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Naphthazarin and methylnaphthazarin cause vascular dysfunction by impairment of endothelium-derived nitric oxide and increased superoxide anion generation

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Abstract

The effects of the naphthoquinone analogue, naphthazarin (Nap), and its derivative, methylnaphthazarin (MetNap), on vascular reactivity were studied using isolated rat aortic rings and human umbilical vein endothelial cells (HUVECs). In this study, we determined vessel tension, nitric oxide (NO) formation, endothelial nitric oxide synthase (eNOS) activity, eNOS protein expression, and superoxide anion ($O_2^{\bullet-}$) generation in an effort to evaluate the effect of Nap and MetNap on the impairment of the NO-mediated pathway. Lower concentrations of Nap (0.01–1 μM) and MetNap (1–10 μM) concentration-dependently enhanced phenylephrine (PE)-induced vasocontraction and abrogated acetylcholine (ACh)-induced vasorelaxation in an endothelium-dependent manner. On HUVECs, both Nap and MetNap concentration-dependently inhibited NO formation induced by A23187, and also partially inhibited nitric oxide synthase (NOS) activity. eNOS protein expression by HUVECs was not affected by treatment with Nap or MetNap, even within 24 h. These data suggest that Nap and MetNap might act as inhibitors of nitric oxide synthesis in the endothelium. In addition, Nap and MetNap were also shown to generate $O_2^{\bullet-}$ on HUVECs with short-term treatment. We concluded that Nap and MetNap inhibited agonist-induced relaxation and induced vasocontraction in an endothelium-dependent manner, and these effects might have been due to modification of the NO content by inhibition of NOS activity and bioinactivation through $O_2^{\bullet-}$ generation.

Keywords: Naphthazarin; Nitric oxide synthase; Nitric oxide; Superoxide anion

1. Introduction

Quinoid compounds are widespread in nature (Thomson, 1991) and have several different roles in organisms; ubiquinone and vitamin K1 act as defensive compounds in biochemical systems. The pigments, alkannin, shikonin, and naphthazarin, have been proven to possess wound healing, antibacterial, anti-inflammatory, and anticancer activities (Papageorgiou, 1980). In addition, some quinoid-like chemicals are used as dyes or are environmental pollutants (Schuetzle, 1983). Although, as quinoid compounds, anthracyclines or anticancer agents (doxorubicin, idarubicin, aclarubicin, and naphthazarin) have limited clinical uses because the side effect of life-threatening cardiomyopathy upon chronic administration, they are still under

Abbreviations: Nap, naphthazarin; MetNap, methylnaphthazarin; PE, phenylephrine; ACh, acetylcholine; HUVECs, human umbilical vein endothelial cells; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

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consideration for their anti-inflammation or antioxidation functions (Anufriev et al., 1998; Kourounakis et al., 2002) in pharmacochemical investigations. This fact should be taken into consideration and rise the important of unwanted toxicity in the use of this potential pharmaceutical drug. Exposure of cells to quinoid compounds may lead to several deleterious cellular consequences. Acute exposure to these compounds may induce cellular damage and even cell death. Furthermore, chronic exposure to various quinoid compounds may cause carcinogenic and mutagenic insults (Price et al., 1975; Tikkanen et al., 1983; Chesis et al., 1984). During recent decades, considerable effects have been made to understand the mechanism underlying the biotoxicity of quinoid compounds.

Naphthazarin, a natural redox-cycling quinoid, and its structural analogue, doxorubicin, were found to have cardioactive effects under in vivo ischemic-reperfusion (Anufriev et al., 1998), to induce antiplatelet aggregation (Ko et al., 1990), formation of glutathione conjugates (Ollinger et al., 1989), and inhibition of DNA topoisomerase-I activity (Song et al., 2000), and to have antibacterial (Papageorgiou, 1980) and anticancer activities (Kyong-Up et al., 1997). In addition to the desired pharmacological actions, naphthazarin has been shown to produce some adverse effects on cellular systems. For example, this natural compound can induce intracellular oxidative stress (Cohen and d'Arcy Doherty, 1987; Ollinger and Brunmark, 1991), cytotoxic effects in hepatocytes (Ollinger and Brunmark, 1991), apoptotic cell death by damaging the lysosomal membrane (Ollinger and Brunk, 1995; Roberg et al., 1999), and genotoxic action on the Ames test (Tikkanen et al., 1983).

The vascular endothelium is important in the maintenance of vascular homeostasis. Endothelial NO plays an important role in the regulation of vascular tone and blood pressure (Luscher and Vanhoutte, 1999). However, NO reacts rapidly with superoxide anions leading to inactivation and loss of its vasodilator activity (Gryglewski et al., 1986). Consequentially, its derived products, including peroxynitrite ($ONOO^-$) and hydrogen peroxide, as permeants and potent oxidants can also cause vasculopathies such as vascular cell injury (Berry et al., 2001; Cai and Harrison, 2000). In particular, vasculotoxic compounds such as homocysteine have been identified as endothelial toxins; their action may involve superoxide-induced impairment of NO-mediated vasorelaxation and oxidative damage to the vascular endothelium (Weiss et al., 2003). On the other hand, indomethacin was recently found to have the vascular action of augmenting ACh-induced vasorelaxation through an increase in reactive oxygen species, particularly $ONOO^-$ (De Angelis et al., 2004).

In the present study, the effects of Nap and MetNap on vascular rings and human umbilical vein endothelial cells (HUVECs) were studied. We found that Nap and MetNap augmented PE-induced vasocontraction and suppressed ACh-induced relaxation in the aorta at lower concentrations in an endothelium-dependent manner. These effects might have been due to the inhibition of nitric oxide synthase activity and to $O_2^{\bullet-}$ generation.

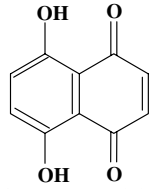
2. Materials and methods

2.1. Chemicals

Nap and MetNap (see Fig. 1) were isolated following a procedure previously described by Cheng et al. (1995). Cell culture reagents including M-199 medium, L-glutamine, penicillin, streptomycin, and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specified.

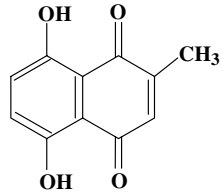
2.2. Thoracic aortic ring preparation

Wistar rats (weighing 200–300 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. Rats were sacrificed, and the descending thoracic aorta of each rat was carefully removed in normal Krebs solution with the following composition (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). After excess fat and connective tissue were removed, the aorta was cut into rings (about 5 mm in length) in 10-ml organ baths containing Krebs' solution (constantly gassed with $95\%O_2 + 5\%CO_2$ at 37 ± 0.5 °C) and attached to a force transducer (Grass FT.03) to measure the isometric contraction as previously described (Cheng et al., 2003). The aortic rings were equilibrated in Krebs solution and maintained



5, 8-dihydroxy-1, 4-naphthalenedione

(Naphthazarin)



(Methylnaphthazarin)

Fig. 1. Chemical structures of naphthazarin and methylnaphthazarin.

under an optimal tension of 2 g for 90 min, with three changes of the solution, before the experimental procedures were begun. Contractions were recorded isometrically via a force–displacement transducer connected to a MacLab/8 e (AD Instruments, Castle Hill, Australia). The presence of functional endothelium was tested in all preparations by determining the ability of acetylcholine (ACh, 10 μM) to induce more than 80% relaxation of the rings precontracted with phenylephrine (PE, 3 μM). In the denuded aorta experiments, the endothelium was mechanically removed by gently rubbing the ring intima surface with a cotton ball, and the absence of ACh-induced relaxation was taken as an indicator of successful denudation.

2.3. HUVEC isolation and culture

Human umbilical cords were obtained from National Taiwan University Hospital, 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 of 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 a typical cobblestone morphology and exhibited uniform staining for human von Willebrand factor (vWF) (Hu et al., 2004) as assessed by indirect immunofluorescence.

2.4. Determination of cell viability by MTT assay

Viability of cells was assessed using the 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by measuring mitochondrial dehydrogenase activity (Carmichael et al., 1987). Viable cells with active mitochondria caused cleavage of the tetrazolium ring into a visible dark blue formazan reaction product. MTT was dissolved in M-199 complete medium, and the stock solution was 2 mg/ml. Cells were plated in 96-well microtiter plates at 1×10^5 ml^{-1} cells per well in a final volume of 200 μl in M-199 complete medium at 37 °C overnight. Cells were treated with different concentrations of Nap and MetNap for another 30 min and 24 h. After incubation, 50 μl MTT was added and incubated at 37 °C for 3 h, and then the medium was gently removed. Cells and dye crystals were dissolved in 100 μl DMSO, and the absorption was measured at 570 nm in an ELISA reader (MRX-TC; Dynex Technology, Chantilly, VA, USA).

2.5. NO determination

HUVECs cultured in 12-well plates were washed twice in HEPES buffer (in mM: HEPES 10, NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Na_2HPO_4 1, and glucose 10; pH 7.4) and then incubated at 37 °C in the same buffer for 30 min with various concentrations of Nap (0.001–1 μM) and MetNap (0.01–3 μM). A23187 (2.5 μM) was used as the 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 spectra. The chemiluminescent signals were detected by a nitric oxide analyzer (NOA280, Sievers Instruments, Boulder, CO, USA) (Ewing and Janero, 1998). Cells were detached and homogenized for protein determination. For calculation of concentrations, the area under the curve was converted to nanomolar NO using a $NaNO_3$ standard curve, and the final data were expressed as a percent relative to the positive control.

2.6. NOS activity measurement

Endothelial nitric oxide synthase (eNOS) activity in HUVECs was determined by measuring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline which is sensitive to L-NAME (Bredt and Synder, 1990). Confluent HUVECs were washed twice with HBSS buffer and incubated in 500 μl HBSS buffer supplemented with arginine, L-[2,3,4-^{3}H]monohydrochloride (1 μCi/ml) at 37 ± 0.5 °C for 30 min. Test compounds (Nap, 0.01–3 μM and MetNap, 1–10 μM) were then added and incubated for another 30 min at 37 ± 0.5 °C (A23187, 2.5 μM, was used as a positive control) (Lee et al., 1998). The supernatant was aspirated and washed twice with cold stop buffer (50 mM HEPES and 5 mM EDTA) to stop the reaction. Cells were denatured with 100 μl 95% ethanol. After evaporating the ethanol, the soluble components were dissolved in 1 ml of stop buffer. L-Citrulline and L-arginine in the supernatants were separated using a Dowex AG 50WX-8 (Na^+ form) column and eluted with 2 ml of stop buffer. The eluted solution (1 ml) was mixed with 3 ml of scintillation cocktail, and the radioactivity was determined with an α-counter (Model 2200; Beckman, Palo Alto, CA, USA). Cells were lysed with 400 μl 0.5 N NaOH, and protein was determined by the Bradford (Bradford, 1976) method. The final data was expressed as a percent relative to the control.

2.7. Western blotting

After incubation with the Nap and MetNap in 60-mm dishes, cells were washed twice with ice-cold

phosphate-buffered saline. Then, 200 μl lysis buffer (50 mM Tris–HCl, 1% NP-40, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.5 M $NaVO_4$; pH 7.4) was added to each dish; cells were scraped from the dishes and then incubated in Eppendorf tubes for 30 min at 4 °C. This was centrifuged at 14,000 rpm and 4 °C for 20 min, and the supernatant was collected. Aliquots were mixed with 4X protein sample buffer (31.25 mM Tris–HCl (pH 6.8), 1% SDS, 25% β-mercaptoethanol, 0.00625% bromophenol blue, and 50% (v/v) glycerol) and boiled for 5 min. Protein samples were run on 10% SDS-PAGE gels, and electrophoresed proteins were transferred to PVDF membranes. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween and incubated with the primary antibody (anti-eNOS, Cell Signaling, Beverly, MA, USA). Horseradish peroxidase-labeled secondary antibody was used, and bands were detected with chemiluminescent reagents following the manufacturer's instruction (Perkin Elmer Life Science, Boston, MA, USA) and then exposed to X-ray film.

2.8. Analysis of $O_2^{\bullet-}$ production by flow cytometry

We used dihydroethidium (DHE), an oxidative fluorescent dye, to detect the formation of $O_2^{\bullet-}$ in HUVECs (De Angelis et al., 2004). Basically, cells were collected, washed twice with ice-cold PBS, and incubated with 0.1 mM DHE (Molecular Probes) for 30 min at 37 °C in the dark. Various concentrations of Nap and MetNap were added and analyzed by FACScalibur (Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.9. Statistical analysis

Data are expressed as the mean ±SEM for the number of experiments indicated. Statistical analysis of the data was performed using one-way ANOVA and Student's *t*-test, and $p < 0.05$ was considered to indicate a significant difference. Values of concentrations which caused 50% cell death (DC_{50}) were calculated and obtained from five regression lines; each regression line was constructed from three to five points. Values of inhibition of these points ranged between 20% and 80%.

3. Results

3.1. Effect of Nap and MetNap on vasoreactivity

The effects of Nap and MetNap on vessel reactivity were studied using rat thoracic aorta. PE (3 μM) was used to induce contractions in the aortic ring with (intact) or without (denuded) endothelium (Cheng et al., 2003). As shown in Fig. 2A, Nap, at lower concentrations (0.01–0.1 μM) significantly enhanced PE-induced vasocontraction with intact endothelium, and this response then declined at higher concentrations (>1 μM). Furthermore, MetNap augmented the vasocontraction at concentrations of 1–10 μM (Fig. 2B). The maximal contraction induced by PE (3×10^5 M) was taken as 100% (Fig. 2A and B). Nap (0.1 μM) and MetNap (10 μM) markedly potentiated the maximal contraction in response to PE as indicated by its increase from 100% to 179.3 ± 6.4% ($n \geq 5$, $p < 0.001$) (Fig. 2A) and from 100% to 210.5 ± 9.3% ($n \geq 5$, $p < 0.001$) (Fig. 2B), respectively. Both compounds potentiated PE-induced vasocontraction only in intact, not denuded,

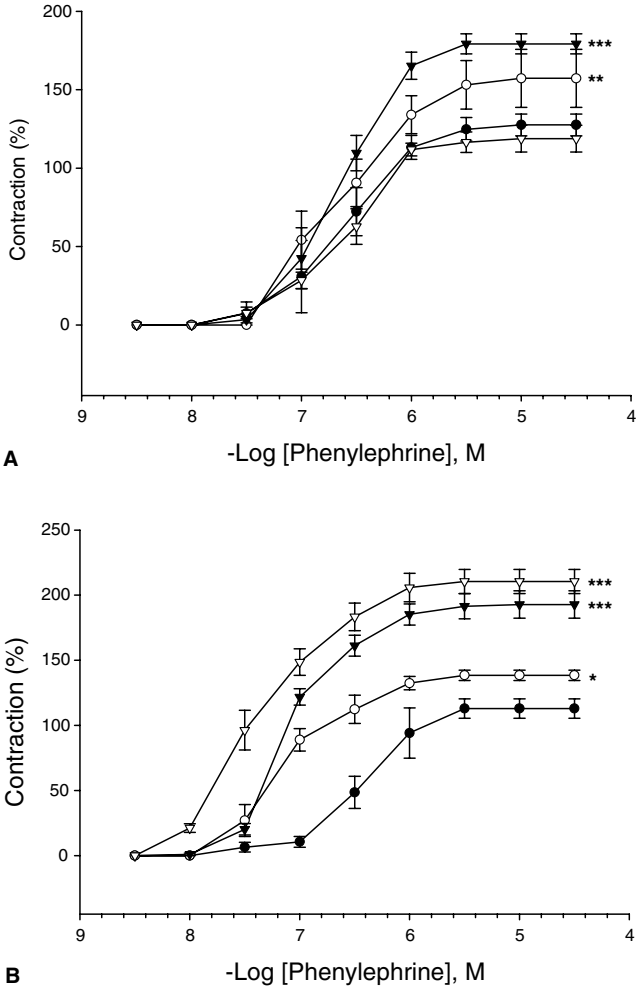


Fig. 2. Cumulative concentration–response curve of phenylephrine in the presence of Nap and MetNap in rat aortic rings. (A) DMSO: (0.1%) (●), or Nap: 0.01 μM (○), 0.1 μM (▼) or 1 μM (□) were preincubated with aortic rings for 5 min before the addition of PE. (B) DMSO (0.1%) (●), or MetNap: 1 μM (○), 3 μM (▼) or 10 μM (□) was also preincubated with aortic rings as previously described. Data are presented as the mean ± SEM ($n \geq 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to the response of the maximal concentration of PE (3×10^5 M).

aorta (data not shown). These results suggest that endothelium might be responsible for both Nap and MetNap's enhancement of vasocontraction. In addition, acetylcholine produced concentration-dependent relaxation of PE-precontracted control vessels (Fig. 3A and B). The maximal relaxation averaged more than 80%. The vasorelaxation evoked by ACh was attenuated by Nap and MetNap in concentration-dependent manners, as shown by the rightward shifts in the curves (Fig. 3). The maximal concentration of Nap (1 μM) and MetNap (10 μM) markedly blocked the maximum relaxation in response to ACh as indicated by its decrease from $80.3 \pm 3.8\%$ to $16.4 \pm 8.7\%$ ($n \geqslant 5$, $p < 0.001$) (Fig. 3A) and from $82.9 \pm 3.9\%$ to $20.1 \pm 5.5\%$ ($n \geqslant 5$, $p < 0.001$) (Fig. 3B), respectively.

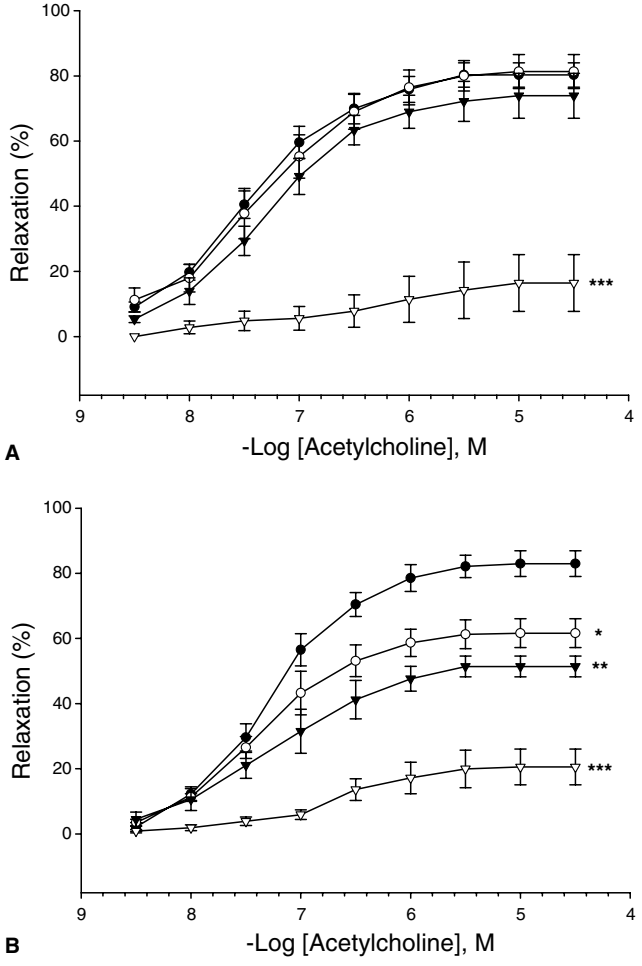


Fig. 3. Cumulative concentration–response curve of acetylcholine in the presence of Nap and MetNap in precontracted rat aortic rings. (A) DMSO (0.1%) (●), or Nap: 0.01 μM (○), 0.1 μM (▼), or 1 μM (□) was preincubated with aortic rings for 5 min before the addition of PE and Ach. (B) DMSO (0.1%) (●), or MetNap; 1 μM (○), 3 μM (▼), 10 μM (□) was treated as previously described. Relaxation is expressed as a percent reduction in the tension from that produced by PE. Data are presented as the mean ± SEM ($n \geqslant 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to the response of the maximal concentration of ACh (3×10^5 M).

3.2. Cytotoxic effect of Nap and MetNap on HUVECs

We used the MTT assay to obtain the viability curve and used the results as an indicator to select suitable concentrations in the subsequent cellular assays. Results from Fig. 4 shows that both compounds exerted no cytotoxic damage in 30 min treatment up to the concentration of 30 μM Nap, and 100 μM MetNap.

3.3. Effects of Nap and MetNap on NOS activity and NO formation

According to related studies, PE-induced contraction is augmented in the presence of NOS inhibitors, such as L-NAME or L-MMA (Ollinger and Brunmark, 1991; Cheng et al., 2003). Therefore, the direct effects of Nap and MetNap on endothelial NO production were investigated in isolated HUVECs. For NO formation, A23187 (2.5 μM) significantly increased the NO content as compared to the control. Both Nap (0.001–1 μM) and MetNap (0.01–3 μM) markedly and concentration-dependently suppressed NO production induced by A23187 in HUVECs (Fig. 5A and B). At the same concentration (1 μM), Nap produced $47.4 \pm 6.1\%$ inhibition of NO production, but only $34.2 \pm 3.0\%$ inhibition was produced by MetNap. For NOS activity measurements, as shown in Fig. 6, the effects of Nap (0.1–3 μM) and MetNap (1–3 μM) on NOS activity were partially and significantly inhibited in concentration-dependent manners. Nap (1 μM) and MetNap (1 μM) produced $9.1 \pm 1.1\%$ and $4.4 \pm 1.7\%$ inhibition of

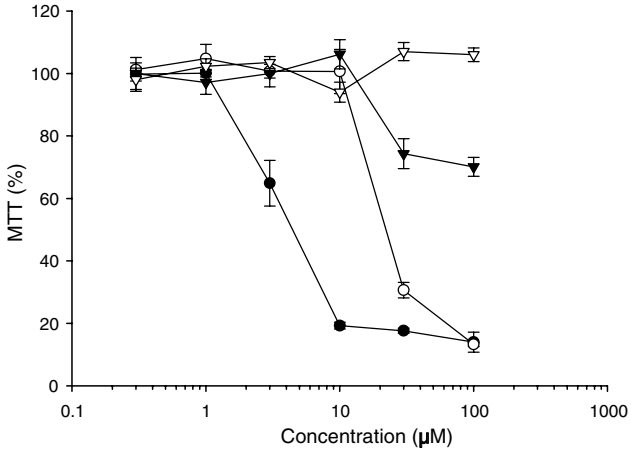


Fig. 4. Cytotoxic effects of Nap and MetNap on HUVECs. HUVECs were treated with various concentrations of Nap and MetNap for 30 min and 24 h. Nap 24 h (●), MetNap 24 h (○), Nap 30 min (▼), MetNap 30 min (∇). Cell viability was determined by MTT assay as described in Section 2. Data points were expressed as mean ± SEM ($n \geqslant 5$).

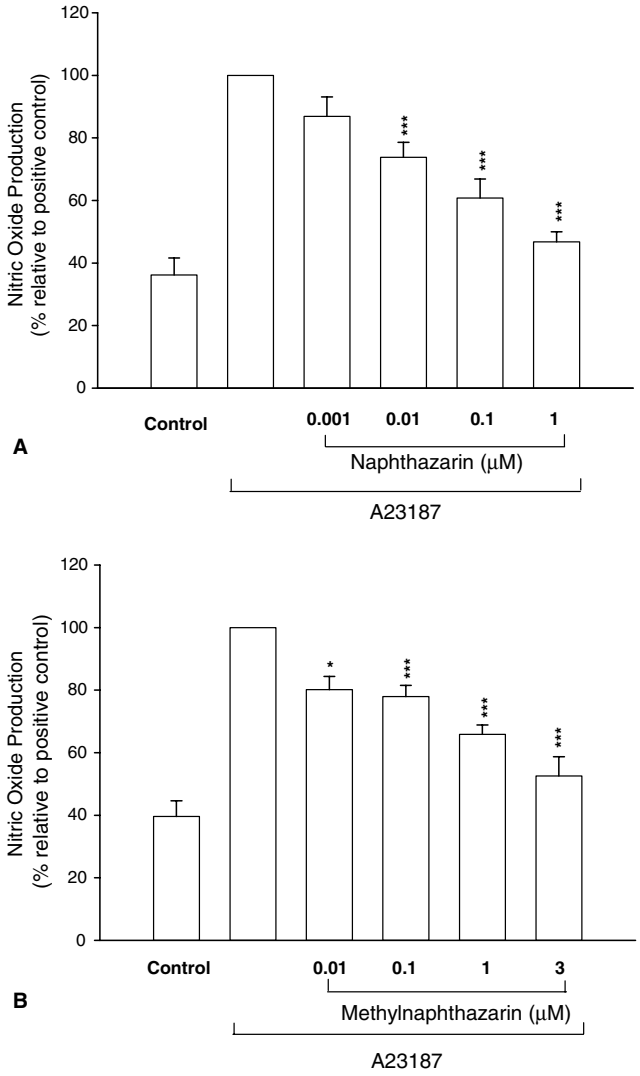


Fig. 5. Effects of Nap and MetNap on the production of nitric oxide induced by A23187. Various concentrations of Nap (0.001–1 μM) and MetNap (0.01–3 μM) were tested in A23187-treated HUVECs within 30 min. Data are expressed as the percent of the positive control (A23187, 2.5 μM). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ ($n \geqslant 8$).

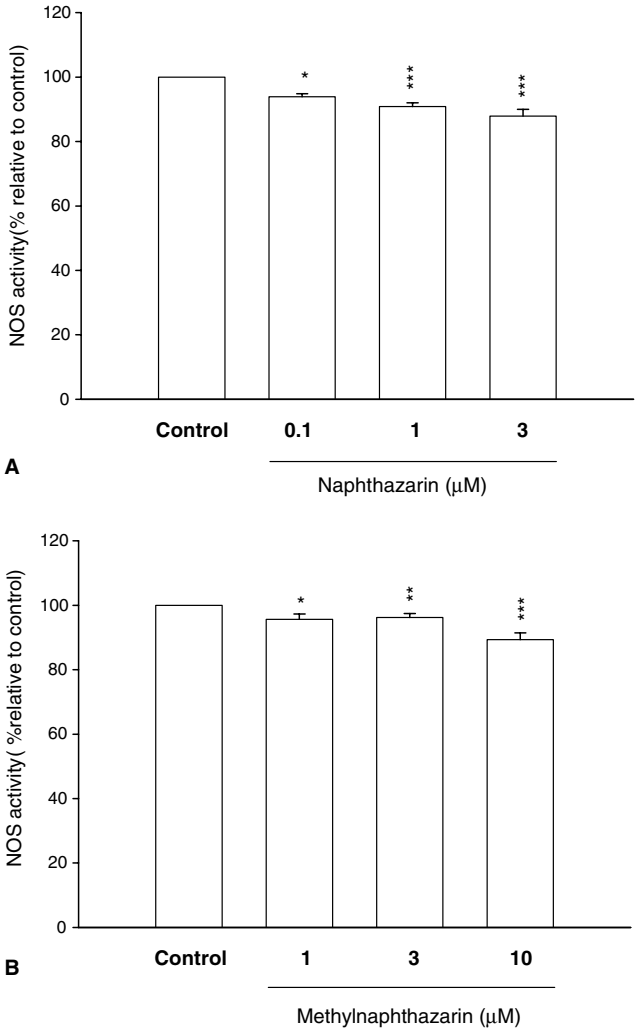


Fig. 6. Effects of Nap and MetNap on eNOS activity. All specific cofactors and substrates were incubated with various concentrations of Nap (0.1–3 μM) and MetNap (1–3 μM) at 37 °C for 30 min. Data are expressed as a percent of the control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ ($n \geqslant 8$).

NOS activity, respectively, relative to the control. Both NO production and NOS activity were significantly inhibited by Nap and MetNap in concentration-dependent manners, suggesting that the promotion of vasocontraction and inhibition of vasodilation might be involved in the decreases in NO formation and NOS enzyme activity.

3.4. Effects of Nap and MetNap on eNOS protein expression

HUVECs were incubated with various concentrations of Nap (0.01–3 μM) and MetNap (0.01–10 μM) for 24 h, then treated cells were harvested for Western blotting. Neither Nap nor MetNap altered the eNOS protein expression within 24 h (Fig. 7A and B).

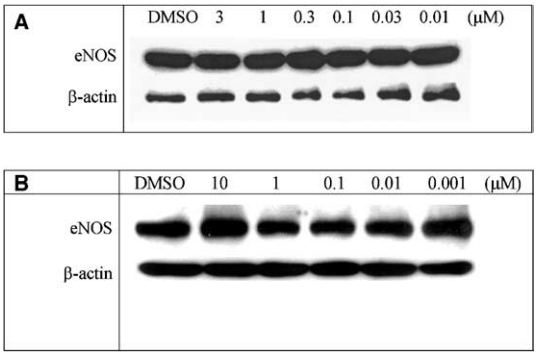


Fig. 7. Effects of Nap and MetNap on eNOS protein expression. HUVECs were incubated with Nap and MetNap at various concentrations as indicated. (A) Expression of eNOS protein after incubation with Nap for 24 h. (B) Expression of eNOS protein after incubation with MetNap for 24 h. β-Actin was used as the internal control. Data were repeated at least three times.

Table 1
Effect of Nap and MetNap in the production of the superoxide anion

Control	Naphthazarin	Methylnaphthazarin
	100 (%)	100 (%)
0.001 μM	102.2 ± 3.1	100.7 ± 2.1
0.01 μM	107.0 ± 3.5	119.9 ± 3.2*
0.1 μM	114.5 ± 1.7*	128.1 ± 2.1**
1.0 μM	128.7 ± 2.3**	130.8 ± 3.4***
3.0 μM	151.5 ± 1.0***	133.1 ± 2.8**

HUVECs were preincubated with dihydroethidium for 10 min, then different concentrations of naphthazarin (0.01–3 μM) were added to induce the production of superoxide. Superoxide production was analyzed by flow cytometry. Data were expressed as a percent of the control.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ ($n \geqslant 7$).

3.5. Effects of Nap and MetNap on the formation of $O_2^{\bullet-}$

We used the superoxide anion fluorescence dye, dihydroethidium (DHE), to detect the generation of $O_2^{\bullet-}$. Data shown in Table 1 indicate that both Nap and MetNap significantly induced superoxide anion formation in concentration-dependent manners (0.001–3 μM). Nap at 3 μM induced 151.5 ± 1.0% superoxide generation relative to the control, while MetNap induced 133.1 ± 2.8% at the same concentration.

4. Discussion

Our previous report demonstrated that various concentrations of naphthoquinone compounds, such as alkannin and shikonin, can alter vascular tone (Hu et al., 2004). Although the results from our previous study suggested that shikonin and its derivatives might act through inhibition of NO synthesis, we do not know, however, whether these compounds directly interact with eNOS or act through other possible pathways. However, no evidence has been shown that naphthoquinones cause similar effects in the vascular endothelium. The actual mechanism still remains unknown.

In the present study, we found that Nap and its derivative, MetNap, caused enhancement of vascular contraction and inhibition of vasorelaxation in an endothelium-dependent manner. It has been shown that stimulation of the vasomotor system by ACh or serotonin, evokes vasorelaxation (Furchgott and Zawadaki, 1980; Luscher and Vanhoutte, 1999) through the production of an endothelium-derived relaxing factor, also known as nitric oxide (NO) (Palmer et al., 1987). L-NAME, an NOS inhibitor, promotes constrictor-induced vasoconstraction and suppresses ACh-induced relaxation by reducing NOS activity (Schiffrin, 1995; Cheng et al., 2003). Therefore, Nap and MetNap might exert their vasocontraction actions and anti-vasorelaxing effect through a similar mechanism. NO is produced from the amino acid, L-arginine, by nitric oxide synthase (NOS) catalyzation (Cobb, 1993; Palmer, 1993) in Ca^{2+}-dependent (Moncada et al., 1991) or Ca^{2+}-independent manners (Dimmeler et al., 1999; Fulton et al., 1999). The NO-mediated vasorelaxation of bioactive agents is endothelium-dependent and possibly occurs through the NO-cGMP pathway (Rapoport and Murad, 1983). Whether Nap and MetNap can interfere with the formation of NO and the activity of NOS might be related to their vasoactive functions. Our results indicated that both Nap and MetNap have the greater ability to inhibit NO formation than to alter NOS activity. At the same concentration (1 μM), Nap and MetNap produced 47.4 ± 6.1% and 34.2 ± 3.0% inhibition in A23187-induced NO production, respectively. But they only have 9.1 ± 1.1% and 4.4 ± 1.7% inhibition in NOS activity. On the other hand, both compound did not involved in the protein expression of eNOS within 24 h. According to previous data, the inhibition of NOS activity seems not the main reason for the response of vasoreactivity to Nap and MetNap.

Two main features of quinines are now generally accepted as being critical for their cytotoxicity. One is the capacity of quinines to produce oxygen free radicals, and the other is the electrophilicity of quinines, which enables them to form adults to cellular constituents. The intracellular free radical production by quinines is thought to be a consequence of $O_2^{\bullet-}$ reduction by semiquinone intermediates. We found that Nap and MetNap are really able to induce $O_2^{\bullet-}$ in concentration-dependent manners. Thus, $O_2^{\bullet-}$ generation might play the major role in these effects. A body of references shows that superoxide anions (O_2^-) reduce NO bioavailability, thus leading to impaired endothelial vasorelaxation and augmented vasoconstriction (Galle et al., 1995; Kyong-Up et al., 1997). Thus, reducing NO availability can result from decreased activity of the NO-production pathway or increased oxidative inactivation of NO induced by O_2^-. Superoxide anions may directly inactivate NO (Gryglewski et al., 1986; Dobrucki et al., 2000), and the product of this reaction, peroxynitrite ($ONOO^-$), can hydroxylate nitrate aromatic compounds and induce cellular injury (Ronson et al., 1999). The longterm treatment (24 h) of MTT data can support the cytotoxic effect. The possibility of impairment of NO's action and $ONOO^-$-induced cytotoxicity is suggested for following reasons. (1) The ability to induce O_2^- formation by Nap was greater than that by MetNap; thus, the final production of $ONOO^-$ by Nap might be greater than that by MetNap. (2) At the same concentration (1 μM), Nap produced 47.4 ± 6.1% inhibition of NO production, but this was only 34.2 ± 3.0% of the effect of MetNap. (3) The cytotoxicity of Nap was much greater than that of MetNap; DC_{50} values of 24 h for

Nap and MetNap were 4.6 ± 0.8 and 25.8 ± 1.3 μM, respectively. From above reasons, O_2^- might play a major role in the impairment of the NO-mediated vasoactive function. We did not phase out the possibility that our observation in Nap and MetNap might be due to other endothelial-dependent vasocontraction mechanism, like TXA2, leukotriene and endothelin (Miller and Vanhoutte, 1985; Pang et al., 1989). We need further investigation to understand the role of these factors in the future study.

In summary, we conclude that Nap and its derivative (MetNap) potentiate PE-induced contractions and inhibit ACh-induced relaxation of the rat aorta in an endothelium-dependent manner, possibly through NO inactivation by inhibition of NO synthesis and superoxide production. Evidence suggests that elevated oxidative stress contributes to the endothelial dysfunction associated with atherosclerosis, hypertension, and heart failure (Cai and Harrison, 2000; Berry et al., 2001; Warnholtz et al., 2001). These data provide information on the cardiovascular adverse effects of quinoid compounds in pharmacochemical investigations.

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