Effect of antioxidant treatments on nitrate tolerance development in normotensive and hypertensive rats

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Objectives **To investigate the effect of chronic antioxidant treatments on the development of nitrate tolerance in spontaneously hypertensive (SHR) and normotensive Wistar–Kyoto (WKY) rats by evaluating (i) coronary vascular reactivity, (ii) lipid peroxidation (malondialdehyde), and (iii) peroxynitrite formation (3-nitrotyrosine).**

Methods **Tolerance was induced in 16-week-old male SHR and WKY, by 4 days of continuous treatment with nitroglycerin patches. Two groups were orally pre-treated (2-weeks) with antioxidants: *N*-acetyl-L-cysteine (NAC) or melatonin. Effects of serotonin (5-HT) and sodium nitroprusside (SNP) perfusion were tested in isolated Langendorff-perfused hearts. 3-nitrotyrosine levels were measured in coronary sinus effluent and malondialdehyde in plasma.**

Results **Nitrate tolerance reduced SNP-induced dilation in both strains. This alteration was differently improved by antioxidants: melatonin was effective in SHR, whereas NAC was effective in WKY. Tolerance also reduced 5-HT-mediated vasodilation in WKY, which was reversed by both antioxidants. By contrast, nitrate tolerance enhanced the vasoconstriction to 5-HT in SHR and both antioxidants prevented this response. Furthermore, tolerance was associated with higher malondialdehyde levels in both strains and with higher 3-nitrotyrosine levels in SHR. These changes were reversed by both antioxidants.**

Conclusions **A participation of oxidative stress was suggested during nitrate tolerance development, since antioxidants prevented the increase in lipid peroxidation and improved vascular responses to SNP and 5HT. Differential effects of antioxidants on SNP-induced vasodilation in SHR and WKY may suggest distinct mechanisms of tolerance development in hearts from hypertensive and normotensive rats. An increased peroxynitrite generation, expressed by higher 3-nitrotyrosine levels, could contribute to nitrate tolerance in the coronary circulation of SHR. *J Hypertens* 2000, 18:187–196 © Lippincott Williams & Wilkins.**

Journal of Hypertension 2000, **18**:187–196

Keywords: antioxidants, coronary vessel reactivity, free 3-nitrotyrosine, free radicals, malondialdehyde, melatonin, N-acetyl-L-cysteine, nitrate tolerance, spontaneously hypertensive rats

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Sponsorship: This research was supported by a grant from the Medical Research Council of Canada. J. deC is the holder of an associateship from the J.C. Edward Foundation, A.C is the holder of a fellowship from Rotary International Foundation, J.F.B is the recipient of a studentship from Le Fond de Recherche en Santé du Québec, E.C.D is the recipient of a studentship from Le Fond de Formation de Chercheurs et l'Aide à la Recherche and M.Le.J is the recipient of a fellowship from Medical Research Council–Canadian Society of Hypertension.

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Received 10 May 1999 **Revised** 10 September 1999
Accepted 2 November 1999

Introduction

Arterial hypertension represents a risk factor for the development of coronary artery disease as well as an aggravating factor, once symptomatic ischaemic heart disease develops. Coronary flow reserve may be negatively affected by hypertension, through the development of left ventricular hypertrophy, the acceleration of epicardial coronary atherosclerosis, and coronary microvascular alteration [1], thereby making the myocardium more vulnerable to ischaemia. Nitrate vasodilators are often used in association with beta-blocking drugs and calcium-channel antagonists in the management of ischaemic and hypertensive patients, but little is known about development of nitrate tolerance under those conditions. The major limitation of nitrate use in clinical practice to treat ischaemic normotensive patients is that the rapid development of tolerance towards their anti-ischaemic and haemodynamic effects has been widely described following chronic administration of nitrates [2]. Moreover, in *in vivo* studies, a cross-tolerance against inorganic nitrovasodilators and

Part of this work was presented at the 17th Meeting of the International Society of Hypertension held in Amsterdam, The Netherlands, 7–11 June 1998.

endothelium-dependent vasodilators was demonstrated in association with nitrate tolerance [3]. Although the exact mechanism of tolerance remains unknown, several mechanisms have been proposed [4]. Recent experimental observations have shown an increased generation of the vascular superoxide anion ($\cdot O_2^-$) in nitrate tolerance, which could enhance nitric oxide (NO) degradation [4,5]. The interaction between $\cdot O_2^-$ and NO, endothelium-derived or exogenously administered such as during nitrate therapy, produces, *in vivo*, peroxynitrite anion ($ONOO^-$) [6]. $ONOO^-$, which is a highly reactive moiety, can also alter vascular reactivity and favour vascular injury by inducing peroxidation of lipids [7] and tyrosine nitration of proteins [8] as well as by depleting cellular glutathione stores [9]. Moreover, an over-production of $\cdot O_2^-$ and a concomitant decrease of antioxidant levels have also been reported to occur in human essential hypertension [10] and in spontaneously hypertensive rats (SHR) [11,12]. These factors could facilitate tolerance development when ischaemic conditions are associated with hypertension during prolonged nitrovasodilator treatment.

The aim of the present study was to evaluate in SHR and in their normotensive Wistar–Kyoto controls (WKY) the effects of two chronic antioxidant treatments, *N*-acetyl-L-cysteine (NAC) or melatonin, on nitrovasodilator tolerance development in the coronary resistance vascular bed. This was achieved by measuring the coronary microcirculation reactivity to sodium nitroprusside (SNP), an exogenous and inorganic NO donor, to serotonin (5-HT), an endothelium-dependent NO-releasing drug, and to papaverine (PAP), an inhibitor of phosphodiesterase that increases cAMP and relaxes vascular smooth muscle. Sixteen-week-old SHRs were used because, at this age, hypertension is well-established and cardiac hypertrophy with reduced coronary reserve flow has developed. NAC, a thiol donor, which increases the glutathione stores, and melatonin, the pineal neurohormone and an endogenous antioxidant, were chosen for their recognized efficacy to scavenge different species of oxygen free radicals [13] including $ONOO^-$ [14,15]. In addition, the effects of antioxidant treatments on plasma levels of malondialdehyde, a known index of lipid peroxidation, and of free 3-nitro-L-tyrosine formation in coronary vascular beds, an index of $ONOO^-$ production [8], were measured in these studies.

Methods

Animals, treatments, and *in vivo* nitrate tolerance

All animals were housed and treated in accordance with Canadian Council on Animal Care guidelines and the protocols were approved by the Local Institutional Animal Ethics Committee. Male SHR and WKY were obtained from Charles River Laboratories (Charles River, St Constant, Quebec, Canada). Animals were housed under a 12 h light–dark cycle, at constant humidity and temperature, with free access to standard laboratory rat chow (Basal Purified Diet 5755C, Purina Mills Inc., St Louis, Missouri, USA) and drinking water. Nitrate tolerance was induced in 16-week-old rats by the continuous application of nitroglycerin patches (the size of nitroglycerin patch was adjusted to the body weight to release a dose *in vivo* of 1.5 μg/h per g body mass) to the shaved dorsal skin between the scapulae over a period of 4 days. Two groups from each strain were orally pre-treated for 2 weeks with antioxidants: either NAC (4 g/kg per day) or melatonin (30 mg/kg per day). A high dose of NAC was used in the present study because of its poor oral biovailability (4–10%) [13]. NAC and melatonin at these concentrations, in previous experiments, induced similar reductions in plasma lipid peroxidation (plasma malondialdehyde) in Sprague–Dawley normotensive rats exposed to oxidative stress. NAC was dissolved in drinking water whereas melatonin was dissolved in absolute ethanol and then added to the drinking water (the final ethanol concentration was 0.3%). NAC and melatonin solutions were freshly prepared daily and administered in dark bottles in the evening (between 2000 and 2200 h) to respect the physiological circadian rhythm of pineal hormone. No differences in daily water intake were observed between NAC-treated and melatonin-treated groups. Indirect tail-cuff measurements of systolic blood pressure (BP) were made in conscious rats by plethysmography (Harvard Apparatus Ltd, South Natick, Massachusetts, USA) during antioxidant treatments and nitrate tolerance development and recorded on a Maclab/8 system (AD Instruments Ltd, Castle Hill, New South Wales, Australia): four to five measurements were taken in each rat and then averaged. The experimental groups comprise both nitrate-tolerant and non-tolerant rats from both strains.

Experiments on non-tolerant animals

Experiments were performed on six groups of rats: control groups (SHR $n = 7$, WKY $n = 7$), NAC-treated groups (SHR-NAC $n = 9$, WKY-NAC $n = 7$) and melatonin-treated groups (melatonin-treated-SHR $n = 8$, melatonin-treated-WKY $n = 7$).

Experiments on nitrate-tolerant animals

Eight groups of rats were used: control groups (SHR $n = 7$, WKY $n = 7$), nitrate-tolerant groups: tolerant SHR ($n = 4$), tolerant WKY ($n = 4$), tolerant NAC-treated SHR ($n = 4$), tolerant NAC-treated WKY ($n = 4$), tolerant melatonin-treated SHR ($n = 4$), and tolerant melatonin-treated WKY ($n = 4$).

Heart preparation

At the end of the fourth day of nitroglycerin patch application, rats from each group were anaesthetized with CO_2 and rapidly decapitated. The chest was

quickly opened and the heart excised and immersed in ice-cold heparinized buffer (10 IU/ml). The heart was immediately mounted on the experimental set-up and perfused (Langendorff model) at constant flow by means of a digital roller pump. A 20 ml compliance chamber along the perfusion line ensured a continuous flow. The flow rate was adjusted during the stabilization period to obtain a coronary perfusion pressure of 75 mmHg and was held constant throughout the study. The flow rate was measured throughout the experiment with an in-line ultrasonic flow probe and meter (Transonic System Inc., model T106) and maintained constant during the whole experiment. The heart was perfused with a modified Krebs–Henseleit–tyrosine buffer (K-H-tyr) containing (in mmol/l): NaCl 118, KCl 4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1, $NaHCO_3$ 24, D-glucose 5, sodium pyruvate 2, and L-tyrosine 0.3. To avoid precipitation, L-tyrosine was dissolved in 1.5 ml of NaOH (0.1 mol/l) and added to 6 l of K-H-tyr buffer. The pH was maintained rigorously at 7.40. The solution was continuously gassed with 95% O_2–5% CO_2 and warmed at 37°C. L-tyrosine was added to the Krebs–Henseleit buffer in order to induce its nitration by the $ONOO^-$ released from the coronary vascular bed to form 3-nitrotyrosine measured in the coronary effluent from the heart. No detectable amount of 3-nitrotyrosine was measured when hearts were perfused with a Krebs–Henseleit buffer without L-tyrosine. The addition of L-tyrosine to the buffer did not cause alteration in coronary vascular reactivity, since similar results were observed using L-tyrosine-free Krebs–Henseleit buffer in the organ chamber. Coronary perfusion pressure was measured with a pressure transducer connected to a sidearm of the aortic perfusion cannula. All drugs were administered through a Y connector in the aortic cannula with a syringe pump (Harvard Apparatus Ltd, model 11) at 1–3% of the coronary flow rate. Adequate mixing of the drugs was ensured by the turbulent flow created in the reverse drop-shaped aortic cannula. All quoted concentrations refer to the final concentration after mixing. Data were recorded on a polygraph system (AG-620, Nihon-Kohden America Inc., Irvine, California, USA).

Experimental protocol

In this study, nitroglycerin was not used to test nitrate tolerance development in coronary microcirculation because organic nitrates such as nitroglycerin did not relax this vascular bed or did so only to a lesser degree and at very high doses because the enzymes responsible for release of NO from organic nitrates are not expressed in resistance coronary vessels [16]. Consequently, we measured coronary vascular responses to an inorganic, endothelium-independent nitrovasodilator, SNP, (3×10^{-7} to 10^{-4} mol/l) and to 5-HT (10^{-7} to 3×10^{-5} mol/l), an endothelium-derived NO releasing drug, by creating a concentration–response curve after an equilibration period of 20 min. The effect of PAP, an inhibitor of phosphodiesterase of cAMP, was also tested at a concentration (3×10^{-5} mol/l) which induced near-maximal vasodilation.

3-Nitrotyrosine: extraction and measurement

Coronary effluent (4 ml) was collected before and during determination of dose–responses curves to drugs. 3-nitrotyrosine was extracted and concentrated 40-fold to be in the detection range of this substance, which is above 0.15 μmol/l using high-performance liquid chromatography (HPLC) coupled to an ultraviolet detection system. Briefly, columns for solid-phase extraction were used; samples were injected, after adjusting the pH of the solution to 5.65 (isoelectric point of the L-tyrosine), through a conditioned C 18 cartridge (Bond Elut LRC 1211-3027, Varian, Palo Alto, California, USA), and 3-nitrotyrosine was finally eluted with 1 ml of HPLC-grade methanol. After evaporation of the solvent in a speed vacuum, the extract was redissolved in a final volume of 100 μl (equal volumes of methanol and purified water). Aliquots (30 μl) were injected into the HPLC system by a SIL-9A autoinjector (Shimadzu Scientific Instruments Inc., Columbia, Maryland, USA). The formation of 3-nitrotyrosine from L-tyrosine was analyzed by separation in HPLC (Primesphere C18 5 μm, 25 cm × 4.6 mm, Phenomenex Inc., Torrance, California, USA), detected by ultraviolet absorbance at 274 nm (Shimadzu SPD-6AV), and recorded on a Chromatography Data System (Shimadzu C-R6A). The mobile phase consisting of 220 mmol/l KH_2PO_4–H_3PO_4 (pH 3.01), 0.86 mmol/l sodium dodecyl sulphate and 10% methanol (v/v), was pumped by an isocratic system at a flow rate of 0.8 ml/min. Peaks of *p*-tyrosine, *m*-tyrosine, *o*-tyrosine and 3-nitro-*p*-tyrosine were identified on the spectrum of the co-elution, and quantified by peak height using external standards.

Plasma lipid peroxidation: assay of malondialdehyde using fluorometry

A modification of the analytical method described by Yagi involving the fluorimetric detection of malondialdehyde was used [17]. To avoid amplification of peroxidation during storage (−80°C) and assay, analysis was performed within 1 week from sampling, and 10 μl of 3 mmol/l butylated hydroxytoluene and 10 μl of 2 mmol/l desferoxamine were added to the blood sample (1 ml) collected at the time of sacrifice. Briefly, 2 ml of 4 mol/l H_2SO_4 and 0.25 ml of 10% (w/v) phosphotungstic acid were added to a tube containing 10 μl of plasma; this mixture was left at room temperature for 10 min before being centrifuged at 2000 *g* for 10 min. The supernatant was then discarded and the pellet was resuspended in a solution of 1 ml of 4 mol/l H_2SO_4 and 0.15 ml of 10% (w/v) phosphotungstic acid followed by a second centrifugation (at 2000 *g* for 10 min). The pellet was then resuspended in 2 ml of

water to which 0.5 ml of a mixture of equal volumes of 0.67% thiobarbituric acid aqueous solution and glacial acetic acid was added. This solution was heated at 95°C for 60 min in a water bath. After cooling the tubes on ice, 2.5 ml of *n*-butanol was added and the mixture was agitated on a vortex for 30 s. Finally, the solution was centrifuged again (2000 *g* for 10 min at room temperature) and the butanol layer transferred for fluorometric detection (Fluorimeter Model 650, Perkins-Elmer, Norwalk, Connecticut, USA) at a wavelength of 553 nm with excitation at 515 nm against an appropriate blank. A standard curve of malondialdehyde was prepared from different concentrations of tetraethoxypropane, which yielded equimolar amounts of malondialdehyde. All the assays were done in triplicate.

Statistics

All values are expressed as means ± SEM. The statistical comparison of results was done by one-factor repeated-measures analysis of variance or by Student's paired or unpaired *t* test when appropriate. Concentration–response curves were analysed in order to compare EC_{50} and maximal relaxation response by a curve-fitting analysis program (GraphPad Prism 2.01, GraphPad Inc., USA). EC_{50} values were expressed as the negative logarithm of the concentration, which produces 50% of the maximal response to each drug. $P < 0.05$ was considered statistically significant.

Chemicals

Nitroglycerin patches were obtained in the form of commercially available preparations (Top-Nitro patch, Schering-Plough Spa, Milan, Italy). All other drugs and components of solutions were purchased from Sigma Chemical Co. (St Louis, Missouri, USA).

Results

Blood pressure, heart weight and body weight

Systolic arterial blood pressure was higher in 16-week-old SHR than in WKY ($P < 0.01$) and was not affected by tolerance development or by antioxidant treatments in both strains, as indicated in Table 1. Mean body weight was significantly lower in all SHR groups than in age-matched WKY ($P < 0.05$); 2 weeks of NAC and melatonin treatment did not alter body weights in either strains (Table 1). Heart/body weight ratio was significantly increased in all groups of SHR as compared to WKY ($P < 0.05$), suggesting that cardiac hypertrophy is well established in SHR at this age (Table 1).

Lipid peroxidation: plasma malondialdehyde and effect of NAC and melatonin

Tolerance development induced a significant rise in plasma malondialdehyde in both strains (tolerant SHR $+31 \pm 7\%$ versus SHR, $P < 0.05$; tolerant WKY $+12 \pm 2\%$ versus WKY, $P < 0.05$) as shown in Figure 1. The plasma malondialdehyde level was similar in untreated 16-week-old SHR and WKY. NAC treatment significantly decreased malondialdehyde levels both in tolerant and non-tolerant hypertensive (tolerant NAC-treated SHR versus tolerant SHR, $P < 0.01$; NAC-treated SHR versus SHR, $P < 0.01$) and normotensive rats (tolerant NAC-treated WKY versus tolerant-WKY, $P < 0.01$; NAC-treated WKY versus WKY, $P < 0.01$) (Fig. 1). Melatonin also significantly reduced plasma malondialdehyde in both strains (tolerant melatonin-treated SHR versus tolerant SHR, $P < 0.05$; melatonin-treated SHR versus SHR, $P < 0.05$; tolerant NAC-treated WKY versus tolerant WKY, $P < 0.05$; NAC-treated WKY versus WKY, $P < 0.05$) as depicted in Figure 1.

3-Nitrotyrosine in coronary effluent: effect of NAC and melatonin

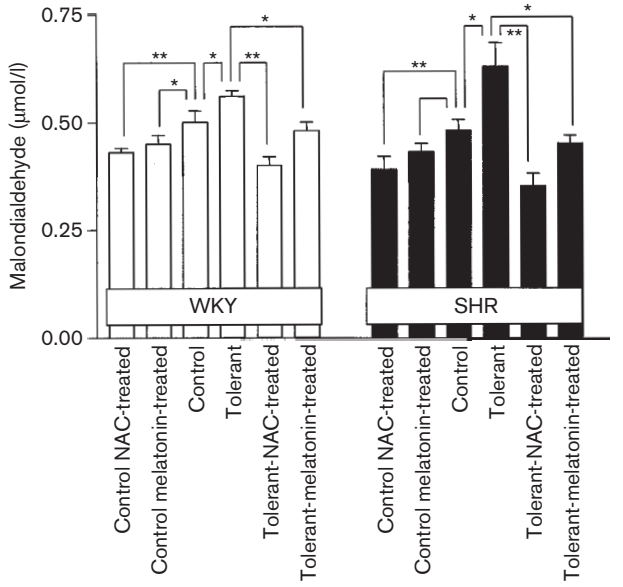
No differences in 3-nitrotyrosine formation were detected among untreated, NAC-treated and melatonin-treated WKY and SHR (as shown in Fig. 2). Tolerance

Table 1 Systolic blood pressure, heart weight, body weight and heart–body weight ratio in all groups

Groups	Systolic blood pressure (mmHg)	Heart weight (mg)	Body weight (g)	Heart/body weight ratio (mg/g)
WKY	128.9 ± 1.1	2001 ± 66	332 ± 6.6	6.0 ± 0.2
NAC-treated WKY	126.3 ± 1.8	2068 ± 91	339 ± 7.5	6.1 ± 0.2
Melatonin-treated WKY	128.9 ± 1.1	1961 ± 87	323 ± 5.8	6.1 ± 0.2
Tolerant WKY	127.8 ± 2.0	2087 ± 96	336 ± 10.3	6.2 ± 0.1
Tolerant NAC-treated WKY	127.7 ± 1.4	1957 ± 186	333 ± 5.1	5.9 ± 0.5
Tolerant melatonin-treated WKY	126.5 ± 0.7	2112 ± 83	338 ± 7.8	6.2 ± 0.1
SHR	187.7 ± 2.8**	1814 ± 64	258 ± 5.6*	7.0 ± 0.2*
NAC treated SHR	184.1 ± 2.1**	1850 ± 81	266 ± 7.3*	6.9 ± 0.3*
Melatonin-treated SHR	190.6 ± 2.9**	1798 ± 54	255 ± 4.8*	7.0 ± 0.2*
Tolerant SHR	185.7 ± 1.1**	1812 ± 92	254 ± 6.8*	7.1 ± 0.5*
Tolerant NAC-treated SHR	185.6 ± 2.0**	1787 ± 79	251 ± 4.2*	7.1 ± 0.3*
Tolerant melatonin-treated SHR	183.6 ± 2.1**	1881 ± 60	251 ± 3.7*	7.5 ± 0.3*

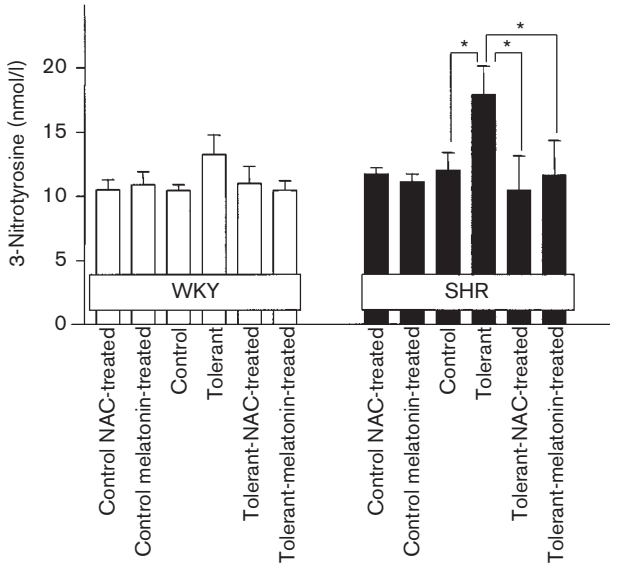
Values are mean ± SEM. * $P < 0.05$, ** $P < 0.01$, significantly different from respective WKY group.

Fig. 1



Bar graph showing plasma malondialdehyde in all studied groups of 16-week-old Wistar–Kyoto rats (WKY, open bars) and age-matched spontaneously hypertensive rats (SHR, solid bars). * $P < 0.05$, ** $P < 0.01$.

Fig. 2



Bar graph showing free 3-nitrotyrosine levels in coronary effluent from isolated hearts of all studied groups of 16-week-old Wistar–Kyoto rats (WKY, open bars) and age-matched spontaneously hypertensive rats (SHR, solid bars). * $P < 0.05$, ** $P < 0.01$.

development in SHR induced a 50% rise in free 3-nitrotyrosine levels in coronary effluent (tolerant SHR versus SHR, $P < 0.05$) whereas no significant differences were found between tolerant WKY and WKY (Fig. 2). In tolerant NAC-treated SHR and tolerant melatonin-treated SHR significant decreases ($P < 0.05$) in free 3-nitrotyrosine formation were measured as compared to tolerant SHR, suggesting a protective role of both antioxidants in reducing $ONOO^-$ release and consequently the tyrosine nitration (Fig. 2). By contrast, melatonin and NAC did not significantly alter 3-nitrotyrosine levels in tolerant WKY. Moreover, no significant differences in free 3-nitrotyrosine were observed during 5-HT, PAP and SNP perfusions in all groups.

Effects of NAC and melatonin on endothelium-independent reactivity

Response to sodium nitroprusside

Coronary vascular relaxations to SNP in isolated perfused hearts from all studied groups are represented as dose–response curves in Figure 3, and the respective parameters are shown in Table 2. An increased maximal relaxation to SNP, without a change in sensitivity, was observed in control untreated SHR as compared to WKY (Fig. 3 and Table 2). NAC and melatonin treatments did not affect dose–response curves as compared to their respective non-tolerant SHR and WKY. Tolerance development induced a rightward shift of the dose–response curves in both WKY and SHR strains suggesting the presence of cross-tolerance against the inorganic nitrovasodilator SNP. Chronic NAC treatment improved the sensitivity without changes in maximal relaxation in tolerant SHR (Fig. 3, right panel and Table 2). A complete prevention of tolerance development was achieved by NAC in tolerant NAC-treated WKY, improving both the sensitivity and maximal relaxation induced by tolerance (Fig. 3, left panel and Table 2). By contrast, melatonin treatment almost completely reversed the negative effect of tolerance development in tolerant SHR on maximal relaxation and sensitivity (Fig. 3, right panel and Table 2). However, no protective effect was observed with melatonin treatment in tolerant WKY (Fig. 3 and Table 2).

Response to papaverine

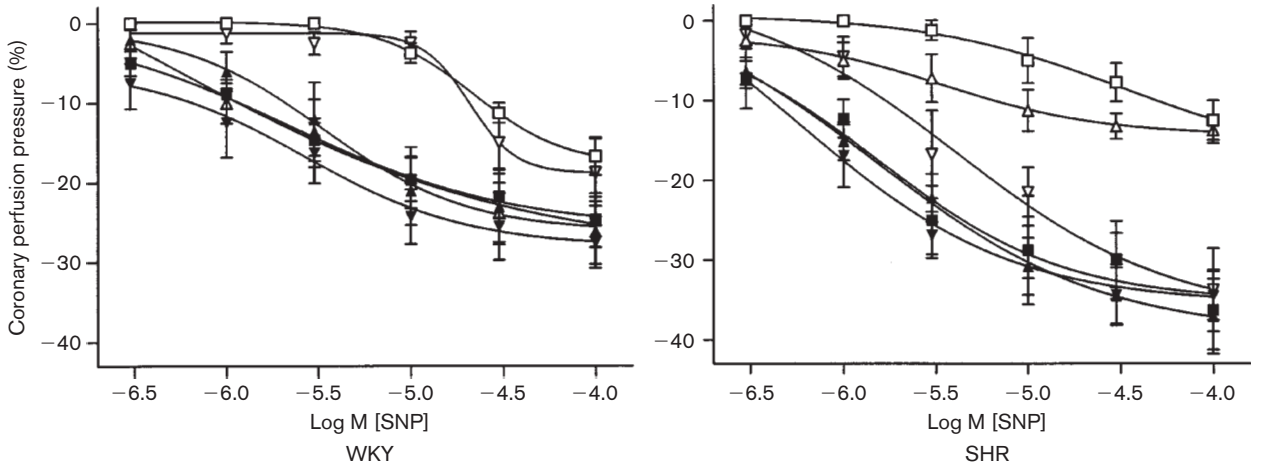
The degree of coronary relaxation by PAP, which relaxes vascular smooth muscle by inhibiting cAMP-phosphodiesterase, did not differ significantly among tolerant, non-tolerant, NAC-treated and melatonin-treated WKY and SHR as represented in Table 2.

Effects of NAC and melatonin on endothelium-dependent reactivity

Response to serotonin

In the hearts of non-tolerant WKY, 5-HT induced a dilation of coronary microcirculation that was unchanged by chronic NAC and melatonin treatments. The 5-HT-induced coronary vasodilation observed in non-tolerant WKY was completely abolished by tolerance development and replaced by a vasoconstrictive response: both antioxidants prevented the development

Fig. 3



Dose–response curves showing endothelium-independent responses to sodium nitroprusside (SNP) in hearts from 16-week-old Wistar–Kyoto rat (WKY) groups (left panel: WKY (■); NAC-treated WKY (▲); melatonin-treated-WKY (▼); tolerant WKY (□); tolerant NAC-treated WKY (△); tolerant melatonin-treated WKY (▽) and age-matched spontaneously hypertensive rat (SHR) groups (right panel: SHR (■); NAC-treated SHR (▲); melatonin-treated SHR (▼); tolerant SHR (□); tolerant NAC-treated SHR (△); tolerant melatonin-treated SHR (▽).

Table 2 NAC and melatonin effects on coronary reactivity (EC_{50}: expressed as the negative logarithm of the concentration, and maximum relaxation or contraction as percentage of the basal tension) to nitroprusside, papaverine and serotonin in all groups

	Sodium nitroprusside		Papaverine	Serotonin	
Groups	EC_{50} (−log)	Maximum relaxation (%)	Maximum relaxation (%)	EC_{50} (−log)	Maximum relaxation (r) or contraction (c) (%)
WKY	$5.66 \pm 0.11^{**}$	$24.3 \pm 2.0^{*}$	10.9 ± 2.2	5.65 ± 0.17	13.6 ± 3.7 (r)**
NAC-treated WKY	5.49 ± 0.14	26.0 ± 4.2	9.1 ± 2.3	5.71 ± 0.11	15.9 ± 3.5 (r)
Melatonin-treated WKY	5.40 ± 0.09	27.5 ± 3.2	10.7 ± 1.1	5.83 ± 0.08	14.0 ± 3.3 (r)
Tolerant WKY	4.66 ± 0.03	16.2 ± 2.4	11.3 ± 2.3	5.72 ± 0.27	11.7 ± 4.8 (c)
Tolerant NAC-treated WKY	$5.74 \pm 0.17^{**}$	$24.7 \pm 2.3^{*}$	11.3 ± 1.2	5.49 ± 0.14	17.5 ± 4.3 (r)**
Tolerant melatonin-treated WKY	4.68 ± 0.07	12.2 ± 5.5	9.9 ± 2.0	5.12 ± 0.43	12.2 ± 4.4 (r)**
SHR	$5.83 \pm 0.18^{**}$	$36.1 \pm 5.0^{**,\dagger}$	11.2 ± 1.2	6.39 ± 0.13	32.1 ± 2.8 (c)***
NAC treated SHR	5.81 ± 0.10	37.0 ± 4.7	12.6 ± 1.0	$5.80 \pm 0.04^{\ddagger}$	18.0 ± 3.4 (c)‡
Melatonin-treated SHR	5.75 ± 0.13	34.5 ± 3.1	10.5 ± 2.1	$5.74 \pm 0.03^{\ddagger}$	15.1 ± 3.2 (c)‡
Tolerant SHR	4.75 ± 0.07	12.5 ± 2.3	12.7 ± 2.2	6.26 ± 0.22	64.4 ± 4.1 (c)
Tolerant NAC-treated SHR	$5.41 \pm 0.13^{*}$	13.7 ± 1.6	10.7 ± 3.6	6.14 ± 0.16	26.2 ± 4.3 (c)***
Tolerant melatonin-treated SHR	$5.40 \pm 0.08^{*}$	$33.7 \pm 5.2^{*}$	13.2 ± 3.3	5.93 ± 0.12	37.5 ± 5.9 (c)**

Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from respective tolerant WKY ot tolerant SHR; $^{\dagger}P < 0.05$ from WKY; $^{\ddagger}P < 0.05$ from SHR.

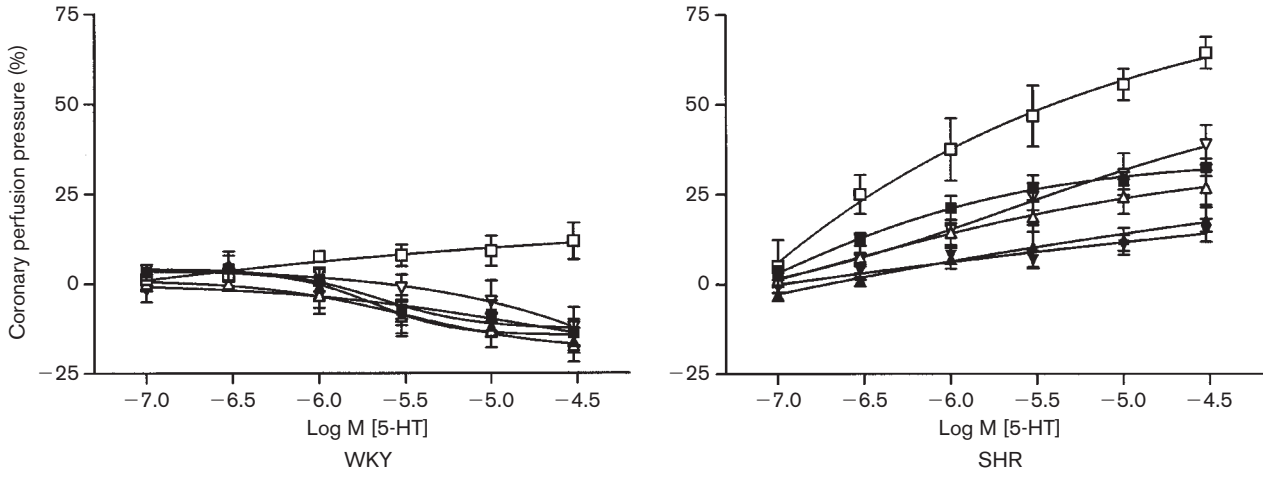
of 5-HT-induced constriction and restored the vasorelaxant effects of 5-HT (Fig. 4, left panel and Table 2). By contrast, in SHR, 5-HT induced a constriction of the coronary circulation that was reduced by the antioxidant treatments; the vasoconstriction observed in non-tolerant SHR was enhanced by tolerance development but this potentiation was prevented by both antioxidant treatments (Fig. 4, right panel and Table 2).

Discussion

The present study evaluated the effects of nitrate tolerance on coronary resistance circulation under both normotensive and hypertensive conditions. Despite the limitation of extrapolating the present findings obtained in rat to other species, the dose of nitrates used in this study may have clinical relevance to the dose of nitrovasodilator used in unstable angina and in heart failure. To date, the majority of experimental and human studies have evaluated the phenomenon of tolerance in conductance vessels, aortas or large epicardial arteries [3,18,19]. In fact, coronary resistance vessels are generally insensitive or less sensitive to nitroglycerin and other organic nitrates than conductance and capacitance vessels. For this reason, in this study, an inorganic nitrovasodilator such as SNP, which directly releases NO, without the enzymatic biotransformation required by nitroglycerin, was used [16].

An increased maximal coronary vasodilation to SNP was observed in non-tolerant SHR compared with WKY. The increase in expression of the soluble guanylate cyclase, the target enzyme of NO, reported by

Fig. 4



Dose–response curves showing endothelium-dependent responses to serotonin (5-HT) in hearts from 16-week-old Wistar–Kyoto rat (WKY) groups (left panel: WKY (■); NAC-treated WKY (▲); melatonin-treated WKY (▼); tolerant WKY (□); tolerant NAC-treated WKY (△); tolerant melatonin-treated WKY (▽) and age-matched spontaneously hypertensive rat (SHR) groups (right panel: SHR (■); NAC-treated SHR (▲); melatonin-treated SHR (▼); tolerant SHR (□); tolerant NAC-treated SHR (△); tolerant melatonin-treated SHR (▽).

Papapetropoulos *et al.* [20], in vascular smooth muscle cells of SHR as compared to WKY, supports our data in coronary resistance vessels. This finding may represent a compensatory response against the increased coronary vascular resistance that is present in 16-week-old SHR. In contrast to our observations, other studies have reported decreased or unchanged relaxation responses to SNP in SHR, but those observations were made in conductance arteries [21,22]. Moreover, we also demonstrated that chronic *in vivo* antioxidant treatments either with NAC or melatonin did not alter the relaxation to SNP in the coronary microcirculation of both non-tolerant SHR and WKY. These findings are in line with previous reports showing that other free radical scavengers, such as ascorbic acid and glutathione, did not change endothelium-independent SNP-mediated vasorelaxation [23].

The present study shows that 4 days of continuous nitroglycerin treatment produced, in coronary resistance vessels of both strains, a cross-tolerance to SNP, as well as to serotonin, an endothelium-dependent NO-releasing drug. The cross-tolerance phenomenon against different nitrovasodilators has been debated for a long time and this phenomenon generally occurs when nitrates are administered *in vivo* [3,5]. Previous evidence has suggested that an over-production of superoxide anion and free radicals could be responsible for the reduced biological life of SNP-derived NO during nitrate tolerance development in both strains, but even more in SHR, resulting in a lesser activation of vascular smooth muscle guanylate cyclase and thus in a reduced relaxation [4,11,12]. This hypothesis is supported by our data demonstrating for the first time an increased generation of peroxynitrite anion, the product of the reaction between NO and $\cdot O_2^-$, in the coronary vascular bed of tolerant SHR as reflected by the higher level of 3-nitrotyrosine in the coronary effluent. The increased generation of $ONOO^-$, which is a weaker stimulator of guanylate cyclase than NO, could result in a reduced production of cyclic GMP and in an impaired vasodilation of coronary bed of those animals [24].

Differences in the effects of the antioxidant treatments were observed for SNP-mediated vasodilation between SHR and WKY: NAC was more effective in preventing the impaired vasodilation to SNP in tolerant WKY than in tolerant SHR, and, conversely, melatonin was efficient in preventing the impaired vasodilation only in tolerant SHR. Although NAC has been shown, in some studies, to reverse nitrate tolerance, controversies still remain about its efficacy in preventing nitrate tolerance development under different pathological conditions [25,26]. Results from *in vivo* experiments in normotensive animals demonstrated that the depletion of vascular thiols, previously thought to be one of the mechanisms responsible for the nitrate tolerance, is not involved, but that an alteration in the cellular thiol turnover could be implicated [27]. Apart from the early studies by Needleman *et al.* [28] who first proposed the presence of thiol as a necessary mechanism for NO donor-mediated vasodilation, little is known about the enzymatic or alternative pathways for formation of *in vivo S*-nitrosothiols, which are the likely intermediate metabolites responsible for the guanylate cyclase activation [29]. Whether the biochemical pathways of *S*-

nitrosothiol production in tolerant SHR are less efficient because of reduced availability of a specific thiol subcellular pool secondary to rapid oxidation by peroxynitrite remains to be clarified. However, since NAC could prevent the rise in free 3-nitrotyrosine levels from coronary effluent in tolerant SHR, this may suggest that reactive oxygen or nitrogen species other than peroxynitrite are probably involved in this phenomenon.

Melatonin, which was recently proven to be a very efficient direct endogenous scavenger of different reactive species including hydroxyl radical and peroxynitrite [14,15], did not affect SNP-mediated relaxation in non-tolerant SHR and WKY and prevented the development of cross-tolerance to SNP in SHR but not in WKY. The beneficial effects of melatonin, whose plasma levels were found to be decreased in SHR [30], could depend on its capacity to increase the activity and the expression of cytoplasmic and mitochondrial superoxide dismutases and glutathione peroxidase, particularly in situations of increased oxidative stress [31]. By contrast, the lack of efficacy of melatonin in preventing the impairment of vasodilation to SNP in tolerant WKY may be due to the fact that the antioxidant enzymatic defences are less stimulated by tolerance, or that different reactive species are generated in tolerant WKY.

A cross-tolerance to serotonin, a drug that produces its vasodilator effects in coronary resistance vessels via the endothelium-dependent release of NO, was also observed. The loss of NO-dependent vasodilation resulted in a coronary vasoconstriction in tolerant WKY rather than the vasodilation observed in non-tolerant WKY. A similar mechanism may be responsible for the increased vasoconstriction observed in the coronary circulation of tolerant SHR as compared to non-tolerant SHR. Since the vascular effects of serotonin depend on the presence and integrity of the endothelium, the vasoconstriction to serotonin observed in non-tolerant SHR is consistent with the impaired vasodilatory function of coronary microvascular endothelium in this model of hypertension. Moreover, in this study, we observed a reduction of the 5-HT-induced vasoconstriction in non-tolerant SHR treated with antioxidants, whereas no differences in 5-HT-induced vasodilation were found among treated and untreated non-tolerant WKY. The increased free radical production seems to be responsible for the enhanced vasoconstriction to serotonin observed in both strains after tolerance development, but also in non-tolerant SHR, because protective effects were demonstrated with both antioxidant treatments. Several mechanisms may be postulated for the protection achieved by antioxidants on the endothelium-dependent vascular reactivity: first, the possibility of an increase in the biological activity of NO and in prostacyclin synthesis, which are both negatively affected by free radicals [32]; second, possibly by reduction of the malondialdehyde level which has been recently reported to improve the endothelium-dependent relaxation in rat tail arteries [33]; third, through the repletion of antioxidant enzymatic (superoxide dismutases and glutathione peroxidase) and non-enzymatic (thiol stores) cellular systems by treatment with NAC and melatonin [31].

In contrast to the above findings, the development of cross-tolerance and antioxidant treatments did not alter coronary vessel dilatation to PAP, which relaxes smooth muscle by inhibiting phosphodiesterase and by increasing cAMP. This latter finding is concordant with previous studies on conductance arteries which showed that forskolin and other agents acting via adenylyl cyclase are not affected by nitroglycerin tolerance or increased free radical generation [4,5].

In the present experiments, an increased generation of $ONOO^-$, expressed by the enhanced free 3-nitrotyrosine concentration, is reported for the first time in the coronary vascular bed of tolerant SHR but not in tolerant WKY. However, no differences in basal free 3-nitrotyrosine levels were found between non-tolerant SHR and WKY. Nevertheless, a difference in $ONOO^-$ release between SHR and WKY cannot be excluded, since the protein-bound 3-nitrotyrosine concentration was not assessed in the present study and an increase in protein-bound 3-nitrotyrosine in aortas from aortic banding-induced hypertensive rats was recently demonstrated [34]. Neither treatment with NAC nor melatonin changed the free 3-nitrotyrosine level in non-tolerant rats of both strains, but they prevented the rise of free 3-nitrotyrosine in coronary effluent of tolerant SHR. These results obtained after the chronic oral administration of antioxidants are in agreement with recent *in vitro* studies showing a protection by thiol donors and melatonin against peroxynitrite-induced tissue toxicity [15] and specifically the nitration of tyrosine [35].

An increase in plasma lipid peroxidation as reflected by higher malondialdehyde levels may be suspected in both tolerant rat groups. The rise was greater in tolerant SHR (+31%) than in tolerant WKY (+12%), whereas no differences in malondialdehyde were observed between non-tolerant SHR and WKY. These observations suggest that free radical production is increased after tolerance development since antioxidants reversed the rise of malondialdehyde observed in tolerant rats. A smaller decrease in malondialdehyde levels was also observed in control rats following antioxidant therapies. Previous studies reporting a protective effect of NAC and melatonin against lipid peroxidation induced by exogenous oxidative stressors

support our observations [36,37]. Although a higher oxidative stress was postulated in SHR, SHR showed plasma malondialdehyde levels comparable to their WKY counterparts in our study. Other investigators have reported similar basal levels of malondialdehyde in SHR and WKY tissues [38], although increased lipid peroxidation in plasma and vascular intima was observed in SHR [39]. Some methodological and biological factors could explain these apparent contradictions in SHR. In fact, no single analytical method can give an accurate account of total lipid peroxidation even when optimizing the measure by fluorometric detection used in the present study. Further, a variation in tissue oxidative balance may yield different values for malondialdehyde release from different tissues to plasma in SHR and WKY, but individual differences would be attenuated by a dilution in the plasma volume.

In summary, our results show in the coronary resistance circulation that a cross-tolerance towards exogenous or endogenous NO-dependent vasodilators develops in SHR and WKY after chronic treatment with nitroglycerin. Tolerance is associated with an increased plasma lipid peroxidation both in SHR and WKY and with an increased generation of peroxynitrite in the coronary vascular bed of hypertensive rats. The clinical relevance of these observations is not yet known, but these findings might suggest differences in the development of tolerance in normotensive and hypertensive conditions.

Acknowledgements

The authors gratefully acknowledge Mrs Jo-Anne Le Guerrier and Diane Papin for their expert technical assistance and Mrs Carole Champagne for her editorial assistance.

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