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Vascular endothelial growth factor increases heme oxygenase-1 protein expression in the chick embryo chorioallantoic membrane

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1 Vascular endothelial growth factor (VEGF) is a potent angiogenic factor. It has been recently suggested that the inducible heme oxygenase (HO-1) isoform may play a role in angiogenesis.

2 The aims of this study were to determine, in chicken embryo chorioallantoic membranes (CAM), whether VEGF increases HO-1 protein expression, and, if so, by which molecular mechanism, and whether HO-1 activity is required for VEGF-induced angiogenesis.

3 Treatment of CAMs with VEGF for 48 h caused a significant increase in HO-1 protein expression, simultaneously with angiogenesis.

4 VEGF-stimulated angiogenesis in CAMs was markedly attenuated by the HO inhibitor zinc mesoporphyrin (ZnMP). This inhibitory effect of ZnMP was not observed with copper mesoporphyrin (CuMP), a metalloporphyrin that has a similar structure to ZnMP but does not inhibit HO enzymatic activity.

5 Overexpression of HO-1 protein elicited by VEGF in CAMs was significantly attenuated by the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM). The effects of BAPTA-AM were, in turn, compensated by the calcium ionophore A-23187.

6 In addition, the protein kinase C inhibitor staurosporine significantly attenuated, in a dose-dependent manner, the VEGF-stimulated HO-1 induction observed in CAMs.

7 These results demonstrate, for the first time, that VEGF upregulates HO-1 protein expression *in vivo* in CAMs by a mechanism dependent on an increase in cytosolic calcium levels and activation of protein kinase C. Our findings also suggest that HO-1 activity is necessary for VEGF-induced angiogenesis in CAMs.
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Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; CAM, chorioallantoic membrane; CuMP, copper mesoporphyrin; DMSO, dimethyl sulfoxide; HO, heme oxygenase; VEGF, vascular endothelial growth factor; ZnMP, zinc mesoporphyrin

Introduction

Vascular endothelial growth factor (VEGF) is a soluble glycoprotein that plays an essential role in angiogenesis (reviewed in Ferrara, 2001). Angiogenesis, the process by which new blood vessels are formed from pre-existing vessels, occurs during embryonic development, and during several physiological and pathological conditions in adult life, including ovulation, wound healing, ischemia, chronic inflammation, and tumor growth (reviewed in Folkman, 1982). The biological effects of VEGF are mediated by at least two tyrosine kinase receptors at the plasma membrane of endothelial cells, VEGF receptor-1 and VEGF receptor-2. Binding of VEGF to its receptors triggers a signaling cascade that results in tyrosine phosphorylation of phospholipase Cγ1, leading to increases in intracellular levels of inositol 1,4,5-trisphosphate and elevation of cytosolic calcium (reviewed in Ferrara, 2001). It has been suggested that the vasodilators, nitric oxide and prostacyclin, play important roles in mediating some biological functions of VEGF (reviewed in Zachary & Gliki, 2001).

Heme oxygenase (HO) has recently attracted great interest as a regulator of cell and organ functions (reviewed in Elbirt & Bonkovsky, 1999). Microsomal HO is the rate-controlling enzyme for heme degradation in mammals and other organisms. HO breaks down heme to equimolar amounts of carbon monoxide, free iron, and biliverdin. In most mammals, biliverdin is rapidly reduced to bilirubin by the abundant cytosolic enzyme biliverdin reductase (Stocker *et al*., 1987). HO has inducible (HO-1) and constitutive (HO-2) isoforms (reviewed in Elbirt & Bonkovsky, 1999). The expression of

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HO-1 is transcriptionally activated, not only by its substrate heme (Srivastava *et al*., 1993; Fernandez & Bonkovsky, 1999), but also by a variety of physical and chemical factors, including shear stress, bacterial endotoxins, and cytokines (reviewed in Elbirt & Bonkovsky, 1999). Recently, evidence of a second constitutive isoform (HO-3) has been found (McCoubrey *et al*., 1997), although its physiological role more likely involves heme binding or heme sensing than heme catabolism, since its specific enzymatic activity is very low. All products of HO activity are biologically active. Iron is a regulator of gene expression (reviewed in Elbirt & Bonkovsky, 1999). Biliverdin and bilirubin are both potent-free radical scavengers with antioxidant properties (Stocker *et al*., 1987). Carbon monoxide is a potent vasoactive molecule that mediates various physiological functions, such as smooth muscle relaxation and vasodilatation (reviewed in Wang *et al*., 1997).

Based on recent evidence suggesting that HO-1 induction may promote *in vitro* endothelial cell proliferation and angiogenesis (Deramaudt *et al*., 1998; Malaguarnera *et al*., 2002), we hypothesize that HO-1 may contribute to mediate the vascular effects of VEGF. Our specific aims were to determine whether VEGF increases the expression of HO-1 protein, and, if so, by which molecular mechanism, and whether HO-1 activity is required for VEGF-induced angiogenesis. These studies were performed in a reproducible and extensively used *in vivo* model of angiogenesis, namely, the chicken embryo chorioallantoic membrane (CAM) (reviewed in Folkman, 1985).

Methods

Materials

Human recombinant $VEGF_{165}$ was from Chemicon International (Temecula, CA, U.S.A.). Zinc mesoporphyrin-IX (ZnMP) and copper mesoporphyrin-IX (CuMP) were obtained from Porphyrin Products (Logan, UT, U.S.A.). The intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was from Molecular Probes (Eugene, OR, U.S.A.). Discs of glass fiber filters and nitrocellulose membranes were from Schleicher & Schuell (Keene, NH, U.S.A.). Paraformaldehyde was from Mallinckrodt-Baker (Paris, KY, U.S.A.). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and prestained protein standards were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The enhanced chemiluminescence Western blotting system was from New England Nuclear (Boston, MA, U.S.A.). All other reagents and chemicals were from Sigma Chemical (St Louis, MO, U.S.A.).

Preparation and treatment of the CAMs

Fertilized Barred Rock chicken eggs (Spafas Inc., Norwich, CT, U.S.A. and Carousel Farms, MA, U.S.A.) were used in these studies. All experiments were approved by the UMass Animal Use Committee and were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health, Publication No. 85-23, revised in 1985). Eggs were incubated from the start of their embryogenesis in a humidified incubator, at 37°C. On the 10th day of the embryo's development, the eggs were taken from the incubator. Shells were washed with 70% ethanol, allowed to air-dry, and wiped with a solution of povidone-iodine, to minimize contamination from the shell surface. Then, a 1-cm^2 window was opened in the egg shell, at the air sac, exposing the CAM. A volume of 20 μl of test substances, or an equal volume of solvent, was then applied to sterile glass fiber filter discs (7 mm in diameter). The impregnated filters were immediately placed upside down on the top of the exposed CAM surface through the window. The shell window was then closed with a transparent adhesive tape, and the eggs were returned to the incubator.

The following substances were tested: the angiogenic factor VEGF (50 ng per egg) (Wilting *et al*., 1993); the HO inhibitor ZnMP-IX (20, 40, and 80 nmol per egg) (Russo *et al*., 1995; Fernandez *et al*., 2001); CuMP-IX (20, 40, and 80 nmol per egg), a metalloporphyrin that does not inhibit HO (Cable *et al*., 1993); the intracellular calcium chelator BAPTA-AM (1 nmol per egg) (Terry *et al*., 1999); the calcium ionophore A-23187 (20 pmol per egg) (Terry *et al*., 1999) and the protein kinase C inhibitor staurosporine (0.2, 2, and 10 nmol per egg) (Oikawa *et al*., 1992). All solutions were freshly prepared on the day of use. Substances were first dissolved in a small volume of dimethyl sulfoxide (DMSO), and then further diluted in 40 mM Tris-HCl (pH 7.4) to give the appropriate final concentrations (final DMSO concentration was 0.5%). ZnMP and CuMP were protected from light during its preparation and use. Control CAMs were treated with an identical volume of solvent (0.5% DMSO solution). In preliminary studies, we found that the concentration of 0.5% DMSO did not cause any significant effect either on HO-1 protein expression or vascular responses, as compared with untreated and saline-treated CAMs. On average, 30 eggs per substance or combination of substances were tested. Vascular responses and HO-1 protein expression were assessed in CAMs 48 h after treatments, as described below.

Evaluation of vascular responses in CAMs

Vascular responses were assessed in CAMs, as previously described (reviewed in Folkman, 1985). In brief, freshly prepared fixative solution (4% paraformaldehyde in 0.1 M phosphate-buffered solution; pH 7.4) was injected between the CAM and the yolk sac to fix the CAM. At 10 min after injection, the shell was removed from the bottom side of the egg, and the embryo and yolk were gently drawn from the egg. The major blood vessels connecting the embryo with the CAM were then cut. The CAM was gently peeled away from the shell, and immediately submerged in fresh fixative for an additional 15 min period. After fixation, CAMs were rinsed in sterile saline (0.9% NaCl), and spread flat on a glass microscope slide. Vascular responses in CAMs were assessed under a stereomicroscope at $\times 10$ magnification. A positive angiogenic response was characterized by increased number and looping of blood vessels beneath as well as towards the loaded samples. To quantitate angiogenic responses, a grid with squares of 1-mm edge length was placed on top of CAM photomicrographs, and the number of blood vessels was counted in 30 randomly chosen squares, at a magnification of $\times 10$. Results were expressed as the number of blood vessels per mm^2.

Analysis of HO-1 protein expression in CAMs

The Western blot technique was used for detection of HO-1 immunoreactive protein, as previously described (Fernandez & Bonkovsky, 1999). In brief, CAM portions surrounding the site of implantation of substances were excised, and mixed with an equal volume of double-strength sample buffer (250 mM Tris-HCl, 4% sodium dodecyl sulfate, 10% glycerol, 2% β-mercaptoethanol, and 0.006% bromophenol blue (pH 6.8)). Samples were then boiled for 5 min, and centrifuged at $10{,}000 \times g$ for 10 min. The resulting supernatants were collected and subjected to 12% sodium dodecyl sulfate – polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, and blocked in Tris-buffered saline (TBS) with 0.05% polyoxyethylenesorbitan monolaureate (Tween 20; TBS-T buffer) containing 5% (wt vol^{-1}) nonfat dry milk. The membranes were then incubated with polyclonal monospecific antibodies against chicken HO-1 (25 μg ml^{-1} in TBS-T with 5% milk), which were prepared as described below. Blots were subsequently washed in TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 : 10,000 dilution in TBS-T with 5% milk). Blots were reprobed with monoclonal antibodies against α-tubulin (1 : 2000 dilution), as an internal control for protein loading. All incubations were carried out for 2 h at room temperature. The enhanced chemiluminescence Western blotting system was used for detection of proteins. Quantification of protein signals was performed using computer-assisted densitometry. Molecular weight was calculated with prestained standards.

Polyclonal monospecific antibodies against chicken HO-1 protein were prepared following a method developed in our laboratory (Bonkovsky *et al*., 1990; Greene *et al*., 1991). In brief, purified chicken HO-1 protein was separated by 12% sodium dodecyl sulfate – polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and visualized by Ponceau staining. The nitrocellulose containing the major band corresponding to purified HO-1 (34 kDa) was cut into small pieces and incubated, for 2 h at room temperature, with serum from rabbits immunized with chicken HO-1 protein. After several washes with Tris-buffered solution (pH 7.4), the purified antibodies were eluted by incubation for 5 min with 200 mM glycine (pH 2.8), and then neutralized with 0.5 M Tris-base (pH 10.0). The final pH was about 8.0. The protein concentration of the antibody preparation was estimated from its absorbance at 280 nm. To confirm that these antibodies retained their binding activity, they were used to detect purified chicken HO-1 protein on blots.

Statistical analysis

Results are presented as mean $\pm$ s.e.m. Preliminary evaluation of data showed that they were normally distributed. Therefore, parametric statistical procedures were used (two-way ANOVA followed by the Tukey – Kramer test for multiple comparisons). Statistical significance was accepted at $P<0.05$.

Results

Effects of VEGF on HO-1 protein expression in CAMs

We first determined the effect of VEGF on HO-1 protein expression in CAMs. For this purpose, CAMs were treated for 48 h with 50 ng VEGF or a 0.5% DMSO control solution. Then, HO-1 protein expression was examined using the Western blot technique and monospecific antibodies against chicken HO-1. A representative immunoblot is shown in Figure 1a, and results from densitometric analysis of protein signals are represented in Figure 1b. Treatment of CAMs with VEGF caused a significant increase in HO-1 protein expression (3.5-fold increase), as compared with control CAMs.

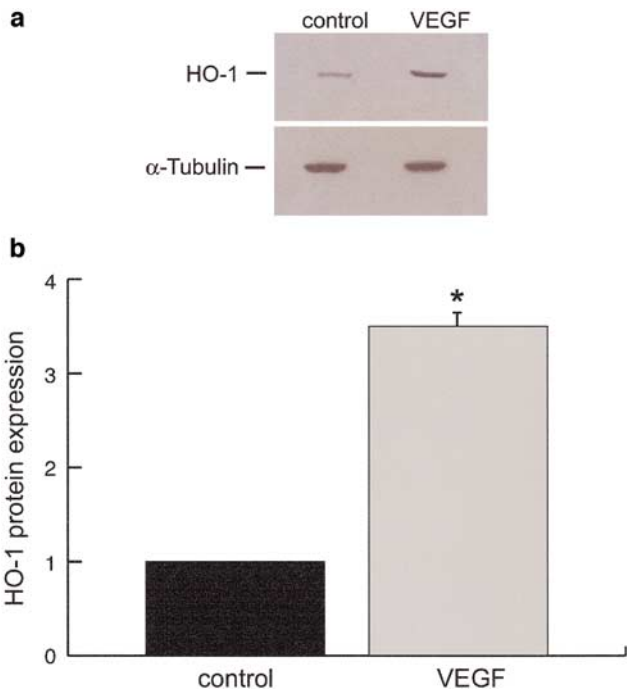

Figure 1 Expression of HO-1 protein in CAMs after treatment for 48 h with 50 ng VEGF or a 0.5% DMSO control solution. (a) Representative Western blot. (b) Densitometric quantification of HO-1 protein expression normalized to α-tubulin. Data are shown as fold increase in HO-1 protein expression above control values (mean $\pm$ s.e. of five separate experiments). $^*P<0.05$ vs control CAMs.

Effects of HO-1 activity inhibition on VEGF-induced angiogenesis in CAMs

We further inquired whether HO-1 is required for *in vivo* angiogenesis in response to VEGF. For this purpose, CAMs were treated with either VEGF alone (50 ng per egg), ZnMP alone (20, 40, and 80 nmol per egg), CuMP alone (20, 40, and 80 nmol per egg), or with a combination of VEGF plus ZnMP, or VEGF plus CuMP. Control CAMs were treated with a 0.5% DMSO solution. Vascular responses were assessed 48 h later. As shown in Figure 2a, CAMs treated with DMSO displayed the typical vascular pattern of a 12-day-old normal CAM, with thin vessels running parallel to each other in a leaf-like pattern. VEGF alone stimulated a clearly visible angiogenic response in CAMs, with the formation of tortuous blood vessels, loops, and fusion of some vessels in the area of treatment (Figure 2b), as well as a significant increase in

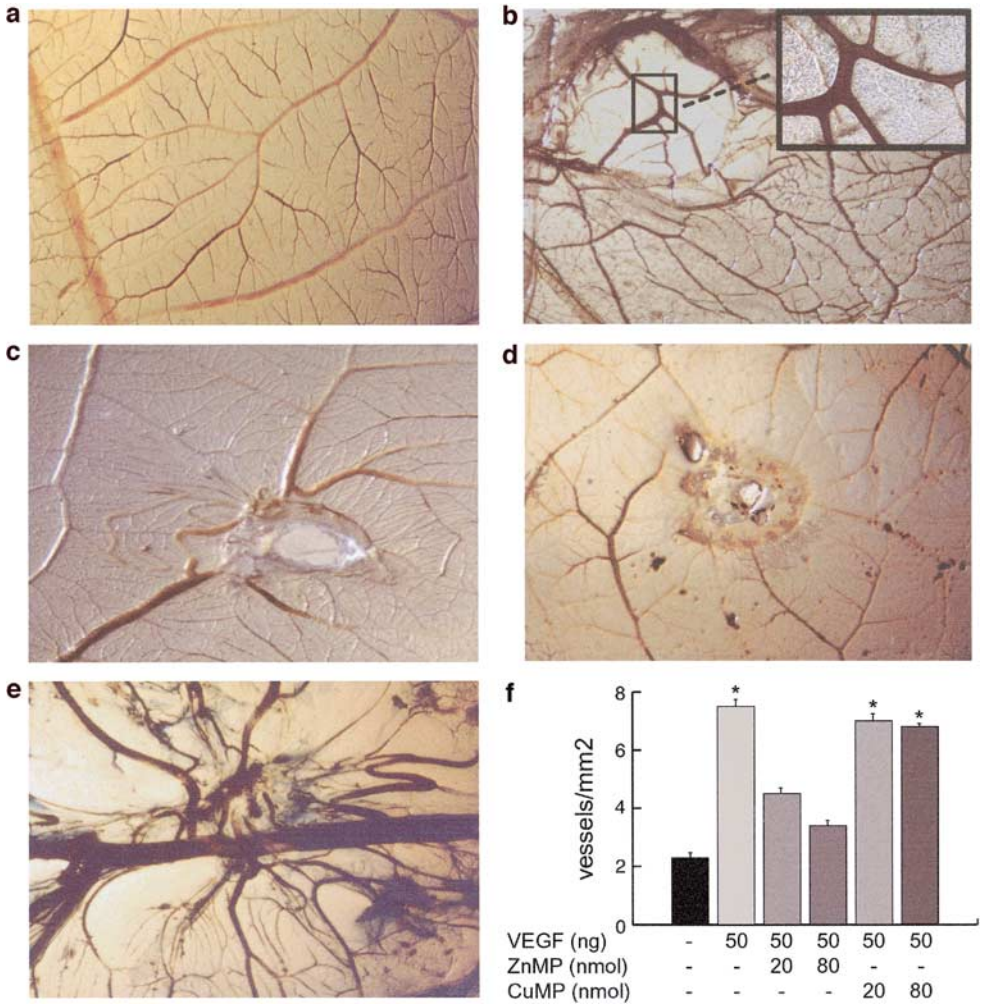

Figure 2 Representative photomicrographs of CAMs treated with VEGF alone (50 ng) (b), VEGF plus increasing doses of the HO-1 inhibitor ZnMP: 20 nmol (c) and 80 nmol (d), or VEGF plus 80 nmol CuMP (e), a metalloporphyrin that does not inhibit HO-1 activity. Control CAMs were treated with a 0.5% DMSO solution (a). Magnification $\times 10$. Fusion of blood vessels in the area of treatment is shown in the inset of Figure 2b ($\times 40$ magnification). Quantification of angiogenic responses in CAMs is shown in panel (f). Results are expressed as number of blood vessels per mm^2. *$P<0.05$ vs CAMs treated with VEGF alone.

vascularity, as compared with control CAMs (7.5 ± 0.4 vs 2.3 ± 0.3 blood vessels mm^{-2}, in VEGF-treated and control CAMs, respectively, $P<0.05$; Figure 2f). Interestingly, this angiogenic response was markedly and dose-dependently attenuated by the HO-1 inhibitor ZnMP (Figures 2c and d), with significantly lower number of blood vessels in CAMs treated with VEGF plus ZnMP than in those receiving VEGF alone (VEGF plus 20 nmol ZnMP: 4.5 ± 0.2 blood vessels mm^{-2}; VEGF plus 80 nmol ZnMP: 3.4 ± 0.3 blood vessels mm^{-2}; $P<0.05$ vs VEGF-treated CAMs; Figure 2f). This inhibitory effect of ZnMP was not observed with CuMP, a metalloporphyrin that has a similar structure to ZnMP but does not inhibit HO activity (Figures 2e and f). In addition, no vascular effects were observed in CAMs treated with ZnMP or CuMP alone, that is, in the absence of VEGF (data not shown). Taken together, these results suggest that HO-1 activity is necessary for VEGF-induced angiogenesis in CAMs.

Effects of intracellular calcium chelation on VEGF-induced HO-1 protein overexpression in CAMs

Next, we wanted to determine by which molecular mechanism VEGF increased HO-1 protein expression in CAMs. We focused, first, on calcium, which is a known mediator of the vascular effects of VEGF (reviewed in Ferrara, 2001). To test whether increases in intracellular calcium levels are necessary for HO-1 protein overexpression in response to VEGF, we used two different tools: the intracellular calcium chelator BAPTA-AM alone (1 nmol per egg) and the calcium ionophore A-23187 (20 pmol per egg). As shown in Figure 3, treatment of CAMs with VEGF alone increased HO-1 protein expression (3.5-fold increase), as compared with control CAMs. Interestingly, this increase was significantly attenuated when BAPTA-AM was included in the treatment (80% inhibition). In addition, the effects of BAPTA-AM were, in turn, compensated by the calcium ionophore A-23187. More-

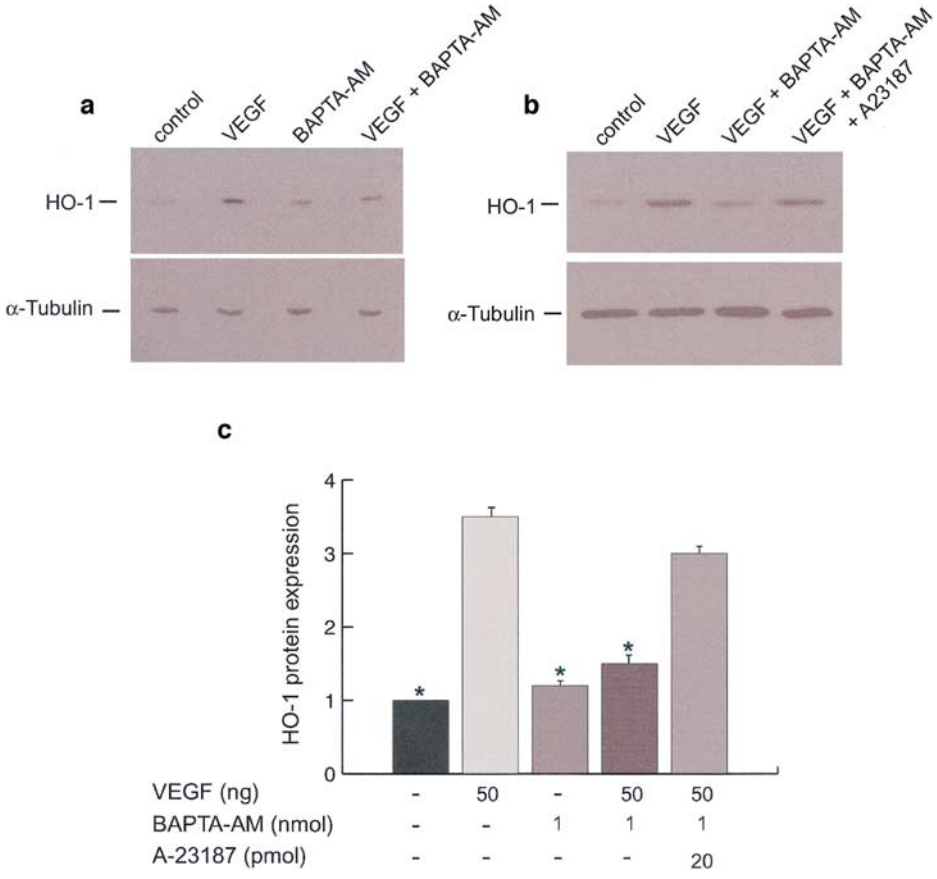

Figure 3 Expression of HO-1 protein in CAMs after treatment for 48 h with VEGF (50 ng) in the presence or absence of the intracellular calcium chelator BAPTA-AM (1 nmol) and the calcium ionophore A-23187 (20 pmol). Control CAMs were treated with a 0.5% DMSO solution. (a, b) Representative Western blots. (c) Densitometric quantification of HO-1 protein expression normalized to α-tubulin. Data are shown as fold increase in HO-1 protein expression above control values (mean ± s.e. of five separate experiments). $*P<0.05$ vs CAMs treated with VEGF alone.

over, no significant effects on HO-1 protein expression were observed in CAMs treated with BAPTA-AM alone (Figure 3). These results suggest that the increase in HO-1 protein expression elicited by VEGF in CAMs depends on an elevation of intracellular calcium levels.

Effects of protein kinase C inhibition on VEGF-stimulated HO-1 protein overexpression in CAMs

We also assessed whether activation of protein kinase C was involved in the signaling pathway by which VEGF increases HO-1 protein expression in CAMs. For this purpose, we used the protein kinase C inhibitor staurosporine (0.2, 2, and 10 nmol per egg). As shown in Figure 4, staurosporine inhibited, in a dose-dependent manner, the VEGF-stimulated HO-1 induction observed in CAMs. Thus, the dose of 0.2 nmol per egg of staurosporine caused a 54% inhibition on VEGF-stimulated HO-1 protein expression, the dose of 2 nmol per egg caused a 77% inhibition, and the dose of 10 nmol per egg led to a 94% inhibition. Taken together, these results suggest that an increase in intracellular calcium levels and activation of protein kinase C are necessary for HO-1 protein induction by VEGF in CAMs.

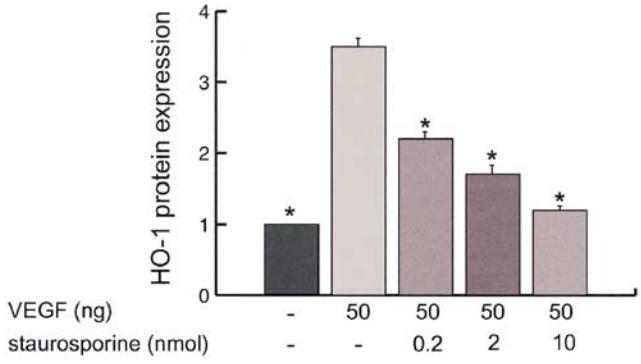

Figure 4 Expression of HO-1 protein in CAMs after treatment for 48 h with VEGF (50 ng) in the presence or absence of increasing doses of the protein kinase C inhibitor staurosporine (0.2, 2, and 10 nmol). Control CAMs were treated with a 0.5% DMSO solution. Densitometric quantification of HO-1 protein expression normalized to α-tubulin. Data are shown as fold increase in HO-1 protein expression above control values (mean ± s.e. of five separate experiments). $*P<0.05$ vs CAMs treated with VEGF alone.

Discussion

The present study provides novel *in vivo* evidence showing that VEGF increases the expression of HO-1 protein, simulta-

neously with angiogenesis, in the CAM of the chick embryo. The molecular mechanism by which VEGF upregulates HO-1 protein expression in CAMs involves an increase in intracellular calcium levels and activation of protein kinase C. Our results also suggest that HO-1 is involved in the signaling pathway(s) by which VEGF stimulates *in vivo* angiogenesis in CAMs, since this effect was markedly attenuated by the HO-1 inhibitor ZnMP.

Recent *in vitro* studies have also provided evidence in support of a possible role of HO-1 in angiogenesis. Thus, overexpression of HO activity by HO-1 gene transfer into endothelial cells causes a significant increase in *in vitro* cell proliferation and capillary formation (Deramaudt *et al*., 1998). In addition, the inducible HO-1 gene is upregulated by members of the Ets family of proteins (Deramaudt *et al*., 1999), which are known to play an important role in the control of vasculogenesis and angiogenesis, under both normal and pathological conditions (Wernert *et al*., 1992). Moreover, it has been shown that prolactin-mediated angiogenesis and cell proliferation is dependent on HO-1 gene expression (Malaguarnera *et al*., 2002). A direct correlation between HO-1 expression and angiogenesis has also been suggested in brain tumors (Hara *et al*., 1996; Nishie *et al*., 1999) and malignant melanomas (Torisu-Itakura *et al*., 2000).

The pathophysiological role of HO-1 in modulating the VEGF-stimulated angiogenesis could be related to the fact that this enzymatic system is responsible for the endogenous production of the powerful vasodilator carbon monoxide (reviewed in Elbirt & Bonkovsky, 1999). The vascular effects of carbon monoxide can be mediated by activation of soluble guanylate cyclase, and the consequent rise in intracellular cyclic guanosine monophosphate (cGMP) levels in vascular smooth muscle cells. Other mechanisms of action of carbon monoxide, independent of cGMP, are also possible, including inhibition of a cytochrome P450-dependent monooxygenase system, and/or activation of calcium-dependent potassium channels (reviewed in Wang *et al*., 1997). In addition, other products of heme metabolism by HO-1 (i.e., biliverdin, bilirubin and free iron), which also possess biological activities that influence vascular functions, could play a role in mediating VEGF biological actions.

Besides the possible role of HO-1 in VEGF-stimulated angiogenesis, HO-1 induction may also represent an adaptive defense mechanism against vascular injury caused by VEGF. The protective effect of HO-1 could be mediated through the ability of HO-1 to enzymatically break down heme, thereby mitigating the hazardous cellular effects of this pro-oxidant (reviewed in Elbirt & Bonkovsky, 1999). In addition, bilirubin, which is formed from biliverdin by biliverdin reductase, is a potent antioxidant (Stocker *et al*., 1987). Moreover, carbon monoxide may also stimulate several functions that may be protective for blood vessels, including inhibition of both smooth muscle cell proliferation (Morita *et al*., 1997) and platelet aggregation (Brüne & Ullrich, 1987), as well as anti-inflammatory (Otterbein *et al*., 2000) and antiapoptotic effects (Brouard *et al*., 2000).

In conclusion, we have demonstrated, for the first time, that VEGF upregulates HO-1 protein expression *in vivo* in CAMs, by a mechanism dependent on an increase in cytosolic calcium levels and protein kinase C activation. Our results also indicate that HO-1 activity is required for the angiogenic properties of VEGF in CAMs. Further investigation is necessary to fully characterize the pathophysiological significance of HO-1 induction on VEGF-induced angiogenesis.

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