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Antiangiogenic effects of *S*-nitrosocaptopril crystals as a nitric oxide donor

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Abstract

Angiogenesis is the formation of new capillaries from preexisting vessels by migration and proliferation of endothelial cells, which produce a cellular signaling messenger, nitric oxide (NO). The purpose of the present study was to examine the effects of exogenous NO donors on angiogenesis by using a novel crystalline NO donor, *S*-nitrosocaptopril. The characteristic X-ray diffraction pattern of *S*-nitrosocaptopril was demonstrated for the first time. On primary capillary endothelial cells pretreated with vascular endothelium growth factor (VEGF), *S*-nitrosocaptopril (1–500 μM), but not captopril, produced a dose-dependent inhibition of endothelial proliferation. On chick embryos of entire living eggs, gelatin sponges adsorbed with VEGF were implanted on the embryo chorioallantoic membrane to promote vascular growth activity within the sponges. Addition of *S*-nitrosocaptopril crystals (0.1 mg) to the gelatin sponges markedly reduced vascular density around the sponges, whereas captopril did not inhibit neovascularization. The vascular hemoglobin content surrounding each of the gelatin sponges was determined as a confirmatory test. *S*-nitrosocaptopril, but not captopril, significantly decreased the hemoglobin content of the embryo tissues immediately surrounding the gelatin sponges. In conclusion, *S*-nitrosocaptopril exerts an inhibitory effect on angiogenesis. This newly discovered function of *S*-nitrosocaptopril appears to be governed by distinct structural NO moiety.

Keywords: Angiogenesis; *S*-nitrosocaptopril; Captopril; Nitric oxide (NO); Capillary endothelium; Chorioallantoic membrane; Chick embryo; Hemoglobin

1. Introduction

Major discoveries in the past have proven that the vascular endothelium is more than a lining for blood vessels. The endothelial cells locally produce endothelium-derived relaxing factor (EDRF), which is now recognized as nitric oxide (NO) (Furchgott, 1988). These normally quiescent cells can rapidly proliferate with a turnover time of 5 days during angiogenesis (Folkman, 1995), which is a highly regulated phenomenon and is under the control of angiogenic stimulators and inhibitors. Unregulated angiogenesis is seen under pathological conditions of tumor growth, diabetic retinopathy, and psoriasis. Angiogenic growth factors, such as vascular endothelium growth factor (VEGF), act as autocrine or paracrine growth factors to induce angiogenesis (Ferrara and Davis-Smith, 1997). VEGF has been shown to induce EDRF-dependent vasorelaxation in a dose-dependent fashion (Ku et al., 1993).

NO has been implicated as a modulator of blood flow, motility, electrolyte and water transport, and in the function of endothelial cells, platelets, mast cells, and macrophages. Although the physiological and pathophysiological functions of NO have been extensively studied, its effects on angiogenic signal transduction pathways are poorly understood and a little information is available concerning the indirect activity of NO on angiogenic models (Pipili-Synetos et al., 1994, 1995). An interesting development in NO donors has been the realization that NO can exert both beneficial and deleterious effects in different tissues and under different conditions.

S-nitrosothiols can elicit many of the various physiological regulatory functions attributed to EDRF/ NO (Myers et al., 1990), including vascular and nonvascular smooth muscle relaxation (Jia and Furchgott, 1993; Jia and Stamler, 1999), inhibition of platelet aggregation (Mathews and Kerr, 1993) and inflammation (Granger and Kubes,

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1996), as well as neurotransmission (Kendrick et al., 1997). It has been well known that *S*-nitrosothiols decompose to yield NO and the corresponding disulfide RSSR (Singh et al., 1996), the latter could then be reduced by either an intracellular spontaneous recovery mechanism (Clancy et al., 1994), or a general protein disulfide reductase (Nikitovic and Holmgrent, 1996). Therefore, a *S*-nitrosothiol composed of an NO group and another pharmacological active component would have considerable theoretical and practical significance.

S-nitrosocaptopril (1-[(2*S*)-3-nitrosomercapto-2-methyl-1-oxopropyl]-L-Proline), an exemplary *S*-nitrosothiol, exhibits many NO-like activities such as direct relaxation of blood vessel both in vivo and in vitro (Jia and Blantz, 1998; Lin et al., 1998), and the inhibition of platelet aggregation (Loscalzo et al., 1989; Amano et al., 1994). *S*-nitrosocaptopril nonenzymetically releases NO in a manner similar to most of *S*-nitrosothiols (Jia et al., 1999). There have been many efforts to make a preparation of *S*-nitrosocaptopril in a powder form, however, the molecular entity is reported to be too unstable to isolate as pure crystals (Loscalzo et al., 1989). This made it impossible to extensively explore the mechanism of actions of the compound or its therapeutic implication. Despite of earlier hundreds of failure in purifying and crystallizing the compound, we have most recently synthesized the red flake crystals with a good yield. The novel crystals provide us with opportunities to explore pilot information regarding the overall function of NO in angiogenesis. Additionally, owing to the inadequacy of biochemical quantitative analyses of amounts of blood vessels in tissues, we extended our previous technique of hemoglobin measurement (Jia et al., 1996) to quantify the vascular existence in the chick embryo chorioallantoic model of entire eggs. The effects of *S*-nitrosocaptopril on the angiogenic models were examined in comparison with the corresponding parent compound captopril.

2. Methods and materials

2.1. X-ray powder diffraction of S-nitrosocaptopril crystals

The red plate-like crystals of *S*-nitrosocaptopril were prepared at 20°C and 40–50% humidity. In an attempt to improve randomness in the orientation of crystallite, the plate-like crystals were slowly and gently ground in a mortar to a fine powder. The X-ray powder diffraction was immediately measured using a diffractometer (D/max-2500) equipped with a Geiger–Müller detector, and nickel-filtered Cu Kα radiation (15, 42 nm). The characteristic diffraction pattern was recorded including values for 2θ that ranges from as near to 0° as possible to 40°. Care was taken to check the diffraction pattern of the ungrounded sample to ensure that no phase transformation occurred during the preparation.

2.2. Preparation of capillary endothelial cells

Male Wistar rats were given Brevital (E. Lilly, Indianapolis, IN) for anesthesia (70 mg/ kg, i.p.), and surgery was conducted under sterile condition. The rat adrenal glands (30 mg/wet tissue) were removed, and washed repeatedly with sterile saline solution. Each gland was bisected and the adrenal cortex was extricated from the capsule. The preparation of capillary endothelial cells was similar to that described by Folkman et al. (1979). Briefly, the adrenal cortex was cut into 1–2 mm pieces using scalpel blades. The tissue pieces were incubated in phosphate buffered saline with 0.75% collagenase D and 0.5% bovine albumin at 37°C for 1 h in a polyethylene tube. The suspension was pipetted with a large-bore pipette to break the tissue into smaller pieces. The suspension was then passed through sterile 75 μm and then 40 μm cell strainers (Becton Dickinson, NJ), respectively. The 40-μm cell strainer was washed thoroughly with culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin (50 units/ ml), and streptomycin (50 μg/ ml). The cells retained on the 40-μm cell strainer (Becton Dickinson Labware, MA) were collected by turning the screen over and flushing from the back with a stream of DMEM. The suspension was centrifuged at 650 rpm for 10 min at 4°C. The pellet was resuspended and washed three times with the culture medium. The final cell suspension was evenly plated into 35-mm gelatinized culture dishes, which were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Capillary segments and endothelial cell aggregates were the first to adhere to the substratum. Adrenal cortical cells and fibroblasts continued to float, and most were removed by aspirating the supernatant between 1 and 3 h after plating. The gelatinized dishes were replenished with fresh medium and incubated undisturbed for 5 days. The evidence that these cells were capillary endothelial cells was obtained from the observation of their characteristic appearance of these cells under an Olympus CK 2 microscope, such as the formation of the endothelial tubes.

2.3. Proliferation assays

When endothelial cells reached confluence the preparation was passaged by nonenzymatic cell dissociation solution. The cells were seeded in 24-well culture plates, in triplicate, at 100 cells/ well. VEGF was added to the cultures at a final concentration of 100 ng/ml to promote growth activity. Cell numbers were determined on days 0, 4, 7 and 10 by counting the dispersed cells in a hemocytometer. The second passage of endothelial cells was treated with *S*-nitrosocaptopril and Captopril, respectively, at final concentrations of 10, 100 and 500 μM twice per day for 2 days. These drugs were diluted with culture medium DMEM immediately before use, and control wells received equal volumes of the culture medium.

2.4. Bioassay of chick embryo chorioallantoic membrane

Fertilized white Leghorn chicken eggs were purchased from a poultry farm. The eggs were incubated in an egg incubator TX 7 (Lyon Electric, CA) under conditions of $50 \pm 1\%$ relative humidity and 37°C. An automatic turner attached to the incubator turned the eggs once per hour to help developing embryos grow in vigor and strength. On the seventh day of incubation, a micromotor drill equipped with a dental drill bit (1 mm) was used to drill a hole at the waist of the egg. Then, under sterile conditions, a surgical cauterization pen (Surgicare, CT) was used to open a window (15×15 mm) through the hole in the egg shell. A sterile gelatin sponge was cut by hand into the desired dimensions ($2 \times 3 \times 3$ mm), and implanted on the embryo chorioallantoic membrane of each egg under a dissecting microscope. Then 20 μl of VEGR (500 μg/ml) were pipetted onto the gelatin sponge. Sterile saline alone was used as negative controls. The window was then sealed with the 3M blenderm™, and the eggs were returned to the incubator.

On day 8 of incubation, *S*-nitrosocaptopril crystals or captopril powder (both 0.1 mg) were added to the surface of the gelatin sponge through the window once per day for 3 days. 3 μl of sterile saline was pipetted onto the gelatin sponge as controls. Care was taken to avoid any light and heat degradation on *S*-nitrosocaptopril when it was added. The advantage of using the crystals, instead of solution of *S*-nitrosocaptopril, is to localize the effects of *S*-nitrosocaptopril at the gelatin sponge, and release the NO topically. On day 11, blood vessels growing vertically into the gelatin sponge and at the boundary between the gelatin sponge and the surrounding embryo mesenchyme were examined under a microscope. The gelatin sponge and the immediately adjacent embryo chorioallantoic membrane were photographed in vivo under a double-headed photomicroscope (Leitz-Dialux 20, Leitz, Wetzlar, Germany). The sponge was then removed for measurement of hemoglobin content. The photographs were scanned and collected as black-and-white images with Adobe Photoshop 5.0 (Adobe Systems, CA) by using a Macintosh PowerPC. The digital images were converted to grayscale and final photomicrographs were generated by using a Kodak XLS 8600 printer.

2.5. Angiogenic quantification by hemoglobin assay

The quantification of angiogenesis on the gelatin sponge implanted in the chick chorioallantoic membrane was performed by measuring the hemoglobin content in the area immediately surrounding the sponge. Three days after drug treatment, the gelatin sponges were harvested from the underlying and the immediately adjacent area of chorioallantoic membrane (approximately $10 \times 10 \times 2$ mm), including newly formed blood vessels and albumen. The gelatin sponge and the adjacent membrane area were removed with a large opening pipette to an Eppendorf tube and the sample was weighed (0.2 g). Fourfold excess volume of phosphate buffer saline (pH 7.4, 10 mM) was added to the sample for wash. The sample was then centrifuged at 5000 g for 5 min and the wash was aspirated. After three cycles of wash–centrifugation–aspiration, 500 μl of the distilled water was added to the pellet. The pellet was homogenized with a motor-driven glass/Teflon® homogenizer for 5 min. 500 μl of phosphate buffer saline was added to the homogenate. The mixture was then centrifuged at $14{,}000 \times g$ for 30 min. The hemolysate supernatant was removed to a cuvette and scanned in a spectrophotometer (Shimadzu UV1601 PC) for absorption between 500 and 650 nm. Hemoglobin concentration in the hemolysate supernatant was determined as previously described by Jia et al. (1996). The concentration of oxygenated hemoglobin in the supernatant was determined using the millimolar extinction coefficient of 14.6 for the peak at 540 nm, which is independent of pH between 5.5 and 10 (Assendelft, 1970; Jia et al., 1996).

2.6. Materials and statistical analyses

Non-enzymatic cell dissociation solution and gelatin were purchased from Sigma (St. Louis, MO). Gelatin sponge (also called Gelform) was obtained from Pharmacia and Upjohn (Kalamazoo, MI). The preparation of *S*-nitrosocaptopril crystals was similar to that described previously (Jia et al., 1999), and now available from Calbiochem-Novabiochem (La Jolla, CA). VEGF was purchased from R&D Systems (Minneapolis, MN). The gelatinized culture dishes were prepared by dissolving gelatin in distilled H_2O (1.5%) above 50°C and pouring the gelatin solution on the culture dishes. After being dried at room temperature in a sterile hood for 3–5 h, the dishes were used immediately or within 2 days. Statistically significant differences between the mean values of the different groups were determined by Student's *t*-test for unpaired data in the present study.

3. Results

3.1. Characteristic powder diffraction of S-nitrosocaptopril

The plate-like crystal form of *S*-nitrosocaptopril produced its own characteristic X-ray diffraction pattern (see Fig. 1). The figure clearly demonstrated the sharply defined diffraction pattern of the anhydrous form of crystals as opposed to the noncrystalline powder. The first established diffraction for *S*-nitrosocaptopril crystals could be used to support a specific powder as being truly representative of a single phase on the basis of the similar density and absorption characteristics.

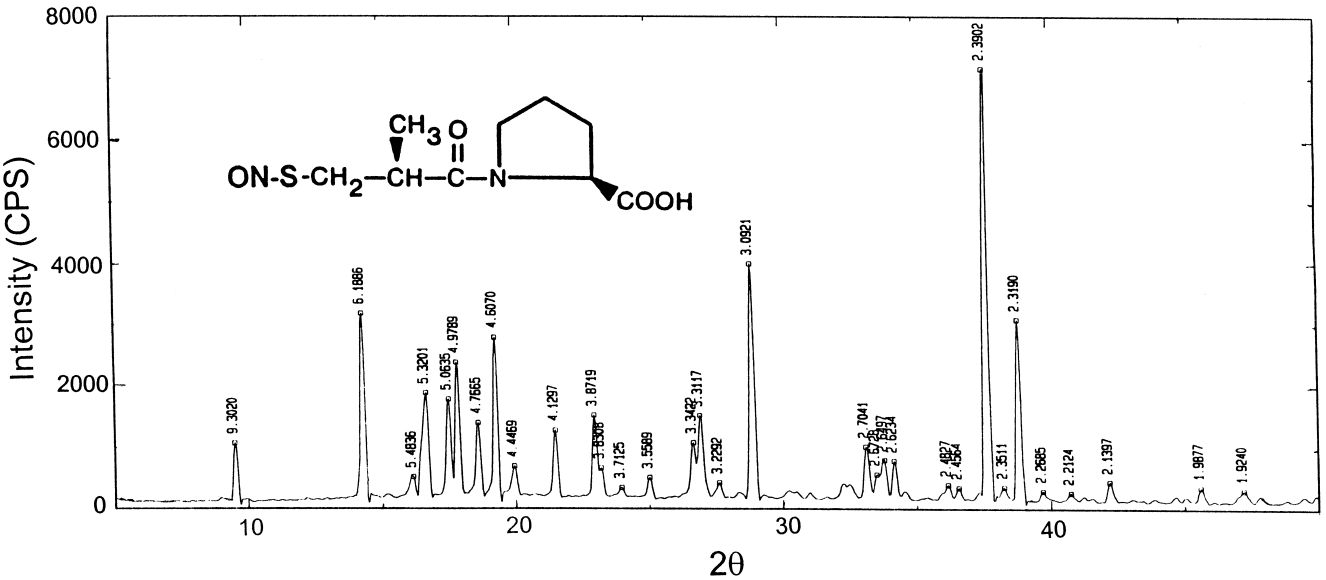

Fig. 1. A representative X-ray powder diffraction of *S*-nitrosocaptopril crystals. The typical powder pattern was obtained from red plate-like *S*-nitrosocaptopril crystals after grinding. θ denotes the angle of diffraction and the numbers above peaks indicate the corresponding interplanar spacing, i.e., *d*-value.

3.2. Effect of S-nitrosocaptopril on capillary endothelial proliferation

In primary cultures of most capillary cells, the earliest appearance of tubes occurred around the 20th day under our conditions. When the original monolayer of capillary endothelial cells formed a small colony, capillary tubes began to form in the middle zone of the colony. Branches began to appear after cells had become connected and, within a week, an entire network of tubes was established. We used the in vitro angiogenesis model to determine if *S*-nitrosocaptopril was acting directly on endothelial cells rather than on accessory cells such as mast cells and macrophages that can be responsible for the development of an angiogenic response in vivo. In this experiment *S*-nitrosocaptopril was able to inhibit VEGF-induced proliferation at concentrations ranging from 10 to 500 μM. *S*-nitrosocaptopril at 1 μM did not significantly affect the endothelial proliferation in comparison with the untreated control cells. As the concentration of *S*-nitrosocaptopril was increased, the formation of the organized tube network was reduced. Treatment with *S*-nitrosocaptopril reduced the endothelial tube formation in a concentration-dependent manner. This inhibition appears to be independent of activity of angiotensin converting enzyme inhibitor because captopril at the same concentration did not show appreciable effects on the cell proliferation (Fig. 2).

3.3. Effects of S-nitrosocaptopril on chick embryo chorioallantoic membrane

On incubation day 11, the VEGF-treated gelatin sponges were surrounded by vessels that developed radially toward the implants. At microscopic level, a highly vascularized tissue was recognizable among the trabeculae of gelatin sponges. The tissue consisted of newly formed blood vessels growing perpendicularly to the plane of the chorioallantoic membrane. These blood vessels were mainly capillaries with a diameter ranging from 3 to 10 μm. The chicken embryo has large interstitial vessels and a sinusoidal mesh of capillaries as well. Vascular density appeared to be higher in the VEGF-treated gelatin sponges ($n = 7$) than the saline-treated sponges ($n = 6$). In contrast, vascular density was very scarce at the center of the sponges treated with *S*-nitrosocaptopril crystals in the presence of VEGF, and 75% of the avascular gelatin sponges were found in this group ($n = 8$). In addition, the numbers

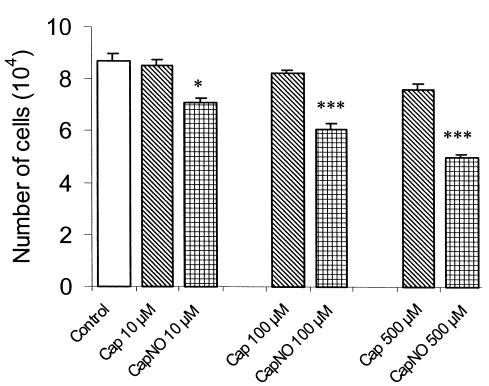

Fig. 2. Effects of *S*-nitrosocaptopril (CapNO) and captopril (Cap) on proliferation of rat capillary endothelial cells. The cells were seeded in gelatin-coated 24-well culture plates. VEGF was added to induce a maximal increase in cell number. The number of cells was determined in five cultures by a hemocytometer 10 days after VEGF treatment. Data represent the means ± S.E.. * and *** denote $P < 0.05$ and < 0.01 vs. control, respectively.

of macroscopical vessels at the boundary between the sponges and the embryo mesenchymes were significantly less in the *S*-nitrosocaptopril-treated group than those in the captopril-treated group ($n = 7$) and in the saline-treated control group. The latter two groups showed numerous capillaries frequently penetrated into the sponges. *S*-nitrosocaptopril exhibited greater anti-angiogenic activity than captopril (Fig. 3).

3.4. Effects of S-nitrosocaptopril on hemoglobin content

The spectrophotometer scan of the hemolysate supernatants prepared from the gelatin sponges ($n = 4$ per group) and the immediately adjacent membrane area exhibited two characteristic peaks at 540 and 577 nm (Fig. 4), which are proven to be representative of the oxygenated hemoglobin (Assendelft, 1970; Jia et al., 1996). The concentrations (μM, mean ± S.D.) of oxygenated hemoglobin in the supernatants were 21.8 ± 1.25 for the saline control group; 23.9 ± 4.37 for the VEGF-pretreated group; 17.8 ± 2.75 for the captopril group pretreated with VEGF; and 10.4 ± 4.32 for the *S*-nitrosocaptopril group pretreated with VEGF. *S*-nitrosocaptopril significantly decreased hemoglobin concentration in the embryo area surrounding the gelatin sponges in comparison with the hemoglobin concentration in the saline control group ($P < 0.05$) and VEGF-pretreated group ($P < 0.05$).

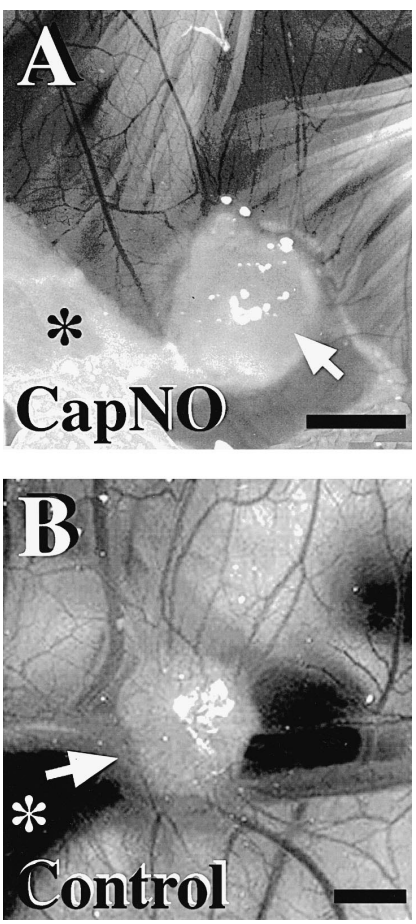

Fig. 3. Inhibition of *S*-nitrosocaptopril (CapNO) on angiogenesis in the chick embryo chorioallantoic membrane. A gelatin sponge (see the arrow) adsorbed with 1 μg of VEGF was implanted on the surface of the membrane on day 8 of incubation. After 3 consecutive days of addition of sterile saline or CapNO crystals to the gelatin sponge, the embryo of entire egg was directly exposed and photographed under a double-headed photomicroscope (Leitz-Dialux 20, Leitz, Wetzlar, Germany). (A) Avascular zone around the CapNO-treated sponge. * indicates the wrinkled membrane of the embryo in vivo. (B) New capillaries penetrated into the sponge treated with VEGF as positive control. Note the presence of small branches of capillaries within the gelatin sponge. * indicates uneven surface of the membrane caused by movements of the embryo while being photographed. Scale bar = 3 mm.

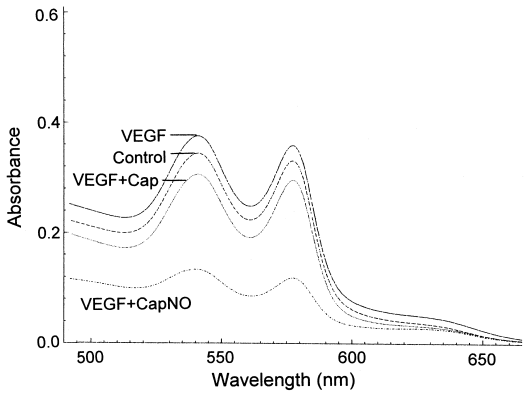

Fig. 4. Representative UV–visible absorption spectra of hemolysate supernatants prepared from chick embryo tissues surrounding the gelatin sponges. The gelatin sponges adsorbed with VEGF were administered with sterile saline (Control), captopril (Cap) and *S*-nitrosocaptopril (CapNO), respectively. The presence of oxygenated hemoglobin is evidenced by the λ_{max} at 540 and 577 nm. Inhibition of *S*-nitrosocaptopril on the neovascularization was shown by reducing spectrum absorbance corresponding to hemoglobin content of the hemolysate supernatant.

4. Discussion

As far as we know, there has been no report on the synthesis of flake *S*-nitrosocaptopril crystals with high purity and stability. Loscalzo et al. (1989) claimed the synthesis of lyophilized *S*-nitrosocaptopril, which was in a red amorphous form with somewhat hygroscopicity and a melting point of 98–104°C. In our preparation, we found that *S*-nitrosocaptopril was unstable and hygroscopic only when the compound was impure. The impure and thus hygroscopic form of *S*-nitrosocaptopril was easily decomposed to the corresponding disulfide with the formation of brown fumes of NO_2 at room temperature. The decomposition of the *S*-nitrosocaptopril powder proceeds as a heat-producing chain reaction, which completes within seconds when the sealed powder is exposed to air. The components mixed together with *S*-nitrosocaptopril (a carboxylic acid) may include sodium carboxylate of *S*-nitrosocaptopril, captopril disulfide and inorganic salts. However, the chemical

stability of *S*-nitrosocaptopril can be significantly improved via a process of purification and dehydration. After the process the crystals become stable and are not hygroscopic. The purified crystals could be stored in the dark at room temperature for at least two months and 4°C for at least 1 year without a significant decay. The melting point of *S*-nitrosocaptopril crystals was determined in the range of 49–52°C (Jia et al., 1999), instead of 98–104°C reported before (Loscalzo et al., 1989), which may be an error of determination. The S—NO bond is so vulnerable to heat and strong light that the bond could be broken when temperature rises. We found that when the testing temperature rose above the range of 49–52°C, the particular red color of *S*-nitrosocaptopril diminished immediately, indicating the breakdown of the S—NO bond.

This is the first report using a characterized NO donor to directly observe the effects of NO on commonly used angiogenic models (Folkman 1995; Folkman et al., 1979) in the presence of VEGF. VEGF has been shown to play a key role in angiogenesis. We found that *S*-nitrosocaptopril inhibited proliferation and tube formation of capillary endothelial cells in vitro. A more direct study of the ability of *S*-nitrosocaptopril to inhibit angiogenesis was afforded by the chick embryo chorioallantoic assay. In such experiments, topical application of crystalline *S*-nitrosocaptopril to small foci, i.e., gelatin sponges adhered to the embryo surface, was found to inhibit angiogenesis in vivo as well. To further assess whether the gelatin sponge model was suitable for the evaluation of the angiogenesis and the anti-angiogenic potential of *S*-nitrosocaptopril, hemoglobin content surrounding the sponge area was quantitatively determined (Fig. 4). The decreases in hemoglobin content extracted from the gelatin sponge area treated with *S*-nitrosocaptopril were in direct proportion to the avascular zone around the sponges, whereas captopril did not significantly block capillary growth into the sponges, thus confirming the inhibitory effect of *S*-nitrosocaptopril on angiogenesis. The finding strongly suggests that the anti-angiogenic activity of *S*-nitrosocaptopril is dependent on the NO moiety because the anti-angiogenic activity is virtually eliminated in captopril, in which an NO group is absent. Although the precise inhibitory mechanisms of *S*-nitrosocaptopril in these reactions have not been elucidated, the involvement of NO in the inhibition of energy consumption cannot be excluded. NO inhibits cell growth by binding to certain enzymes that are involved in cellular respiration and electron transport in mitochondria, and voltage-dependent calcium channels (for review, see Lancaster, 1992). These events play important roles in the proliferation of various cells so it is possible that NO donors act to inhibit these processes in endothelial cells. Two *S*-nitrosothiols, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine, were reported to be cytostatic (Wink et al., 1997), and this may participate in the inhibition of cell migration, invasion of tissues, proliferation, and formation of a new underlying basement membrane matrix. The mechanism by which *S*-nitrosocaptopril inhibits angiogenesis may also involve its inhibition of platelet activation (Amano et al., 1994), as demonstrated by Pipili-Synetos et al. (1994, 1995) that increasing the availability of NO resulted in inhibition of angiogenesis in the chorioallantoic membrane of the chick embryo partly via inhibition of platelet activation.

Although our results strongly support the proposal that the NO released from *S*-nitrosocaptopril can account for the inhibition of angiogenesis, it is still possible that a minor part of the inhibition is due to a direct anti-angiogenic effect of captopril. Captopril may be produced upon the decomposition of *S*-nitrosocaptopril and then reduction by either an intracellular spontaneous recovery mechanism (Clancy et al., 1994), or a general protein disulfide reductase (Nikitovic and Holmgrent, 1996). It has also been shown by Volpert et al. (1996) that captopril can directly block neovascularization induced in the rat cornea, and inhibit endothelial chemotaxis in the millimolar range. The inhibition of the migration of endothelial cells was not dependent on ACE inhibition, but may be the result of the ability of captopril to inhibit the activity of the Zn^{2+}-dependent metalloproteinases that endothelial cells require to respond to an angiogenic stimulus. Although we observed a slight inhibition of captopril (500 μM) on endothelial proliferation (Fig. 2) whether the above-mentioned type of reaction occurred to any extent in the present study is not known.

The chick embryo chorioallantoic assay has the advantage of being simple and inexpensive. Practically, it can be used as a large scale screening assay. The major modification of the embryo model performed in the present study was using an entire egg with a small window on the shell for inserting gelatin sponges and administering drugs, rather than using shell-less embryos. Under shell-less condition, the chick embryos may develop abnormally. Although many efforts have been made to quantify the results of the assay, the major disadvantages of the previous chick embryo chorioallantoic assay are that first, the drug vehicles are placed on preexisting vessels, and newly formed blood vessels grow within the embryo mesenchyme. The actual neovascularization can hardly be distinguished from a falsely increased vascular density due to the rearrangement of preexisting vessels that follows contraction of the membrane. In contrast, by using the gelatin sponge in the present design, the newly formed blood vessels grow perpendicularly to the plane of the embryo chorioallantoic membrane inside the sponge that does not contain preexisting vessels. To this respect, the proposed assay may resemble the cornea assay in which neovascularization occurs in an avascular stroma. However, the gelatin sponge assay is much easier, faster and cheaper than the corneal assay. A collagen gel combined with sucralfate to prevent growth factors from decay has been used to induce angiogenesis (Nguyen et al., 1994). However, preparation of the collagen gel appears to be time-consuming compared to

the simple cutting by hand of the dry gelatin sponges. Secondly, a common problem associated with angiogenesis assays is the maintenance of the drug to be tested at the site of administration. In our gelatin sponge assay, the tested drug is held within the sponge which, in turn, firmly adheres to the embryo surface. Angiogenic active drugs might be tested by this method. Thirdly, the previous embryo assay may cause a secondary vasoproliferative response due to the nonspecific inflammatory reaction. In contrast, the present gelatin sponge assay appears to be well tolerated and very little, if any, inflammatory reaction has been observed in all the embryos examined so far.

Angiotensin II, the product of the angiotensin converting enzyme, has been demonstrated to induce neovascularization in chick embryo chorioallantoic membrane (Fernandez et al., 1985; Le Noble et al., 1991, 1993) and enhance microvessel density in the cremaster muscle (Hernandez et al., 1992). *S*-nitrosocaptopril, but not captopril, directly antagonizes the vasoconstriction of angiotensin II (Cooke et al., 1989). In addition, *S*-nitrosocaptopril has various pharmacological effects, including inhibition of platelet aggregation (Loscalzo et al., 1989; Amano et al., 1994) and blood vessel dilation in normal and hypertensive rats (Jia and Blantz, 1998; Jia et al., 1999). Thus, this compound has potential clinical implication in the treatment of many forms of cardiovascular diseases as a combined NO donor and an angiotensin converting enzyme inhibitor.

References

Amano, M., Takahashi, M., Kosaka, T., Kinoshita, M., 1994. Differential inhibition of platelet aggregation and calcium mobilization by nitroglycerin and stabilized nitric oxide. J. Cardiovasc. Pharmacol. 24, 860–866.

Assendelft, O.W.V., 1970. Spectrophotometry of haemoglobin derivatives. 1st Royal VanGorcum, The Netherlands.

Clancy, R.M., Levartovsky, D., Leszczy-Piziak, J., Yegudin, J., Abramson, S.B., 1994. Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: evidence for *S*-nitrosoglutathione as a bioactive intermediary. Proc. Natl. Acad. Sci. U. S. A. 91, 3680–3684.

Cooke, J.P., Andon, N., Loscalzo, J., 1989. *S*-nitrosocaptopril: II. Effects on vascular reactivity. J. Pharmacol. Exp. Ther. 249, 730–734.

Fernandez, L.A., Twickler, J., Mead, A., 1985. Neovascularization produced by angiotensin II. Lab. Clin. Med. 105, 141–145.

Ferrara, N., Davis-Smith, T., 1997. The biology of vascular endothelial growth factor. Endocr. Rev. 18, 4–24.

Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other diseases. Nat. Med. 1, 27–31.

Folkman, J., Haudenschild, C.C., Zetter, B.R., 1979. Long-term culture of capillary endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 76, 5217–5221.

Furchgott, R.F., 1988. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that acid-activatable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor (EDRF) is nitric oxide. In: Vanhoutte, P.M. (Ed.), Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium. Raven, New York, pp. 401–414.

Granger, D.N., Kubes, P., 1996. Nitric oxide as antiinflammatory agent. Methods in Enzymology 269, 434–442.

Hernandez, I., Cowley, A.W., Lombard, J.H., Greene, A.S., 1992. Salt intake and angiotensin II alter microvessel density in the cremaster muscle of normal rats. Am. J. Physiol. 263, H8664–H8667.

Jia, L., Blantz, R.C., 1998. The effects of *S*-nitrosocaptopril on renal filtration and blood pressure in rats. Eur. J. Pharmacol. 354, 33–41.

Jia, L., Furchgott, R.F., 1993. Inhibition by sulfhydryl compounds of vascular relaxation induced by nitric oxide and endothelium-derived relaxing factor. J. Pharmacol. Exp. Ther. 267, 371–378.

Jia, L., Stamler, J.S., 1999. Dual actions of *S*-nitrosylated derivative of vasoactive intestinal peptide as a vasoactive intestinal peptide-like mediator and a nitric oxide carrier. Eur. J. Pharmacol. 366, 69–78.

Jia, L., Bonaventura, C., Bonaventura, J., Stamler, J.S., 1996. *S*-nitrosoheamoglubin: a dynamic activity of blood involved in vascular control. Nature 380, 221–226.

Jia, L., Yang, X., Guo, W., 1999. Physicochemistry, pharmacokinetics and pharmacodynamics of *S*-nitrosocaptopril crystals, a new nitric oxide donor. J. Pharm. Sci. 88, 981–986.

Kendrick, K.M., Guevara-Guzman, R., Zorrilla, J., Hinton, M.R., Broad, K.D., Mimmack, M., Ohkura, S., 1997. Formation of olfactory memories mediated by nitric oxide. Nature 388, 670–673.

Ku, D.D., Zaleski, J.K., Liu, S., Brock, T., 1993. Vascular endothelial growth factor induces EDRF-dependent relaxation of coronary arteries. Am. J. Physiol. 265, H586–H592.

Lancaster, J.R., 1992. Nitric oxide in cells. Am. Sci. 80, 248–259.

Le Noble, F.A., Hekking, J.W., Van Straaten, H.W., Slaaf, D.W., Struyker-Boudier, H.A., 1991. Angiotensin II stimulates angiogenesis in the chorioallantoic membrane of the chick embryo. Eur. J. Pharmacol. 195, 305–306.

Le Noble, F.A., Schreurs, N.H.J.S., Van Straaten, H.W.M., Slaaf, D.W., Smits, J.F.M., Struyker-Boudier, H.A., 1993. Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane. Am. J. Physiol. 264, R460–R465.

Lin, M.J., Yang, X., Wang, J., Jia, B.J., Jia, L., 1998. Inhibitory effects of *S*-nitrosocaptopril on vasomotor tone. Acta Pharmacol. Sin. 19, 485–488.

Loscalzo, J., Smick, D., Andon, N., Cooke, J., 1989. *S*-nitrosocaptopril: I. Molecular characterization and effects on the vasculature and on platelets. J. Pharmacol. Exp. Ther. 249, 726–729.

Mathews, R.W., Kerr, S., 1993. Biological activity of *S*-nitrosothiols: the role of nitric oxide. J. Pharmacol. Exp. Ther. 267, 1529–1537.

Myers, P.R., Minor, R.L., Guerra, R. Jr., Bates, J.N., Harrison, D.G., 1990. Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide. Nature 345, 161–163.

Nikitovic, D., Holmgrent, 1996. *S*-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. J. Biol. Chem. 271, 19180–19185.

Nguyen, M., Shing, Y., Folkman, J., 1994. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. Microvasc. Res. 47, 31–40.

Pipili-Synetos, E., Sakkoula, E., Haralabopoulos, G., Andriopoulou, P., Peristeris, P., Maragoudakis, M.E., 1994. Evidence that nitric oxide is an endogenous antiangiogenic mediator. Br. J. Pharmacol. 111, 894–902.

Pipili-Synetos, E., Papageorgiou, A., Sakkoula, E., Sotiropoulou, G., Fotsis, T., Karakiulakis, G., Maragoudakis, M.E., 1995. Inhibition of angiogenesis, tumour growth and metastasis by the NO-releasing vasodilators, isosorbide mononitrate and dinitrate. Br. J. Pharmacol. 116, 1829–1834.

Singh, R.J., Hogg, N., Joseph, J., Kalyanaraman, B., 1996. Mechanism of nitric oxide release from *S*-nitrosothiols. J. Biol. Chem. 271, 18596–18603.

Volpert, O.V., Ward, W.F., Lingen, M.W., Chesler, L., Solt, D.B., Johnson, M.D., Molteni, A., 1996. Captopril inhibits angiogenesis and slows the growth of experimental tumors in rats. J. Clin. Invest. 98, 671–679.

Wink, D.A., Cook, J.A., Christodoulou, D., Krishna, M.C., Pacelli, R., Kim, S., DeGraff, W., Gamson, J., Vodovotz, Y., Russo, A., Mitchell, J.B., 1997. Nitric oxide and some nitric oxide donor compounds enhance the cytotoxicity of cisplatin. Nitric Oxide Biol. Chem. 1, 88–94.