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Keywords : Cardiovascular diseases¡BNitric Oxide¡BVasorelaxation¡BAnti-thrombsis¡B Antioxidant¡BAnti-genotoxicity¡BAnti-angiogenesis¡Bα-Naphthoflavone

The effect of α-naphthoflavone (α-NF) on vascular function was studied in thoracic aorta isolated from rat and primary cultured human umbilical vein endothelial cells (HUVECs). α-NF dose-dependently induced relaxation of the phenylephrine pre-contracted aorta in endothelium-dependent and independent manner at lower and higher concentrations, respectively. The cGMP, but not cAMP, content was increased significantly in α-NF treated aorta. Pretreatment with L-NAME or methylene blue significantly attenuated both α-NF induced vasorelaxation and the increase of cGMP content. The increase of cGMP content induced by α-NF was also inhibited when the extracellular Ca^{2+} was chelated with EGTA. These results suggested that the endothelium-dependent vasorelaxation induced by α-NF was most likely through the activation of nitric oxide synthase and guanylyl cyclase in Ca^{2+} dependent manner. In HUVECs, α-NF dose-dependently induced formation of NO and Ca^{2+} influx. The NO formation induced by α-NF was abolished when the extracellular Ca^{2+} was removed or when the HUVECs were pretreated with Ca^{2+} channel blockers, SKF 96365 and Ni^{2+}, but not by L-type Ca^{2+} channel blocker verapamil. The Ca^{2+} influx, as measured by the $^{45}Ca^{2+}$ uptake, induced by α-NF was also inhibited by SKF 96365 and Ni^{2+}. Our data concluded that α-NF, at lower concentrations, induced endothelium-dependent vasorelaxation by promoting the extracellular Ca^{2+} influx in endothelium and the activation of the NO-cGMP pathway.

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Alpha-naphthoflavone induces vasorelaxation through the induction of extracellular calcium influx and NO formation in endothelium

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Abstract The effect of α-naphthoflavone (α-NF) on vascular function was studied in isolated ring segments of the rat thoracic aorta and in primary cultures of human umbilical vein endothelial cells (HUVECs). α-NF induced concentration-dependent relaxation of the phenylephrine-precontracted aorta endothelium-dependently and -independently at lower and higher concentrations, respectively. The cGMP, but not cAMP, content was increased significantly in α-NF-treated aorta. Pretreatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) or methylene blue attenuated both α-NF induced vasorelaxation and the increase of cGMP content significantly. The increase of cGMP content induced by α-NF was also inhibited by chelating extracellular Ca^{2+} with EGTA. These results suggest that the endothelium-dependent vasorelaxation induced by α-NF is mediated most probably through Ca^{2+}-dependent activation of NO synthase and guanylyl cyclase. In HUVECs, α-NF induced concentration-dependent formation of NO and Ca^{2+} influx. α-NF-induced NO formation was abolished by removal of extracellular Ca^{2+} and by pretreatment with the Ca^{2+} channel blockers SKF 96365 and Ni^{2+}, but not by the L-type Ca^{2+} channel blocker verapamil. The Ca^{2+} influx, as measured by $^{45}Ca^{2+}$ uptake, induced by α-NF was also inhibited by SKF 96365 and Ni^{2+}. Our data imply that α-NF, at lower concentrations, induces endothelium-dependent vasorelaxation by promoting extracellular Ca^{2+} influx in endothelium and the activation of the NO-cGMP pathway.

Keywords Naphthoflavone-Endothelium-NO-Ca^{2+}

Introduction

The endothelium plays an important role in the vascular system. Not only is it a barrier between the blood and tissue, it also produces a variety of vasoactive agents that are important in controlling the body's homeostasis under normal and many pathological conditions (Jaffe *1985*; Vanhoutte et al. *1986*). In the vascular system, the endothelium, when stimulated by neurotransmitters, hormones, substances derived from platelets and the coagulation system, can evoke vasorelaxation (Furchgott and Zawadzki *1980*; Luscher et al. *1988*) through the production of NO (Palmer et al. *1988*). NO is synthesized from the amino acid L-arginine by oxidation of its terminal guanidine nitrogen by the endothelial cell enzyme nitric oxide synthase (eNOS) (Cobb et al. *1993*; Moncada and Higgs *1993*) in a calcium-dependent manner (Moncada et al. *1991*). Besides its vasorelaxing property, NO released from endothelium is also important in preventing the aggregation of platelets (Furchgott et al. *1984*; Furchgott *1984*) and inhibiting smooth muscle proliferation (Ignarro et al. *2002*; Gewaltig and Kojda *2002*).

Flavonoids are substances occurring naturally in fruit, vegetables, grains, barks, roots, stems, flowers, tea and wine (Middleton *1998*). More than 4,000 different flavonoids have been identified, many of which are responsible for the attractive colours of flowers, fruits, and leaves (De Groot and Rauen *1998*). These natural products are known for their beneficial effects on health, especially for protection against vascular disease and cancer (Birt et al. *1986*; Wei et al. *1990*; Heo et al. *1992*). One of their therapeutically relevant effects on the vascular system may be attributed to their ability to interact with the NO-generating pathway in vascular endothelium (Balestrieri et al. *2003*; Duffy and Vita *2003*; Youdim et al. *2002*). The most frequently studied flavonoid, quercetin, has biological properties consistent with its protective effect on the vascular system (Lanza et al. *1987*; Gryglewski et al. *1987*; Tzeng et al. *1991*; Frankel et al. *1993a*, *1993b*). The specific effects on vascular system might also come from the broad spectrum of modulating effects of flavonoids as antioxidants (Hanasaki et al. *1994*; Kerry and Abbey *1997*), and inhibitors of ubiquitous enzymes such as lipoxygenase (Alcaraz and Hoult *1985*; Moroney et al. *1988*), cyclooxygenase (Moroney et al. *1988*), phospholipase A2 (Alcraz and Hoult *1985*; Fawzy et al. *1988*) and protein kinase C (Ferriola et al. *1989*). They also inhibit LDL oxidation (De Whalley et al. *1990*; Rankin and Leake *1988*) and platelet aggregation (Gryglewski et al. *1987*; Lanza et al. *1987*) and promote vasodilation (Duarte et al. *1993a*, *1993b*). All these findings have led researchers to use flavonoids as the starting material for drug or health-food development aimed at reducing the risk factor for vascular disease (Formica and Regelson *1995*). In addition to the beneficial

effect on the vascular system, flavonoids also have antiviral and carcinostatic properties (Buening et al. *1981*; Guengerich and Kim *1990*; Cholbi et al. *1991*; Li et al. *1994*; Siess et al. *1995*; Sousa et al. *1985*). The anti-carcinogenicity of some flavonoids has been attributed to modulation of the cytochrome P450 enzymes that metabolize procarcinogens to their activated form (Benson et al. *1980*; Gordon et al. *1991*; Kanazawa et al. *1998*; Nijhoff et al. *1993*; Rodgers and Grant *1998*).

α-Naphthoflavone (α-NF) is a prototype flavone that belongs to a group of phytochemicals and is a normal component of human diets (Shou et al. *1994*; Das et al. *1994*). Their ability to modulate P450-mediated activities was first reported over three decades ago (Diamond and Gelboin *1969*). Most of these studies have assessed the effect of α-NF on P450-mediated hydroxylation of benzo(a)pyrene (BP), an environmental pollutant present in cigarette smoke and polluted air that is carcinogenic in experimental animals (Kinoshita and Gelboin *1972*). In addition to its inhibition on P450s, α-NF is also an antagonist at the aromatic hydrocarbon receptor (AhR), the cellular receptor of BP and other polycyclic aromatic hydrocarbons (PAHs) (Dong et al. *2001*; Jeon et al. *2002*). The α-NF isomer, β-naphthaflavone (β-NF), on the other hand, is a strong agonist at the AhR (Staples et al. *1998*; Jeon et al. *2002*).

The aim of the present study was to investigate the mechanism underlying α-NF-induced vasorelaxation. This question was addressed in thoracic aorta ring segments isolated from rats and in primary cultures of human umbilical vein endothelial cells (HUVECs). We found that α-NF induced endothelium-dependent vasorelaxation in a Ca^{2+}-dependent manner.

Materials and methods

Chemicals α-NF, β-NF, phenylephrine (PE), acetylcholine (ACh), sodium nitroprusside, trichloroacetic acid (TCA), EGTA and 3-isobutylmethylxanthine (IBMX) were obtained from Sigma (St. Louis, Mo., USA). Cell culture reagents including M-199 medium, L-glutamine, penicillin, streptomycin and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, N.Y., USA). cAMP and cGMP enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, Mich., USA). ^{3}H-labelled L-arginine and $^{45}Ca^{2+}$ were purchased from Amersham Life Sciences (Arlington Heights, Il., USA). All other chemicals were from Sigma. When drugs were dissolved in dimethylsulphoxide (DMSO), the final concentration of DMSO in the bathing solution did not exceed 0.1%, a concentration not interfering with muscle contraction or other measurements.

Aortic ring studies Male Wistar rats (250–300 g) were purchased from the Animal Centre of the College of Medicine, National Taiwan University, Taipei, Taiwan. The thoracic aorta was removed carefully after the rat had been killed by stunning followed by exsanguination. Fat and connective tissues were dissected away in normal Krebs' solution (in mM: NaCl 118.5, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1 and $CaCl_2$ 2.5; pH 7.4). The aortae were then cut into rings about 5 mm long in a 10-ml organ bath gassed continuously with 95% O_2/5% CO_2 at 37±0.5 °C. Two L-shaped stainless-steel hooks were inserted into the aortic lumen; one was fixed at the bottom of the bath and the other connected to a force transducer (Hu et al. *2001*). The aortic rings were equilibrated in Krebs' solution and maintained under an optimal tension of 1 g for 45 min. During this period the organ baths were perfused with fresh (37 °C) buffer solution for 45 min. Once at their optimal length, the segments were allowed to equilibrate for 30 min before experimentation. Contractions were recorded isometrically via a force-displacement transducer (Grass FT.03) connected to a MacLab/8e recorder (ADInstruments, Castle Hill, NSW, Australia). The presence of functional endothelium was assessed by determining the ability of 10 μM ACh to induce more than 80% relaxation of rings precontracted with 3 μM PE. The endothelium was removed by rubbing the luminal surface gently with a cotton ball. Successful removal of endothelium was confirmed by the absence of ACh-induced relaxation. The denuded aorta was also challenged with PE and a high $[K^+]$ (60 mM) to ensure that the vessel had not been damaged during denudation. Aortic rings with a normal contractile response were then used for experiments.

α-NF and β-NF concentration/response curves were obtained by adding increasing concentrations of these substances (0.1–100 μM) to rings precontracted with 3 μM PE after the response to the previous concentration had stabilized. To examine the effect of NOS inhibition, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 300 μM) was added 10 min before PE.

Rat aorta cAMP and cGMP measurement Rat aorta cyclic nucleotide contents were determined according to Kauffman et al. (*1987*). Depending on the purpose of the experiment, the aorta rings were either placed in Ca^{2+}-free Krebs' (containing EGTA 2.5 mM) buffer or pretreated with the inhibitors L-NAME (300 μM) or methylene blue (10 μM) for 10 min. Test compounds, such as sodium nitroprusside (10 μM), ACh (10 μM), forskolin (10 μM), α- or β-NF (10–100 μM) were added and the ring incubated for 5 min. After incubation with test compounds, the rat aortic rings were frozen rapidly in liquid N_2 and stored at –70 °C. For assay, the tissue was

homogenized in 0.5 ml 10% TCA in a Potter glass homogenizer. The homogenates were centrifuged at 10,000 *g* for 5 min and supernatants removed and extracted 4 times with 3 vol ether. cAMP and cGMP contents were then assayed using enzyme immunoassay kits. The precipitates were used for protein determination (Lowry et al. *1951*).

HUVEC isolation and culture Human umbilical cords were obtained from the Hospital of the National Taiwan University, Taipei, Taiwan. HUVECs were isolated by enzymatic digestion from 20-cm-long umbilical cord vein segments filled with 0.1% collagenase (Rosenkranz-Weiss et al. *1994*). After 15 min incubation at 37 °C, the vein segments were perfused with 30 ml medium 199 containing 10 U/ml penicillin and 100 µg/ml streptomycin to collect the cells. After centrifugation for 8 min at 900 *g*, the cell pellet was resuspended in the same medium supplemented with 20% heat-inactivated FBS, 30 µg/ml endothelial cell growth supplement (ECGS) and 90 µg/ml heparin. Confluent primary cells were detached using trypsin-EDTA (0.05:0.02% v/v), and HUVECs from passage 2 were used in the present study. Cultures had typical cobblestone morphology and stained uniformly for human von Willebrand factor (vWF) (Janel et al. *1997*) as assessed by indirect immunofluorescence.

NO determination HUVECs cultured in 12-well plates were washed twice with in a HEPES buffer (in mM: HEPES 10, NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Na_2HPO_4 1, glucose 10, pH 7.4) and then incubated at 37 °C in the same buffer for 30 min with various concentrations of α-NF (1~100 µM) or ACh (30 µM) as positive control. Supernatants were collected and then injected into the nitrogen-purge chamber containing vanadium (III) chloride in HCl at 91 °C. All NO metabolites are liberated as gaseous NO and react with ozone to form activated nitrogen dioxide that luminesces in the red and far-red spectrum. The chemiluminescent signals were detected by a nitric oxide analyser (NOA280, Sievers Instruments, Boulder, Colo., USA) accordingly (Ewing and Janero *1998*). The cells were detached and homogenized for protein determination. For calculation of concentrations, the area under the curve was converted to nanomolar NO using an $NaNO_3$ standard curve and the final data was expressed in picomoles/milligram protein.

$^{45}Ca^{2+}$ uptake The $^{45}Ca^{2+}$ influx measurement was modified from Cheng and Kang (*1997*). Confluent HUVECs cultured in 6-well plates were washed twice with HEPES buffer and then incubated in the same buffer containing $^{45}Ca^{2+}$ (10 µCi/ml) and treated with test compounds with or without the blockers for 5 min. Two concentrations of α-NF (50 and 100 µM) and blockers, including the receptor-operated Ca^{2+} channel blocker, SKF96365 (30 µM), the non-specific Ca^{2+} (channel blocker Ni^{2+} 1 mM) and the L-type Ca^{2+} channel blocker verapamil (2 µM) were used. After incubation, the

supernatants were aspirated and the cells washed 3 times with cold HEPES buffer containing 10 mM $LaCl_3$. Cells were lysed with 0.01 N NaOH and the cell $^{45}Ca^{2+}$ content measured by scintillation counter (Model 2200; Beckman, Palo Alto, Calif., USA).

Statistical analysis Data are expressed as means±SEM from *n* experiments. The significance of differences between means was established using Student's *t*-test, with $P<0.05$ being considered significant. EC_{50} values were calculated from five regression lines. Each regression line was constructed with between three and five points. These points corresponded to response magnitudes of 20–80%.

Results

Vasorelaxant effect of α-naphthoflavone in rat aorta

A transient phasic contraction followed by a tonic contraction was induced by PE (Fig. 1A) and the contracted aorta could be relaxed by addition of ACh (10 µM) through the induction of NO formation in endothelium-intact but not in the denuded (endothelium removed) aortic rings. As seen in Fig. 1, 1 and 50 µM α-NF induced vasorelaxation of intact aorta precontracted with PE (3 µM) by 48.58±10.7 and 74.72±6.2%, respectively. The vasorelaxation induced by α-NF was greatly attenuated in denuded aorta, suggesting that most of the relaxation caused by α-NF was endothelium dependent. Pretreatment with the NOS inhibitor L-NAME (Fig. 1B) or the guanylyl cyclase inhibitor methylene blue (data not shown) also attenuated the α-NF-induced vasorelaxation, suggesting that this vasorelaxation effect of α-NF might be due to the activation of NO synthesis in the endothelium.

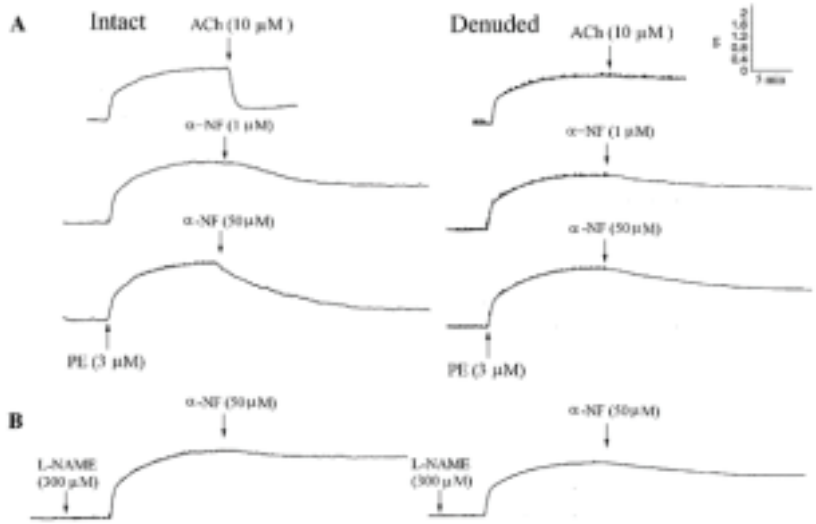

Fig. 1A, B Effect of α-naphthoflavone (*α-NF*) on contractility of isolated rat aortic rings. **A** Phenylephrine (*PE*, 3 µM)-precontracted, intact (*left*) or denuded (*right*) rings. **B** N^{ω}-nitro-L-arginine methyl ester (*L-NAME*, 300 µM)-pretreated rings. In denuded

aorta, the endothelium was removed by rubbing with a cotton ball, and the absence of 10 μM acetylcholine (*ACh*)-induced relaxation was taken as an indicator of successful denudation. The experiments were repeated with at least three different preparations

Both α-naphthoflavone and β-naphthoflavone induce vasorelaxation in the rat aorta

Increasing concentrations of α-NF or β-NF were added cumulatively (0.1–100 μM) to induce relaxation of the precontracted, intact or denuded aorta or the aorta pretreated with L-NAME (Fig. 2). α-NF induced vasorelaxation in the intact aorta concentration dependently with an EC_{50} of 0.95±0.13 μM. The α-NF-induced vasorelaxation was largely prevented (~80%) in denuded or L-NAME-treated aortae. α-NF also induced vasorelaxation in denuded or L-NAME-treated aortae, however at much higher concentrations (Fig. 2A). Compared with α-NF, the isomer β-NF had a much weaker effect vasorelaxant effect (EC_{50}>100 μM, Fig. 2B).

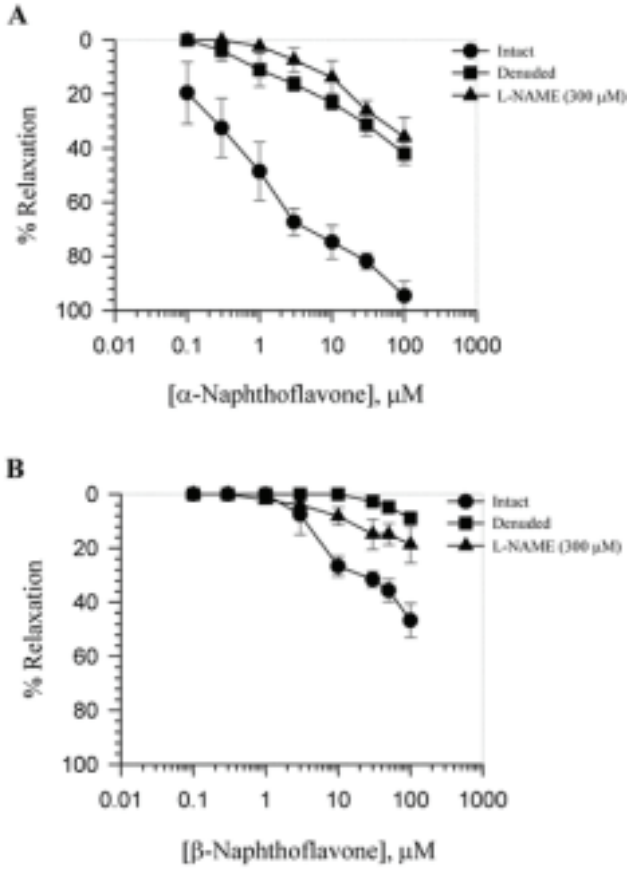

Fig. 2 Concentration/response curves for α-NF (**A**) and β-NF (**B**)-induced vasorelaxation of PE-precontracted rat thoracic aortic rings with (●) or without (■)

endothelium, or in the presence of L-NAME 300 μM (▲). Means±SEM, *n*=6

Effect of α-naphthoflavone on cAMP and cGMP content in rat aorta

The above data suggest that the endothelium-dependent vasorelaxation induced by α-NF at lower concentrations was due to activation of eNOS. NO, once generated, can activate guanylyl cyclase, with subsequent generation of cGMP in many cells, including smooth muscle (Moncada and Higgs *1993*). The effects of α-NF and β-NF on cyclic nucleotide formation in aortic rings were thus investigated and data are summarized in Table 1. Sodium nitroprusside, an NO donor, and forskolin, an adenylyl cyclase activator, increased cGMP and cAMP contents in aorta, respectively. α-NF concentration-dependently increased cGMP but not cAMP content. β-NF also increased cGMP content, but to a lesser degree.

Table 1 Effects of α-naphthoflavone and β-naphthoflavone on cAMP and cGMP contents of isolated rat aortic rings. cAMP and cGMP contents were measured as described in Methods. Means±SEM, *n*=6 individual experiments (*ND* not determined)

Treatment		Cyclic GMP	Cyclic AMP
		(pmol/mg protein)	
Control		0.14±0.06	1.33±0.25
Sodium nitroprusside	10 μM	1.72±0.56***	ND
Forskolin	10 μM	ND	9.57±1.56***
α-Naphthoflavone	10 μM	0.31±0.07	1.73±0.28
	50 μM	1.09±0.37*	2.18±0.26
	100 μM	1.48±0.23**	1.98±0.32
β-Naphthoflavone	10 μM	0.13±0.08	1.00±0.21
	50 μM	0.36±0.11	1.18±0.28
	100 μM	0.55±0.09	1.73±0.31

*P<0.05, **P<0.01, ***P<0.001 vs. control (Student's *t*-test)

ACh (10 μM) increased cGMP formation significantly in intact (2.15±0.09 pmol/mg protein) but not in denuded aorta (0.09±0.02 pmol/mg protein) relative to control (0.11±0.03 pmol/mg protein) (Table 2). ACh-induced cGMP formation was inhibited in aorta pretreated with L-NAME (300 μM) (0.15±0.02 pmol/mg protein) or methylene blue (0.53±0.10 pmol/mg protein). Induction of cGMP formation by α

-NF (100 μM, 1.48±0.23 pmol/mg protein) was also abolished in the denuded aorta (0.21±0.17 pmol/mg protein) and in the aorta treated with 300 μM L-NAME (0.25±0.11 pmol/mg protein) or 10 μM methylene blue (0.53±0.22 pmol/mg protein). Interestingly, the increase of cGMP content induced by α-NF was also diminished when extracellular Ca^{2+} was chelated with EGTA (Table 2), suggesting that α-NF-induced cGMP formation was dependent on both Ca^{2+} and the endothelium.

Table 2 Effects of blockers on acetylcholine- and α-naphthoflavone-induced cGMP formation. Means±SEM, *n*=6 individual experiments. Intact aorta was used in all experiments unless specified

Treatment		Cyclic GMP (pmol/mg protein)
Control		0.11±0.03
ACh (10 μM)		2.15±0.09***
ACh (Denuded, 10 μM)		0.09±0.02
L-NAME (300 μM)		0.07±0.07
Methylene blue (10 μM)		0.13±0.04
Ca^{2+}-free (2.5 mM EGTA)		0.05±0.01
ACh (10 μM)		2.15±0.09***
	+L-NAME (300 μM)	0.15±0.02
	+Methylene blue (10 μM)	0.53±0.10
α-NF (100 μM)		1.48±0.23***
	+L-NAME (300 μM)	0.25±0.11
	+Methylene blue (10 μM)	0.53±0.22
	+Ca^{2+}-free (2.5 mM EGTA)	0.18±0.02
α-NF (Denuded, 100 μM)		0.21±0.17

****P*<0.001 vs. control (Student's *t*-test)

Effect of α-naphthoflavone on NO formation in HUVECs

The direct effect of α-NF on endothelial cells was investigated further in primary cultures of HUVECs and the data are summarized in Fig. 3. α-NF concentration-dependently induced NO formation in HUVECs, with maximal induction at 100 µM (Fig. 3A). ACh (30 µM) also induced significant NO formation in HUVECs both in normal and Ca^{2+}-free (5 mM EGTA) HEPES buffer (8.79±0.68 and 8.07±1.58 pmol/mg protein) (Fig. 3B). The NO formation in HUVECs induced by α-NF (50 µM: 8.90±1.26, 100 µM: 10.72±1.74 pmol/mg protein) was attenuated significantly in Ca^{2+}-free HEPES buffer (2.60±0.66 and 2.30±0.32 pmol/mg protein respectively), suggesting that α-NF induced NO formation was dependant on extracellular Ca^{2+}. α-NF-induced NO formation was blocked by pretreatment with the receptor-operated Ca^{2+} channel blocker SKF96365 (30 µM) and the non-specific Ca^{2+} channel blocker, Ni^{2+} (1 mM), but not the L-type Ca^{2+} channel blocker verapamil (2 µM) (Fig. 3C). In contrast, β-NF did not induce NO formation at concentrations up to 100 µM (data not shown).

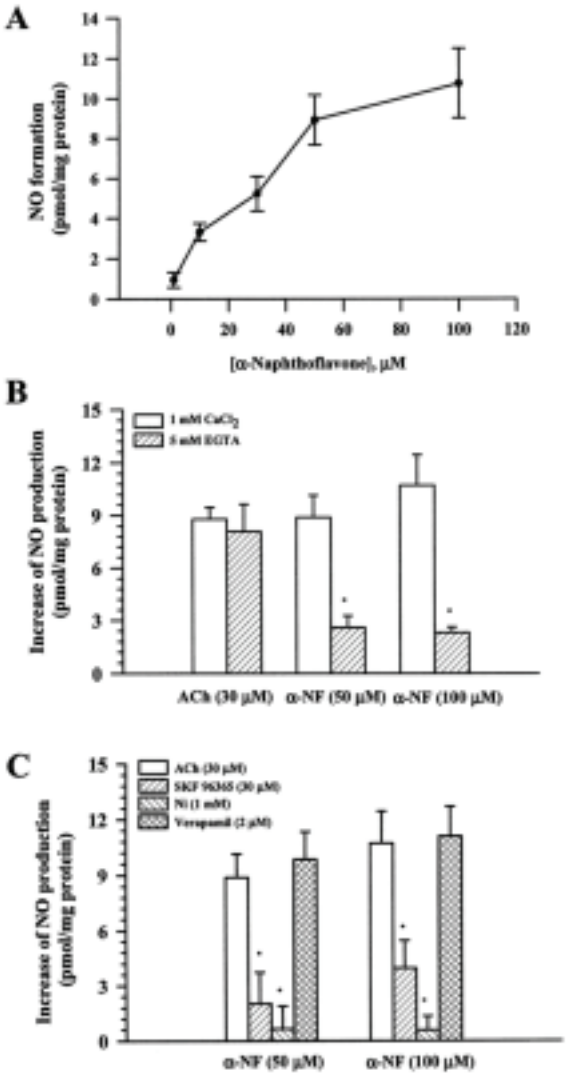

Fig. 3A–C Effect of α-NF on NO formation in human umbilical vein endothelial cells (HUVECs). **A** HUVECs were treated with 50 or 100 µM α-NF. **B** HUVECs were treated with ACh (30 µM) or α-NF (50 or 100 µM) for 30 min in normal HEPES buffer with 1 mM $CaCl_2$ or in Ca^{2+}-free HEPES buffer (containing 5 mM EGTA with no added $CaCl_2$). **C** Effect of pretreatment with various substances: acetylcholine (ACh, 30 µM); SKF96365 (30 µM); Ni (1 mM); verapamil (2 µM) as indicated. Means±SEM, *n*=3 independent experiments. **P*<0.05 vs. respective control

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α-Naphthoflavone induces $^{45}Ca^{2+}$ influx in HUVECs

Both α-NF-induced cGMP (Table 2) and NO (Fig. 3B) formation were attenuated in the absence of extracellular Ca^{2+}, suggesting that α-NF might exert its effect through induction of extracellular Ca^{2+} influx. The effect of α-NF on Ca^{2+} flux was investigated using the $^{45}Ca^{2+}$ loading method in adherent HUVECs. As seen in Fig. 4, a 30~40% increase of Ca^{2+} flux was observed in α-NF treated HUVECs. This was inhibited by SKF96365 and Ni^{2+}, but not verapamil. As seen with NO formation, β-NF did not induce $^{45}Ca^{2+}$ influx at concentrations up to 100 µM (data not shown).

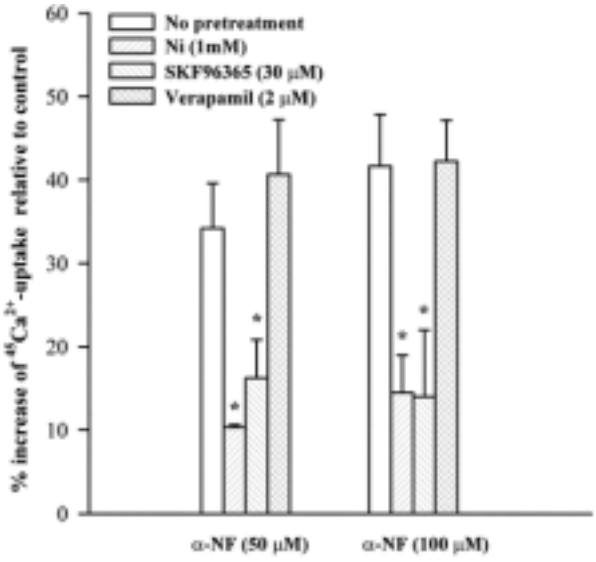

Fig. 4 Effect of α-NF on $^{45}Ca^{2+}$-influx. HUVECs were incubated in HEPES buffer containing $^{45}Ca^{2+}$ (10 µCi/ml) and treated with test compounds for 5 min: α-NF (50 or 100 µM) with or without blockers (SKF96365, 30 µM; Ni, 1 mM and verapamil, 2 µM) as indicated. Means±SEM, *n*=3 independent experiments. **P*<0.05 vs. respective control

Discussion

In the vascular system, NO is synthesized by eNOS after the latter's activation in endothelial cells and stimulates cGMP production by activating soluble guanylyl

cyclase in the adjacent smooth muscle (Palmer et al. *1987*). Increased cGMP causes contracted muscle to relax, possibly through lowering the intracellular [Ca^{2+}], most likely by increasing Ca^{2+} efflux to the extracellular space and Ca^{2+} reuptake into intracellular stores (Lincolin et al. *1990*; Lincolin and Cornwell *1991*; Ganitkevich et al. *2002*) or by dephosphorylation of myosin light-chain kinase (Ganitkevich et al. *2002*; Silveira et al. *1998*). Flavonoids exert physiological actions on various biological systems including the vascular system. Studies into the correlation between the low mortality rate due to cardiovascular disease and the red wine consumption in Mediterranean populations (Renaud and DeLorgeril *1992*) indicates that the flavonoids in red wine are responsible, at least in part, for this effect (Formica and Regelson *1995*).

The present study showed that α-NF relaxed the endothelium-intact rat aorta with an EC_{50} of 0.95±0.13 µM, a value lower than flavonoid EC_{50}s reported previously (Fitzpatrick et al. *1993*; Jimenez et al. *1999*; Kim et al. *2000*). Endothelial denudation significantly attenuated α-NF-induced relaxation, suggesting that most of the vasorelaxant effect of α-NF was endothelium dependent. Pretreatment with L-NAME, a NOS inhibitor, or methylene blue, an inhibitor of guanylyl cyclase, inhibited the α-NF-induced relaxation to an extent similar to that seen in the endothelium-denuded preparations. These findings suggest that activation of NOS might be responsible for the endothelium-dependent vasorelaxation induced by α-NF. Consistent with this is the increased cGMP content in the α-NF-treated aorta. Whilst α-NF at 1 µM induced nearly 50% relaxation of the isolated aorta, a significant increase in cGMP content was achieved only in aortae exposed to α-NF concentrations exceeding 10 µM. Several possibilities might explain this discrepancy. First, although the increase in cGMP content in the aorta treated with 10 µM α-NF was not significant, the trend was still apparent compared with the basal level. Without knowing the exact correlation between intracellular cGMP content and the relaxation effect, it is difficult to compare concentration/responses relationships from the two measurements. It is possible that only a small amount of cGMP is needed to induce vasorelaxation. Second, cGMP content was measured using an enzyme immunoassay kit, the sensitivity of which for cGMP is limited, especially with respect to the latter's extraction from whole tissue. Finally, we have shown also that α-NF induces endothelial-independent relaxation. The EC_{50} obtained from muscle relaxation measurement can therefore be expected to be lower than that obtained from cGMP measurement. The steric isomer β-NF also induced vasorelaxation, however, at much higher concentrations with an EC_{50}>100 µM. The endothelium-dependent

vasorelaxation induced by β-NF is most probably through the NO-cGMP pathway, as for α-NF, since β-NF treatment also augmented the cGMP formation in aorta.

Three NO synthase (NOS) isoforms have been identified to date (Bredt et al. *1991*; Sessa et al. *1992*; Xie et al. *1992*). eNOS and neural NOS (nNOS) have been shown to be Ca^{2+}/calmodulin-dependent and expressed are constitutively mainly in endothelial cells and neurons, respectively. eNOS and nNOS are activated mainly by an increase of cytosolic [Ca^{2+}] (Mayer et al. *1989*). Another NOS isoform, inducible NOS (iNOS), can be induced by endotoxin and cytokines in immune cells and is independent of Ca^{2+} (Kerwin and Heller *1994*; Iyengar et al. *1985*). In the present study, the α-NF-induced increase of cGMP content was attenuated when extracellular Ca^{2+} was chelated with EGTA. This implies that the activation of NOS may have been due to an α-NF-induced influx of extracellular Ca^{2+} into the endothelium. The results from the experiments with HUVECs support this contention. First, α-NF treatment augmented NO release from HUVECs, a response that was abolished when extracellular Ca^{2+} was chelated. Second, Ca^{2+} influx, as evident by the increase of $^{45}Ca^{2+}$ uptake, in HUVECs was induced by α-NF.

Both the increase of NO formation and Ca^{2+} influx induced by α-NF in HUVECs were inhibited by the Ca^{2+} channel blockers Ni^{2+} and SKF96365. These results suggest that α-NF treatment induced Ca^{2+} influx through an SKF96365- and Ni^{2+}-sensitive Ca^{2+} channel in the endothelium. SKF 96365 inhibits the Ca^{2+} influx through the non-selective cationic channel activated by internal Ca^{2+} store depletion by endoplasmic reticulum Ca^{2+}-ATPase inhibitors or receptor agonists (Low et al. *1996*; Millanvoye-Van Brussel et al. *1999*). However, the SKF 96365-sensitive Ca^{2+} channel can also be activated directly without depletion of internal Ca^{2+} store (Inazu et al. *1995*). In addition, SKF 96365 inhibits the unidentified Ca^{2+} channel activated by mechanical stress in endothelial cells (Yao et al. *2000*). Ni^{2+}, on the other hand, is a non-specific Ca^{2+} channel blocker and inhibits the Ca^{2+} channel activated in endothelial cells by the agonist ACh (Wang et al. *1996*), the Ca^{2+} pump inhibitor cyclopiazonic acid (Li and van Breemen *1996*) and blood flow (Yao et al. *2000*). It is interesting to note that β-NF treatment also induces (Xie et al. *2002*) or potentiates (Graier et al. *1995*) capacitative Ca^{2+} influx in endothelial cells, possibly through the formation of P450 metabolite 5,6-epoxyeicosatrienoic acid (5,6-EET), a calcium influx factor (CIF). However, our data showed that α-NF, a P450 inhibitor, is a more potent inducer of vasorelaxation and Ca^{2+} influx in endothelial cells. These results

imply that formation of CIF through P450 metabolism might not be involved in the induction of Ca^{2+} influx by naphthoflavone compounds. A previous study has also shown that flavonoids can induce Ca^{2+} influx in endothelium and hence vasorelaxation through activation of tetraethylammonium-sensitive K^{+}-channels (Kim et al. *2000*). The inability of tetraethylammonium to inhibit the α-NF induced vasorelaxation (data not shown) also precludes this possibility. Moreover, the L-type voltage-gated Ca^{2+} channel blocker verapamil did not inhibit the α-NF induced NO formation and Ca^{2+} influx. This implies that either the Ca^{2+} influx induced by α-NF is not through L-type voltage-gated Ca^{2+} channel or that there are no L-type voltage-gated Ca^{2+} channels in HUVECs. The presence of L-type voltage-gate Ca^{2+} channels in endothelial cells is controversial. Although lack of voltage-gated Ca^{2+} channels has been reported in endothelial cells isolated from the porcine coronary artery (Uchida et al. *1999*) and the rabbit and rat aorta (Muraki et al. *2000*), the voltage-gated Ca^{2+} channels have been demonstrated in freshly isolated capillary endothelial cells from bovine adrenal glands (Bossu et al. *1992a*, *1992b*) and cerebral microvascular endothelial cells from newborn pig brain cortex (Yakubu et al. *2002*). However, to our knowledge, L-type Ca^{2+} channels have not been demonstrated in human endothelial cells (Ding and Vaziri *2000*). Further investigation will be needed to understand the exact mechanism of how or which Ca^{2+} channel was activated by α-NF in endothelium.

Our study showed also that higher concentrations of α-NF also induced vasorelaxation in the endothelium-denuded aorta. The EC_{50} for this endothelium-independent effect was estimated to be 200-fold higher than that of endothelium-dependent effect. Flavonoids such as dioclein (Trigueiro et al. *2000*) induce endothelium-independent vasorelaxation by inhibiting voltage-dependent Ca^{2+}-influx and the release of intracellular Ca^{2+} store in rat aorta. Eriodictyol, a flavonoid obtained from *Satureja obovata* also induces vasodilatation by inhibiting Ca^{2+} influx in rat aorta (Ramon Sanchez de Rojas et al. *1999*). α-NF's endothelium independent vasorelaxation may be mediated by the same mechanism. This however, awaits further investigation.

Flavonoids, including genistein (3–100 µM); kaempferol (3–60 µM) and quercetin (1–100 µM), increase intracellular cAMP content in uterine smooth muscle (Revuelta et al. *1999*). An increase of cAMP content in smooth muscle also induces vasorelaxation (Lincolin et al. *1990*; Lincolin and Cornwell *1991*). In the present study, however, the cAMP content, unlike cGMP, was not increased in α-NF treated aorta. This suggests that cAMP might not play an important role in α-NF induced vasorelaxation. In addition to the effect on vascular endothelial cells, preliminary

findings show that α-NF also inhibits the platelet aggregation induced by collagen, arachidonic acid, platelet activation factor and ADP (Y.-W. Cheng, C.-H. Li, C.-C. Lee, J.-J. Kang, unpublished data).

In conclusion, the present study demonstrates that the flavonoid compound α-NF promotes the influx of extracellular Ca^{2+} and release of NO by vascular endothelium. In addition to its vasorelaxant effect, NO is considered an important anti-atherogenic factor by virtue of its inhibitory effect on platelet aggregation (Furchgott *1984*) and smooth muscle proliferation (Ignarro et al. *2002*). α-NF also exerts anti-carcinogenic effects by virtue of its inhibition of P450 (Andries et al. *1990*; Shimada et al. *1998*; Tassaneeyakul et al. *1993*) and antagonism at the AhR (Dong et al. *2001*; Jeon et al. *2002*). The results of this study provide evidence for a further beneficial effect of α-NF on the vascular system.

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