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ORGAN TOXICITY AND MECHANISMS

Effects of chronic 4-n-nonylphenol treatment on aortic vasoconstriction and vasorelaxation in rats

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Abstract 4-Nonylphenol (para-nonylphenol, 4-NP), metabolites including linear and branched isoforms of nonylphenol (n-NP and t-NP, respectively), has been considered an endocrine disrupting substance resulting in reproductive dysfunction and increasing reactive oxygen species production in testis, liver, kidney, and brain. However, to date, whether vasculature is susceptible to NP exposure remains to be unclear. In this study, we have investigated the effects of chronic in vivo 4-n-NP exposure on vasoconstriction and vasorelaxation in male rats. After a 20-week 4-n-NP treatment orally at the dosage of 10 and 50 μM in the drinking water, phenylephrine- and potassium chloride-induced concentration-dependent responsiveness assessed by wire myograph were both significantly higher in aorta isolated from 4-n-NP-treated rats compared with control rats, but acetylcholine-induced vasorelaxation was similar between these two groups. In addition, systemic oxidative stress and vascular, but not intestinal, oxidant enzyme activities assessed by lucigenin-amplified chemiluminescence were all markedly higher in 4-n-NP-treated rats. In conclusion, our results suggested that chronic in vivo 4-n-NP exposure augments vascular contractile responsiveness through enhanced vascular oxidant enzyme activity.

Keywords Nonylphenol · Vascular function · Oxidative stress

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Introduction

4-Nonylphenol (*para*-nonylphenol, 4-NP), one of the major degradation metabolites derived from alkylphenol polyethoxylates (AP_nEs) used as plastic additives or surfactants, is first discovered in wastewater treatment plants (Giger et al. 1984) and it is considered an xenoestrogen or endocrine disrupting substance (Sonnenschein and Soto 1998). Furthermore, the alkyl chains in 4-NP can exist as either linear or complex branched alkyl isomers called normal nonylphenol (n-NP) or technical nonylphenol (t-NP), respectively. In addition, the evidence has suggested that branched and non-branched 4-NP interact differently with ligand-binding cavity of the estrogen receptor (Tabira et al. 1999).

4-NP has been found ubiquitous (Ekelund et al. 1993) and its accumulation in plants and animals has been demonstrated to influence sexual and reproductive development (Ahel et al. 1993; Ekelund et al. 1990; Nagao et al. 2000). 4-NP also migrates from polyvinyl chloride food packing films into foods (Inoue et al. 2001). The average daily intake of 4-NP is different among countries with markedly higher intake in Taiwan compared with that in Germany and New Zealand (Lu et al. 2007). Increasing evidences have shown that 4-NP exposure elevates oxidative stress in various tissues such as testes, liver, kidney, brain, and neutrophils (Gong and Han 2006; Obata and Kubota 2000; Okai et al. 2004). However, whether vascular system is susceptible to 4-NP exposure has not been elucidated.

Increased production of reactive oxygen species (ROS), such as hydroxyl radicals, peroxynitrite, superoxide anion and hydrogen peroxide, contributes to an elevated oxidative stress which is a well-documented risk factor in the development of cardiovascular diseases (Faraci 2006). Nicotinamide adenine dinucleotide phosphate (NADPH)-related oxidase (Li and Shah 2003) and xanthine oxidase (Cai 2005) are intracellular oxidant enzymes responsible for ROS generation, and their activities can be represented indirectly by the ROS production in the presence of NADPH or xanthine using lucigenin-based chemiluminescence assay (Berry et al. 2000; Yu et al. 2006). Although other studies have demonstrated that 4-NP increased oxidative stress, whether 4-NP exposure affects vascular NADPH-related oxidase or xanthine oxidase activity remains unclear.

Enhanced vascular contractile responsiveness and decreased vascular endothelium-dependent relaxation both contribute to vascular dysfunction (Luscher 1994). Increasing evidence has shown that ROS contributes to vasoconstriction induced by phenylephrine (Matsumoto et al. 2006) or by a voltage-gated calcium channel opener (Gordeeva et al. 2003). Moreover, elevated ROS levels could diminish acetylcholine-mediated endothelium-dependent vasorelaxation. Therefore, altering vascular oxidative stress status will affect vascular function.

The objectives of this study were to investigate that whether chronic in vivo 4-n-NP exposure affects vascular function (vasoconstriction and vasorelaxation) and whether vascular NADPH-related and xanthine oxidase are involved in the reaction.

Materials and methods

Animals

Thirteen male Sprague Dawley (SD) rats were obtained from BioLASCO Taiwan Co., Ltd (Ilan, Taiwan, ROC), and housed in a 12:12 h light–dark cycle. Owing to the low solubility of 4-n-NP, it was dissolved in ethanol as a 50-mM stock solution and then added in the drinking water at the dosage of 10 and 50 μM for 4-n-NP-treated rats ($N = 8$). Control rats ($N = 5$) were treated with vehicle solvent (0.1% ethanol) added in the drinking water. Based on our preliminary studies, both in vitro and in vivo 0.1% ethanol treatment had little effects on isolated aortic vasoconstriction and vasorelaxation (data not shown). Eight-week old rats were treated with 4-n-NP or vehicle for 20 weeks. At the end of the study, peripheral venous blood from the tail was obtained to assess the oxidative stress index. In addition, testes were harvested for the measurement of the testes to body weight ratio, and the aorta was isolated for the evaluation of vasoconstriction and vasorelaxation. Aorta and intestine were also collected for the quantification of tissue ROS contents. All animals were humanely treated throughout the experiment which was approved by the Committee on the Use and Care of Animals, National Pingtung University of Science and Technology, Taiwan.

Aortic contractile and relaxant response

Thoracic aorta was isolated, cleaned, and cut into slices of 2 mm in length for evaluating the contractile and relaxant response as previously reported (Yen and Lau 2002). Briefly, aortic rings were carefully mounted on an isometric force transducer (XDFT05, Singa, Taiwan) with a tension of 1.8 g, and placed in an organ chamber filled with Krebs solution (NaCl, 99.01 mM; KCl, 4.69 mM; $CaCl_2$, 1.87 mM; $MgSO_4$, 1.20 mM; K_2HPO_4, 1.03 mM; glucose, 11.1 mM) maintained at pH 7.4 and bubbled with 95% O_2–5% CO_2. After an equilibration of 90 min, 10 μM of phenylephrine (PE) was added to the organ chamber for the assessment of contractile activity, and then 30 μM of acetylcholine (ACh) was added to assess the endothelial integrity. Rings with PE-induced tension <0.3 g or ACh-induced relaxation <10% of pre-contractile tension were discarded. After washing and a re-equilibration for 30 min, a cumulative potassium chloride dose (from 10 to 110 mM) was applied to obtain a concentration-dependent contractile curve. After washing and re-equilibration, PE (1 μM) was used to induce vasoconstriction, and then a cumulative ACh dose (from 10 nM to 30 μM) was added to the organ chamber to obtain a concentration-dependent relaxant curve. Similarly, a cumulative PE dose (from 0.1 nM to 10 μM) was applied to obtain a concentration-dependent contractile curve. All data were acquired and analyzed using the XctionView system (XctionView, Singa, Taiwan).

Oxidative stress index measurement

After a 20-week treatment, blood was collected from the tail veins in the conscious state for the measurement of total

peroxides level and total antioxidant capacity using free-oxygen radicals test (FORT) and free-oxygen radical defense test (FORD) kit according to the manufacture's instruction, respectively. Briefly, 10 μL of whole blood was mixed with Reagent 2 of FORT to produce a colorimetric reaction based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides into derivative radicals according to Fenton's reaction. Samples were then mixed with Reagent 1 containing chromogen, an amine derivative. Derivative radicals were trapped by the chromogen and developed, in a linear kinetic-based reaction at 37°C, fairly long-lived colored radical cations that were photometrically detectable. Therefore, the intensity of the color directly correlated with the levels of radical compounds produced. The absorbance values were then converted into conventional units, called FORT units. FORD test, on the other hand, is a colorimetric test based on the ability of antioxidants present in plasma produced by mixing 50 μL of whole blood with Solution 1 in the FORD kit to reduce a preformed colored radical cation (i.e. purple) from mixing Solution 2 with Solution 3, and the assessment of the degree of decoloration of the final mixture which was proportional to the amount of antioxidants present in the blood sample. The oxidative stress index was represented by the total peroxides level (FORT)/total antioxidant capacity (FORD) ratio.

Lucigenin-based chemiluminescence assay

The ROS content in tissue was determined using chemiluminescence assay as described previously (Yu et al. 2006). Briefly, rats were killed to obtain thoracic aorta that was cut into 2 mm in length. The isolated aortic rings were incubated in Krebs–HEPES solution (containing Na-HEPES, 20 mM) at pH 7.4 and bubbled with 95% O_2–5% CO_2 for 20 min. Then, aortic rings were transferred into the 96-well plate containing 100 μM lucigenin for measuring the ROS signal using a luminometer (Plate CHAMELEON, Hidex, Finland). Afterwards, tissue ROS content was measured in the presence of 100 μM of NADPH or xanthine auto-injected into the indicated wells.

Chemicals

4-n-NP (CAS No. 104-40-5) was purchased from Sigma-Aldrich with 99.9% purity (Fluka, Germany). All other chemicals and solvents were of analytical grade, and were obtained from commercial suppliers.

Statistical analysis

Data were expressed as mean ± SEM, with significance at $P < 0.05$. Statistical comparisons between two groups were made using unpaired *t* test, and statistical comparisons between concentration-dependent curves were performed using repeat measure mixed model two-way ANOVA.

Results

Characteristics of 4-n-NP-treated rats

After a 20-week treatment with 4-n-NP, the ratio of testes to body weight was similar between these two groups (3.0 ± 0.1 and 2.8 ± 0.2% in control and 4-n-NP-treated rats, respectively). In addition, whole blood was used to demonstrate in vivo oxidative stress index, the ratio of total peroxides level to total antioxidant capacity, and we found that systemic oxidative stress was significantly higher in 4-n-NP-treated rats compared with controls (199.2 ± 18.9 and 133.0 ± 15.2, respectively) ($P < 0.05$).

Chronic effects of 4-n-NP on vasoconstriction and vasorelaxation

The responsive curve of cumulative concentration of phenylephrine, an alpha-1 adrenergic receptor agonist, was markedly higher in aorta isolated from 4-n-NP-treated rats compared with that from control animals ($P < 0.05$, Fig. 1). Similar results were obtained in potassium chloride,

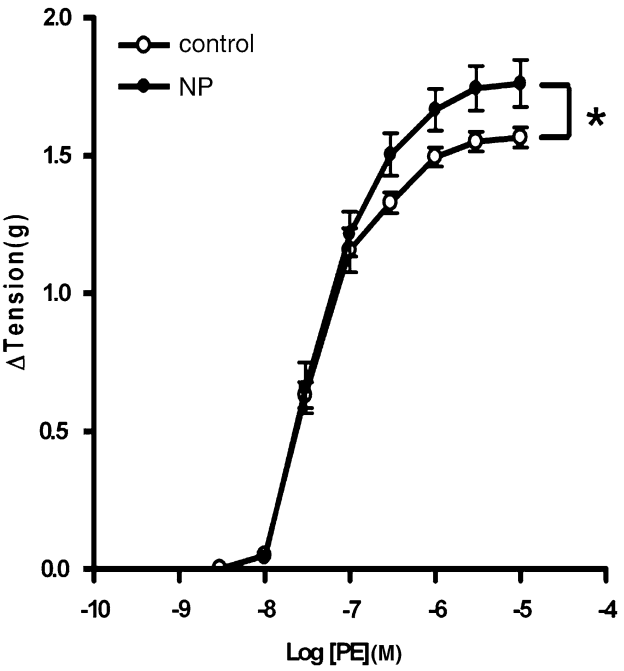

Fig. 1 Dose responsive curve of phenylephrine in control (*open circle* $N = 5$) and 4-n-NP-treated (*closed circle* $N = 8$) rats. The data are expressed as mean ± standard error. Statistical significance was determined by comparison with the controls. *$P < 0.05$

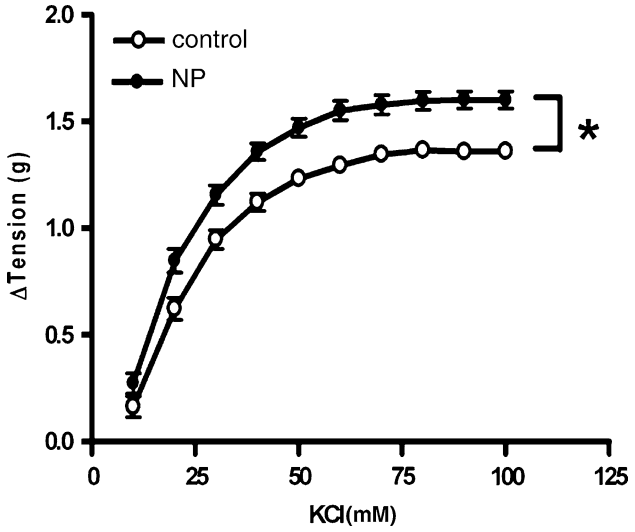

Fig. 2 Dose responsive curve of potassium chloride in control (*open circle* $N = 5$) and 4-n-NP-treated (*closed circle* $N = 8$) rats. The data are expressed as mean ± standard error. Statistical significance was determined by comparison with the controls. *$P < 0.05$

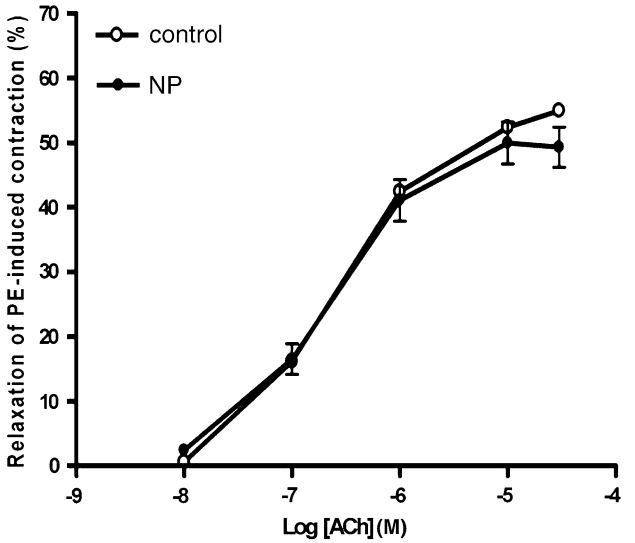

Fig. 3 Dose responsive curve of acetylcholine in control (*open circle* $N = 5$) and 4-n-NP-treated (*closed circle* $N = 8$) rats. The data are expressed as mean ± standard error

a voltage-gated calcium channel opener, induced dose-dependent contractile responsive curve ($P < 0.05$, Fig. 2). However, dose responsive curves of endothelium-dependent relaxation inducing by acetylcholine, a muscarinic receptor agonist, were not significantly different between control and 4-n-NP-treated rats ($P > 0.05$; Fig. 3).

Chronic effects of 4-n-NP on oxidant enzyme activity

After an in vivo chronic treatment of 4-n-NP for 20 weeks, aortic NADPH-related oxidase activity was evaluated indirectly via lucigenin-based chemiluminescence method using NADPH as substrate. In the absence of NADPH, the aortic ROS content was similar between control and 4-n-NP-treated rats (37 ± 3 and 43 ± 2 cpm, respectively). In the presence of NADPH, nevertheless, the aortic ROS content was significantly higher (high as 1.8-fold) in 4-n-NP-treated rats compared with control animals ($P < 0.05$). The similar result was observed in the presence of xanthine showing that the tissue ROS content was significantly higher in 4-n-NP-treated animals compared with that of controls ($P < 0.05$). We also assessed ROS contents in duodenum and found that NADPH-mediated ROS production ($P > 0.05$) and xanthine-mediated ROS production ($P > 0.05$) were both comparable between these two groups. The results are summarized in Table 1.

Discussion

4-Nonylphenol (4-NP), a metabolite of alkylphenol polyethoxylates, has been demonstrated to disrupt the development of the reproductive system, and to enhance reactive oxygen species generation in striatum (Obata and Kubota 2000), testes (Chitra and Mathur 2004; Gong and Han 2006), and neutrophils (Okai et al. 2004). In addition, this study clearly revealed that a chronic 4-n-NP treatment significantly increased systemic and local oxidative stress (see the first paragraph in the "Results" section; Table 1, respectively).

Average intake dosage for 4-n-NP-treated rats in this study was 0.2 and 0.8 mg/kg/day, and we found that the ratio of testes to body weight was similar between control and 4-n-NP-treated rats as above mentioned in the results. This suggests a lack of remarkable genital toxicity on a 20-week 4-n-NP exposure in 4-n-NP-treated rats in this study. This observation is not contradictory to those from previous studies showing that high dose (500 mg/kg/day) of 4-NP exposure disrupts the development of gonads in male and female rats (Nagao et al. 2000).

Although previous reports suggest that 4-NP has a weak-estrogenic effect on the reproductive system, it seems to have little estrogenic effect on vascular system. In our preliminary study, aorta isolated from male rats treated with 4-n-NP at the dosage of 2 mg/kg/day for 10 weeks exhibited altered vasomotor activity (data not shown). Therefore, the present study further investigated whether male rats exposing under low concentration of 4-n-NP (i.e. 0.8 mg/kg/day) for a longer time (i.e. 20 weeks) still showed modified aortic vasoconstrictive or vasorelaxing responses. Chronic effect of 4-n-NP on vascular tension observed in this study showing enhanced phenylephrine- and KCl-induced vasoconstriction (Figs. 1, 2, respectively) is not consistent with that observed in in vitro studies. Acute

Table 1 Chronic effects of 4-n-NP on oxidant enzyme activity

	Aorta		Duodenum	
	Control (CPS)	NP (CPS)	Control (CPS)	NP (CPS)
+NADPH (100 μM)	965 ± 76	1,717 ± 240*	13,370 ± 1,318	13,180 ± 1,338
+Xanthine (100 μM)	75 ± 6	138 ± 18*	1,344 ± 218	1,091 ± 168

NADPH- or xanthine-induced reactive oxygen species production in aorta and duodenum isolated from control ($N = 5$) and NP-treated ($N = 8$) rats were measured using lucigenin-based chemiluminescence assay

The data are expressed as mean ± standard error

Statistical significance was determined by comparison with the controls in the same induction

* $P < 0.05$

4-NP exposure evokes endothelium-independent vascular smooth muscle relaxation via inhibiting L-type calcium channels (Ruehlmann et al. 1998), which is similar to that induced by 17β-estradiol (Castillo et al. 2006; Nakajima et al. 1995). Similar inconsistencies have also been observed in the investigation of in vivo chronic effect and in vitro acute effect of 17β-estradiol on vascular smooth muscle tension (Cheng and Gruetter 1992; Colucci et al. 1982; Ravi et al. 1994), suggesting that acute NP effect cannot totally reflect its chronic influence on the regulation of vascular tension.

In addition, the chronic 4-n-NP exposure had little effect on ACh-induced endothelium-dependent relaxation in this study (Fig. 3). However, the previous results have demonstrated that 17β-estradiol treatment enhances endothelium-dependent vasorelaxation via nitric oxide-cyclic GMP pathway (Hayashi et al. 2000; Yen et al. 2004). These observations suggest that 4-n-NP dose not have estrogenic effect on the regulation of vascular endothelium function.

In conclusion, while the chronic low-dose 4-n-NP exposure had little effect on the reproductive system in this study, it did significantly alter vascular contractile responsiveness and systemic and local oxidative stress status. Furthermore, this is the first study clearly showing that chronic 4-n-NP exposure enhanced vascular, but not intestinal or oxidant enzymes activity. Because vascular dysfunction is the hallmark of cardiovascular disease, it is reasonable to suggest that chronic 4-n-NP exposure may be a risk factor to cardiovascular diseases in mammals.

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