



In vitro and *in vivo* assessment of cellular permeability and pharmacodynamics of S-nitrosylated Captopril, a nitric oxide donor

*^{1,2}Lee Jia & ¹Hong Wong

¹Institute For Drug Development, 14960 Omicron Dr. San Antonio, Texas, TX 78245-3217, U.S.A. and ²Department of Clinical Pharmacology, East Hospital, Fuzhou, Fujian 350025, China

1 The present studies were aimed at testing the hypothesis that S-nitrosylated captopril (CapNO), a novel crystalline nitric oxide (NO) donor, readily permeates both *in vitro* and *in vivo* endothelial monolayers, resulting in its pharmacodynamic effects.

2 CapNO and Captopril (Cap) were added to apical side of endothelial monolayers formed on microporous membranes, and the permeated drugs were collected from basolateral side and detected by a HPLC method. The permeability coefficient (P_{app} ; cm sec^{-1}) of CapNO across the endothelial monolayers was 6.0×10^{-5} , higher than that of Cap (3.13×10^{-5}), indicating the enhancement effect of the attached NO group in CapNO on cellular permeability. The P_{app} of CapNO and Cap across Caco-2 cells were 3.15×10^{-5} and 1.53×10^{-5} , respectively. The low P_{app} of CapNO to Caco-2 cells may be attributed to the high membrane resistance of Caco-2 cells.

3 A bolus injection of CapNO to epicardial coronary artery of chronically-instrumented awake dogs caused significant increases in coronary blood flow and coronary diameters dose-dependently without significant changes in aortic pressure. In contrast, the equimolar doses of Cap did not produce haemodynamic responses.

4 Intravenous CapNO caused an instant increase in the regional cerebral blood flow determined by H_2 -clearance, whereas the equimolar doses of Cap did not enhance the cerebral blood flow.

5 These results conclude that the NO group, an active component of CapNO, enhances both *in vitro* and *in vivo* endothelial permeability to the entire compound, resulting in instant increases in blood flow and vascular diameters. In contrast, the equimolar Cap does not have the instant vascular effects.

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Abbreviations: Cap, Captopril; CapNO, S-nitrosylated captopril; HPLC, high performance liquid chromatography; NO, nitric oxide; P_{app} , permeability coefficient; RS-NOs, S-nitrosylated molecules

Introduction

For discovering nitric oxide (NO) as a signalling molecule in the cardiovascular system, Furchgott and Ignarro received the 1998s Nobel Prize in Physiology and Medicine. They and others had noted that S-nitrosocysteine showed close physiological and pharmacological resemblance to NO (Furchgott *et al.*, 1992; Ignarro *et al.*, 1981, Myers *et al.*, 1990; Feelisch *et al.*, 1994). In the course of comparing the similarities between S-nitrosocysteine and NO as relaxants of vascular smooth muscle, Jia & Furchgott (1993) found that S-nitrosocysteine was a powerful relaxing agent being equal to or somewhat greater in potency than NO on rings of rabbit aorta in bioassay. This finding has generated an interest in linking NO to thiol-containing molecules to synergistically improve the efficacy of the parent thiol-containing molecules, and thus creating them dual capacities of both a NO donor and a parent molecule without the

introduction of toxicity. Led by this hypothesis, we had synthesized both S-nitrosylated haemoglobin (Jia *et al.*, 1996; Stamler *et al.*, 1997) and S-nitrosylated vasoactive intestinal peptide (Jia & Stamler, 1999). Both molecules exhibited NO-like vascular relaxing activity without the loss of their original bioactivities. Noting that S-nitrosylated molecules (RS-NOs, where R can be any one of a large range of chemical entities) serve as the body's endogenous storage and transport forms for NO, many efforts have been made to convert the SH- group of captopril (Cap) into a S-NO group, and synthesize a powder form of S-nitrosylated captopril, i.e., S-nitrosocaptopril (1-[(2S)-3-nitrosomercapto-2-methyl-1-oxopropyl]-L-Proline; CapNO) (Loscalzo *et al.*, 1989; Park, 1992; Amano *et al.*, 1994). This compound can be used as both an NO-containing drug and an angiotensin converting enzyme inhibitor.

We are the first who synthesized red crystalline CapNO in a large scale (Lin *et al.*, 1998; Jia & Blantz, 1998), systematically characterized the physicochemical properties of the novel crystals (Jia *et al.*, 1999; 2000), and revealed the

*Author for correspondence at: Institute For Drug Development, 14960 Omicron Dr. San Antonio, Texas, TX 78245-3217, U.S.A.
E-mail: Ljia@saci.org

LD50 of oral CapNO at 2078 ± 100 mg kg⁻¹ without adverse effects after subchronic toxicity study (Jia *et al.*, 2001). In isolated blood vessels, we found that CapNO at μ M levels produced quick and sustained vasorelaxation (Lin *et al.*, 1998), suggesting that the compound promptly permeates through the endothelial monolayers and acts on the underlying smooth muscle to produce vasorelaxation. To test the aforementioned hypothesis, we established both an *in vitro* endothelial model and *in vivo* dog and rat models. The human endothelial cells were cultured on microporous membranes (Transwell) that represent a more physiological environment to allow substances to transport across cellular monolayers. In addition, CapNO was also locally administered to animals to observe the corresponding pharmacodynamic responses triggered by CapNO after permeating through the endothelial luminal surface. The animal protocol was approved by the Institutional Animal Care and Use Committee. The present studies were performed in comparison of CapNO effects with that of Cap. The results of these studies are reported here.

Methods

Biostability studies of CapNO

To ensure that CapNO did not undergo biomatrix-catalyzed decomposition, we first determined the stability of CapNO in a serum-free Dulbecco's modified Eagle medium (DMEM) at 37°C for 6 h using a HPLC method (Jia *et al.*, 1999) with minor modifications. CapNO crystals were dissolved in the nonbicarbonate-based DMEM medium to make a final concentration of 1 mM, which was then maintained at 37°C in a well-humidified incubator containing 5% CO₂. An aliquot of the medium was withdrawn at various times and centrifuged at $14,000 \times g$ for 10 min. Forty microliters of the supernatant were directly injected into a reversed-phase C18 column (Luna 3 μ m, 100×4.6 mm i.d., Phenomenex, Torrance, CA, U.S.A.) through a HPLC system (Model HP 1100, Agilent Technologies, CA, U.S.A.). The column was maintained at 25°C, and protected with a C18 octadecyl guard cartridge (4×3.0 mm i.d., Phenomenex). A mobile phase composed of phosphoric acid and HPLC-grade water (0.1%, v v⁻¹) was pumped onto the column at the rate of 0.7 ml min⁻¹ for 12 min. The u.v. absorbance of CapNO was monitored at 220 nm.

Cell culture and CapNO cellular permeability assay

The CapNO cellular permeability assay was implemented on endothelial monolayers and analysed by the aforementioned HPLC method. Both human umbilical endothelial cells and Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The endothelial cells were grown in Kaighn's F12K medium supplemented with 2 mM of L-glutamine, 1.5 mg ml⁻¹ of sodium bicarbonate, 20% foetal bovine serum, 0.1 mg ml⁻¹ of heparin, 0.3 mg ml⁻¹ of endothelial cell growth supplement, 50 U ml⁻¹ of penicillin and 50 μ g ml⁻¹ of streptomycin. The Caco-2 cells were cultured in DMEM plus 25 mM of glucose, 10% foetal bovine serum, 10 mM of HEPES, 10 μ g ml⁻¹ of transferrin, and the same concentrations of L-glutamine,

penicillin, streptomycin, and sodium bicarbonate as used for the endothelial cells. The cells were incubated at 37°C and 5% CO₂ in culture flasks with growth area of 75 cm² and a density of 10^5 cells cm⁻². The medium was replaced with fresh medium three times per week until the cells formed confluent monolayers. The cells were harvested from the flasks with a mixture solution of 0.05% trypsin and 0.53 mM of EDTA-4Na in Hank's balance salt solution when the cells reached confluence. The cells were then transferred and seeded onto clear Transwell inserts (Catalogue No. 3460; Corning Costar Corp., Cambridge, MA, U.S.A.). The inserts are featured as polyester membrane filters with a pore size of 0.4 μ m and a diameter of 12 mm. Half of the Transwells acted as control wells without cells seeded, and the other half were plated with endothelial cells at a density of 10^5 cells per cm². Cells were allowed to grow for 7–10 days until a monolayer was formed on the membrane. On the day of experiment, the medium was aspirated. With the inserts suspended in the wells, serum-free DMEM (pH 7.3) containing CapNO was added to the apical chamber to give a final concentration of 100 μ M while the basolateral chamber was filled with prewarmed DMEM. An aliquot (50 μ l) of the DMEM was removed from the basolateral chamber at 0.5, 1 and 2 h, and immediately stored in -80°C until the HPLC analysis.

The apparent permeability coefficient (P_{app}) of CapNO was determined according to Artursson (1990) equation:

$$P_{\text{app}} = \frac{\Delta Q / \Delta t}{60 \cdot A \cdot C_0} (\text{cm} \cdot \text{sec}^{-1})$$

where $\Delta Q / \Delta t$ is the permeability rate ($\mu\text{M s}^{-1}$) of the drug across the microporous membrane, which is calculated from the initial straight slopes. A is the surface area of the membrane (i.e., 1 cm²), and C_0 is the initial CapNO concentration in the donor chamber at $t=0$ (μM). The results of experiments performed in six replicates are presented as mean \pm s.d.mean.

Preparation for awake dogs with chronically coronary artery catheterization

Mongrel dogs (21–27 kg) were anaesthetized with intravenous thiamylal sodium (60–80 mg kg⁻¹) and subjected to left thoracotomy in the fourth intercostal space. The left circumflex coronary artery distal to the left atrial appendage was minimally dissected. A pair of 7-MHz piezoelectric crystals (1.5 \times 2.5 mm, 15–20 mg) was attached to a Dacron backing and sutured to the adventitia on opposite surfaces of the dissected vessel segment with 6-0 prolene. Oscilloscope monitoring and on-line sonomicrometry were used to ensure proper crystal position. A pulse Doppler flow probe was implanted distal to the crystals. An inflatable balloon occluder was placed distal to the flow probe. All branches of the circumflex artery between the crystals and the occluder were ligated. Heparin sodium-filled polyvinyl catheters were inserted into the left ventricular cavity *via* the apex, into the left atrium *via* the atrial appendage, and into the ascending aorta *via* the left internal thoracic artery. The catheters, tubing, and wires were tunnelled to a subcutaneous pouch at the base of the neck. After a 10- to 15-day recovery period, the catheters and wires were exteriorized under general anaesthesia.

Haemodynamic measurement in awake dogs after dosing

Dogs were trained to lie awake in the lateral recumbent position while loosely restrained. The laboratory was kept dimly illuminated and quiet. Aortic pressure, left ventricular end-diastolic pressure, dP/dt, external coronary diameter, and coronary flow were monitored continuously. CapNO and Cap were diluted in sterilized saline, and administered, respectively, to the dogs (100 and 500 $\mu\text{mol kg}^{-1}$) via the left atrial catheter by a bolus injection. To verify potential effects of vehicle on vasculature, 0.1-ml saline were injected as vehicle control. Between injections, phasic coronary blood flow and coronary artery diameter were allowed to return to preinjection levels. Allowing a 15-min period between injections resulted in no modification of repeated dose injections. Epicardial coronary diameter, coronary blood flow, heart rate and aortic and left ventricular end-diastolic pressure were compared before and after each drug injection. To compare the time course of effects of CapNO and Cap on coronary vasodilation, the times of increases in epicardial coronary diameter and coronary blood flow were measured.

Rat surgery for measuring brain regional blood flow

Adult male Wistar rats (295–340 g) were anaesthetized with sodium pentobarbital (50 mg kg^{-1} , i.p.), intubated and ventilated on room air with a small animal respirator at a rate and tidal volume sufficient to maintain normal values of PaCO₂ (35–45 mmHg). The rats were catheterized with a PE50 tube in a femoral artery to monitor blood pressure and obtain blood samples, and in a femoral vein to administer supplemental anaesthetics and saline. Aliquots of arterial blood (200 μl) were withdrawn periodically to monitor blood gas tensions and pH using a blood gas/pH analyzer. The blood was replaced intravenously with sterilized saline. The rats were placed on a heating pad, and the rectal temperature was monitored and maintained at 37°C.

Measurement of cerebral blood flow of anaesthetized rats

H₂ clearance was used in the present studies for multiple *in situ* determinations of cerebral blood flow from tissues in which a small electrode was implanted. The method was similar to that described previously (Stamler *et al.*, 1997). The tissue PO₂ was measured continuously with polarographic platinum microelectrodes (50 μm outer diameter, coated with a hydrophobic gas permeable Nafion membrane to protect the electrode from fouling) mounted on a micromanipulator. The rat's head was immobilized in a Kopf stereotaxic frame. The lambda and the bregma were first localized. The microelectrodes were implanted, through a small hole in the skull after puncture of the dura mater, into the parietal cortex, caudate putamen nucleus (AP, +0.8 mm; ML, -2.5 mm, DV, -5.2 mm), and substantia nigra (AP, -5.3 mm; ML, -2.4 mm, DV, -8.2 mm) for measurement of regional blood flow. The PO₂ electrodes were polarized to -0.65 V against an Ag/AgCl reference located on the tail, and arterial PO₂ was adjusted by changing the inspired O₂ concentration and atmospheric pressure. The current flow was measured using a low-impedance nanoampere meter during and after the inhalation of gas mixture of 2.5% H₂ for 1 min. The H₂-clearance and O₂ tracings were recorded by

using PC WINDAQ version 4.1 software program. Cerebral blood flow was calculated with the initial slope method by converting coefficient K to blood flow per volume of tissue when the tissue/blood partition coefficient of H₂ is known (Aukland *et al.*, 1964). Regional blood flow was monitored for 30 min before and after administration of CapNO or Cap (both 1 and 10 $\mu\text{mol kg}^{-1}$, i.v.).

Results

CapNO permeability across endothelial and Caco-2 monolayers

Biostability testing of CapNO incubated with DMEM at 37°C for 6 h revealed only 14.2% of CapNO decomposed (Figure 1). The decomposition rate of CapNO was dependent on temperature and initial concentrations of the drug (Jia *et al.*, 1999). To maintain the decomposition of CapNO within the given degree, the following permeability experiment was conducted under the same conditions as the biostability.

The integrity of each batch of cell monolayers was first verified by microscopy, and then tested by measuring the leakage of [¹⁴C]D-mannitol in representative cell monolayers. D-mannitol is a molecule known to traverse cellular sheet exclusively *via* the paracellular route through tight junctions (zonula occludens) (Mullin *et al.*, 1986). The apical to basolateral flux for this paracellular marker did not exceed values of 0.5% penetrated per hour. Both the morphological microscopic evaluation and mannitol leakage test indicated the integrity of the cell monolayers. The permeability rate of CapNO across endothelial monolayers seeded on Transwell was determined by the HPLC method, and shown in Figure 2. When applied to the apical side (donor chamber) of endothelial monolayers, CapNO (100 and 250 μM) was detectable on the basolateral side (collect chamber) at time point of 30 min. However, attempts to use 50 μM of CapNO for the study ran into difficulties related to the u.v. detection limit of HPLC, particularly, at early time points of 0.5 and 1 h. The time course for CapNO to appear in the collect chamber of control Transwell (cell-free) was close to that of Transwell seeded with cells, suggesting the relatively high permeability rate of CapNO across the endothelial cells.

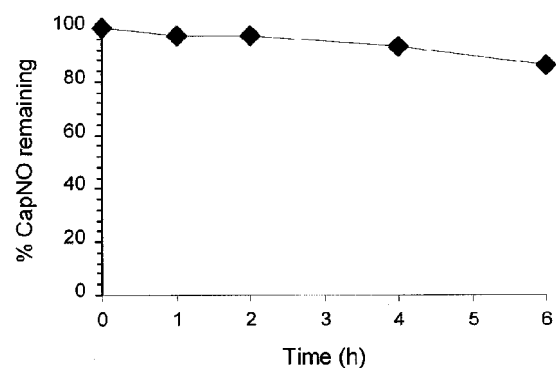


Figure 1 Biostability of CapNO (1 mM) incubated in DMEM culture medium at 37°C. The remaining amount of CapNO in the DMEM culture medium was determined by the HPLC method. Each point represents the mean \pm s.d. of three separate measurements.

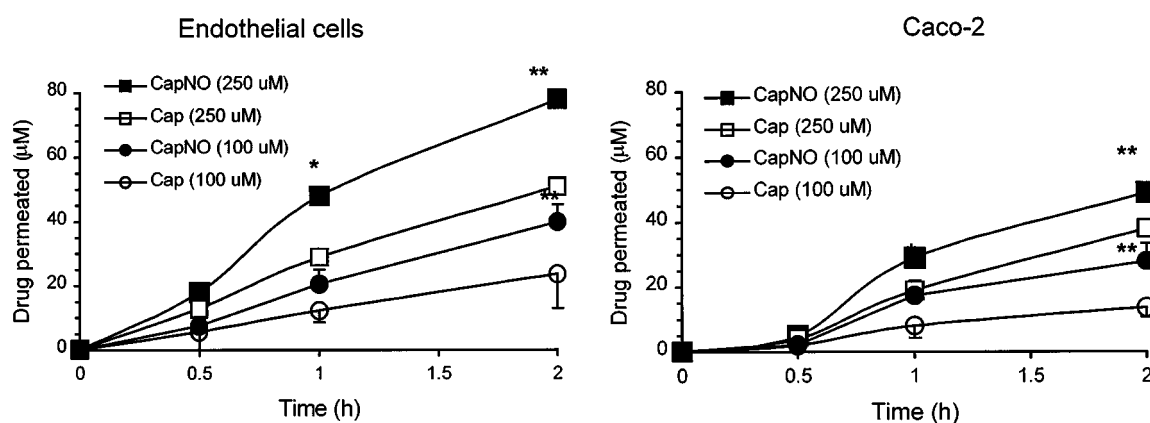


Figure 2 Cellular permeability of CapNO and Cap across human umbilical endothelial monolayers and Caco-2 monolayers. CapNO and Cap were added to the apical chambers of Transwell at final concentrations of 100 and 250 μM . The permeated CapNO and Cap were determined from the basolateral chambers by the HPLC method. The data represent the mean \pm s.d. ($n=6$), and evaluated for statistically significant differences by one way analysis of variance (ANOVA). * $P < 0.05$ and ** $P < 0.01$, compared with CapNO. For clarity, only representative error bars were shown.

Under the well-controlled conditions, we found that the P_{app} (cm s^{-1}) of CapNO (100 μM) across endothelial monolayers was 6.0×10^{-5} , which was significantly higher than those of drugs with comparable molecular weight, such as Cap (3.13×10^{-5}), Taxol, and carbendazim (unpublished observation) tested in this laboratory. Since the experiment was conducted by using the serum-free DMEM, the effects of the drugs bound to serum protein on the permeability rate could be avoided. Therefore, the permeability rate of individual drugs is comparable to each other without interference from plasma protein. Figure 2 also shows permeation of CapNO and Cap through Caco-2 cells, a model system of intestinal epithelial permeability. Comparing the results obtained from endothelial monolayers, it was seen that Caco-2 cells possessed more resistance against CapNO than endothelial cells. The P_{app} of CapNO and Cap through the Caco-2 cells were 3.15×10^{-5} and 1.53×10^{-5} cm s^{-1} , respectively. Measurements of transcellular resistance in culture medium using a Millipore Millicell-ERS system (Millipore Corporation, Bedford, MA, U.S.A.) revealed that the transcellular resistance of Caco-2 and endothelial cells was about 420–500 and 15–25 $\Omega \cdot \text{cm}^2$, respectively. The differences in resistance between the two cell lines appear to affect the corresponding cellular permeability to the drugs.

Comparison of effects of CapNO and Cap on hemodynamics of awake dogs

Bolus injections of CapNO (100 and 500 nmol kg^{-1}) into the left atrium of the awake dogs produced immediate haemodynamic changes in a dose-dependent manner, including epicardial vasodilation, an increase in coronary diameter and blood flow, a transient decrease in aortic pressure, and a transient increase in heart rate and left ventricular dP/dt. Figure 3 illustrates a direct tracing of the hemodynamic responses to a bolus injection of CapNO (500 nmol kg^{-1}). The increase in coronary blood flow and the concomitant decrease in aortic pressure represent a net decrease produced by CapNO in the resistance of vessels that regulates flow. Compared with the blood flow of regulatory resistance vessels

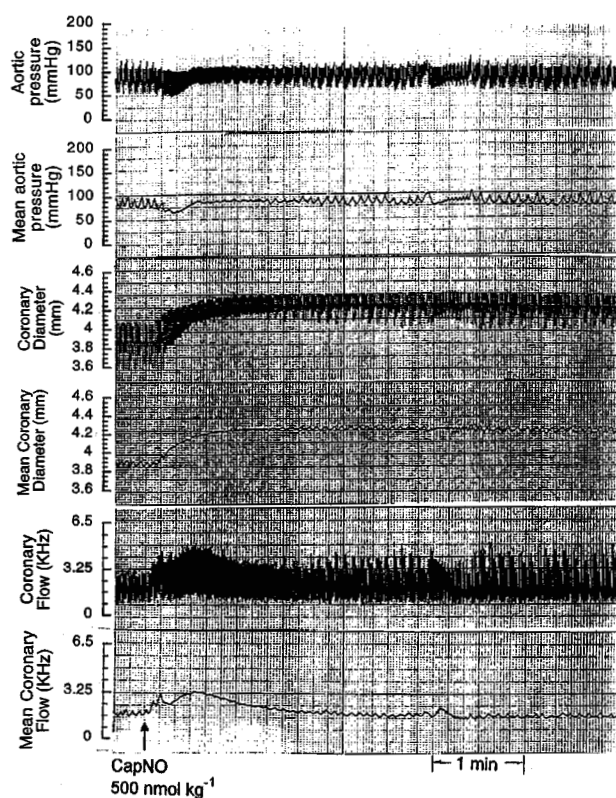


Figure 3 Representative haemodynamic responses of a chronically-instrumented awake mongrel dog to a bolus injection of CapNO (500 nmol kg^{-1}) into left atrium. The arrow indicates the injection time. The haemodynamic parameters represent four individual experiments. Note the immediate and sustained increases in canine coronary diameter caused by CapNO.

(represented by blood flow), the coronary conductance vessels (represented by circumflex dimensions) (Zhang *et al.*, 1993) were more sensitive to the effects of CapNO. The effects of CapNO on coronary dimension were sustained, lasting about

36 min (100 nmol kg^{-1}) and 60 min (500 nmol kg^{-1}) after aortic pressure, heart rate and left ventricular end-diastolic pressure had returned to control levels. In contrast, the changes in coronary blood flow were brief, lasting about 1 min, indicating marked differential effects of CapNO on the duration of vasodilation of proximal and distal coronary vessels. There were no significant differences in the changes in dimension with and without constant blood flow, indicating that the responses of the conductance vessels to CapNO resulted from a direct effect. At equimolar doses the epicardial vasodilation responses to Cap (100 and 500 nmol kg^{-1}) were not significant. Basically, Cap did not cause appreciable changes in coronary diameter, blood flow, mean aortic pressure and left ventricular end-diastolic pressure. Table 1 tabulates haemodynamic changes before and after bolus injections of CapNO or Cap into left atrium of the awake dogs. Peak effects were measured. The table showed that CapNO (500 nmol kg^{-1}) caused a $\sim 23\%$ increase in coronary flow, and $\sim 11\%$ increase in vessel dimensions that represented a $>20\%$ increase in cross-sectional area. Whereas, Cap (500 nmol kg^{-1}) did not show any significant effects on haemodynamic parameters of the chronically-instrumented awake dogs (Table 1)

Comparison of effects of CapNO and Cap on rat cerebral blood flow

The basic H_2 -clearance in the parietal cortex, caudate nucleus, and substantia nigra of the rats was determined three times during and after inhalation of $2.5\% \text{ H}_2$ for 1 min. Inhalation of the $2.5\% \text{ H}_2$ gas did not change the H_2 content in the three regions. Immediately after the third basic determination, the rats were intravenously given CapNO or Cap (1 and $10 \mu\text{mol kg}^{-1}$), and H_2 -clearance was determined at 3, 10 and 20 min post dose. Figure 4 dynamically shows the time course of increases in blood flow caused by CapNO in the regions of parietal cortex, substantia nigra and caudate nucleus. Figure 5 displays the dose-dependent peak effects of CapNO on cerebral blood flow. Concomitant with the increase in brain regional blood flow, CapNO produced a significant decrease in the mean blood pressure. For instance, the mean blood pressure of the rats ($n=6$) was $121 \pm 7 \text{ mmHg}$ prior to dosing. Intravenous administrations of 1 and $10 \mu\text{mol kg}^{-1}$ of CapNO decreased blood pressure to $107 \pm 8 \text{ mmHg}$ ($P < 0.01$) and $92 \pm 7 \text{ mmHg}$ ($P < 0.001$), respectively. Attempt to use even higher dose of CapNO ($50 \mu\text{mol kg}^{-1}$) failed because this intravenous dose of

CapNO caused almost 50% drop in blood pressure. The maximum decrease in blood pressure was observed by 5 min after CapNO injections. In contrast to CapNO, intravenous Cap at equimolar doses (1 and $10 \mu\text{mol kg}^{-1}$) did not cause significant changes in the cerebral blood flow and mean blood pressure (Figure 4).

Discussion

The endothelium is a single layer of epithelioid cells that lines, as one cell thick, on the luminal surface of all blood vessels and lymphatics. In a normal adult, endothelial cells weigh in excess of 100 g and occupy a surface area of more than 1000 m^2 . Due to its key location, its sensitivity to changes in mechanical (haemodynamic) forces and in chemical and neurohumoral environment, and its ability to transmit these changes into compensatory adjustments of vascular smooth muscle tone, endothelium plays a vital role in cardiovascular homeostasis. The endothelium also serves as a permeability barrier and determines what substances penetrate the vessel wall. Commercial availability of the microporous membrane (Transwell) has enhanced reproducibility of drug cellular permeation studies. These tissue culture-treated microporous membranes represent a more physiological environment, which allows the exchange of substances across both the apical and basolateral membranes rather than just across the apical membrane as is the case with cells grown on plastic dishes. In conducting transport studies with cells cultured on microporous membranes, it is essential that control experiments be conducted using the same Transwell as shown in the present studies, and thus the results of these experiments will assure that the solute is freely permeable through the microporous membrane and the supporting matrix, and that the diffusion barrier is provided by the cell monolayers only. Under the well-controlled conditions that included using serum-free medium to avoid a protein-binding effect and monitoring CapNO decomposition during the period of incubation in DMEM, we demonstrated that CapNO permeates both endothelial cells and Caco-2 cells at a rate relatively faster than Cap (Figure 2), Taxol, and carbendazim tested in this laboratory. With a diffusible NO group attached to the compound, CapNO is conferred upon the high permeability through the cells. That may be a rational explanation why CapNO permeates through the cellular membrane faster than its parent Cap. We have reported that CapNO possesses the oral bioavail-

Table 1 Effects of CapNO and Cap on haemodynamic parameters of chronically-instrumented awake mongrel dogs

	CapNo (100 nmol kg^{-1})		Cap (100 nmol kg^{-1})		CapNO (100 nmol kg^{-1})		Cap (100 nmol kg^{-1})	
	Baseline	Response	Baseline	Response	Baseline	Response	Baseline	Response
Heart rate (beats min^{-1})	65 ± 11	71 ± 9	70 ± 8	74 ± 18	60 ± 12	$74 \pm 10^*$	78 ± 20	87 ± 23
Mean aortic pressure (mmHg)	105 ± 11	101 ± 8	103 ± 12	99 ± 14	106 ± 14	99 ± 7	105 ± 0	105 ± 4
Mean coronary diameter (mm)	3.6 ± 0.2	$3.9 \pm 0.1^*$	3.5 ± 0.2	3.6 ± 0.3	3.7 ± 0.1	$4.2 \pm 0.2^*$	3.6 ± 0.1	3.6 ± 0.1
Mean coronary flow (KHz)	1.5 ± 0.4	2.2 ± 0.5	1.6 ± 0.3	1.7 ± 0.2	1.6 ± 0.4	$3.2 \pm 0.5^{**}$	1.6 ± 0.4	1.6 ± 0.4
LV dp/dt max (mmHg s^{-1})	1911 ± 101	2001 ± 112	1923 ± 102	1975 ± 100	1852 ± 74	$2007 \pm 116^*$	1925 ± 106	1952 ± 117
LVEDP (mmHg)	11 ± 1	9 ± 1	11 ± 1	10 ± 1	11 ± 1	$8 \pm 1^{**}$	10 ± 0	10 ± 0

LVEDP, left ventricular end-diastolic pressure, LV dp/dt max, maximal rate of rise. $^*P < 0.05$, $^{**}P < 0.01$, compared with the baseline values (mean \pm s.d., $n=4$) using paired t -test.

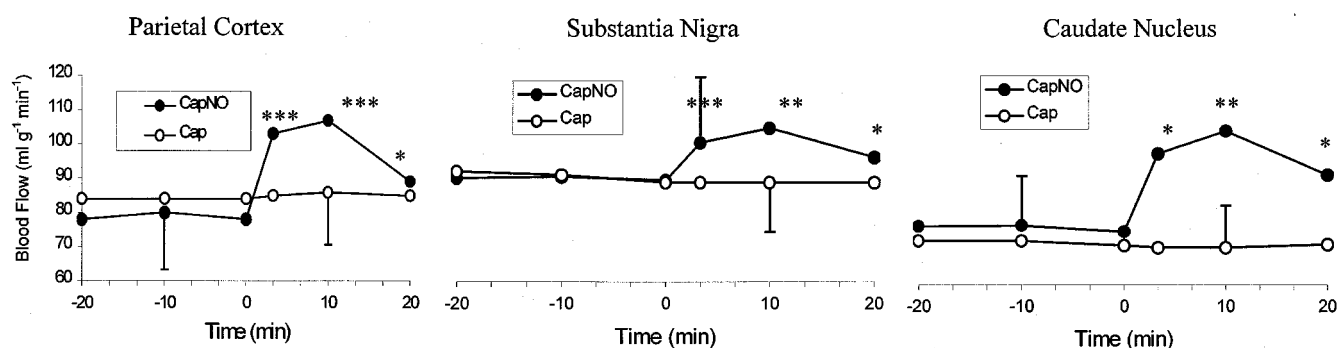


Figure 4 Comparison of effects of intravenous injections of CapNO ($1 \mu\text{mol kg}^{-1}$, $n=6$) and Cap ($1 \mu\text{mol kg}^{-1}$, $n=6$) on cerebral blood flow in rat parietal cortex, substantia nigra and caudate nucleus. The drugs were administered at 0 min. Each point represents the mean. Only one standard deviation bar is shown for clarity. Statistically significant differences compared with responses to the rats administered with Cap by one-way ANOVA: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

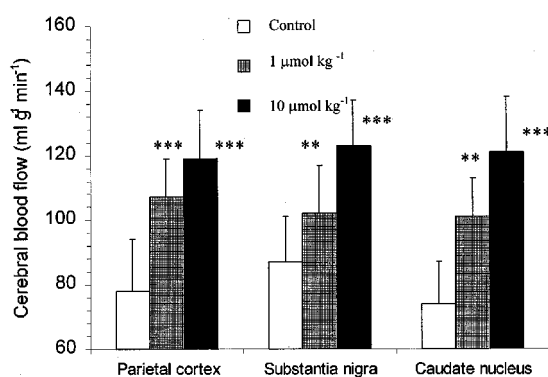


Figure 5 Peak effects of intravenous CapNO on cerebral blood flow of rat parietal cortex, substantia nigra and caudate nucleus. The effects were obtained at 10 min after dosing. The control represents blood flow before CapNO administration. The data represent the mean \pm s.d. ($n=6$), and evaluated for statistically significant differences by *t*-test: ** $P < 0.01$; and *** $P < 0.001$.

ability of 22–25% after administration to rats (Jia *et al.*, 1999). The results seem to be consistent with the present study of CapNO permeation through Caco-2 cells (Figure 2), a functional and anatomic feature characteristic of absorptive small intestinal enterocytes (Hidalgo *et al.*, 1989). The NO donor showed a certain degree of *in vitro* and *in vivo* permeation through intestinal barrier. The differences in cellular permeability to CapNO between Caco-2 and endothelial cells may result from the difference in transmembrane resistance between the two cell lines: Caco-2 cells show higher transmembrane resistance, and hence lower permeation to CapNO. In addition, Caco-2 cells contain P-glycoprotein, an apically polarized transmembrane protein excluding drugs from cells (Gao *et al.*, 2001). Whereas, endothelial cells may not contain P-glycoprotein. The discrepancy in P-glycoprotein content between the two cell lines may be attributed to the permeability variation.

To produce its characteristic effects (vasodilation), CapNO must be present in appropriate concentrations at its sites of action (vascular smooth muscle). Although obviously a function of the amount of CapNO administered, the concentrations attained also depend upon the extent and

rate of CapNO passage across endothelium into smooth muscle. In general, drugs cross membranes either by passive diffusion along a concentration gradient or by mechanisms involving the active participation of components of the membrane. It seems probable that the observed increase in cellular permeability to CapNO in the present experiment was due to the passive diffusion mechanism because we have demonstrated that the absorption amount of oral CapNO into systemic circulation is proportional to the amount of CapNO dosed (Jia *et al.*, 1999), although the precise permeability mechanism is not presently clear. Using 5 mM S-nitroso-N-acetylpenicillamine (one of RS-NO analogues), Salzman *et al.* (1995) have reported that NO reduced cellular ATP levels and reversibly increases the permeability of tight junctions in cultured Caco-2 cells. Although the concentrations used in their studies were too high, the possibility that NO increases cellular permeability can not be excluded.

The bolus injection technique provided *in vivo* assessment of the direct effect of CapNO on conductance and blood flow regulatory vessels (Figure 3), as well as cerebral blood flow (Figures 4 and 5) while minimizing the effects of pressure and heart rate. The present study was conducted by administering drugs *via* the left atrial catheter into awake dogs, differently from the experiment done by Shaffer *et al.* (1990), in which CapNO was injected *via* the femoral vein of anaesthetized dogs. CapNO caused increases in vascular dimension and blood flow *in vivo* (Figures 3–5). The facts suggest that CapNO could somehow permeate through the local tunica intima consisting of a lining of endothelial cells, and directly produce vasorelaxation of smooth muscle. It is possible that the bolus local injection causes rapid vasodilation that briefly exceeds the local autoregulatory forces which rapidly return blood flow to control values (Figure 3). In contrast, epicardial vessels are not exposed to the same autoregulatory forces, and consequently, unopposed vasodilation of epicardial vessels is sustained. Myogenic constriction of small vessels is an additional factor that may have influenced brief duration of dilation of flow regulatory vessels by CapNO because blood flow regulatory vessels are under continuous modulation and regulation by the adjacent contracting myocardium. It may be hypothesized that close coupling of blood flow to

myocardial metabolic needs would tend to attenuate and/or abbreviate response to a direct vasodilator stimulus that occurs in the resistance vasculature.

Differently from other endothelial cells, the brain capillary endothelium lacks aqueous pores (Pardridge *et al.*, 1990). Drugs and solutes enter the brain based on their molecular size and shape, degree of ionization, and relative lipid solubility of their ionized and nonionized forms. Intravenous injections of CapNO produced a significant increase in cerebral blood flow, whereas the parent Cap did not. Again, the difference in pharmacodynamic effects between the two compounds can only be attributed to the NO group presented in CapNO.

In general, NO donors categorize into different groups, and each of them behaves differently. Some are NO donors and permeable to cells, and the others are NO sinks (Simon *et al.*, 1996; Feelisch & Stamler, 1996). The biochemical differentiations among them rely on not only the mechanisms by which NO is released, but also their transmembrane permeability

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