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European Journal of Pharmacology 477 (2003) 59–68
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Nitric oxide-releasing aspirin inhibits vasoconstriction in perfused tail artery of normotensive and spontaneously hypertensive rats

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Received 19 May 2003; received in revised form 30 July 2003; accepted 5 August 2003

Abstract

The aim of this study was to investigate the capacity of the 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX 4016), a nitric oxide (NO)-releaser derivative of aspirin, to decrease blood pressure in spontaneously hypertensive rats (SHR) and to counteract the adrenergic vasoconstriction in perfused tail artery of these animals. Oral treatment for 10 consecutive days with NCX 4016 (100 μmol/kg) in SHR and their genetic controls Wistar Kyoto (WKY) rats resulted in a reduction of blood pressure in SHR but not in WKY rats. In SHR, the NCX 4016 treatment increased the serum nitrite/nitrate and diminished the serum thromboxane B_2, whereas aspirin did not change blood pressure but abolished the serum thromboxane B_2. Perfused tail arteries excised from vehicle-treated SHR exhibited a significant impairment of endothelium-dependent vasorelaxant function. These vessels, prepared from SHR or WKY rats treated orally with NCX 4016 (10, 30 and 100 μmol/kg for 7 consecutive days), revealed a dose-dependent decrease in vasoconstriction in response to transmural nerve stimulation and norepinephrine, whereas aspirin was ineffective. Furthermore, in tail arteries of both SHR and WKY rats treated orally with NCX 4016 (100 μmol/kg for 7 consecutive days), the cGMP increased significantly. In conclusion, NCX 4016, by releasing NO and increasing cGMP in vascular tissue, reduces sympathetic-mediated vasoconstriction in resistance vessels and lowers blood pressure in SHR.

Keywords: NCX 4016; Hypertension; Vasoconstriction; Nitric oxide (NO); cGMP; Spontaneously hypertensive rat; Tail artery

1. Introduction

For decades, hypertension has been the subject of extensive studies, but its etiopathogenic mechanism is still unclear. A reduction in intravascular size such as that occurring during vasoconstriction, or an increase of intravascular volume is the basic haemodynamic parameters leading to remodelling process and marking systemic hypertension. Over the years, nitric oxide (NO) has emerged as a critically important factor in the normal maintenance of blood pressure (Moncada et al., 1988; Rees et al., 1989). Synthetized principally by endothelial cells NO-synthase, NO modulates vasoconstriction and controls vessel tone with its potent vasorelaxant activity (Lüscher, 1991; Moncada et al., 1991). Consequently, an impairment of NO vascular action is a central feature of endothelial dysfunction leading to hypertension (Calver et al., 1992).

Among the variety of animal models developed to mimic human essential hypertension, the spontaneously hypertensive rat (SHR) is one of the most widely used. In this model, supplementation of NO by NO-donating compounds may reduce high blood pressure by improving the altered endothelial-dependent relaxant function. The use of NO-synthase inhibitors, such as N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA), has shown that NO contributes to basal blood pressure in a similar way in both SHR and Wistar Kyoto (WKY) rats (Arnal et al., 1993; Fozard and Part, 1991). However, in vitro studies indicate a decreased NO-dependent relaxation in skeletal muscle arterioles (Huang and Koller, 1996), in aorta (Gil-Longo et al., 1996) and many other vessels

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doi:10.1016/j.ejphar.2003.08.004

(Crabos et al., 1997; Dohi et al., 1990; Matrougui et al., 1997) of SHR. These considerations prompted us to investigate the activity of 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX 4016), an NO-releasing derivative of aspirin, in the control of elevated systemic blood pressure of SHR with a particular focus on the effect in perfused tail artery preparations submitted to neurogenic stimulation. The tail artery was chosen as the area of study because this vessel possesses a thick media layer and it is comparable in a sense that it has resistance vessels (Machkow et al., 1997). In this regard, it has been reported that the tail artery of SHR may have the potential for producing a heightened peripheral resistance in these animals (Cassis et al., 1985, 1988; Head, 1989) in view of the hypernoradrenergic innervation consistent with a greater number of nerve axon bundles influencing neuroeffector mechanisms.

NCX 4016 is a chemical combination of aspirin with an NO donor (Del Soldato et al., 1999; Ignarro et al., 2002) which has been shown to display antiaggregatory and antithrombotic activity by a dual mechanism of action involving inhibition of cyclooxygenase and release of NO, the latter acting on soluble guanylate cyclase in both platelets and vascular smooth muscle cells (Minuz et al., 1998; Wallace et al., 1999). The nitroester of aspirin has been shown to improve post-ischemic ventricular dysfunction in the rabbit (Rossoni et al., 2000) and to reduce infarct size caused by ischemia–reperfusion in anaesthetized rat (Rossoni et al., 2001) and pig (Wainwright et al., 2002). This occurs largely through NO donation, which modulates a number of cellular events leading to inflammation, obstruction of coronary microcirculation, arrhythmias and myocardial tissue necrosis (Rossoni et al., 2001). Recently, Muscarà et al. (2001) reported that NCX 4016, given orally over a 2-week period to rats with L-NAME-induced hypertension, significantly reduced the elevated blood pressure. These authors suggested that the beneficial effect of NCX 4016 on this model of hypertension may be mediated through a possible interference with the effects of endogenous pressor agents and by the direct vasodilatatory action of NO released by this compound.

The experiments performed using perfused tail arteries of SHR, where the sympathetic nervous system is a contributing factor to the mechanism responsible for initiation and maintenance of high blood pressure (Head, 1989), will shed new light on the mode of action of NCX 4016 in reducing elevated blood pressure of SHR.

2. Materials and methods

2.1. Animals

Male normotensive WKY rats and age-matched (16–17 weeks) SHR were used in these experiments. Animals were purchased from Charles River Italia (Calco, LC, Italy), housed under standard conditions (four rats per cage; temperature, 22 ± 1 °C; humidity, 55 ± 10%) and maintained on 12/12-h light/dark cycle with the light on from 0700 h. Rats were fed standard chow (Mucedola, Settimo Milanese, MI, Italy), with water ad libitum. All experimental procedures were approved by the Animal Care Committee of the University of Milan, Italy, and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Protocols

Three groups of SHR and WKY rats ($n = 10$ animals per group) were treated orally by gavage with polyethylene glycol 400 (PEG 400; 2 ml/kg), NCX 4016 (100 μmol/kg) or aspirin (100 μmol/kg) once a day for 10 consecutive days. Two hours after each treatment, the animals were submitted to systolic blood pressure and heart rate measurements in a conscious state (see Section 2.3). From these experiments, it has been observed that a period of 7 consecutive days of treatment with NCX 4016 caused the maximal antihypertensive effect (-32%, $P < 0.01$; see Results) in SHR, whereas 10 consecutive days of oral treatment did not result in a further decrease of blood pressure.

To determine the serum thromboxane B_2 and nitrite/nitrate levels, and to measure the tissue cGMP, different groups of SHR and WKY rats ($n = 8$ animals per group) were treated orally by gavage with vehicle (PEG 400, 2 ml/kg), NCX 4016 (100 μmol/kg) or aspirin (100 μmol/kg) once a day for 10 consecutive days. At the end of the prefixed time, the animals were anaesthetized (thiopentone sodium, 60 mg/kg i.p.), the blood was collected for serum thromboxane B_2 and nitrite/nitrate determinations (see Section 2.4) and the central tail artery was dissected for cGMP assay (see Section 2.5).

To investigate the activity of these compounds on acetylcholine-induced vasodilatation and on transmural nerve stimulation-induced vasoconstriction, different groups of SHR and WKY rats ($n = 7$ animals per group) were treated orally by gavage with vehicle (PEG 400, 2 ml/kg), NCX 4016 (10, 30 and 100 μmol/kg) or aspirin (100 μmol/kg) once a day for 7 consecutive days. Two hour after the last treatment, the central tail artery was isolated free under anaesthesia and prepared for in vitro perfusion experiments (see Section 2.6).

2.3. Blood pressure measurement

Rats were weighed and blood pressure was measured in the conscious state by the tail-cuff method using apparatus (model 8006) from U. Basile (Comerio, VA, Italy). Before the tail-cuff blood pressure determination, animals were placed into a warming cupboard (30 °C) for 30 min. Blood pressure values for individual rats were obtained from the average of three consecutive measurements and were considered valid only when these readings did not differ by more than 5 mm Hg. Heart rate was also calculated from the blood pressure tracing.

2.4. Serum thromboxane B_2 and nitrite/nitrate determinations

At the end of 3, 6, 12 h and 1, 3, 5, 7, 10 days of the oral treatment with vehicle (PEG 400, 2 ml/kg), NCX 4016 (100 μmol/kg) or aspirin (100 μmol/kg), blood samples were taken from the abdominal aorta of SHR and WKY rats under anaesthesia. The blood samples were clotted at room temperature and the sera were separated by centrifugation (10 min at 2000 × *g*) and stored at −20 °C. Serum thromboxane B_2 and nitrite/nitrate determinations were performed using a commercially available enzyme immunoassay and colorimetric kits, respectively.

2.5. Tissue cGMP determination

cGMP was directly measured in tail arteries taken from SHR and WKY rats treated orally with vehicle (PEG 400, 2 ml/kg) or NCX 4016 (100 μmol/kg) once a day for 10 consecutive days. Two hours after each treatment, the rats were anaesthetized and the tail arteries (10 cm) were rapidly removed (about 1 min), frozen in liquid nitrogen and stored at −70 °C until assay. The frozen artery samples were weighed and homogenized in 1 ml of ice-cold 6% (w/v) trichloroacetic acid with a cell disrupter (Ultra-Turrax 18/10, Ika-Werk, Janke and Kunkel, Staufen, Germany) for 90 s (9 × 10-s bursts with a 20-s delay between bursts). The homogenates were centrifuged at 2000 × *g* for 15 min at 4 °C. Precipitates were used for protein determination by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Supernatant fractions were extracted four times with water-saturated diethyl ether until the aqueous phase reached pH 7.4. The samples were then evaporated under a stream of nitrogen at 60 °C. The residue was dissolved in 50 mM sodium acetate buffer, and cGMP content was assayed in duplicate using a commercially available enzyme immunoassay kit (cross-reactivity with cAMP is less than 0.0001%). Results were expressed as pmol/mg protein of cGMP.

2.6. "In vitro" tail artery preparation

SHR and WKY rats were anaesthetized, and a proximal segment of tail artery (4 cm) was quickly dissected out and placed in oxygenated Krebs Henseleit solution (37 °C, pH 7.4) of the following composition (mM): NaCl, 118; KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; ascorbic acid, 0.3; and EDTA, 0.03. To avoid the involvement of endothelium-derived relaxing factors, in some arteries, the endothelium was removed by gently threading a 5-cm segment of 6–0 silk through the artery lumen followed by 5 min directing a stream of gas mixture (95% O_2 + 5% CO_2) intraluminally (Spokas and Folco, 1984). In these vascular segments, precontracted with norepinephrine (30 nM, peak effect approximately in 5 min), acetylcholine (from 0.01 to 10 μM) was ineffective (data not shown). Proximal segments of tail arteries were cannulated (20-gauge needle) using the procedures described by Berti et al. (1971) and Spokas and Folco (1984). The arteries were bathed (20 ml) extraluminally with gassed (95% O_2 + 5% CO_2) Krebs Henseleit solution at 37 °C, maintained in a vertical position with a small weight (0.5 g) on the distal end. These vascular segments were perfused intraluminally through the needle at a constant flow rate of 5 ml/min (basal perfusion pressure 24 ± 3 mm Hg) with gassed Krebs Henseleit solution via a Minipuls-3 peristaltic pump (Gilson, Villiers Le Bel, France). This perfusion condition was selected on the basis of preliminary experiments where the relationship between flow rate and increase in intraluminal mean perfusion pressure was examined (data not shown). After a 30- to 45-min equilibration period, the experimental protocol was initiated. Changes in perfusion pressure, which represent changes in vascular resistance, were monitored with a Bentley pressure transducer (model 800, U. Basile, Comerio, VA, Italy), and the resulting electrical signals were recorded by a two-channel polygraph (model Gemini, U. Basile).

Vasoconstriction of tail arteries was induced by transmural nerve stimulation or by the addition of norepinephrine to the perfusion stream. Transmural nerve stimulation was delivered with a Grass S88 stimulator (Grass Instruments, Quincy, MA, USA) through platinum electrodes (40 mm long, 0.5 mm diameter; U. Danuso, Bresso, MI, Italy) placed 5 mm away from the vascular tissue. Stimulation parameters were 60 V (supramaximal voltage), 1-ms pulse duration, at various frequencies (from 0.5 to 32 Hz, twofold increments in frequencies). Responses of tail arteries to transmural nerve stimulation were measured using 5-s train duration at 3-min intervals. It was observed that there was no reduction in responses if the time interval between responses was 3 min. In some experiments, the endothelium morphology was examined to determine whether or not transmural nerve stimulation produced any endothelial damage. Scanning electron microscopy confirmed that, at the end of transmural nerve stimulation-experiments, the endothelium was morphologically intact in non-denuded arteries, whereas control denuded arteries were devoid of a homogeneous intact endothelial cell layer (data not shown). Exogenous norepinephrine responses in tail arteries were elicited by increasing concentrations (from 0.01 to 10 μM, threefold increments in concentration) of this mediator. Responses of the preparations to transmural nerve stimulation or norepinephrine were expressed as a percentage of the maximal vasoconstriction of the arteries to 120 mM KCl in individual experiments. The mean maximal KCl response was 152 ± 12 mm Hg.

2.7. Drugs

NCX 4016 was obtained from NicOx S.A. (Valbonne-Sophia Antipolis, France). Aspirin, acetylcholine chloride, norepinephrine bitartrate, PEG 400 and dimethylsulphoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Thiopentone sodium (Pentothal sodium) was obtained from Abbott (Campoverde, LT, Italy). Enzyme immunoassay kits

for cGMP (RPN-226) and thromboxane B_2 (RPN-220) determinations were obtained from Amersham Italia (MI, Italy). Colorimetric assay kit for nitrite/nitrate (cat# 780001) determination was obtained from Cayman Chemical (Ann Arbor, MI, USA).

For in vivo oral treatment, NCX 4016 and aspirin were dissolved in PEG 400 (vehicle). For in vitro experiments, these compounds were first solubilized in 100% DMSO to produce a concentrated stock solution (10 mM) from which final bath dilutions were made. The DMSO (vehicle) concentration did not elicit any effects per se on the parameters tested. The norepinephrine was dissolved in Krebs Henseleit solution with 70 μM ascorbic acid added as an antioxidant. All the drugs were prepared daily.

2.8. Statistical analysis

Result are expressed as mean ± S.E.M. For statistical analysis, one-way analysis of variance (ANOVA) for repeated measures with Dunnett's multiple comparison test as post hoc test, two-way ANOVA and Student's *t*-test were used as appropriate. All analysis were performed using the GraphPad Prism computer-software (GraphPad Software, San Diego, CA, USA). A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Experiments in conscious rats

When NCX 4016 (100 μmol/kg) was given orally to SHR, a reduction in blood pressure was recorded. The effect was dependent on the length of the treatment and was maximal after 7 consecutive days (from 228 ± 9.2 to 155 ± 7.5 mm Hg, $P<0.01$). In fact, no further reduction of blood pressure was observed when this treatment was extended to 10 consecutive days. NCX 4016, at the peak of its antihypertensive activity, did not modify hear rate of SHR (Fig. 1). Aspirin (100 μmol/kg) given orally for 10 consecutive days did not influence either blood pressure or hear rate of SHR. Neither NCX 4016 (100 μmol/kg) nor aspirin (100 μmol/kg) given orally for 10 consecutive days changed basal values of blood pressure and heart rate in WKY rats (Fig. 1).

3.2. Serum thromboxane B_2 concentrations

Both aspirin and NCX 4016 given orally (100 μmol/kg) to SHR significantly reduced the serum thromboxane B_2 levels (Fig. 2). The kinetic profile of the effect of aspirin after a single administration was more rapid than that of NCX 4016 and after 24 h, the basal serum thromboxane B_2 concentrations had decreased by 94% ($P<0.001$) and 51% ($P<0.001$), respectively. When the length of the treatments was increased from 5 to 10 consecutive days, the degree of the inhibition of serum thromboxane B_2 levels stabilized around 97% ($P<0.001$) and 77% ($P<0.001$), respectively, for aspirin and NCX 4016 (Fig. 2).

3.3. Serum nitrite/nitrate concentrations

The kinetic profile of nitrite/nitrate released by NCX 4016 (100 μmol/kg) given orally to SHR and WKY rats shows a similar trend. However, in SHR the basal levels of nitrite/nitrate was twofold higher ($P<0.01$) than that found

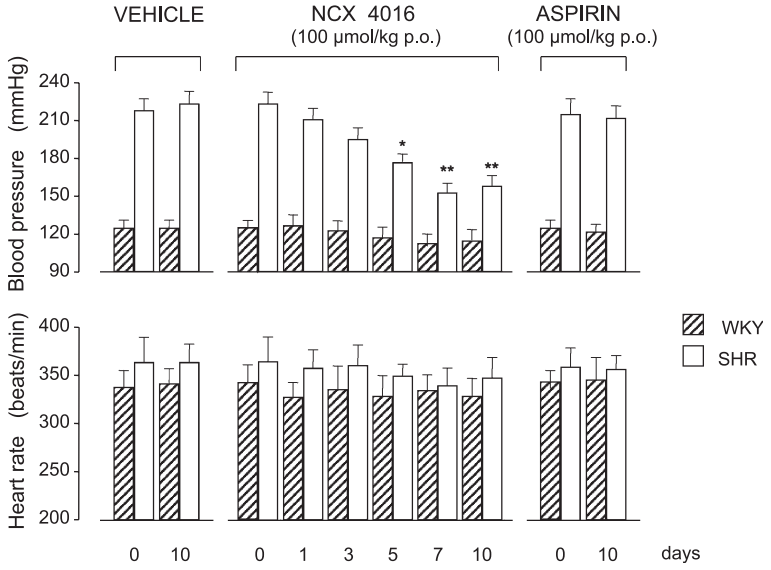

Fig. 1. Effect of NCX 4016 and aspirin treatment on systolic blood pressure and heart rate in normotensive Wistar Kyoto (WKY) rats and in spontaneously hypertensive rats (SHR). Vehicle, NCX 4016 or aspirin were administered orally once a day for 10 consecutive days. Blood pressure and heart rate were measured in the conscious state by tail-cuff method 2 h after each treatment. Values are means ± S.E.M. of ten animals per group. *$P<0.05$, **$P<0.01$ versus the corresponding time 0 (pre-treatment).

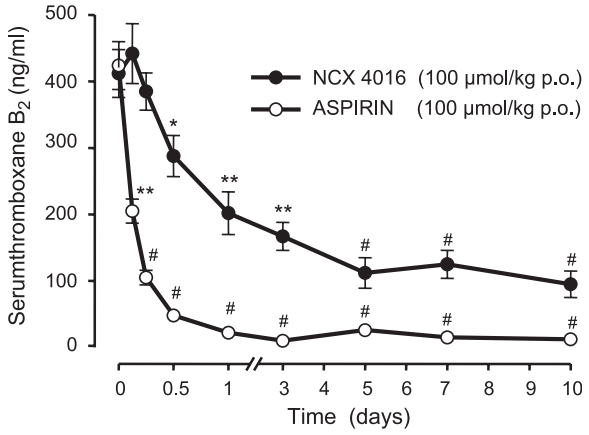

Fig. 2. Time course of the effect of NCX 4016 and aspirin treatment on serum thromboxane B_2-synthesis in spontaneously hypertensive rats. Each point is the mean ± S.E.M. of eight rats per group. *$P<0.05$, **$P<0.01$, #$P<0.001$ versus untreated rats (time 0).

in WKY rats (Fig. 3). Following a single administration of NCX 4016, there was a rapid increase of serum nitrite/nitrate concentrations which peaked at 6–12 h in both SHR (from 47 ± 2 to 222 ± 12 µM, $P<0.001$) and WKY rats (from 24 ± 3 to 178 ± 12 µM, $P<0.001$). Moreover, even when duration of the treatment was prolonged, the serum nitrite/nitrate concentration remained significantly higher than the basal values and approximately in the same µM range in SHR and WKY rats. After 10 consecutive days of NCX 4016 treatment, the serum nitrite/nitrate levels were 3.5-fold ($P<0.001$) and 4.2-fold ($P<0.001$) higher in SHR and WKY rats, respectively, as compared to the corresponding basal values (Fig. 3).

3.4. Tissue cGMP determination

The results relating to a direct cGMP measurement in segments of tail arteries taken from NCX 4016-treated SHR and WKY rats are reported in Fig. 4. NCX 4016 (100 µmol/kg p.o.) given to SHR brings about an increase of cGMP in the vascular tissue which was proportional to the length of the period of administration. In particular, after a period of 5 and 7 consecutive days of NCX 4016 treatment, the concentration of cGMP in the vascular tissue rose 2.7-fold ($P<0.01$) and 3.1-fold ($P<0.01$), respectively, over the basal values (0.32 ± 0.02 pmol/mg protein) measured in tail arteries taken from vehicle-treated SHR. A similar trend of results was obtained in tail arteries of vehicle-treated WKY rats but in these tissues, the basal values of cGMP were 1.6-fold greater ($P<0.05$) than that found in vascular segments taken from vehicle-treated SHR (Fig. 4).

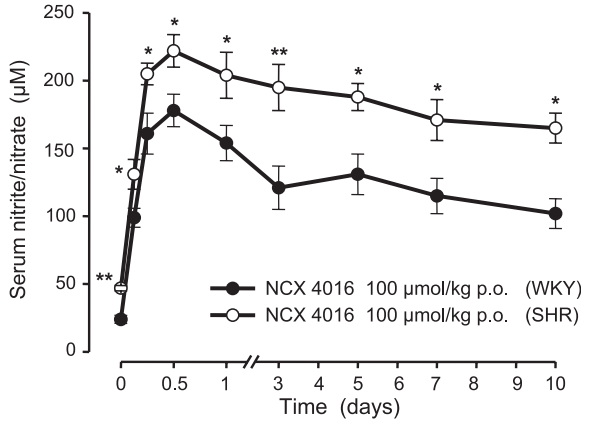

Fig. 3. Time course of the effect of NCX 4016 treatment on serum nitrite/nitrate levels in normotensive Wistar Kyoto (WKY) rats and in spontaneously hypertensive rats (SHR). Each point is the mean ± S.E.M. of eight rats per group. *$P<0.05$, **$P<0.01$ versus the corresponding time in WKY rats.

3.5. Perfused tail artery experiments

It is known that the incremental increase of flow through the arteries results in a parallel increase in arterial diameter: the role of NO in this phenomenon was conclusively demonstrated by Rubanyi et al. (1986). The present experiments, carried out with intact tail arteries explanted from vehicle-treated rats and subjected to increasing sheer forces, clearly show an endothelium-dependent vasorelaxant dysfunction of SHR as compared to WKY rats. In fact, when a number of tail arteries, removed from vehicle-treated SHR, were perfused to 5 ml/min or 10 ml/min, they developed a perfusion pressure which was 68% ($P<0.01$) and 80% ($P<0.01$) higher than that obtained in vascular tissues of WKY rats (data not shown). On the bases of these results, all the tail arteries used in these experiments were perfused at a constant flow rate of 5 ml/min.

The damage to endothelial cell function was also demonstrated in intact tail artery preparations of vehicle-treated SHR and WKY rats precontracted with norepinephrine (30

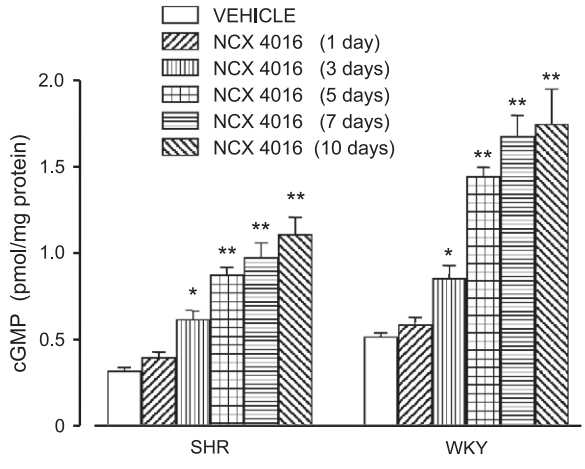

Fig. 4. Time course of the effect of NCX 4016 treatment (100 µmol/kg p.o.) on cGMP concentration (content) in tail arteries taken from spontaneously hypertensive rats (SHR) and in normotensive Wistar Kyoto (WKY) rats. Each column is the mean ± S.E.M. of eight arteries per group. *$P<0.05$, **$P<0.01$ versus vehicle-treated rats. SHR versus WKY: $P<0.05$ for all columns.

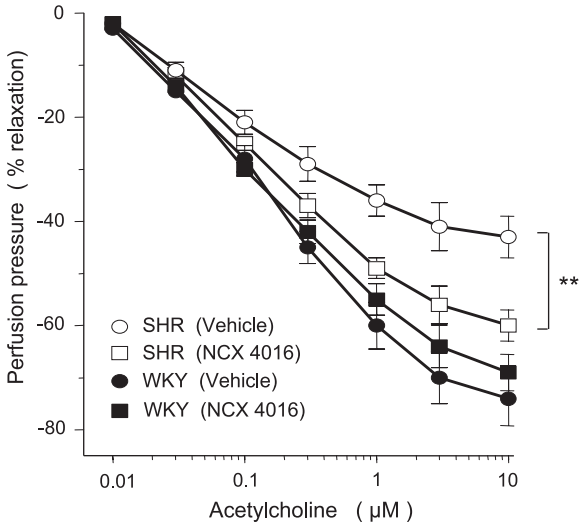

Fig. 5. Effects of NCX 4016 treatment (100 μmol/kg p.o. for 7 consecutive days) on concentration–response vasodilatory effect of acetylcholine in perfused tail artery preparations with intact endothelium from Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Relaxation was expressed as percent of the precontracted tension induced by norepinephrine (30 nM). Data are mean value ± S.E.M. of seven arteries per group. $^{**}P<0.01$.

nM) and challenged with increasing concentrations of acetylcholine (from 0.01 to 10 μM) (Fig. 5). As shown, the dilatory concentration–response curves of tail artery to acetylcholine indicated that the endothelium-dependent relaxation was impaired in arteries from vehicle-treated SHR (maximal relaxation of 42.5 ± 4.1% compared with 74.2 ± 5.2% in vehicle-treated WKY rats; $P<0.001$), whereas the sensitivity to acetylcholine, as reflected by the EC_{50}, was not altered. The NCX 4016 treatment (100 μmol/kg for 7 consecutive days) partially restored the impaired relaxation in SHR by increasing the maximal response to 60.3 ± 3.4% ($P<0.01$ versus vehicle-treated SHR). However, no change in the relaxation was induced by NCX 4016 treatment in WKY rats (Fig. 5). In denuded tail artery preparations of both vehicle-treated SHR and WKY rats, the relaxant activity of acetylcholine was almost completely lost even at the higher concentrations (data not shown).

The results regarding transmural nerve stimulation of perfused tail arteries taken from SHR and WKY rats treated orally for 7 consecutive days with NCX 4016 (10, 30 and 100 μmol/kg) are reported in Fig. 6. When subjected to transmural nerve stimulation (from 0.5 to 32 Hz), the vascular preparations of vehicle-treated SHR developed higher ($P<0.01$) perfusion pressure as compared to the arteries of vehicle-treated WKY rats. For example, at 4 Hz, the increase in perfusion pressure in tail arteries of SHR was 1.5-fold greater (60 ± 4% of the maximal response to KCl) than that obtained with vascular preparations taken from WKY rats (41 ± 2% of the maximal response to KCl). The frequency-dependent increases in perfusion pressure of tail arteries from SHR and WKY rats were antagonized in a dose-related manner by NCX 4016 treatment. The inhibitory effect of NCX 4016 was similar in potency in preparations of both SHR and WKY rats in spite of a greater sensitivity to transmural nerve stimulation of vascular segments from SHR. Particularly, at 4 Hz, the perfusion pressure values of the tail arteries taken from SHR and WKY rats treated orally for 7 consecutive days with the higher dose of NCX 4016 (100 μmol/kg) were reduced by 58% ($P<0.01$) and 46% ($P<0.01$), respectively, as compared to the values obtained in preparations of vehicle-treated SHR and WKY rats.

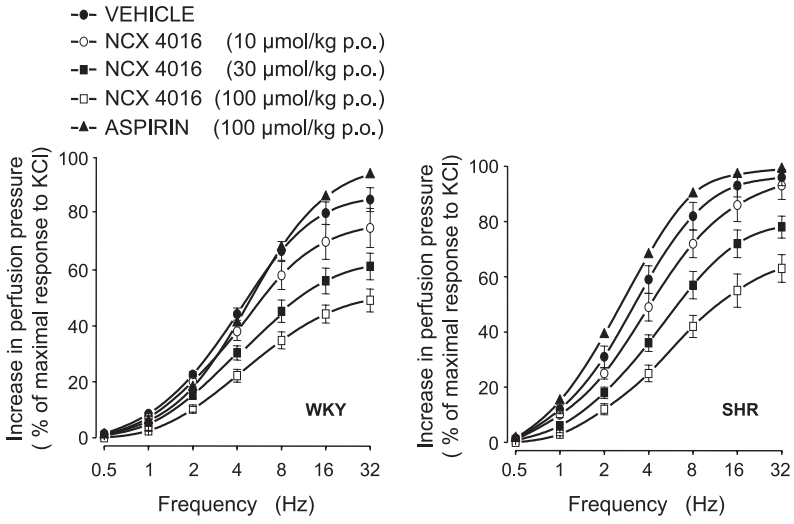

Fig. 6. Effect of NCX 4016 and aspirin treatment on frequencies–response vasoconstriction curves of transmural nerve stimulation in perfused tail artery preparations taken from Wistar Kyoto (WKY) rats (left panel) and spontaneously hypertensive rats (SHR) (right panel). Data are mean value ± S.E.M. of seven arteries per group. The vascular preparations of vehicle-treated SHR developed higher ($P<0.01$) perfusion pressure as compared to the arteries of vehicle-treated WKY rats. Statistical significance for tail arteries taken from both SHR and WKY rats: $P<0.05$ and $P<0.01$ for NCX 4016 30 and 100 μmol/kg p.o., respectively, as compared to the corresponding preparations obtained from vehicle-treated animals.

Treatment of both SHR and WKY rats with aspirin (100 μmol/kg p.o. for 7 consecutive days) did not modify the effects of transmural nerve stimulation in vascular segment preparations (Fig. 6).

Similar results were obtained by challenging the perfused tail arteries with norepinephrine (from 0.01 to 10 μM) (data not shown). In this instance, the sensitivity to norepinephrine of arterial preparations of vehicle-treated SHR was 1.7-fold greater ($P < 0.01$) than that of vascular preparations of WKY rats. In these experiments, NCX 4016 (10, 30 and 100 μmol/kg p.o. for 7 consecutive days) brought about a dose-dependent inhibition of norepinephrine-induced vasoconstriction in arteries of SHR with similar potency to that recorded in vascular preparations taken from WKY rats. Aspirin (100 μmol/kg p.o. for 7 consecutive days) did not modify the vasoconstriction induced by graded concentrations of norepinephrine in either SHR or WKY rats (data not shown).

4. Discussion

It is now well established that, among the various endothelium-derived relaxing factors, NO plays an important role in the maintenance of cardiovascular homeostasis, not only by influencing vascular tone and permeability and modulating the effects of different endothelium-derived contracting factors, but also in the long-term regulation of vascular growth and remodelling (Rudd et al., 2000). A number of studies have demonstrated that endothelium-dependent vasorelaxation is impaired in hypertensive patients (Calver et al., 1992; Panza et al., 1990) and in hypertensive rat models (Küng and Lüscher, 1995). This is particularly true in SHR during the established phase of the disease, where the reduced NO activity, due to an increased oxidative stress (Grumfeld et al., 1995; Ma et al., 2001), is well matched with the hypernoradrenergic innervation of different resistance vessels (Cassis et al., 1985, 1988; Head, 1989). The results of the present experiments clearly demonstrate that oral supplementation of SHR with NO, via the NO-releasing derivative of aspirin NCX 4016, significantly attenuated the elevated blood pressure, probably by overcoming the vascular endothelium dysfunction of these animals. However, long-term treatments with NCX 4016 to evaluate the possible tolerance treatment, which is well documented for NO donors, are needed. The finding that the antihypertensive activity of NCX 4016 in SHR was maximal after 7 days of drug administration or when a significant amount of nitrite/nitrate was present in the blood clearly indicates that NO, released by NCX 4016, was available to exert its influence on the cardiovascular system. In this regard, Carini et al. (2001) detected an increased levels of nitrosylhemoglobin in the venous blood of rats treated orally with NCX 4016 using electron spin resonance analysis.

The bioactivation of NCX 4016 and the consequent release of the aspirin-moiety are indicated by the kinetic profile of the thromboxane B_2 synthesis inhibition in the blood of SHR, an event which was particularly marked from 5 to 10 days after drug administration. This effect and the presumed blockade of prostacyclin generation in a setting of vascular endothelial dysfunction did not interfere with the antihypertensive effect of NCX 4016.

Even aspirin, when given for 10 consecutive days to SHR, did not exacerbate hypertension in spite of a prompt and sustained impairment of cyclooxygenase activity and the full suppression of thromboxane B_2 blood content. These findings are in contrast with the results of Muscarà et al. (2001), which show that aspirin aggravates hypertension induced by L-NMMA. Apart from differences in drug treatment regimen, the discrepancy with the present results could be attributed to a specific and severe dysfunction of vascular endothelium caused by L-NMMA and to a more favourable condition for aspirin to worsen hypertension, being cyclooxygenase activity impaired.

The antihypertensive activity observed in SHR with NCX 4016 may find a partial explanation in the results obtained with the perfused tail artery of these animals. This artery, like many other resistance vessels in SHR, has a larger sympathetic innervation as compared to that of WKY rats (Head, 1989). As regards to development and maintenance of hypertension, the functional significance of the enhanced sympathetic innervation of these blood vessels relates to the well-established phenomenon of vascular smooth muscle cell hypertrophy and hyperplasia, which is believed to be the pathophysiological basis for the increase in peripheral vascular resistance and elevated blood pressure in this animal model (Folkow et al., 1970). As reported in the present study, the tail arteries of SHR are characterized by endothelium-dependent vasorelaxant dysfunction unlike those explanted from the WKY rats. This phenomenon is largely due to impairment of NO production. In fact, when the tail arteries of these animals were subjected to increasing shear forces, they developed a significantly greater perfusion pressure than that obtained in tail arteries taken from WKY rats. The damage by vascular endothelial cells was clearly evident in norepinephrine-precontracted tail arteries of SHR, where the relaxant effect of acetylcholine was significantly reduced compared with the tail artery preparations of WKY rats. In line with these findings, the responses of perfused tail arteries to transmural nerve stimulation or norepinephrine (data not shown) were more significant in vehicle-treated SHR than in vascular preparations of WKY rats. It is relevant that NCX 4016 reduces the vasoconstriction responses due to transmural nerve stimulation at various frequencies or because of the different concentrations of norepinephrine, in both tail artery preparations of SHR and WKY rats. The vasorelaxant effect of NCX 4016 is concentration-dependent, and appears to be mediated by NO donation and by the activation of the soluble guanylate cyclase/cGMP pathway. Moreover, in norepinephrine-precontracted tail arteries of WKY rats, the vasorelaxant activity of NCX 4016 was markedly antagonized by the

inhibition of soluble guanylate cyclase activity via methylene blue or 1*H*-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (Rossoni et al., 2002). In addition, the concentration of cGMP in the vascular tissue of SHR increases with the duration of NCX 4016 treatment: after 7 consecutive days, it is three times the concentration of cGMP detected in the vascular tissue of vehicle-treated animals. Similar results were obtained in tail arteries of WKY rats, but the basal values of cGMP were significantly higher than those measured in vascular tissues of SHR. A decreased responsiveness to endothelium-dependent vasodilatatory substances is characteristically seen in isolated arteries from various models of experimental hypertension. However, the precise status and role of NO/cGMP pathway in these models of hypertension are not clear. Basal aortic cGMP content was found to decrease in the same model of hypertension (Arnal et al., 1993; Otsuka et al., 1988), whereas other studies have reported increased values of cGMP in the carotid arteries of SHR (Mourlon-Le Grand et al., 1992). Recently, Ruetten et al. (1999), investigating the expression and the activity of soluble guanylate cyclase in young and aging SHR, reported that the endothelium-independent relaxation of aortic rings was attenuated in response to soluble guanylate cyclase activation. This was claimed to be associated with a specific reduction of expression of both α_1 and β_1 subunits of heterodimeric soluble guanylate cyclase, with diminished contents of basal cGMP. Furthermore, it has been reported that an excess of superoxide anion production in aorta of SHR is responsible for desensitization (down regulation) of vascular components of soluble guanylate cyclase/cGMP system (Kojda et al., 1998).

Considering the antagonistic effect of NCX 4016 on the vasoconstriction induced in tail arteries of SHR by transmural nerve stimulation, an interference via NO-donation with the prejunctional mechanisms regulating norepinephrine release seems unlikely. Bucher et al. (1992) reported that during transmural nerve stimulation of rat tail artery preparations, the norepinephrine concentration in perfusates was not changed by the NO-donors, 3-morpholino-sidnonimine-*N*-ethylcarbamide (SIN-1) or by the cGMP-phosphodiesterase inhibitor (zaprinast) in spite of the vasoconstriction inhibition. However, since in the present study, the norepinephrine concentration in perfusates was not measured during transmural nerve stimulation of the vessel preparations, a modulation of perivascular adrenergic nerve activity by NCX 4016 cannot be ruled out.

Interestingly, neither aspirin nor the aspirin-moiety of NCX 4016 changed their effect on vasoconstriction, in spite of their ability to impair cyclooxygenase activity and to reduce the formation of relaxant prostaglandins: aspirin remained ineffective and the vasorelaxant effect of NCX 4016 was unmodified. It is therefore reasonable to speculate that when prostacyclin and other eicosanoids generation is inhibited, the abluminal release of NO or supplementation with NO-donor is sufficient to maintain the vascular tonus within the normal range.

The lack of beneficial effect of aspirin observed in the present study deserves a further comment, in view of the antioxidant activity of aspirin and the related restoration of aortic vasorelaxation recently reported by Wu et al. (2002). These authors claimed that through a mechanism still unknown, aspirin (100 mg/kg p.o. for 12 consecutive days) inhibits NAD(P)H oxidase activity with diminution of superoxide anion production and with consequent recovery of acetylcholine vasorelaxation in aortic tissues. The discrepancy between these findings and that obtained in perfused tail artery preparations is difficult to explain, even if the lower dose of aspirin used (100 μmol/kg p.o. for 7 consecutive days = 18 mg/kg p.o. for 7 consecutive days) could be a possible reason. Nevertheless, it is likely that the molecular mechanism underlying the inhibitory effect on NAD(P)H oxidase activity in perfused tail arteries has a different sensitivity to aspirin from that of oxidase operating in aortic vasculature.

Besides the above considerations, the fact that NCX 4016 lowers systemic blood pressure only in SHR and not in WKY rats, when serum nitrite/nitrate and vascular cGMP increase and changes in vascular reactivity after this NO donor treatment are similar in SHR and WKY rats, remains an open question. This discrepancy suggests that the mode of action of NCX 4016 in reducing blood pressure of SHR cannot be confined only to an effect on peripheral vasculature, but may involve other unidentified mechanisms which are particularly active in SHR and sensitive to exogenous NO.

Another important point, in terms of anti-thrombotic activity, emerging from the present results is the inhibitory effect of NCX 4016 observed in whole blood of SHR. NCX 4016 and the related nitro-aspirin 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)butyl ester (NCX 4215) have been reported to inhibit, in a concentration-dependent manner, thrombin-induced aggregation of platelets pre-treated with acetylsalicylic acid. This effect was reversed by oxyhaemoglobin and methylene blue acid, which further implies that the inhibitory effect is related to NO release together with an increase in cGMP levels. NCX 4016 proved to be significantly more potent than NCX 4215 in its role either as a cyclooxygenase inhibitor or as a NO donor (Del Soldato et al., 1999; Wallace et al., 1995).

In conclusion, by releasing the NO-moiety and increasing cGMP of resistance vessels, NCX 4016 reduces the sympathetic-mediated vasoconstriction and lowers the systemic blood pressure of SHR. The direct vasodilatatory activity of NCX 4016, independent of vascular endothelial cells, may not be the sole mode of action of this compound, and the antagonism against other endogenous pressor systems should be further investigated. However, the gastric safety profile (Tashima et al., 2000) and the anti-thrombotic activity (Lechi et al., 1996; Wallace et al., 1995, 1999) of NCX 4016, together with its beneficial effect observed in SHR, may open new therapeutic possibilities in cardiovascular diseases, particularly in the treatment of sustained sympathetic nerve activation.

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