Journal of Cellular Biochemistry 93:197–206 (2004)

Prolactin Increases HO-1 Expression and Induces VEGF Production in Human Macrophages

L. Malaguarnera,* R.M. Imbesi, A. Scuto, F. D'Amico, F. Licata, A. Messina, and S. Sanfilippo

Department of Biomedical Sciences, University of Catania, Italy

Abstract The pituitary hormone prolactin (PRL) is a multifunctional polypeptide which exerts a role on cell proliferation and may also contribute to cell differentiation. PRL is also produced by immune cells and is regarded as a key component of the neuroendocrine–immune loop and as a local regulator of macrophage response. The involvement of PRL in regulating monocyte/macrophage functions is suggested by the presence of PRL receptors in these cells. It has been shown that PRL possess both angiogenic and antiangiogenic effects. Recently, we revealed that augmentation of HO-1 activity enhances PRL-mediated angiogenesis in human endothelial cells. Since macrophages are key participants in angiogenesis our objective was to investigate the effect of PRL also in human macrophages. In vitro treatment of macrophages with PRL was found to increase both heme oxygenase-1 (HO-1) expression and protein synthesis in a time and dose dependent manner as quantified respectively by reverse-transcriptase real-time polymerase chain reaction and Western blot analysis. PRL-treated macrophages also showed an enhanced release of vascular endothelial growth factor (VEGF) as demonstrated by ELISA assay. Furthermore, to determine whether PRL-induced HO-1 activity was required for VEGF production by macrophages, the effect of PRL on the induction of VEGF was studied in the presence of an inducer stannic chloride ($SnCl_2$) and of an inhibitor stannic mesoporphyrin (SnMP) of HO activity. Our observations suggest that PRL may regulate monocyte activation and influences not only immune function but also angiogenesis. J. Cell. Biochem. 93: 197–206, 2004.

Key words: prolactin; heme-oxygenase-1; macrophages; angiogenesis

Prolactin (PRL), a polypeptide hormone secreted by the acidophilic cells of the anterior pituitary gland, is implicated in diverse arrays of physiological functions such as osmoregulation, reproduction, growth, and development [Ben-Jonathan et al., 1996]. PRL synthesis has been demonstrated in extra-pituitary tissue, including endothelial [Clapp et al., 1998], neuronal, and immune cells such as lymphocytes, mononuclear cells, and thymocytes [Ben-Jonathan et al., 1996]. The emerging role of PRL in immunoregulation has led to the concept of a dual function for PRL as both a circulating hormone and cytokine [Bazan, 1989; Ben-Jonathan et al., 1996]. It is structurally related to several cytokines and its receptor (PRL-R) belongs to the superfamily of hematopoietic cytokine receptors [Cosman et al., 1990]. Recent evidence indicates that PRL is involved in regulating monocyte/macrophage functions, this is also supported by the presence of PRL-R in these cells [Gala and Shevach, 1993]. Perturbation of PRL physiology has significant immunological effects in humans. Hyperprolactinemia is associated with malignant diseases and also with autoimmune diseases such as multiple sclerosis, lupus erythematosus, and rheumatoid arthritis [Cosman et al., 1990]. Recent studies showed that PRL possess both angiogenic and antiangiogenic effects [Struman et al., 1999]. The intact human PRL molecule, with a molecular mass of 23 kDa, was found to be angiogenic and increases cell proliferation in bovine brain capillary endothelial cells whereas its respective 16 kDa N-terminal fragment is antiangiogenic [Ferrara et al., 1991]. In addition, the angiogenic effect of PRL was markedly detected in invasive macroprolactinoma [Turner et al., 2000], the most common type of primary pituitary PRL-secreting tumor.

Grant sponsor: AIRC.

*Correspondence to: Lucia Malaguarnera, Via Androne, 83, 95124 Catania, Italy. E-mail: lucmal@mbox.unict.it

Received 13 February 2004; Accepted 12 April 2004

DOI 10.1002/jcb.20167

Published online 29 June 2004 in Wiley InterScience (www.interscience.wiley.com).

Angiogenesis is necessary in several physiological processes, such as the formation of embryonic tissues or placental development, in which induction and cessation of angiogenesis appear to be highly regulated through the expression of angiogenic or antiangiogenic factors. Moreover, angiogenesis is necessary for the continued growth of solid tumors, and acceleration of tumor growth accompanies neovascularization [Fidler and Ellis, 1994]. The stroma of the neoplasm is essential for tumor growth, invasion, and neovascularization. The stroma intermingles with and surrounds neoplastic cellular elements in almost all solid tumor cells and includes interstitial connective tissues, basal lamina, and constituents such as type IV collagen, laminin, fibronectin, and proteoglicans [Dvorak, 1996]. Blood vessels and inflammatory cells such as lymphocytes, neutrophils, macrophages, and natural killer cells are also observed frequently in the stroma. Interaction of stroma with malignant cells is considered to be crucial for development of neovascularity in tumors [Nagy et al., 1988].

Among stromal cells, macrophages carry out various biological functions, including participation in tumor angiogenesis [Sunderkötter et al., 1994]. Macrophages are important among the key angiogenic effector cells that produce a number of growth stimulators and inhibitors, proteolitic enzymes, and promote the expression of vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-α), platelet growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) by vascular endothelial cells, capable of modulating new vessel formation [Yoshida et al., 1997].

Diverse mediators have been implicated in the process of angiogenesis and cell proliferation including members of bFGF, VEGF [Ferrara and Henzel, 1989; Folkman, 1995], and heme oxygenase [Deramaudt et al., 1998; Quan et al., 2001]. Three HO isozymes (HO-1, HO-2, and HO-3), the products of distinct genes, have been described [Shibahara et al., 1993; McCoubrey et al., 1997]. HO-1, which is distributed ubiquitously in mammalian tissue, is induced strongly and rapidly by many compounds that elicit cell injury. HO-1 metabolizes heme to the antioxidant bilirubin and carbon monoxide (CO), and represents a powerful endogenous defensive mechanism against free radicals in many diseases. The natural substrate of HO, heme, is itself a potent inducer of the enzyme [Abraham et al., 1996]. HO-1 is an inducible heat shock protein [Shibahara et al., 1987] and is increased in whole animal tissues and in cultured cells following treatment with heme, inflammatory cytokines, and angiogenic factors [Deramaudt et al., 1998].

Induction of heme oxygenase (HO)-1 during inflammation has been demonstrated in many cell types. In macrophages, HO-1 gene transcription is up-regulated by inflammatory mediators such as LPS and IL-1β [Wiesel et al., 2000]. Moreover, it has been demonstrated that HO-1 gene expression could be a useful marker for macrophage infiltration as well as neovascularization in human gliomas [Nishie et al., 1999] and melanomas [Torisu-Itakura et al., 2000].

Since we have recently observed that in human endothelial cells PRL-mediated angiogenesis was modulated by the level of HO-1 gene expression, the aim of our studies was to investigate the importance of PRL in the functional activation of human monocytes/macrophages as well as the ability of PRL to stimulate the HO-1 expression and the VEGF production in macrophages.

MATERIALS AND METHODS

Cells

Human monocytes–macrophages (HMMs) were isolated from fresh buffy coat of healthy volunteers. The buffy coats were diluted with phosphate-buffered saline (PBS) supplemented with 2.5 mM EDTA and layered onto Ficoll-Hypaque gradients (Invitrogen, Milan, Italy). After 30 min of centrifugation at 400*g* at room temperature, the mononuclear cells were collected, washed twice with PBS, and placed in plastic Petri dishes at a concentration of 1–2×10^6 cells/cm surface areas in Iscove's medium supplemented with 2 mM glutamine, and 50 mg/ml of penicillin/streptomycin. After 2 h of incubation, the non-adherent cells were washed out using PBS. The adherent cells (HMMs) were cultured in Iscove's medium supplemented with 10% fetal calf serum (FCS) 2 mM glutamine, and 1% of penicillin/streptomycin (Invitrogen).

Cell Treatment

For each experimental group, we used 100 mm Petri dishes. The cells were then treated with different concentrations of PRL

(5, 10, 25, 50, 100 ng/ml). Cells were divided into several groups, cell groups were pre-treated with different reagents inducing HO-1 activity stannic chloride ($SnCl_2$) 10 μM and with the inhibitor stannic mesoporphyrin (SnMP) 10 μM for 8 h and then with 25 ng/ml of PRL. Untreated cells were used as control.

Superoxide Anion (O_2^-) Production

The O_2^- production was assayed by the spectrophotometric measurement of ferricytochrome *c* reduction. Cells were harvested 4 h after PRL treatment. Cells were washed once with PBS and incubated with 0.5 ml of reaction mixture consisting of Krebs Ringer phosphate (KRP) buffer containing 80 μM Cytochrome *c*, 2 mM NaN_3, and with or without 100 ng/ml PMA (Sigma Chemical Co., Italy). After 1 h of incubation at 37°C, the supernatants were collected and used to assay the amount of reduced Cytochrome *c* by the difference in adsorbance at 550–468 nm. The O_2^- release was calculated using a coefficient of 0.0245 (the extinction coefficient μml/l of Cytochrome *c* determined at 550–468 nm), and expressed as μmol O_2^-/mg proteins.

RT-PCR

Reverse transcription (RT) was carried out using the Superscript™ One-Step RT-PCR System (Invitrogen, Milan, Italy). Poly-d(T)n was used as reverse transcription primer. Specific primers for the hHO-1 cDNA fragment were as follows: primer 1, 5′-CAGGCAGAGAATGCTGAGTTC-3′; primer 2, 5′-GATGTTGAGCAGGAACGCAGT-3′. PCR was performed using the AmpliTaq PCR (Perkin-Elmer Cetus Instruments, Norwalk, CT). For each RT-PCR, a sample without reverse transcriptase was processed in parallel and served as a negative control. Cycling parameters for amplifying RT products were as follows: 95°C, 1 min; 60°C, 1 min; 72°C, 1–3 min, for 30 cycles, and then extended at 72°C for another 5 min.

GAPDH was used for normalization of data. After amplification, PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Quantitative Real Time PCR

Real-time fluorescence PCR, based on SYBR Green, was carried out in a 30 μl final volume containing 1× SYBR Green PCR Master Mix (Applied Biosystems, Monza, Italy), 200 nM forward and 200 nM reverse primers (Table I) and 20 ng of cDNA. Thermal cycling was performed for each gene (HO1 and HO-2) in triplicate on cDNA samples in MicroAmp Optical 96-well reaction plate (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems) using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplification was carried out with the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles each of 95°C for 15 s and 60°C for 1 min. All data were captured using Sequence Detector Software (Applied Biosystems).

Western Blot

Cells were harvested using cell lysis buffer as described previously [Abraham et al., 1987]. The lysate was collected for Western blot analysis or HO activity measurement. Protein levels were visualized by immunoblotting with antibodies against human HO-1, total immunoreactions HO-1 (rat and human) or HO-2. Briefly, 30 μg of lysate supernatant was separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, Milan, Italy) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 5% milk in 10 mM Tris-HCl (pH 7.4) 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4°C overnight. After washing with TBST, the membranes were incubated with a 1:2,000 dilution of anti-HO-1 or anti-HO-2 antibodies for 1 h at room temperature with constant shaking. Human HO-1 and HO-2 antibodies were kindly provided by Prof. NG Abraham (Dept. of Pharmacology, New York Medical College).

TABLE I. Synthetic Primers for RT-PCR for Gene Verifications

Gene	Primer	Sequence 5′–3′	Fragment length
Target gene HO-1	Forward	CCAGCGGGCCAGCAACAAAGTGC	265
	Reverse	AAGCCTTCAGTGCCCACGGTAAGG	
Internal standard HO-2	Forward	GTGGCCCAGCGAGCACTGAAACTC	255
	Reverse	AGGGAACCCATCCTCCAAGGTCTC	

The filters were then washed and subsequently probed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) at a dilution of 1:2,000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions.

VEGF Assay

Surnatants were analyzed for VEGF using enzyme-linked immunosorbent assay (ELISA) obtained commercially (R&D Systems, Milan, Italy) the assays were performed according to the manufacturer's protocol. VEGF concentrations are expressed in pg/ml. A standard curve was prepared from a group of serially diluted standard samples of VEGF. Absorbance was read against a blank at 450 nm λ using a microtiter ELISA reader.

Immunocytochemical Staining

Indirect immucytochemistry was performed on cells grown on glass coverslips. Cells were fixed in 3.5% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. After quenching in 50 mM ammonium chloride and permeabilization with 0.1% saponin in PBS, cells were incubated for 2 h anti-VEGF (1:100 dilution; Santa Cruz Biotechnologies, Inc., CA) rabbit polyclonal antibodies. Detection was then carried out using the ImmunoCruz™ Staining System (Santa Cruz Biotechnologies, Inc.) according to the manufacturer's instructions. This kit utilizes a horseradish–peroxidase–streptavidin complex and DAB as a chromogen. Finally, a counterstain of Harris' hematoxylin was applied. Control immunocytochemical experiments were performed by substitution of the primary antibodies with normal rabbit serum at the same concentration as that of the respective primary antisera.

Statistical Analysis

Data are expressed as mean ± SE. Significance was assessed by one way analysis of variance (ANOVA) and Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Superoxide Anion (O_2^-) Production

To investigate the mechanisms by which PRL activates HMMs, O_2^- release was carried out by the spectrophotometric measurement of ferricytochrome c reduction. Macrophages were cultured at different times (2, 4, 8, 16, and 24 h) in the absence or the presence of different concentrations (5, 10, 25, 50, and 100 ng/ml) of PRL. As shown in Figure 1 PRL induced O_2^- production in a dose, and time-dependent manner. At 25 ng/ml production of O_2^- was increased 8.7-fold over that of the control within 4 h of PRL treatment ($P < 0.05$). PMA (100 ng/ml) treated macrophages were used as positive control.

HO-1 Expression in Macrophages Following PRL Exposure

In the following sets of experiments, we evaluated the effect of PRL on HO-1 gene

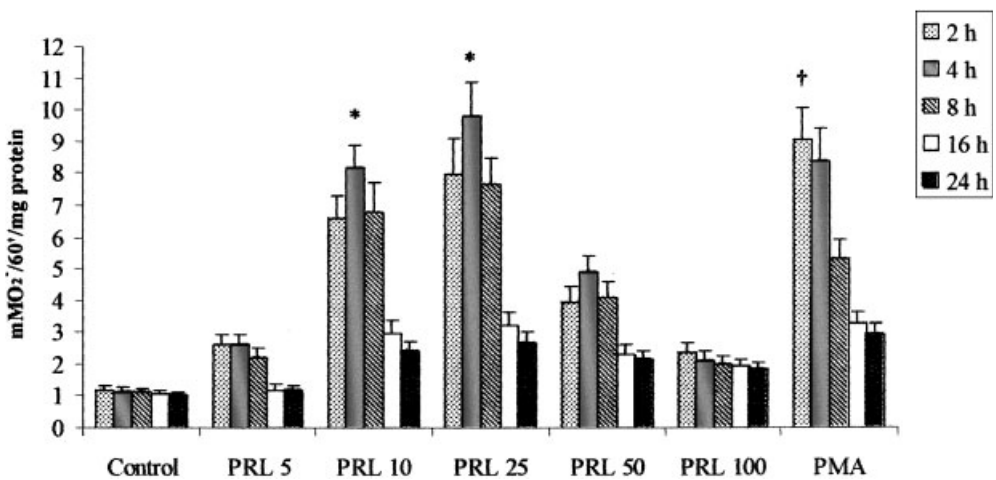

Fig. 1. Time- and dose-dependent effect of PRL on O^-_2 production in human macrophages. PMA (100 ng/ml) treated-macrophages were used as positive control. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t* test; $^*P < 0.05$.

expression by measuring HO-1 mRNA levels. Total mRNA of macrophages treated with different concentrations of PRL (5, 10, 25, 50, 100 ng/ml) for 4 h were subjected to RT-PCR. PRL treatment resulted in a dose-dependent increase in the expression of HO-1 with the higher expression at 25 ng/ml (Fig. 2A lane 4). Semi-quantitative evaluation of HO-1 mRNA level was done by using GAPDH. Densitometry analysis indicated a maximum of 3.05-fold increase (Fig. 2B) in HO-1 mRNA levels in macrophages treated with 25 ng/ml PRL compared to untreated control cells ($P < 0-05$). We then performed a time course to assess the higher HO-1 expression following PRL treatment, as shown in Figure 3, HO-1 mRNA levels were increased at 4 h and reached the higher expression at 8 h of PRL treatment (Lane 3, and 4). After 16 and 24 h of exposure HO-1 expression was undetectable. Macrophages treated for 8 h with 10 μM $SnCl_2$, a potent inductor of HO-1 activity, were used as control (lane 7). Semi-quantitative evaluation of HO-1 mRNA changes by densitometry analysis indicated a 4.5-fold increase in HO-1 mRNA levels in macrophages treated with PRL (25 ng/ml) for 8 h (Fig. 3B) compared to untreated controls. To confirm the time course data, RT preparations were also subjected to real-time PCR. As seen in Figure 3C, the level of HO-1 in macrophages treated for 4 and 8 h were markedly elevated (2.3 and 3.6-fold respectively) compared with untreated control. After 16 and 24 h of PRL treatment, the levels of HO-1 expression HO-1 were markedly low.

Enhancement of HO-1 Protein Synthesis in Macrophages Following PRL Exposure

To determine whether the increase in HO-1 mRNA was associated with elevation of HO-1

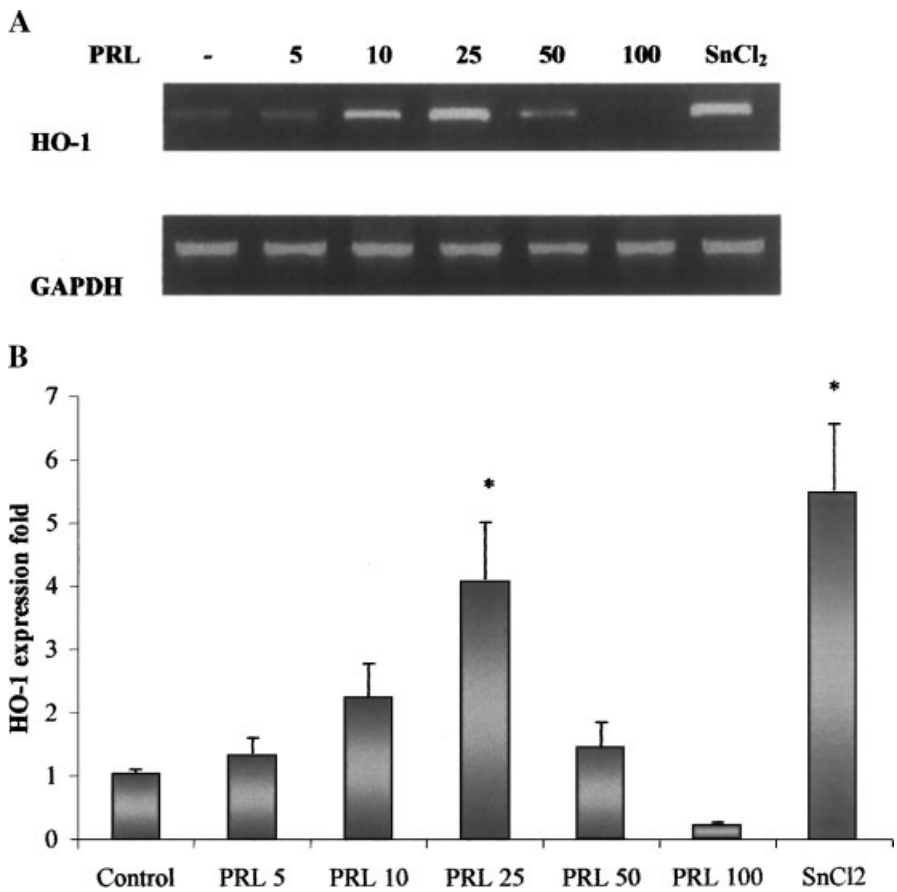

Fig. 2. **A**: Detection of HO-1 expression by RT-PCR of RNA obtained from monocytes/macrophages untreated and treated 4h with different concentration of PRL. Lanes: (**1**) Control; (**2**) PRL 5 ng/ml; (**3**) PRL 10 ng/ml; (**4**) PRL 25 ng/ml; (**5**) PRL 50 ng/ml; (**6**) PRL 100 ng/ml; (**7**) $SnCl_2$ 10 μM. **B**: The level of HO-1 mRNA normalized to GAPDH. Statistical analysis was performed by Student's *t* test; $*P < 0.05$, of treated cells versus control.

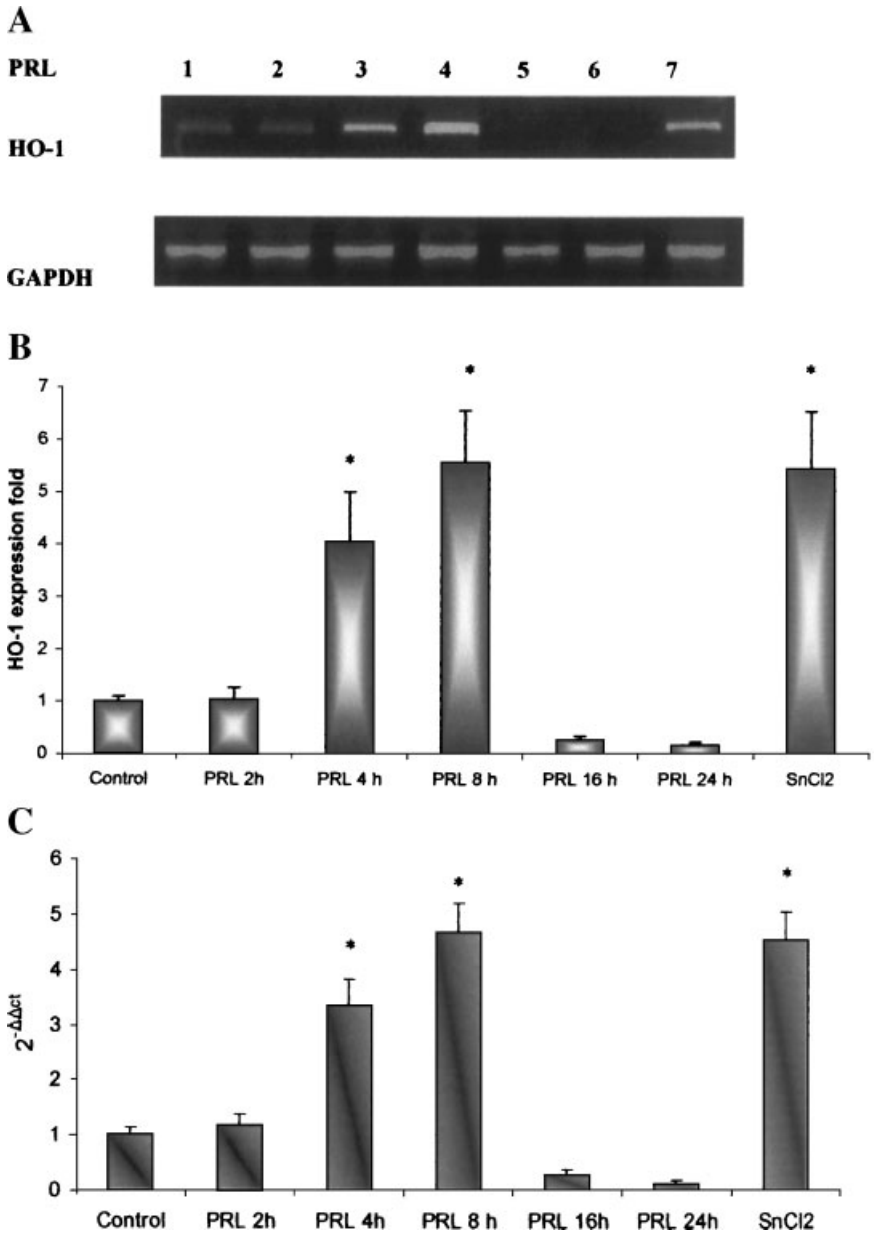

Fig. 3. **A**: Time course of HO-1 expression by RT-PCR of RNA obtained from monocytes/macrophages untreated and treated with PRL (25 ng/ml). Lanes: (**1**) Control; (**2**) 2 h; (**3**) 4 h; (**4**) 8 h; (**5**) 16 h; (**6**) 24 h; (7) $SnCl_2$ 10 μM. **B**: The level of HO-1 mRNA normalized to GAPDH. Statistical analysis was performed by Student's *t* test; $^*P < 0.05$, of treated cells versus control. **C**: Quantification of HO-1 mRNA levels by Real Time PCR. mRNA levels are expressed as $2^{-\Delta\Delta ct}$ value.

protein in macrophages, Western blot analysis was performed on cell lysates obtained from treated and untreated human macrophages using HO antibodies that recognize human HO-1 protein. A time-dependent increase in HO-1 protein was observed, as shown in Figure 4A, following exposure to 25 ng/ml PRL, the cells exhibited a strong signal for HO-1 protein at 8 and 16 h (lanes 3 and 4), at 4 and 24 h the HO-1 levels were comparable to the control untreated cells (lanes 2 and 5). A similar amount of HO-2 protein was observed in each line. Semi-quantitative evaluation of HO-1 protein was measured by scanning densitometry (Fig. 4B). The results showed that HO-1 synthesis was increased 3.9-fold and over control within 16 h of treatment with 25 ng/ml PRL ($P < 0.05$).

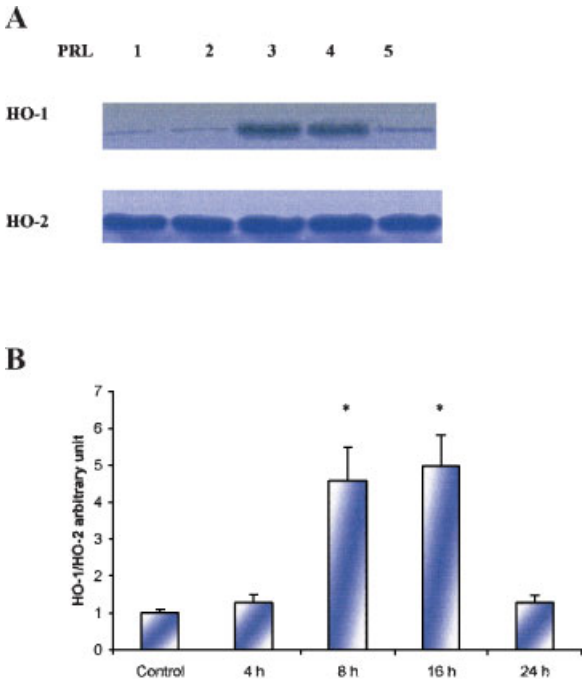

Fig. 4. **A**: Western blot analysis of human macrophages untreated and treated with PRL (25 ng/ml). Lanes: (**1**) Control; (**2**) 4 h; (**3**) 8 h; (**4**) 16 h; (**5**) 24 h. **B**: Level of HO-1 protein normalized to HO-2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

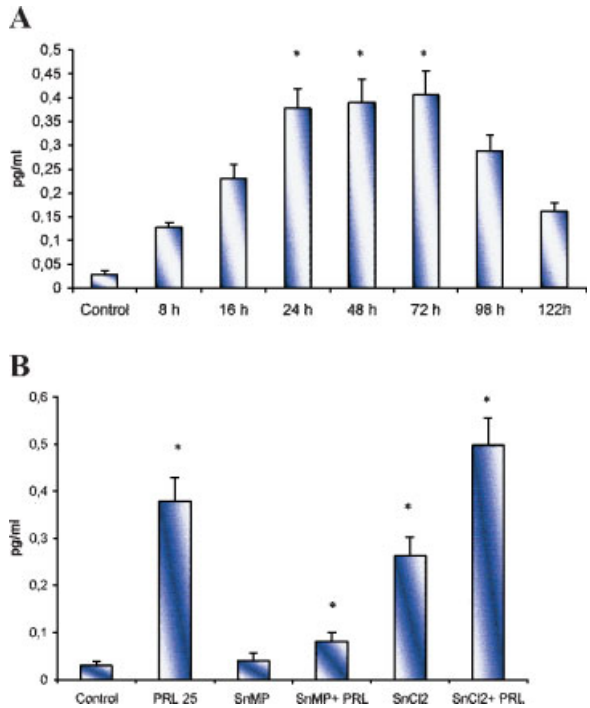

Fig. 5. **A**: Time course of VEGF production in macrophages treated with PRL (25 ng/ml). Data are representative of three independent experiments. **B**: Effect of inducer and inhibitor of HO-1 on PRL mediated VEGF production, cells pre-treated for 8 h with $SnCl_2$ (10 mM), and SnMP (10 mM) and then incubated with PRL (25 ng/ml) for 24 h. Data are representative of three independent experiments. Statistical analysis was performed by Student's *t* test; $^*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effect of PRL on VEGF Production

To verify if PRL was capable to enhance VEGF production, cells were incubated with PRL (25 ng/ml) in 0.5% FBS. As shown in Figure 5, Panel A, at 25 ng/ml, PRL caused an enhancement of VEGF production within 24 h and at 72 h was increased by 3.4 fold, as compared with control cells ($P < 0.05$). To assess whether the effect of PRL on the induction of VEGF production in macrophages was dependent on HO-1, it was essential to evaluate the effect of an inducer and an inhibitor of HO-1 activity. Macrophages were pretreated for 8 h with $SnCl_2$ (10 mM), a potent inducer of HO-1 or SnMP (10 mM) a competitive inhibitor of HO-1 activity, and then PRL 25 ng/ml was added for another 24 h. As seen in Figure 5 panel B, pre-treatment with SnCl2 resulted in a significant increase of VEGF production by 4.6-fold, as compared with control cells ($P < 0.05$). In contrast, pre-treatment with SnMP markedly attenuated (2.9-fold decrease) VEGF production by macrophages as compared with macrophages PRL treated ($P < 0.05$). These findings confirm that PRL is an important inductor of angiogenesis and an important relationship exists between PRL-mediated angiogenesis and the levels of HO-1.

Effects of PRL on Phenotypical Differentiation of Cultured Monocyte/Macrophages

Immunocytochemical staining using anti-VEGF showed that long-term (4–8 days) incubation of cultured monocyte/macrophages with PRL at a concentration of 25 ng/ml phenotypically gave rise to elongated, mononucleated cells showing to form endothelial tube-like structures, in which VEGF-positive cytoplasm seems to delineate a lumen (Fig. 6B). This result suggest that PRL induces monocytes/macrophages to differentiate into endothelial progenitor cells.

DISCUSSION

In the present study, we demonstrate that in vitro treatment of human macrophages with PRL enhances HO-1 gene expression and VEGF production. The finding showing that the

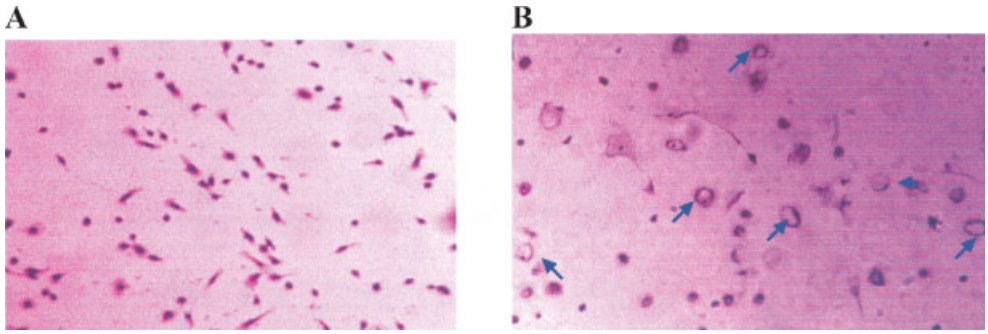

Fig. 6. Immunocytochemical staining using anti-VEGF of cultured monocyte/macrophages with PRL 25 ng/ml. **A**: untrated macrophages; **B**: PRL treated macrophages. 100× magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment with PRL was associated with an increase in the O_2^- content is also of great interest. This result is consistent with the notion that changes in the redox status of the cell are the prerequisite for the upregulation HO-1. Our results demonstrate that HO-1 expression reflects the functional state of activated macrophages.

The addition of PRL resulted in an increase in a dose and time-dependent manner in the expression of HO-1 at the mRNA (Figs. 2, 3) and protein levels (Fig. 4). By producing a number of growth stimulators and inhibitors, cytokines and proteolytic enzymes, macrophages play a key role in the angiogenesis cascade [Sunderkötter et al., 1994]. The PRL-induced HO-1 overexpression in macrophages may have a crucial function of these cells in angiogenesis.

Moreover, we observed that PRL-mediated increase VEGF production is dependent on the levels of HO-1 gene expression as evidenced by modulating HO-1 levels by known inducer or inhibitor. Experimental results demonstrated that pre-treatment of macrophages with $SnCl_2$, an inducer of HO-1, caused a significant increase in PRL-mediated VEGF release. In contrast, SnMP, a competitive inhibitor of HO activity [Kappas et al., 1985, 1988], caused a significant decrease VEGF release, suggesting that the VEGF production induced by PRL treatment was dependent on HO-1 expression. Our results are consistent with other recent reports in which it was demonstrated that HO-1 upregulates growth factors, including VEGF [Abdel-Aziz et al., 2003; Abraham et al., 2003; Jozkowicz et al., 2003].

Macrophages in the stromal compartment of tumors, often called tumor associated macrophages, are closely correlated with neovascularization and prognosis in patients with breast cancer [Leek et al., 1996]. Several reports have demonstrated that HO-1 is upregulated in various types of cancers. Elevated HO activity has been found in renal adenocarcinoma [Goodman et al., 1997, 2002]; in prostate cancer and in solid tumors [Doi et al., 1999]. This expression is consistent with the role of HO-1 in tumor growth as well as in tumor metastasis [Tsuji et al., 1999]. In addition, it has been demonstrated that HO-1 gene expression could be a useful marker for macrophage infiltration as well as neovascularization in human gliomas [Nishie et al., 1999] and melanomas [Torisu-Itakura et al., 2000]. Of note is our recent finding showing that PRL-mediated angiogenesis was modulated by the level of HO-1 gene expression also in human endothelial cells [Malaguarnera et al., 2002] suggesting that in the stromal compartment PRL effect in infiltrating macrophages may have a cumulative effects in the induction of angiogenesis as demonstrated also by immunocytochemical staining. PRL could also act as an angiogenetic signal for monocytes/macrophages cultured in vitro, inducing them to differentiate into endothelial progenitor cells (EPCs). Our present, morphological results confirm previous findings of Fernandez-Pujol et al. and of Schmeisser et al. [Fernandez-Pujol et al., 2000; Schmeisser et al., 2001] pointing out the complex relationship between monocyte/macrophages and endothelial cells.

The mechanism of PRL mediated increases in angiogenesis may be related to the HO products such as CO and bilirubin. In fact, it has been shown that CO, generated by HO-1, enhances endothelial cell proliferation and decreases apoptosis [Brouard et al., 2000]. When HO activity is blocked by SnMP or the action of CO is inhibited by hemoglobin, HO-1 activity no

longer prevents endothelial cell apoptosis [Deramaudt et al., 1998].

Collectively, these findings suggest that the heme oxygenase system, presumably via the production of CO or bilirubin, sub serves mechanisms that prevent growth arrest and apoptosis and may mediate the angiogenesis induced by PRL.

In conclusion, the involvement of PRL in the activation of macrophages and the production HO-1 and its products CO and/or bilirubin is indicative of an integrated cooperative regulation of the component of immune system with that of the endocrine system in the induction of angiogenesis. Additional studies to elucidate the detailed regulatory pathway of PRL on induction of HO-1 gene expression and angiogenesis are necessary to develop strategies for a potential targeted pharmacological modulation of HO-1.

ACKNOWLEDGMENTS

We thank Dr. Gabriella Denti of the S. Immuno-haemathology and Transfusional Medicine, Mr. Michele Tuttobene and Mrs. Antonella Rannisi for preparation of buffy coats.

REFERENCES

Abdel-Aziz MT, el-Asmar MF, el-Miligy D, Atta H, Shaker O, Ghattas MH, Hosni H, Kamal N. 2003. Retrovirus-mediated human heme oxygenase-1 (HO-1) gene transfer into rat endothelial cells: The effect of HO-1 inducers on the expression of cytokines. Int J Biochem Cell Biol 35: 324–332.

Abraham NG, Lin JH, Dunn MW, Schwartzman ML. 1987. Presence of heme oxygenase and NADPH cytochrome P-450 (c) reductase inhuman corneal epithelium. Invest Ophthalmol Vis Sci 28:1464–1472.

Abraham NG, Drummond GS, Lutton JD, Kappas A. 1996. The biological significance and physiological role of heme oxygenase. Cell Physiol Biochem 6:129–168.

Abraham NG, Scapagnini G, Kappas A. 2003. Human heme oxygenase: Cell cycle-dependent expression and DNA microarray identification of multiple gene responses after transduction of endothelial cells. J Cell Biochem 90:1098–1111.

Bazan JF. 1989. A novel family of growth factor receptors: A common binding domain in the growth hormone, prolactin, erythropoietin, and IL-6 receptors, and the p75 IL-2 receptor beta-chain. Biochem Biophys Res Commun 164: 788–795.

Ben-Jonathan N, Mershon JL, Allen DL, Steinmetz RW. 1996. Extrapituitary prolactin: Distribution, regulation, functions, and clinical aspects. Endocr Rev 17:639–669.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP. 2000. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. J Exp Med 192:1015–1026.

Clapp C, Lopez-Gomez FJ, Nava G, Corbacho A, Torner L, Macotela Y, Duenas Z, Ochoa XX, Noris G, Acosta E, Garay E, Martinezd LE. 1998. Expression of prolactin mRNA and of prolactin-like proteins in endothelial cells: Evidence for autocrine effects. J Endocrinol 158:137–144.

Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG, March CJA. 1990. A new cytokine receptor superfamily. Trends Biochem Sci 15:265–270.

Deramaudt BM, Braunstein S, Remy P, Abraham NG. 1998. Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. J Cell Biochem 68:121–127.

Doi K, Akaike T, Fujii S, Tanaka S, Ikebe N, Beppu T, Shibahara S, Ogawa M, Maeda HTI. 1999. Induction of heme oxygenase-1 nitric oxide and ischaemia in experimental solid tumours and implications for tumour growth. Br J Cancer 80:1945–1954.

Dvorak HF. 1996. Wound that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 17:122–137.

Fernandez-Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkievicz J, Elsasser HP, Müller R, Havemann K. 2000. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 65:287–300.

Ferrara N, Henzel WJ. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factorspecific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858.

Ferrara N, Clapp C, Weiner R. 1991. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. Endocrinology 129:896–900.

Fidler IJ, Ellis LM. 1994. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 79: 185–188.

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

Gala RR, Shevach EM. 1993. Identification by analytical flow cytometry of prolactin receptors on immunocompetent cell populations in the mouse. Endocrinology 133: 1617–1623.

Goodman AI, Choudhury M, da-Silva JL, Schwartzman ML, Abraham NG. 1997. Overexpression of the heme oxygenase gene in renal cell carcinoma. Proc Soc Exp Biol Med 214:54–61.

Goodman AI, Li Volti G, Abraham NG, Malaguarnera L. 2002. Role of heme oxygenase in angiogenesis and renal carcinoma. Heme oxygenase in biology and medicine. Kluwer Academic/Plenum Publishers. pp 459–467.

Jozkowicz A, Huk I, Nigisch A, Weigel G, Dietrich W, Motterlini R, Dulak J. 2003. Heme oxygenase and angiogenic activity of endothelial cells: Stimulation by carbon monoxide and inhibition by tin protoporphyrin-IX. Antioxid Redox Signal 5:155–162.

Kappas A, Simionatto CS, Drummond GS, Sassa S, Anderson KE. 1985. The liver excretes large amounts of heme into bile when heme oxygenase is inhibited competitively by Sn-protoporphyrin. Proc Natl Acad Sci USA 82:896–900.

Kappas A, Drummond GS, Manola T, Petmezaki S, Valaes T. 1988. Sn-protoporphyrin use in the management of

hyperbilirubinemia in term newborns with direct Coombs-positive ABO incompatibility. Pediatrics 81: 485–497.

Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. 1996. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56:4625–4629.

Malaguarnera L, Pilastro MR, Quan S, Ghattas MH, Yang L, Mezentsev AV, Kushida T, Abraham NG, Kappas A. 2002. Significance of heme oxygenase in prolactin-mediated cell proliferation and angiogenesis in human endothelial cells. Int J Mol Med 4:433–440.

McCoubrey WK Jr, Huang TJ, Maines MD. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem 247:725–732.

Nagy JA, Brown LF, Senger DR, Lanir N, Van De Water L, Dvorak AM, Dvorak HF. 1988. Pathogenesis of tumor stroma generation: A critical role for leaky blood vessekls and fibrin deposition. Biochim Biophys Acta 948:305–326.

Nishie A, Ono M, Shono T, Fukushi J, Otsubo M, Onue H, Ito Y, Inamura T, Ikezaki K, Fukui M, Iwaki T, Kuwano M. 1999. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. Clin Cancer Res 5:1107–1113.

Quan S, Yang L, Abraham NG, Kappas A. 2001. Regulation of human heme oxygenase in endothelial cells by using sense andantisense retroviral constructs. Proc Natl Acad Sci USA 98:12203–12208.

Schmeisser A, Gerlichs CD, Zang H, Eskafi S, Graffy C, Ludwig J, Strasse RH, Daniel WG. 2001. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel® under angiogenic conditions. Cardiovascular Res 49:671–680.

Shibahara S, Muller RM, Taguchi H. 1987. Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem 262:12889–12892.

Shibahara S, Yoshizawa M, Suzuki H, Takeda K, Meguro K, Endo K. 1993. Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. J Biochem 113:214–218.

Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, Weiner RI, Martial JA. 1999. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: An efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci USA 96:1246–1251.

Sunderkötter C, Steinbrank K, Goebeler M, Bhardwaj R, Sorg C. 1994. Macrophages and angiogenesis. J Leukoc Biol 55:410–422.

Torisu-Itakura H, Furue M, Kuwano M, Ono M. 2000. Co-expression of thymidine phosphorylase and heme oxygenase-1 in macrophages in human malignant vertical growth melanomas. Jpn J Cancer Res 91: 906–910.

Tsuji MH, Yanagawa T, Iwasa S, Tabuchi K, Onizawa K, Bannai S, Toyooka H, Yoshida HTI. 1999. Heme oxygenase-1 expression in oral squamous cell carcinoma as involved in lymph node metastasis. Cancer Lett 138: 53–59.

Turner HE, Nagy Z, Gatter KC, Esiri MM, Harris AL, Wass JA. 2000. Angiogenesis in pituitary adenomas—relationship to endocrine function, treatment, and outcome. J Endocrinol 165:475–481.

Wiesel P, Foster LC, Pellacani A, Layne MD, Hsieh CM, Huggins GS, Strauss P, Yet SF, Perrella MA. 2000. Thioredoxin facilitates the induction of heme oxygenase-1 in response to inflammatory mediators. J Biol Chem 275:24840–24846.

Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M. 1997. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol 17:4015–4023.