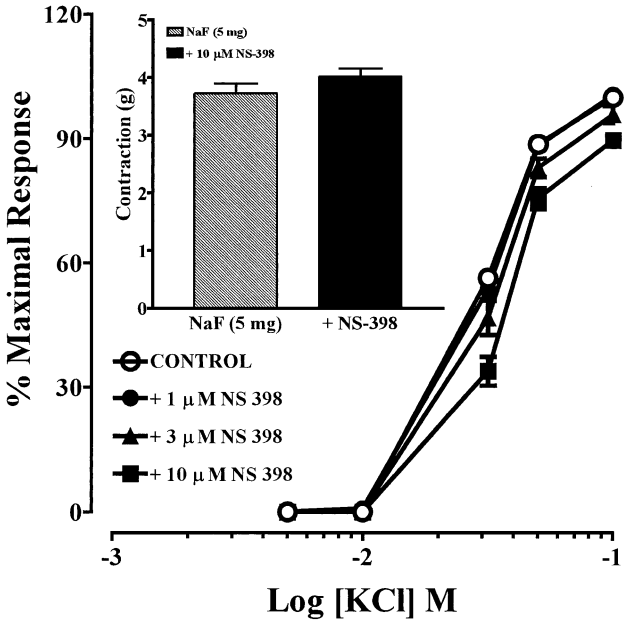

Fig. 3. Effects of NS-398 on contractions of rat aortic rings initiated by KCl or NaF (5 mg; insert). Each data point on the graphs represents the mean ± S.E.M., $n = 7$.

taining (mmol/l): Tris–HCl (pH 7.5) 50, ethylenediaminetetraacetic acid (EDTA) 5, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 10, benzamidine 10 and sodium orthovanadate 1; and (in μg/ml): phenylmethylsulfonyl fluoride (PMSF) 50, aprotinin 10, leupeptin 10, pepstatin A 10 and β-mercaptoethanol 0.3%. Protein concentrations were determined using the method of Bradford (Bio-Rad). Protein samples (60 μg/lane) were electrophoresed in 10% polyacrylamide/SDS gels and transferred by electroblotting onto nitrocellulose membranes. Blots were incubated with either a goat anti-human cyclooxygenase-1 polyclonal antibody (0.6 μg/ml; Santa Cruz Biotechnology) or an anti-rat cyclooxygenase-2 monoclonal antibody (0.5 μg/ml; Transduction Laboratories). The anti-rat cyclooxygenase-2 has been shown to react selectively with cyclooxygenase-2, but not cyclooxygenase-1 (Zimmermann et al., 1999). Blots were subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10,000 dilution; Bio-Rad). Signal detection was facilitated with enhanced chemiluminescence (ECL, Amersham). Proteins extracted from normal rat testes and lipopolysaccharide-treated rat kidney were used as positive controls for cyclooxygenase-1 and -2, respectively. Specific signals were analyzed using a densitometer (Personal Densitometer SI, Molecular Dynamics) and the ImageQuant software (Molecular Dynamics) and compared using paired *t*-test. A *P*-value less than 0.05 was considered as significant.

2.4. Drugs

Norepinephrine bitartrate, 5-hydroxytryptamine maleate, sodium fluoride, acetylcholine bromide, cycloheximide, cyclopiazonic acid, dexamethasone, *N*ω-nitro-L-arginine methyl ester (L-NAME), *N*ω-nitro-D-arginine methyl ester (D-NAME) and sodium nitrite were purchased from Sigma, St. Louis, MO. NS-398 (*N*-(2-cyclohexyloxy-4-nitrophenyl)-methane sulfonamide) was purchased from Cayman Chem., Ann Arbor, MI. Celecoxib (celebrex) and 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone) or L-752860 were obtained courtesy of Dr. Leonardo Clavijo and Merck Res. Labs., Rahway, NJ, respectively. With the exception of stock solutions of norepinephrine and 5-HT which were prepared in 0.1 M hydrochloric acid and diluted as desired with distilled water, all other compounds were dissolved in dimethylsulfoxide.

2.5. Data analysis

Results are expressed as means ± S.E.M. Statistical significance was assessed using the one-way analysis of var-

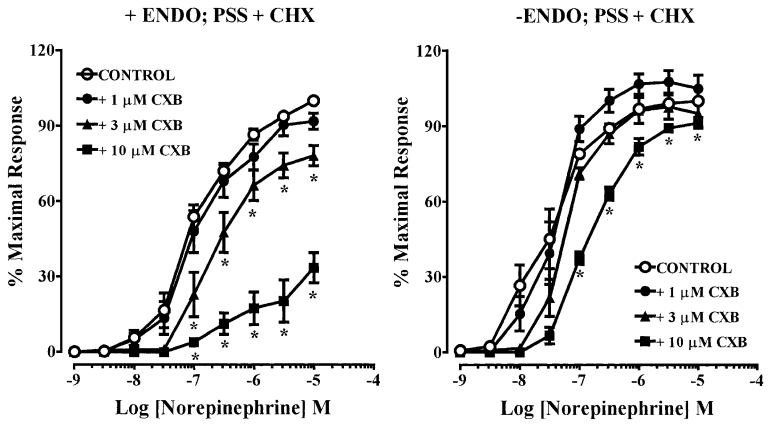

Fig. 4. Effect of celecoxib (CXB) on responses initiated by norepinephrine in endothelium-intact (+ENDO) and denuded (−ENDO) rat aortic rings. The PSS bathing the tissues contained cycloheximide (CHX, 10 μM). (○) represents responses to norepinephrine alone, while (●), (▲) and (■) represent responses to norepinephrine in the presence of 1, 3 and 10 μM CXB, respectively. Each data point on the graphs represents the mean ± S.E.M., $n = 8$; * denote statistical differences ($P < 0.05$) between control and CXB-treated response curves to norepinephrine.

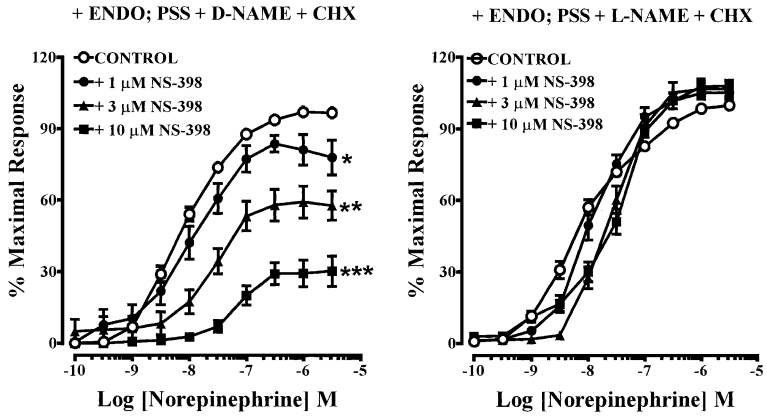

Fig. 5. Influence of a nitric oxide synthesis inhibitor L-NAME (right panel) and its inactive isomer, D-NAME (left panel), on attenuation of norepinephrine contractions by NS-398 in endothelium-intact (+ENDO) rat aortic rings. The tissue bathing PSS also contained cycloheximide (CHX, 10 μM). In both panels, (○) represents responses to norepinephrine alone, while (●), (▲) and (■) represent responses to norepinephrine in the presence of 1, 3 and 10 μM NS-398, respectively. Each data point on the graphs represents the mean ± S.E.M., $n=6$; *, ** and *** denote statistical differences ($P<0.05$) between E_{max} of control and treated tissues to norepinephrine.

iance (ANOVA) statistical program, and the difference between mean values were considered significant when $P<0.05$. pD_2 values, defined as negative logEC_{50} (EC_{50}; i.e., effective concentration of agonist required to produce 50% maximal response), were determined from concentration–response curves which were fitted using a sigmoidal regression with variable slope for each agonist in the absence or presence of NS-398. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of NS-398 on vascular contractile responses

Norepinephrine (0.001–3 μM) initiated contractions of endothelium-intact aortic ring segments and NS-398 (1–10 μM), but not its vehicle, dimethylsulfoxide, concentration-dependently antagonized the contractions. NS-398 significantly decreased norepinephrine pD_2 values at 3 and 10 μM (Table 1), and markedly suppressed the E_{max} responses at all concentrations tested (Fig. 1). 5-HT (0.1–30 μM) also concentration-dependently contracted isolated aortic rings and the responses were antagonized by NS-398 (Fig. 2). As with norepinephrine, NS-398 caused significant ($P<0.05$) suppression of 5-HT E_{max} at all concentrations of the compound used in our study. E_{max} values for 5-HT were more profoundly suppressed (80.8 ± 5.4% and 93.9 ± 2.2%) compared to norepinephrine (42.2 ± 3.7% and 60.2 ± 3.6%) at 3 and 10 μM NS-398, respectively. However, contractions initiated by KCl (10–100 mM) and also by 5 mM sodium fluoride (NaF) were relatively unaffected by NS-398 (Fig. 3).

Two other chemically distinct cyclooxygenase-2-selective inhibitors, celecoxib (1–10 μM) and compound L-752860 (10–100 μM), also concentration-dependently blocked norepinephrine induced contractions. As with NS-398, celecoxib (Fig. 4) and also compound L-752860 (data not shown) decreased norepinephrine pD_2 and E_{max} values significantly.

Table 2
Treatment with sodium nitrite (50 μM) restores the inhibitory effectiveness of NS-398 on norepinephrine (NE) and cyclopiazonic acid (CPA) induced contractions of rat aorta

Contraction force (g)			
Experimental conditions	Control	+3 μM NS-398	+10 μM NS-398
+ Endo aorta; PSS + L-NAME			
NE (0.1 μM)	2.30 ± 0.30	2.20 ± 0.50	2.50 ± 0.40
NE (1 μM) during $NaNO_2$ (50 μM)	1.60 ± 0.50	0.60 ± 0.02[a]	0.24 ± 0.01[a]
CPA (1 μM)	1.03 ± 0.01	1.10 ± 0.01	1.08 ± 0.02
CPA (10 μM) during $NaNO_2$ (50 μM)	0.53 ± 0.01	0.26 ± 0.01[b]	0.08 ± 0.02[b]
− Endo aorta			
NE (0.1 μM)	2.42 ± 0.30	2.58 ± 0.22	2.62 ± 0.38
NE (1 μM) during $NaNO_2$ (50 μM)	1.48 ± 0.44	0.94 ± 0.14[c]	0.17 ± 0.05[c]
CPA (1 μM)	0.71 ± 0.06	0.68 ± 0.03	0.75 ± 0.08
CPA (10 μM) during $NaNO_2$ (50 μM)	0.48 ± 0.16	0.12 ± 0.01[d]	0.04 ± 0.01[d]

Data represent the means ± S.E.M. ($n=7$).

[a] Statistical difference ($P<0.05$) from the corresponding value for norepinephrine (NE) or cyclopiazonic acid (CPA) in the absence of sodium nitrite.

[b] Statistical difference ($P<0.05$) from the corresponding value for norepinephrine (NE) or cyclopiazonic acid (CPA) in the absence of sodium nitrite.

[c] Statistical difference ($P<0.05$) from the corresponding value for norepinephrine (NE) or cyclopiazonic acid (CPA) in the absence of sodium nitrite.

[d] Statistical difference ($P<0.05$) from the corresponding value for norepinephrine (NE) or cyclopiazonic acid (CPA) in the absence of sodium nitrite.

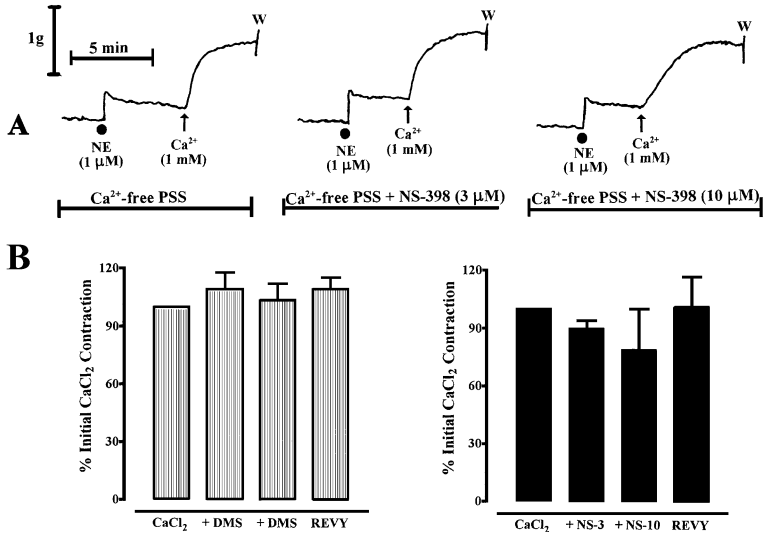

Fig. 6. (A) Effects of NS-398 at 3 or 10 μM (NS-3 and NS-10, respectively) on contractions of rat aortic rings initiated by $CaCl_2$ during priming with 1 μM NE. (B) Bar graphs depicting the effects of DMSO, vehicle for NS-398 (20 and 60 μl DMS, respectively; left panel), or NS-398 itself (NS-3 and NS-10, respectively; right panel) on aortic contractions initiated by $CaCl_2$ during priming with 1 μM NE. Aortic rings were bathed with Ca^{2+}-free PSS (CF-PSS). Data plotted in each column represents the mean ± S.E.M., $n = 5$.

3.2. Influence of protein synthesis inhibition and of endothelium on NS-398/agonist interactions

Aortic rings bathed continuously in PSS containing cycloheximide (10 μM) exhibited significantly ($P < 0.05$) enhanced reactivity to norepinephrine, but not to 5-HT. Norepinephrine pD_2 values were 7.65 ± 0.05 (normal PSS) versus 8.00 ± 0.03 (PSS + cycloheximide); 5-HT pD_2 values on the other hand were 5.78 ± 0.08 (normal PSS) versus 5.80 ± 0.05 (PSS + cycloheximide). Dexamethasone

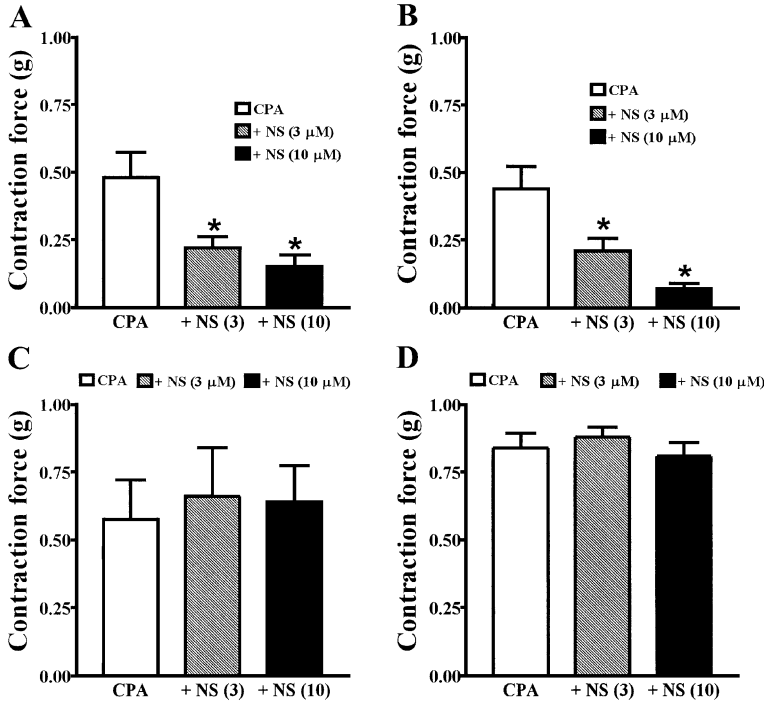

Fig. 7. Effects of NS-398 at 3 or 10 μM, NS (3) and NS (10), respectively, on contractions of endothelium-intact (panels A, B and D), or denuded (panel C) rat aortic rings initiated by cyclopiazonic acid (CPA; 10 μM—panels A and B, or 1 μM—panels C and D). Experiments in panels A and C were performed with normal PSS, while those in panels B and D were performed with normal PSS + D-NAME and normal PSS + L-NAME at 100 μM, respectively. Data plotted in each column represents the mean ± S.E.M., $n = 9$; * denotes statistical difference ($P < 0.05$) between responses to CPA alone or to CPA in the presence of either 3 or 10 μM NS-398, i.e., NS (3) and NS (10), respectively.

(1 μM) treatment did not significantly alter pD_2 values of control vessel rings for either agonist. In the presence of either cycloheximide or dexamethasone, as with normal PSS, contractions to both agonists were inhibited concentration dependently by NS-398 (see Table 1).

As would be expected, vessel rings denuded of endothelium, or treated with L-NAME (100 μM) did not relax to acetylcholine (1 μM). The pD_2 values for norepinephrine (see Table 1) and also to 5-HT were significantly higher. 5-HT pD_2 values were: 6.18 ± 0.07 (denuded) versus 5.78 ± 0.08 (intact) vessels rings. NS-398 (1–10 μM) significantly decreased norepinephrine pD_2 values in intact aortic rings bathed with normal PSS, but minimally altered norepinephrine pD_2 values in denuded, or L-NAME treated rings (Table 1). NS-398 did not alter E_{max} to either norepinephrine or 5-HT in vessel rings deprived of endothelium (Figs. 1 and 2), or treated with L-NAME (Fig. 5). However, treatment of endothelium-intact vessel rings bathed with PSS + L-NAME, or endothelium-denuded vessel rings with sodium nitrite (50 μM) restored the inhibitory effectiveness of NS-398 on norepinephrine-induced contractions (Table 2).

3.3. Effects of NS-398 on contractions induced by receptor-mediated Ca^{2+} entry and by cyclopiazonic acid

Aortic rings bathed with external Ca^{2+}-free PSS responded reproducibly to successive applications of norepinephrine (1 μM), and when precontracted with the agonist, the vessel rings further contracted to addition of 1 mM $CaCl_2$. Preincubation with NS-398, at 3 and 10 μM, but not its vehicle, dimethylsulfoxide, blocked contractions obtained to norepinephrine in external Ca^{2+}-free PSS. However, NS-398 had no effects on contractions initiated by readmission of 1 mM $CaCl_2$ in aortic rings primed with norepinephrine (Fig. 6).

Cyclopiazonic acid (CPA), a Ca^{2+}-ATPase blocker, initiated a slow, sustained but tachyphylactic contractile response of endothelium-intact aortic rings bathed with normal PSS. CPA (10 μM) initiated contractions of endothelium-intact vessel rings bathed with PSS, or PSS + D-NAME (100 μM). Preincubation of vessel rings with PSS containing NS-398 (3 or 10 μM), reduced the CPA-induced contractile response in a concentration-dependent manner (Fig. 7A and B). Endothelium denudation or treatment with L-NAME (100 μM) enhances aortic contractions to many agonists. Thus, we had to reduce CPA concentration from 10 to 1 μM in order to elicit statistically similar levels of force under these conditions. NS-398 (3 or 10 μM), which concentration-dependently blocked CPA-induced contractions in endothelium-intact vessel rings, failed to alter the response to this agonist in denuded tissues (Fig. 7B), and also in tissues bathed with PSS + L-NAME (100 μM) to inhibit nitric oxide synthesis (Fig. 7D). However, NS-398 blocked CPA-induced aortic contractions of denuded rings, and of L-NAME treated vessel rings that were exposed to sodium nitrite (50 μM) (see Table 2).

3.4. Cyclooxygenase-2 expression in aortic tissue

If cyclooxygenase-2 protein is the target for NS-398 on aortic vessels, it is imperative to determine the cell types, endothelium or smooth muscle, which expresses the protein. Immunoblot analyses using a cyclooxygenase-2 antiserum showed a major immunoreactive protein band of ~ 69 kDa, corresponding to cyclooxygenase-2 expression, in both endothelium-intact and endothelium-denuded aortic tissues (Fig. 8A). The protein expression corresponds to that of the positive control, i.e., stimulated mouse macrophage cell lysate (mac) as supplied by Transduction Laboratories. As could be seen on the densitometric quantification units, cyclooxygenase-2 protein expression is significantly denser and more prominent in vessels with intact, compared to those without, endothelium (Fig. 8B).

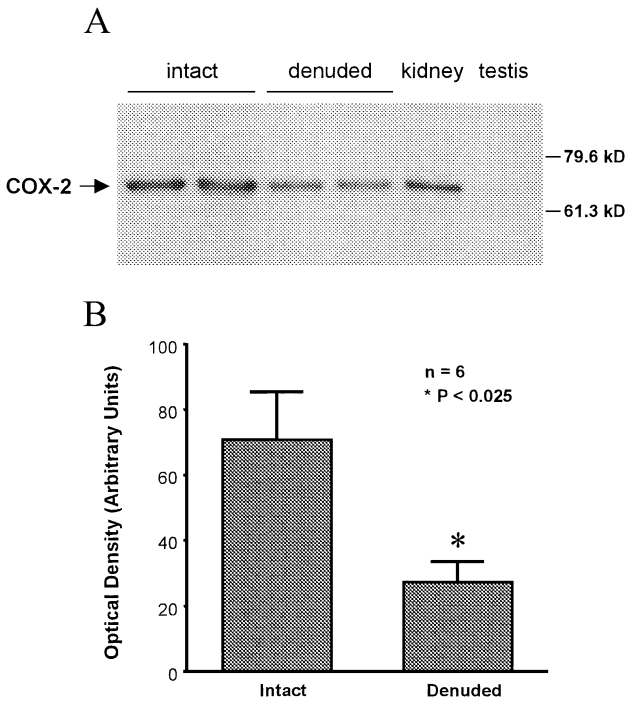

Fig. 8. Western analyses of cyclooxygenase-2 protein expression in rat aorta. (A) A representative blot showing the expression of cyclooxygenase-2 protein in endothelium-intact and denuded rat aorta (60 μg protein/lane). Total cellular proteins extracted from LPS-treated rat kidney (10 μg) were used as a positive control. The anti-cyclooxygenase-2 antibody reacted specifically with cyclooxygenase-2 but not cyclooxygenase-1 as shown by the absence of signal in the lane loaded with total cellular proteins extracted from normal rat testis (10 μg). Conversely, the anti-cyclooxygenase-1 antibody detected a strong signal in testicular proteins but only very weak signals in aortic proteins regardless of the presence or absence of endothelium (data not shown). (B) Quantification of cyclooxygenase-2 protein expression in six rats as assessed with densitometric analyses. Data are presented as means ± S.E.M.; *$P < 0.05$.

4. Discussion

Cyclooxygenase-2 is generally considered an inducible enzyme. Thus, its expression can be induced by proinflammatory cytokines, mitogens or lipopolysaccharides (Cryer and Dubois, 1998). Cyclooxygenase-2-related activity can also be enhanced by stretch-induced changes in intrinsic tone (Charette et al., 1995). The increased levels of eicosanoids associated with its induction can be suppressed by treatment with protein synthesis inhibitors such as cycloheximide and actinomycin D (Masferrer et al., 1994; Akarasereenont et al., 1995), or by glucocorticoids (Cryer and Dubois, 1998). In our current study, NS-398, the prototype cyclooxygenase-2-selective inhibitor, acutely antagonized receptor-mediated contractions of rat aorta in the absence, or in the presence of protein synthesis inhibitor cycloheximide or dexamethasone. Since blockade of aortic contractions by NS-398 is acute and probably did not occur through inhibition of induced synthesis of new proteins, we hypothesize two possible targets of action for this compound in our experimental model. First, cyclooxygenase-2 proteins that are native to rat aortic tissues and thus not amenable to modulation by protein synthesis inhibition in vitro, and/or second, that NS-398 elicits a nonspecific inhibition of a signal transduction process that is common to norepinephrine and 5-HT.

The hypothesis that aortic tissue cyclooxygenase-2 is the target for NS-398 is attractive based on our observation that other chemically distinct inhibitors of this isozyme, namely, celecoxib and L-752860, a highly selective, tetrasubstituted furanone cyclooxygenase-2 inhibitor (Riendeau et al., 1997), also inhibited receptor agonist induced contractions. We further tested the hypothesis by conducting Western blot analyses of cyclooxygenase proteins in freshly isolated aortic vessel segments. Our data show 3-fold greater expression of cyclooxygenase-2 immunoreactive proteins in endothelium-intact, compared to endothelium-denuded aortic segments. This finding indicates that cyclooxygenase-2 is constitutive to both endothelium and smooth muscle cells of rat aorta. Constitutive expression of cyclooxygenase-2 has previously been demonstrated in the aorta of Wistar Kyoto (WKY) rats (Garcia-Cohen et al., 2000) and in rat brain (Yamagata et al., 1993) and kidney (Harris et al., 1994; Vio et al., 1997). Bishop-Bailey et al. (1997) also found cyclooxygenase-2 mRNA in freshly prepared rat aorta with intact or disrupted endothelium. Since cyclooxygenase-2 is expressed in the aorta, our data may be interpreted to support the notion that this isozyme mediates the formation of a factor(s) that contributes to, or facilitates aortic contraction. Connolly et al. (1998) suggested that cyclooxygenase-2 mediate the formation of a vasoconstrictor prostaglandin in rat aortic smooth muscle. Muscara et al. (2000) also showed cyclooxygenase-2 inhibition to reduce the serum concentration of thromboxane B_2 in hypertension.

Further evidence that cyclooxygenase-2 is expressed in rat aortic endothelium, and its product contributes to aortic contraction is shown by comparing NS-398/receptor agonist interactions in vessels rings with/without endothelium, or treated with D- or L-NAME to specifically determine a possible role of endothelium-derived nitric oxide. NS-398 failed to alter responsiveness to norepinephrine or 5-HT in endothelium-denuded and in L-NAME treated vessel rings. Two deductions can be made from these observations. (1) Cyclooxygenase-2, resident in vascular endothelium, serves as the target for NS-398 action. Endothelium denudation results in concomitant decrease in cyclooxygenase-2 protein expression and a loss of product(s) associated with its activity. (2) Endothelium-derived nitric oxide (EDNO) interacts with a cyclooxygenase-2 and/or its putative product(s) to regulate aortic vascular tone.

That NS-398 did not significantly alter contractions to KCl and sodium fluoride suggest that the acute effects of the compound did not involve the voltage-gated Ca^{2+} channels and activation of G-proteins/phosphatase inhibition (Adeagbo and Triggle, 1991), respectively. Therefore, our alternative hypothesis that NS-398 might act to block a regulatory event common to α_{1D}-adrenoceptors and 5-HT_2 receptors deserves consideration. The possible signal transduction processes that may be altered by NS-398 include the receptor-operated Ca^{2+} channels and/or release of Ca^{2+} from an intracellular binding site such as the sarcoplasmic reticulum, or even the protein kinase C/mitogen-activated protein kinases (MAPKs) pathways. We investigated the effects of NS-398 on ligand-gated Ca^{2+} entry by conducting contraction experiments to readmission of Ca^{2+} in aortic rings bathed in Ca^{2+}-free PSS and primed with norepinephrine, or 5-HT. NS-398, or its vehicle dimethylsulfoxide, did not alter Ca^{2+} entry facilitated by norepinephrine. However, 5-HT-mediated Ca^{2+} entry was significantly decreased in the presence of dimethylsulfoxide and more profoundly decreased in the presence of NS-398. Because dimethylsulfoxide and NS-398 effects were partially restored by washing, we deduced that these compounds influenced 5-HT-driven Ca^{2+} entry into aortic smooth muscle. Our study did not examine the effects of NS-398 on protein kinase C and other cellular kinase systems.

Calcium for physiologic function such as vascular smooth muscle contraction is distributed and sequestered into many compartments within the cell. These include the inositol triphosphate (IP_3)-sensitive pool stored in the sarcoplasmic reticulum, a caffeine-sensitive pool and other stores, e.g., in the mitochondria. Since receptors activated by norepinephrine and by 5-HT, α_{1D} and 5-HT_2, respectively, are G-protein coupled and elicit contraction of rat aorta through the phospholipase C-IP_3 pathway, our study focused on the SR as the probable intracellular site of action of cycloxygenase-2 blockade with NS-398. Homeostasis of the cytosolic Ca^{2+} concentration is tightly regulated by the activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) or Ca^{2+} pump. Cyclopiazonic acid selectively inhibits the activity of this pump (Goerger et al., 1988; Seidler et al., 1989) thus promoting leakage of Ca^{2+} from its sarcoplasmic reticulum storage site to initiate a slow contraction of rat aortic rings.

NS-398 blunted aortic contractions initiated by cyclopiazonic acid. The blunting effect of the compound on CPA, as with norepinephrine and 5-HT induced contractions, was eliminated by endothelium denudation, or by treatment with L, but not D-NAME. These data suggest that this nonsteroidal antiinflammatory drug directly inhibits the leakage (release) of Ca^{2+} from the sarcoplasmic reticulum in an endothelium-derived nitric oxide-dependent manner. This assertion is supported by our finding that exposure of endothelium-denuded, or L-NAME-treated aortic rings to sodium nitrite restores NS-398 effectiveness in blocking contractions elicited by cyclopiazonic acid and norepinephrine (see Table 2). Since sodium nitrite generates nitric oxide at physiological pH such as prevails in our experimental conditions, or at acidic pH (Modin et al., 2001), it can be deduced from the present study that endothelium-derived nitric oxide influences Ca^{2+} release/uptake into sarcoplasmic reticulum. This deduction is compatible with the possibility that NS-398 may inhibit the production of an endothelial cyclooxygenase-2-mediated product that facilitates aortic contractility through the regulation of Ca^{2+} homeostasis by the sarcoplasmic reticulum. Connolly et al. (1998) attributed the blockade of rat aortic contractions by nimesulide, a compound chemically related to NS-398, to inhibition of the production of an endogenous facilitator of vascular contractions.

In conclusion, cyclooxygenase-2 is constitutively expressed in rat aortic endothelial and smooth muscle cells and NS-398 modulates aortic contractions principally through an action on endothelial cyclooxygenase-2. Our data strongly suggest that cyclooxygenase-2, in concert with endothelium-derived nitric oxide, regulate the Ca^{2+} pump function in rat aorta.

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