High-dose Morphine Impairs Vascular Endothelial Function by Increased Production of Superoxide Anions

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Background: The effects of high-dose morphine on vascular endothelial function have not been previously shown. The authors hypothesized that the pro-oxidant effect of high-dose morphine impairs vascular endothelial function.

Methods: Mice were subjected to placebo or morphine (20 mg/kg intraperitoneal) injection for consecutive 14 days. Aortas were harvested for assessment of vasomotor function by isometric force recordings. Protein expression of p47phox was determined by Western blotting. Generation of superoxide anions were detected under a confocal microscope.

Results: Compared with controls, contraction response to phenylephrine was significantly enhanced in the aorta of mice treated with high-dose morphine (maximal contractions were 150 ± 26 vs. 261 ± 32 mg, respectively; n = 5 or 6, P = 0.04). Endothelium-dependent relaxations to acetylcholine (10−10 to 10−5 M) were significantly reduced in morphine-treated animals but were normalized by superoxide scavenging. Fluorescent densities of dihydroethidium were increased in the aorta of morphine-treated mice. Aorta of mice treated with morphine expressed higher levels of p47phox (a major subunit of nicotinamide adenine dinucleotide phosphate oxidase). In cultured endothelial cells, morphine enhanced production of reactive oxygen species.

Conclusions: Collectively, the authors' results showed that high-dose morphine impairs vascular endothelial function via attenuation of biologic activity of endothelium-derived nitric oxide. Chemical antagonism between superoxide anions generated by nicotinamide adenine dinucleotide phosphate oxidases may be the molecular mechanism responsible for the inactivation of endogenous nitric oxide after treatment with high-dose morphine.

Materials and Methods

Animals and Drug Treatment

Mice (C57BL/6J, 8–10 weeks old) were obtained from the Animal Center of the National Cheng Kung University (Tainan, Taiwan). The animals were housed under controlled temperature of 21° ± 0.5°C in wire-mesh cages, with free access to food and water. Mice were randomly assigned to control or morphine-treated group and received intraperitoneal injection of normal saline or morphine (20 mg·kg−1·day−1, the National Bureau of Controlled Drugs, Department of Health, Taipei, Taiwan), respectively, for 14 consecutive days, as described in our previous study.11 The dose of morphine used in the current study was arrived from the published reports in morphine-dependent rats or mice.3,12–14 At the end of treatment, mice were killed by injection of pentobarbital (250 mg/kg intraperitoneal). Thoracic and abdominal aortas were obtained from each animal. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (The National Cheng Kung University College of Medicine, Tainan, Taiwan).
**Histology Examinations**

Biopsies of formalin-fixed aortic segments were embedded in paraffin wax and sectioned (5 μm). Sectioned tissues were stained with hematoxylin and eosin.

**Measurement of Vascular Reactivity**

Aortic rings (approximately 2 mm long) were isolated and mounted in organ chambers containing 10 ml Krebs solution. The chambers were maintained at 37°C and aerated continuously with 94% O₂–6% CO₂. Changes in isometric force were recorded continuously using an isometric force-displacement transducer (Grass FT03; Grass Instrument, West Warwick, RI). Each ring was gradually stretched to 1.5 g. After a 45-min equilibration period, the rings were contracted by cumulative addition of phenylephrine (10⁻⁹ to 10⁻⁵ M; Sigma-Aldrich, St. Louis, MO). To study the relaxation, isolated aortic ring was first contracted with an EC₅₀ (the concentration required to induce 50% of maximum contraction) of phenylephrine. Concentration-response curves were then obtained by cumulative addition of acetylcholine (10⁻⁹ to 10⁻⁵ M; Sigma-Aldrich) and a nitric oxide (NO) donor (DEA-NONOate, 10⁻⁹ to 10⁻⁵ M; Sigma-Aldrich) during the contraction to EC₅₀ of phenylephrine. Some of the preparations were incubated for 15 min before each contraction with a cell-permeable superoxide dismutase mimetic (Mn-III-tetrakis-4-benzoic acid-porphyrin; MnTBAP; 10⁻⁴ M; Biomol, Plymouth Meeting, PA). Papaverine (3 × 10⁻⁴ M; Sigma-Aldrich) was used to induce complete relaxation of the vessels. All experiments were performed in vessels with intact endothelium, and in the presence of indomethacin (10⁻⁵ M; Sigma-Aldrich) to prevent the possible influence of vasoactive prostaglandins.

**Western Blot Analysis**

Soluble protein (30 μg) extracted from mouse aorta was loaded into polyacrylamide gels (9–12%) and transferred onto polyvinylidene fluoride membranes. Mouse monoclonal antiendothelial nitric oxide synthase (eNOS) antibodies (1:1,000; BD Transduction Labs, San Jose, CA) or anti-p47phox (1:1,000; BD Transduction Labs) antibodies were used. After washing, the membranes were incubated with 1:2,000 dilution of horseradish peroxidase–linked secondary antibodies, and bands were visualized by enhanced chemiluminescence. Protein levels were quantified by scanning densitometry (Scion Image, Frederick, MD).

**Dihydroethidium Assay for Detection of Superoxide Anions**

The unfixed frozen aortic segments were sectioned with a cryostat and placed on glass slides. Dihydroethidium (1 μM; BD Transduction Labs) was applied to each tissue section, and then the sections were covered-slipped. The slides were incubated in a light-protected humidified chamber at 37°C for 30 min before measurement of red fluorescence labeling by a laser scanning confocal imaging system.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Bioscience (Walkersville, MD). HUVECs were cultured in six-well plates with the supplement of endothelial growth medium 2 (EGM-2; Cambrex) in a 95% air-5% CO₂ environment at 37°C. Cells between passages 5 and 8 were used in this study. When cells reached an 80% confluence, each well was washed with PBS and incubated with endothelial cell basal medium 2 (EBM-2) overnight at 37°C. Subsequently, HUVECs were washed and reincubated in EBM-2 containing variable concentrations of morphine (10⁻¹⁰ to 10⁻⁴ M) for another 12 h.

*Human umbilical vein endothelial cells were harvested at the end of experiments. Generation of intracellular ROS in HUVECs after treatment with different concentrations of morphine was assessed after incubation with dihydroethidium. When oxidized within the cells, dihydroethidium produces DNA-sensitive fluorochromes, which was quantified by a flow cytometry (BD Biosciences).*

**Statistical Analysis**

Results are presented as the mean ± SEM. Data were compared by an unpaired t test or analysis of variance, as appropriate. Statistical significance was accepted at a level of P < 0.05. The n number represents the number of animals in each treatment group.

**Results**

**Vascular Reactivity and Histology**

Contractions to phenylephrine were significantly enhanced in the aortic rings isolated from mice treated with high-dose morphine (fig. 1). On the other hand, endothelium-dependent relaxations to acetylcholine were significantly impaired in morphine-treated mice, and there was a significant reduction in the maximal relaxation in comparison with controls (73 ± 6 vs. 96 ± 2%, respectively; P = 0.008, n = 4–6; fig. 2A). The impaired relaxation response was normalized by incubation with a superoxide scavenger, MnTBAP (fig. 2A). Endothelium-independent relaxations induced by DEA-NONOate were similar (fig. 2B). Hematoxylin and eosin stain showed no difference in the thickness of intimal and medial layers between the two treatment groups under high-power fields (400×; data not shown).

**Protein Expression**

Because organ bath experiments revealed that morphine-induced endothelial dysfunction was normalized...
by superoxide scavenging, we therefore studied the protein expression of eNOS and NADPH oxidases in the aortas. Compared with controls, expression of eNOS and p47phox (a major subunit of NADPH oxidase) was significantly enhanced in the aorta of mice treated with high-dose morphine (fig. 3).

Detection of Superoxide Anions in Aortas
Levels of vascular superoxide anions were detected by the dihydroethidium assay. Fluorescent densities of dihydroethidium in the aortic rings were increased in morphine-treated animals (fig. 4).

Effects of Morphine on Cultured HUVECs
To further investigate the pro-oxidant effect of high-dose morphine in a more homogeneous and controlled condition, we performed experiments in cultured endothelial cells. Morphine stimulated intracellular production of ROS in a concentration-dependent manner (fig. 5).

Discussion
We presented the first study demonstrating that high-dose morphine impairs vascular endothelial function via increased production of superoxide anions. The reduced endothelium-dependent relaxation is associated with higher levels of superoxide anions, indicating that the endothelium-derived NO is most likely neutralized by superoxide anions and thus impairs endothelial function. We speculated that the up-regulation of NADPH oxidases may be responsible for the generation of vascular superoxide anions after high-dose morphine, although other superoxide-generating genes can also be involved.

In the current study, mice received a high daily dose of morphine injection (20 mg · kg⁻¹ · day⁻¹) in accordance with the previously reported model of morphine dependence¹²–¹⁴ and in our previous study.¹¹ Fourteen days after the administration of morphine, isolated mouse aortas were analyzed for vascular reactivity. Contraction responses to phenylephrine (α₁-adrenergic receptor agonist) were enhanced, and endothelium-dependent relaxation to acetylcholine was reduced in morphine-treated mice. The impaired endothelium-dependent relaxation response was normalized by treatment with a cell-permeable superoxide scavenger, indicating that increased generation of superoxide anions and subsequently attenuated endothelium-derived NO is the major mechanism responsible for the endothelial dysfunction in this experimental model. Therefore, higher contraction of the aorta is well correlated with the reduced basal, nonstimulated levels of endothelium-derived NO after treatment with morphine. We tested the function
of vascular smooth muscle by cumulative addition of NO donor and found that endothelium-independent relaxation responses were similar between the two groups. Together with morphologic findings shown by the hematoxylin and eosin staining, these functional analyses did not favor a significant remodeling process in the vascular smooth muscle cells.

Using the classic pharmacologic approach, endothelial dysfunction induced by high-dose morphine was normalized by means of superoxide scavenging in the vasomotor function experiments. We therefore determined levels of superoxide anions in the mouse aorta using the dihydroethidium assay. Consistent with a previous study in glomerular epithelial cells, in which a dose-dependent elevation of superoxide anions was shown after treatment with morphine ($10^{-14}$ to $10^{-6}$ M), our results clearly demonstrated that the fluorescent densities of dihydroethidium were significantly increased in the aorta of mice treated with high-dose morphine. In our in vitro experiments, we also detected a concentration-dependent increase of intracellular ROS in the cultured endothelial cells after treatment with morphine. Increased generation of superoxide anions in the isolated aorta and ROS in the cultured endothelial cells thus support our observations in the impaired endothelium-dependent relaxation.

Fig. 3. Western blot analysis for protein expression of aorta in control and morphine-treated mice. Expression of endothelial nitric oxide synthase (eNOS) and p47phox (a major subunit of nicotinamide adenine dinucleotide phosphate oxidase). * $P < 0.05$, $n = 4$ or 5 animals in each group. Statistical analysis (unpaired t test) was performed by comparing the relative density of bands quantified by scanning densitometry (Scion Image) in two groups. Data are shown as mean ± SEM.

Fig. 4. Dihydroethidium assay was performed in the frozen mouse aorta to measure the generation of superoxide anions. Increased dihydroethidium fluorescent (red) densities were detected in the aorta isolated from morphine treated mice (B) compared with controls (A). Experiments were performed in 4 or 5 animals for each group.
One of the major sources of superoxide generation in the vasculature is activation of NADPH oxidases. Vascular NADPH oxidase is a multicomponent enzyme, which consists of a membrane-bound p22phox heterodimer and the subunits, including p67phox, p47phox, p40phox, and Rac. The subunit p47phox is critical in modulating enzymatic activity of NADPH oxidase. The NADPH oxidases catalyze the univalent critical in modulating enzymatic activity of NADPH oxidase.22 NO interacts with excessive superoxide radicals in a near diffusion-controlled reaction to form peroxynitrite, which is potent in nitration of cellular proteins. In the current study, protein expression of p47phox indicates the up-regulated NADPH oxidases involved.

On the other hand, expression of eNOS was also significantly enhanced in the aorta of mice treated with morphine. Limited data are currently available to demonstrate the effect of morphine on constitutive NOS isoforms. Stefano et al. showed a two-phase NO release after incubation of macrophages in morphine, in which induction of constitutive NOS and inducible NOS are responsible for the acute and delayed phase of NO release, respectively. Activation of neuronal NOS has also been shown in association with high-dose morphine injection (15 mg/kg) in rats. In cultured human circulating polymorphonuclear leukocytes, messenger RNA levels of eNOS were up-regulated after morphine (1 μg/ml) exposure. However, in vivo regulation of eNOS after morphine treatment has not been previously demonstrated. Furthermore, mechanisms underlying morphine-induced activation of NOS remain unclear. The other potential mechanisms responsible for enhanced eNOS expression with reduced NO bioavailability are that the eNOS enzymatic system is dramatically "switched on" during prolonged exposure to oxidative stress, but balance between vascular protection and damage is still not able to be preserved due to excessive production of superoxide anions, which have been well characterized in animal models of aging and heart failure.

The pro-oxidant effects of morphine have been previously demonstrated in the literature. Morphine causes a dose-dependent increase of superoxide generation in the glomerular mesangial and epithelial cells. At higher concentrations (in the range of 10\(^{-10}\) to 10\(^{-6}\) M), morphine inhibited the proliferation of cultured glomerular epithelial cells, whereas morphine stimulated glomerular epithelial cell proliferation at lower concentrations. The biphasic effect of morphine on cell survival is also seen in cultured endothelial cells. Gupta et al. showed significant cytotoxic effect of morphine at high concentrations up to 10\(^{-2}\) M. Using a mouse model, Zhang et al. demonstrated that morphine-induced (20–30 mg · kg\(^{-1}\) · day\(^{-1}\) for up to 20 days) hepatotoxicity was mediated by increased oxidative stress in the liver, and the hepatic injury was protected by antioxidants, such as ascorbic acid. In accord with these previous reports, our current data support the concept of pro-oxidant effect of high-dose morphine in vascular endothelial function.

In conclusion, high-dose of morphine impairs endothelial dysfunction by increased production of vascular superoxide anions. Activation of NADPH oxidase may be the molecular mechanisms responsible for reduced bioavailability of endothelium-derived NO. This study provides new insight into the pro-oxidant effect of high-dose morphine in the vascular endothelium.

References


Fig. 5. Formation of reactive oxygen species (ROS) in the cultured human umbilical vein endothelial cells after exposure to different concentrations of morphine (10\(^{-10}\) to 10\(^{-4}\) M). Intraacellular levels of ROS were measured by dihydroethidium under flow cytometry. *P < 0.05 in comparison with controls. +P < 0.05 in comparison with 10\(^{-10}\) M morphine. Relative densities of ROS were analyzed by analysis of variance and are shown as mean ± SEM. n = 3 or 4 independent experiments in triplicate.