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Biphasic regulation of angiogenesis by reactive oxygen species

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Reactive oxygen species (ROS) are believed to be important molecules in the regulation of angiogenesis. However, direct evidence is obtained from hydrogen peroxide only. The comparison of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) effects on angiogenesis in one angiogenic model were studied. Tube formation, migration and adhesion of endothelial cells were enhanced with a low concentration of O_2^- generated by 500 μM xanthine (X) and 1 mU/ml xanthine oxidase (XO), but significantly inhibited as the XO increased to 10 mU/ml or more. Low concentrations of H_2O_2 (0.01–1 μM) induced tube formation and the maximal tube formation was achieved at 0.1 μM which also induced cell migration and adhesion, while high concentrations of H_2O_2 (100 μM) inhibited tube formation and cell migration. Both H_2O_2 and O_2^- inhibited cell proliferation at high concentration only. $HO^\bullet$ at low concentration neither inhibited nor stimulated the tube formation, cell proliferation and migration but inhibited at high concentration. The effects of O_2^- were significantly abolished by catalase (CAT) alone or in combination with superoxide dismutase (SOD), but not by inactive CAT or SOD alone. Active CAT, but not inactive CAT, also reversed the effects of H_2O_2. Pretreatment with GSH effectively reversed the inhibitory effects of $HO^\bullet$. Therefore, our results suggest that ROS have biphasic effects on angiogenesis, which indicated that pharmacologically regulating cellular ROS levels might serve as an anti-angiogenic or angiogenic principles. They also provide a theoretical basis for the development and rational use of novel angiogenic and anti-angiogenic drugs.

1. Introduction

Mammalian cells generate reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and their metabolites through their normal metabolic processes. A relatively high concentration of them induces oxidative stress and may have deleterious effects. On the other hand, it has been suggested that a low concentration of ROS might exert an important physiological role as intracellular signaling molecules (Lander 1997; Kunsch and Medford 1999; Irani 2000; Sen and Packer 1996). In practice, ROS can be produced by many drugs through the following processes: 1) during their metabolism by enzymes such as cytochrome P450-dependent monooxygenases or peroxidases; 2) by depleting antioxidant defenses; 3) indirectly by the induction of cytochrome P450 isoenzymes and peroxisome proliferation. The ROS generation seems to be responsible for the action of certain drugs, and, on the other hand, the oxidative stress induced by certain drugs is also related to their side effects (Paolini and Cantelli-Forti 2000).

Angiogenesis, the formation of new capillary from preexisting vessels, plays an important role in physiological processes such as wound healing, embryonic development, and in pathological conditions, such as tumor growth and metastasis, rheumatoid arthritis, proliferate diabetic retinopathy, ischemic coronary artery diseases and brain infarction (Folkman 1995; Griffioen and Molema 2000). The process of angiogenesis consists of several steps, beginning with activation of endothelial cells by growth factor, followed by enzymatic degradation of basement membrane, detachment of endothelial cell from adhesion proteins, endothelial cell migration into the perivascular spaces and proliferation, and finally new vessel formation (Folkman and Shing 1992).

Cultured endothelial (Sundqvist 1991) and tumor cells (Szatrowski and Nathan 1991) are able to produce a low concentration of ROS even under non-stimulated conditions or augmented by hypoxia/reoxygenation (Inauen et al. 1990; Lum et al. 1992; Zweier et al. 1994; Terada 1996). The hypoxic induction of angiogenesis is a hallmark of pathological processes such as wound healing and solid tumor formation (Lelkes et al. 1998). A previous study also reported that H_2O_2 stimulated angiogenesis *in vitro* (Yasuda et al. 1999). The anti-angiogenic effects of many compounds, such as ascorbic acid (Ashino et al. 2003), green tea catechins, vitamin E (Tang and Meydani 2001; Tang et al. 2003) and resveratrol (Lin et al. 2003), are involved to their anti-oxidative properties. Thalidomide exerts its anti-angiogenic properties via the generation of hydroxyl radicals (Sauer et al. 2000).

Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor and stimulates endothelial cell proliferation and migration *in vitro* and angiogenesis *in vivo* (Neufeld et al. 1999). It has been reported that low concentrations of ROS induced VEGF mRNA expression in

human retinal pigment epithelial, human melanoma and rat glioblastoma cells (Kuroki et al. 1996). Moreover, NAD(P)H oxidase, a major source of endothelial superoxide generation, is required for VEGF-induced endothelial cell proliferation and migration (Avid et al. 2000). ROS derived from NAD(P)H oxidase mediated VEGF-induced endothelial cell proliferation, migration and VEGF receptor-2 (KDR) tyrosine phosphorylation (Ushio-Fukai et al. 2002). As the first reactive oxygen product during O_2 metabolism *in vivo*, the superoxide anion (O_2^-) is released constitutively in the nanomolar range by NAD(P)H dependant oxidase in vascular cell (Griendling et al. 2000). Xanthine oxidase activity, one of the sources of O_2^- generation, was found to be much higher in capillary endothelial cells (Jarasch et al. 1986). However, up to date, direct evidence that ROS affect angiogenesis is obtained from H_2O_2 only (Shono et al. 1996; Yasuda et al. 1999). The exact relationship between ROS and angiogenesis is still unclear. In the present study, besides H_2O_2, the effects of O_2^-, and $HO^\bullet$ on angiogenesis were examined in the same angiogenic model. We found that ROS have a biphasic effect on angiogenesis. The activation of angiogenesis may benefit ischemic coronary artery disease, brain infarction, wound healing and even embryonic development. On the other hand, the inhibition of angiogenesis may benefit by suppressing tumor growth and diabetic retinopathy and so on. Thus, understanding the biphasic regulation of angiogenesis by ROS will undoubtedly help pharmacologists to design the optimal therapeutic agents for the prevention and treatment of angiogenesis related diseases.

2. Investigations and results

2.1. Effects of ROS on angiogenesis

Human umbilical vein endothelial cells grown on uncoated dishes developed many cobblestone-like appearances during confluence (Fig. 1A). However, the tube-like structures have to be developed on fibrin gel (Fig. 1B).

As shown in Fig. 2A, treatment with 500 μM xanthine (X) alone or in combination with low concentrations of xanthine oxidase (XO) (XO, 0.1 or 0.2 mU/ml) had no effect on tube formation compared with control. The tube formation can be induced at least when the concentration of XO increased to 0.5 mU/ml. The maximal tube formation appeared at 1 mU/ml XO, and then decreased gradually at 2 and 5 mU/ml XO. As the concentrations of XO increased further within 10–100 mU/ml, tube formation was inhibited even compared with control. So we selected the following critical concentrations of XO 0, 1, 10 and 100 mU/ml for the further experiments.

Hydrogen peroxide showed the same effect on tube formation as O_2^-. Treatment with low concentrations of H_2O_2 (0.0001 or 0.001 μM) had no effect on tube formation. The tube formation was induced at least at 0.01 μM. The maximal tube formation was achieved at 0.1 μM. The inhibitory effect appeared at 100 μM (Fig. 2C). However, the hydroxyl radical did not show the same effects as O_2^- and H_2O_2. As shown in Fig. 2E, in the presence of 100 μM $FeSO_4$, the addition of 1, 10 or 100 μM H_2O_2 inhibited tube formation, while even in a concentration as low as 0.0001 μM, no increasing effects of H_2O_2 were observed. The biphasic effects of O_2^- on tube formation did not disappear when SOD (100 U/ml) or inactive CAT were added before X/XO treatment, but were significantly abolished by CAT (100 U/ml) alone, or combined with SOD (Fig. 2B). The biphasic effects of H_2O_2 were also reversed by pretreatment with CAT (100 U/ml), but not by inactive CAT (Fig. 2D). Pretreatment with 5 mM GSH effectively reversed the inhibitory effect induced by $HO^\bullet$ (Fig. 2F).

2.2. Effects of ROS on cell proliferation

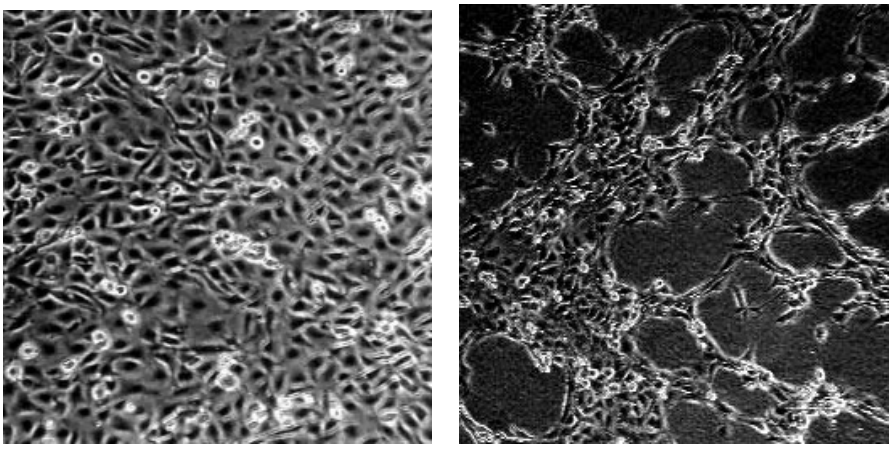
High concentrations of O_2^-, H_2O_2 or $HO^\bullet$ all significantly inhibited the proliferation of endothelial cell, but low concentrations of them did not inhibit nor stimulate the proliferation (Fig. 3).

2.3. Effects of ROS on cell migration

As shown in Fig. 4A, in the presence of 500 μM X, the addition of 1 mU/ml XO significantly increased cell migration by 1.25-fold, while 10 mU/ml XO inhibited migration significantly. CAT alone or combined with SOD downregulated the cell migration to the control level, while pretreatment with SOD, or inactive CAT had no effect on cell migration. H_2O_2 0.1 μM increased cell migration, while 100 μM H_2O_2 inhibited. Both effects can be reversed by CAT, but not by inactive CAT (Fig. 4B). Opposite to O_2^- and H_2O_2, the hydroxyl radical could only inhibit cell migration when the concentration was high enough, and the effect can be reversed by GSH (Fig. 4C).

2.4. Effects of ROS on cell adhesion

Low concentrations of O_2^- (X 500 μM/XO 1 mU/ml) induced cell adhesion about 1.2-fold. Pretreatment with CAT alone or combined with SOD, but not SOD or inac-



A B

Fig. 1:
Human umbilical vein endothelial cells grown on uncoated dishes develop many cobblestone-like appearances (A). While tube-like structures (B) have to be developed on fibrin gel

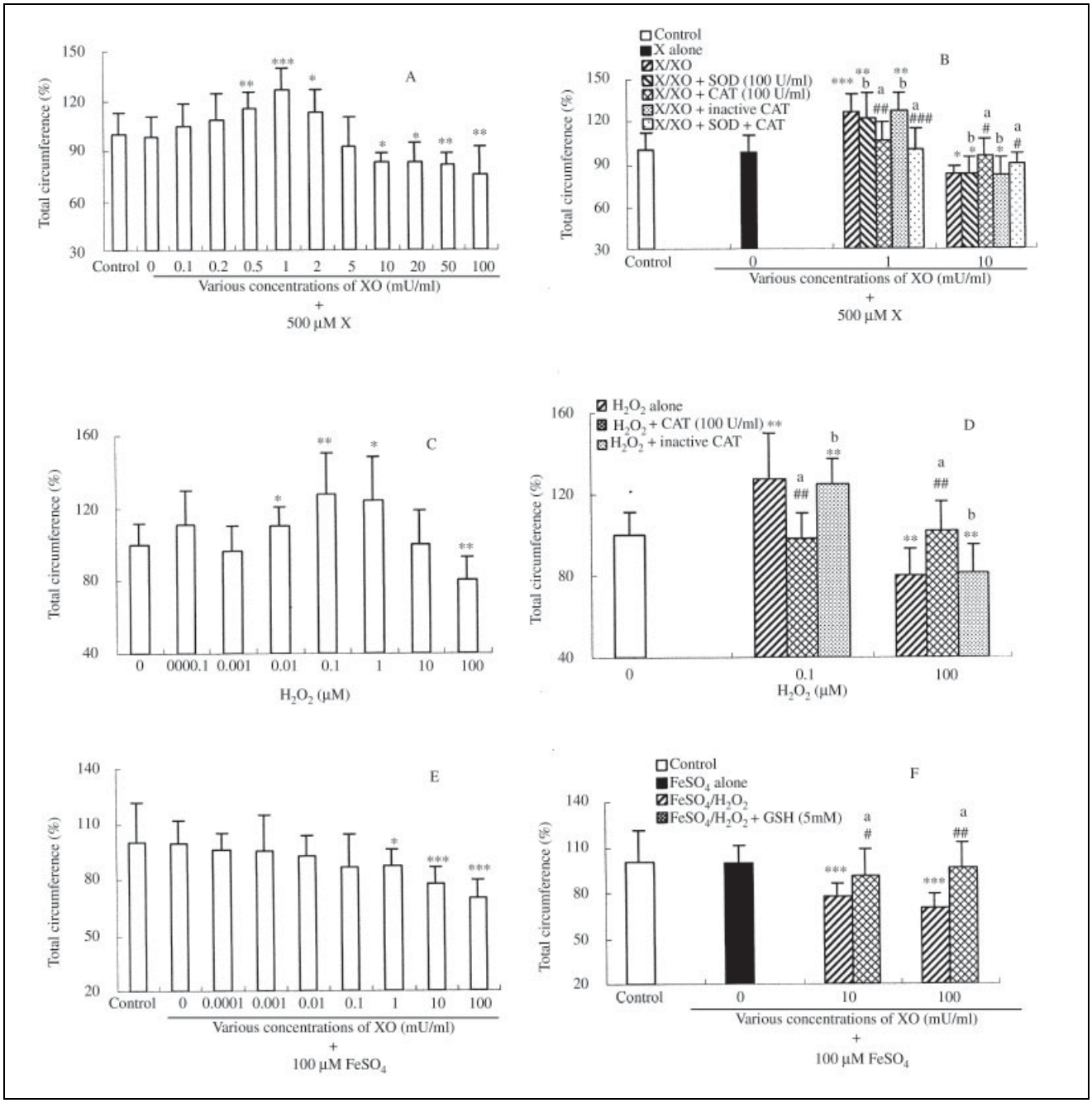


Fig. 2: Effects of ROS on endothelial cell tube formation. Endothelial cells (A) treated by 500 μM xanthine (X) with or without various concentrations xanthine oxidase (XO); (B) pretreated with SOD (100 U/ml), CAT (100 U/ml), SOD combined with CAT or inactive CAT for 30 min, then X/XO were added; (C) treated with or without various concentrations of H_2O_2; (D) pretreated with CAT (100 U/ml) or inactive CAT for 30 min, then H_2O_2 were added; (E) treated by 100 μM $FeSO_4$ with or without various concentrations H_2O_2; (F) pretreated with GSH (5mM) for 30 min then $FeSO_4$/H_2O_2 were added. After 12 h, the total circumference of tube-like structure was measured. Each value is the mean of four cultures from duplicate independent experiments and expressed as percentage of control. ^{a} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs the group treated with X alone (A) and (B); the group without H_2O_2 (C) and (D); and vs the group treated with $FeSO_4$ alone (E) and (F); ^{b} $p > 0.05$, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs the group treated with X/XO (B); treated with H_2O_2 alone (D) or treated with $FeSO_4/H_2O_2$ (F)

tive CAT, completely reversed the cell adhesion while a high concentration of O_2^- (X 500 μM/XO 100 mU/ml) inhibited cell adhesion (Fig. 5A). H_2O_2 0.1 μM induced cell adhesion and the effect can be abolished by pretreatment with CAT, but not by inactive CAT (Fig. 5B). However, HO within the tested concentrations had no effect on cell adhesion at all (Fig. 5C).

3. Discussion

In our present study, we have demonstrated that angiogenesis *in vitro* can be stimulated by low concentrations of O_2^- or H_2O_2, but not $HO^{\bullet}$, while it can be inhibited by high concentrations of all three ROS. The concentrations leading to a maximal stimulation of angiogenesis for O_2^- is 500 μM X/XO 1 mU/ml, for H_2O_2 0.1 μM; however, $HO^{\bullet}$ did not stimulate angiogenesis within the concentration range tested.

It is widely known that O_2^- can be converted to H_2O_2 by SOD. H_2O_2 can be converted to H_2O by CAT. The presence of O_2^- and H_2O_2 facilitates $HO^{\bullet}$ production. $HO^{\bullet}$ can be scavenged by GSH. Pretreatment with SOD could not prevent the superoxide-induced increase in angiogenesis *in vitro*. However, pretreatment with CAT alone or

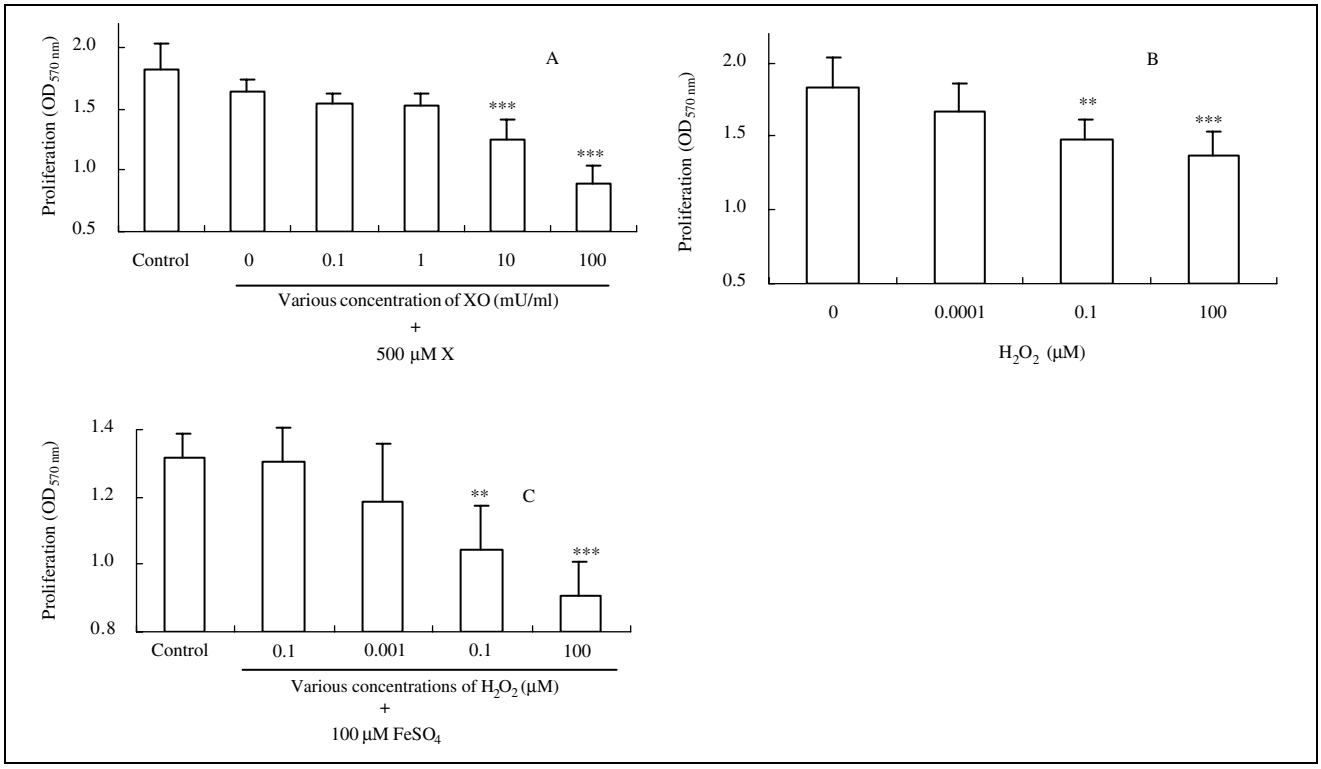


Fig. 3: Effects of ROS on endothelial cell proliferation. Endothelial cells treated by 500 μM X with or without various concentrations of XO (A); by various concentrations of H_2O_2 (B); by 100 μM $FeSO_4$ with or without various concentrations of H_2O_2 (C) for 12 h. Values are expressed as mean ± S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs the group treated with X alone (A); vs the group without H_2O_2 (B) or vs the group treated with $FeSO_4$ alone (C)

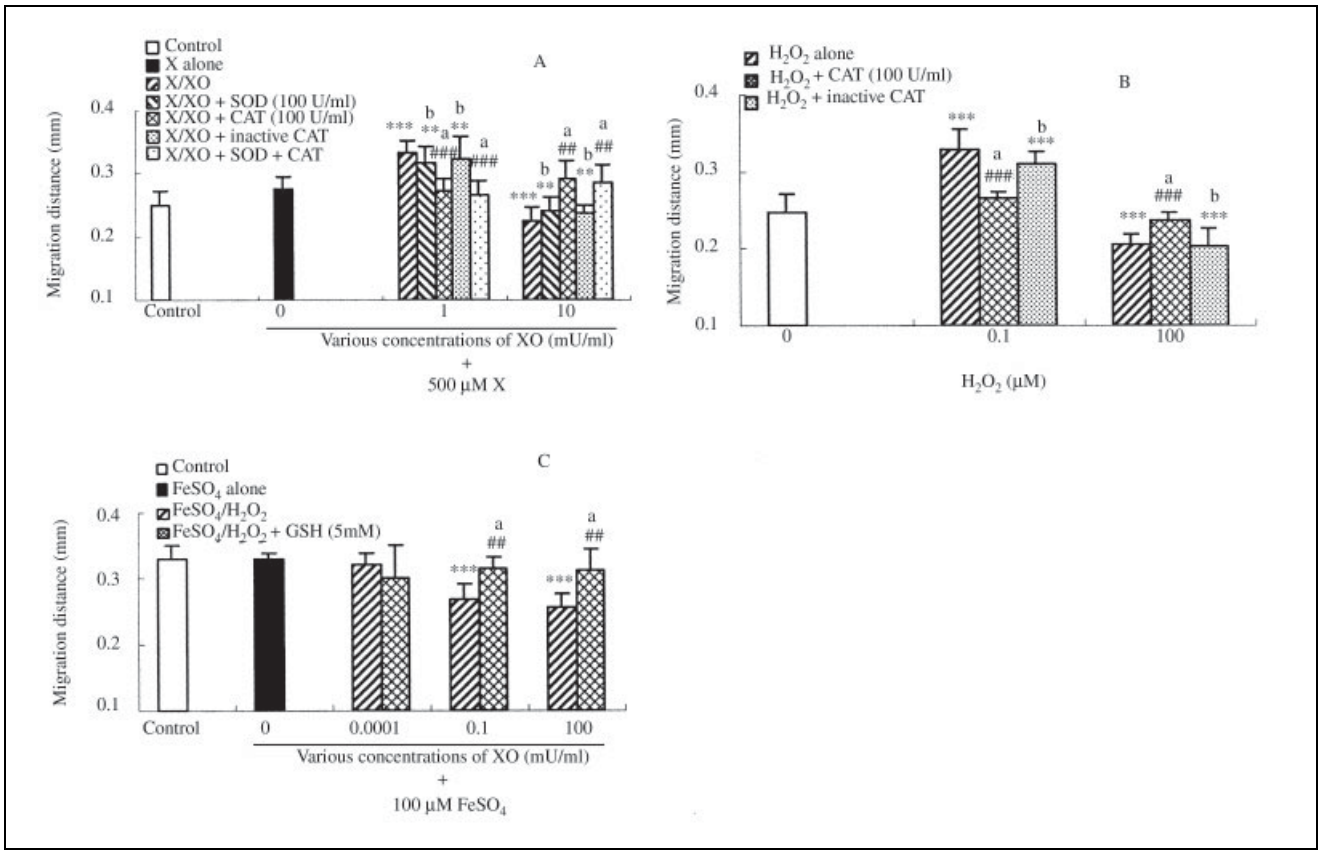


Fig. 4: Effects of ROS on endothelial cell migration. Confluent endothelial cells were scraped and (A) pretreated with SOD (100 U/ml), CAT (100 U/ml), SOD combined with CAT or inactive CAT for 30 min, then X/XO were added; (B) pretreated with CAT (100 U/ml) or inactive CAT for 30 min, then H_2O_2 were added; (C) pretreated with GSH (5 mM) for 30 min then $FeSO_4/H_2O_2$ were added. After incubation for 12 h, the cells were fixed and stained. Cell migration was qualified by measuring distance between wound edges before and after incubation over 5 points per cultured well. Values are expressed as mean ± S.D. of three independent experiments. [a] $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs the group treated with X alone (A), the group without H_2O_2 (B) and vs the group treated with $FeSO_4$ alone (C); [b] $p > 0.05$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs the group treated with X/XO (A), treated with H_2O_2 alone (B) or treated with $FeSO_4/H_2O_2$ (C)

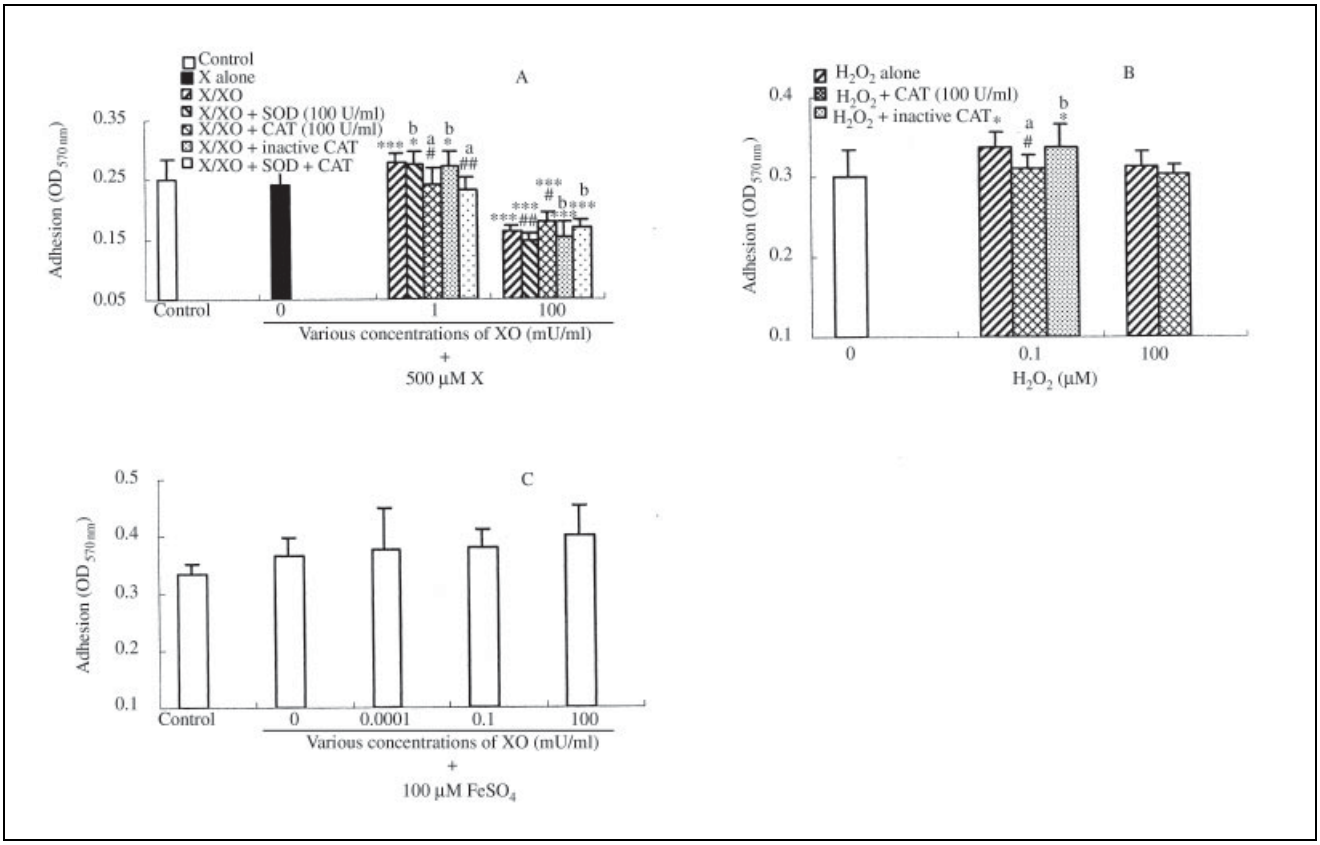


Fig. 5: Effects of ROS on endothelial cell adhesion. Endothelial cells seeded on the wells of 96-well plate precoated with type I collagen were (A) pretreated with SOD (100 U/ml), CAT (100 U/ml), SOD combined with CAT or inactive CAT for 30 min, then X/XO were added; (B) pretreated with CAT (100 U/ml) or inactive CAT for 30 min, then H_2O_2 were added; (C) treated by 100 μM $FeSO_4$ with or without various concentrations H_2O_2. The plates were washed twice with PBS. The attached cells were fixed, stained with 2% crystal violet, solved in 100 μl 2% SDS and the optical density was measured at 570 nm. Each condition was tested in 3 wells and each experiment was done three times ^{a} $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs the group treated with X alone (A), the group without H_2O_2 (B) and vs the group treated with $FeSO_4$ (C); ^{b} $p > 0.05$, $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs the group treated with X/XO (A), treated with H_2O_2 alone (B) or treated with $FeSO_4/H_2O_2$ (C)

combined with SOD entirely suppressed the angiogenic response, while inactive CAT had no effect on it. This might be due to an overproduction of H_2O_2 caused by SOD. Our results show that H_2O_2 stimulated angiogenesis. However, whether the angiogenic response resulted from $HO^{\bullet}$ is still unclear. So we tested the effect of $HO^{\bullet}$ generated from Fe^{2+}/H_2O_2. The results indicated that $HO^{\bullet}$ had no angiogenic activity even if the concentration of H_2O_2 declined to 0.0001 μM. All these results suggested that, among the three ROS, H_2O_2 was the key intermediate for angiogenesis. Our results are consistent with previous reports that H_2O_2, but not O_2^-, mediated activated polymorphonuclear leukocytes- and lymphocytes-induced angiogenesis (Yasuda et al. 2000; Monte et al. 1997). Likewise, although VEGF stimulated O_2^- production via NAD(P)H oxidase is essential for VEGF-induced VEGF receptor-2 (KDR) tyrosine phosphorylation in angiogenesis, KDR phosphorylation can be blocked by CAT (Ushio-Fukai et al. 2002), suggesting the endogenously produced H_2O_2 to act as a signaling molecular in VEGF signal transduction. Nox 1, a homolog of $gp91^{phox}$, the catalytic subunit of NAD(P)H oxidase, is a potent trigger of the angiogenesis switch, increasing vascularity of tumors and inducing VEGF mRNA and VEGF receptor expression. Nox 1 induction of VEGF can be eliminated by coexpression of CAT (Arbiser et al. 2002), indicating that H_2O_2 signals the switch to angiogenesis phenotype.

The stimulation of endothelial cell proliferation is essential for angiogenesis. However, in the present paper although low concentration of ROS did not promote endothelial cell proliferation, while stimulating angiogenesis. This suggested that cell proliferation is not the prerequisite for angiogenesis. NO, another ROS, stimulated angiogenesis *in vitro* but inhibited cell proliferation as well (Shimizu et al. 2004).

Endothelial cell migration following degradation of extracellular matrix and adhesion to matrix proteins are important steps in angiogenesis. Both endothelial cell migration and adhesion were enhanced by incubation with O_2^- or H_2O_2, thus, the stimulation of cell migration and adhesion are likely to be involved in O_2^-- or H_2O_2-induced angiogenesis. Moreover, the O_2^--induced cell migration and adhesion can be abrogated by CAT alone or in combination with SOD, but not by SOD or inactive CAT. The H_2O_2-induced cell migration and adhesion can also be downregulated to the basal level by CAT, but not by inactive CAT. However, both cell migration and adhesion could not be induced by $HO^{\bullet}$.

From the above discussion we could conclude that both O_2^- and H_2O_2 at low concentrations are able to induce angiogenesis *in vitro*, and that H_2O_2 is the key intermediate responsible for stimulating angiogenesis. This indicates that ROS have biphasic effects on angiogenesis. Many diseases, such as cancer, chronic inflammation and diabetes, are dependent on angiogenesis, so these diseases may benefit from a therapeutic inhibition of angiogenesis. While in many ischemic diseases, such as ischemic coronary artery diseases, critical limb ischemia and brain infarction, angio-

genesis may be favorable. The results of the present study suggest that pharmacologically regulating cellular ROS levels might serve as an anti-angiogenic or angiogenic therapeutic principle.

4. Experimental

4.1. Reagents

Xanthine, xanthine oxidase, superoxide dismutase (SOD), catalase (CAT), fibrinogen, thrombin from human plasma, 6-aminohexanoic acid, sulforhodamine B (SRB), type collagen were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Shanghai Pujiang Application Biochemical Institute (Shanghai, China). Sodium doclecyl sulfate (SDS) was the product of Amresco. Cell culture medium RPMI 1640 was purchased from Gibco (CA, USA). Calf serum was the product of Si-Ji-Qing Co. (Hangzhou, China). All other reagents were of analytical grade.

4.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) (obtained from China Center for Type Culture Collection, Wuhan, China) were maintained in RPMI 1640 containing 10% inactivated calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.0 mg/ml $NaHCO_3$ at 37 °C with 5% CO_2 in a humidified atmosphere.

4.3. Treatment protocol and generation of superoxide (O_2^-) and hydroxyl radical ($HO^\bullet$)

The following systems were used to generate or scavenge ROS: (1) O_2^- was generated from a xanthine-xanthine oxidase system (X/XO). In the presence of 500 μM X (the final concentration), the concentration of XO was adjusted to generate different quantities of O_2^-; (2) Generation of $HO^\bullet$ was achieved by a $FeSO_4$–H_2O_2 system. The regular concentration of $FeSO_4$ in the cell culture system was 100 μM, while the concentration of H_2O_2 was adjusted; (3) The antioxidant enzymes, superoxide dismutase (SOD, scavenger of O_2^-), catalase (CAT, scavenger of H_2O_2), and inactive CAT (heated in 56 °C water bath for 30 min) or antioxidant GSH (scavenger of $HO^\bullet$), were added 30 min before ROS treatment.

4.4. In vitro angiogenesis assay

Tube formation of endothelial cells on fibrin gel was performed according to the method of Vailhe et al. (1996; 1997) with modification. Briefly, fibrinogen in PBS was dialyzed against PBS overnight and sterilized with a 0.2-μm syringe filter. To make fibrin gel, 250 μl of 3 mg/ml fibrinogen solution was placed into each well of a 24-well plate, and 50 μg/ml 6-aminohexanoic acid was added to modulate the degradation of gel by plasmin, then human thrombin was added to a final concentration of 0.625 U/ml. The gel was allowed to polymerize overnight at 37 °C. Endothelial cell suspension (1.5×10^5 cells/ml) containing 2% calf serum was seeded onto the surface of the fibrin gel. In the presence or absence of different concentrations of ROS and antioxidant enzymes or compounds, the cells were incubated for 12 h, and then washed three times with PBS and fixed with 2.5% glutaraldehyde in PBS. Subsequently, randomly selected fields of phase-contrast microscopy were photographed. Tube formation was quantified by total circumference of tubular structures in 5 randomly selected fields using a computer-assisted image analyzer (CMIS 8.0, Beijing University of Aeronautics and Astronautics, Beijing, China).

4.5. Cell proliferation assay

The effects of ROS on proliferation of endothelial cell were determined in 96-well plates by the sulforhodamine B (SRB) method (Skehan et al. 1990). Briefly, exponentially growing cells were harvested and seeded in 96-well plates with the final volume 100 μl containing 5×10^3 cells per well. After 24 h incubation, cells were treated with various concentrations of ROS for 12 h. The cultures were then fixed at 4 °C for 1 h by addition of ice-cold 50% trichloroacetic acid to give a final concentration of 10%. Fixed cells were rinsed 5 times with deionized water and stained for 10 min with 0.4% SRB dissolved in 0.1% acetic acid. The wells were washed 5 times with 0.1% acetic acid and left to dry overnight. The absorbed SRB was dissolved in 150 μl unbuffered 1% Tris base solution in water (pH 10.5). The absorbency of extracted SRB at 570 nm was measured on a microplate reader. Each concentration of ROS was tested for three parallel wells.

4.6. Cell migration assay

For detection of cell migration, confluent endothelial cells in a 6-well plate were scraped with a sterile rubber scraper to remove a portion of the cell monolayer. The cultures were washed twice with PBS and incubated with medium containing 2% calf serum in the presence or absence of ROS and antioxidant enzymes or compounds for 12 h at 37 °C. After incubation, the cells were washed with PBS, fixed with 75% ethanol and stained with Giemsa dye. Cell migration from the edge of an injured monolayer was quantified by measuring the distance between wound edges before and after incubation over 5 points per cultured well (Weis et al. 2001).

4.7. Cell adhesion assay

The cell adhesion assay was performed according to Malinda et al. (1999) with modification. Wells of a 96-well plate were coated at room temperature overnight with 2 μg of type collagen in PBS in a final volume of 50 μl. The wells were then washed three times with PBS and blocked for 2 h with 1% bovine serum albumin (BSA) in 100 μl PBS at 37 °C. Additional uncoated wells were incubated with BSA to serve as a negative control. The wells were then washed three times with 100 μl PBS. Endothelial cells (2.5×10^4 cells/100 μl) suspended in RPMI 1640 with or without ROS, antioxidant enzymes or compounds, were added to each well. The plate were incubated for 1 h at 37 °C and then washed twice with PBS. The attached cells were fixed and stained with 2% crystal violet for 10 min and washed with PBS. Cells were solved in 100 μl 2% sodium doclecyl sulfate (SDS) and the optical density was measured at 570 nm.

4.8. Statistical analysis

Results are expressed as means ± SD, and were analyzed using the Student's t test. Values of $P < 0.05$ were considered statistically significant.

References

Arbiser JL, Petros J, Klafter R, Govindajaran B, Mclayghlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS, Lambeth JD (2002) Reactive oxygen generated by Nox 1 triggers the angiogenic switch. Proc Natl Acad Sci USA 99: 715–720

Ashino H, Shimamura M, Nakajima H, Dombou M, Kawanaka S, Oikawa T, Iwaguchi T, Kawashima S (2003) Novel function of ascorbic acid as an angiostatic factor. Angiogenesis 6: 259–269.

Avid MR, Kachra Z, Spokes KC, Aird WC (2000) NAD(P)H oxidase activity is required for endothelial cell proliferation and migration. FEBS Lett 486: 252–256.

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

Folkman J, Shing Y (1992) Angiogenesis. J Biol Chem 267: 10931–10934.

Griendling KK, Sorescu D, Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 86: 494–501.

Griffioen AW, Molema G (2000) Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52: 237–268.

Inauen W, Payne DK, Kvietys PR, Granger DN (1990) Hypoxia/reoxygenation increases the permeability of endothelial cell monolayer: role of oxygen radicals. Free Radic Biol Med 9: 219–213.

Irani K (2000) Oxidant signaling in vascular cell growth, death, and survival: A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 87: 179–183.

Jarasch ED, Bruder G, Heid HW (1986) Significance of xanthine oxidase in capillary endothelial cell. Acta Physiol Scand Suppl 548: 39–46.

Kunsch C, Medford RM (1999) Oxidative stress as a regulator of gene expression in the vasculature. Circ Res 85: 753–766.

Kuroki M, Voest EE, Amano S, Beerepoot LV, Takashima S, Tolentino M, Kim RY, Rohan RM, Colby KA, Yeo KT, Adamis AP (1996) Reactive oxygen intermediates increase vascular endothelial growth factor expression *in vitro* and *in vivo*. J Clin Invest 98: 1667–1675.

Lander HM (1997) An essential role for free radicals and derived species in signal transduction. FASEB J 11: 118–124.

Lelkes PI, Hahn KL, Sukovich DA, Karmiol S, Schmidt DH (1998) On the possible role of reactive oxygen species in angiogenesis. Adv Exp Med Biol 454: 295–310.

Lin MT, Yen ML, Lin CY, Kuo ML (2003) Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. Mol Pharmacol 64: 1029–1036.

Lum H, Barr DA, Shaffer JR, Gordon RJ, Ezrin AM, Malik AB (1992) Reoxygenation of endothelial cells increases permeability by oxidant-dependent mechanisms. Circ Res 70: 991–998.

Malinda KM, Nomizu M, Chung M, Delgado M, Kuratomi Y, Yamada Y, Kleinman HK, Ponce ML (1999) Identification of lamin α1 and β1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. FASEB J 13: 53–62.

Monte M, Davel LE, Sacerdote de Lustig E (1997) Hydrogen peroxide is involved in lymphocyte activation mechanisms to induce angiogenesis. Eur J Cancer 33: 676–682.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z (1999) Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 13: 9–22.

Paolini M, Cantelli-Forti G (2000) Oxidative stress induced by the metabolism of medical and nonmedical drugs. In: Sen CK, Packer L, Hanninen O (ed.): Handbook of Oxidants and Antioxidants in Exercise, Elsevier, p. 1021–1060.

Sauer H, Gunther J, Heschelir J, Wartehkerg M (2000) Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. Am J Pathol 156: 151–158.

Sen CK, Packer L (1996) Antioxidant and redox regulation of gene transcription. FASEB J 10: 709–720.

Shimizu S, Kageyama M, Yasuda M, Sasaki D, Naito S, Yamamoto T, Kiuchi Y (2004) Stimulation of *in vitro* angiogenesis by nitric oxide through the induction of transcription factor ETS-1. Int J Biochem Cell Biol 36: 114–122.

Shono T, Ono M, Izumi H, Jimi S, Matsushima K, Okamoto T, Kohno K, Kuwano M (1996) Involvement of transcription factor NF-κB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. Mol Cell Biol 16: 4231–4239.

Skehan P, Stroreng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.

Sundqvist T (1991) Bovine aortic endothelial cells release hydrogen peroxide. J Cell Physiol 148: 152–156.

Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51: 794–798.

Tang FY, Meydani M (2001) Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cell through suppression of IL-8 production. Nutr Cancer 41: 119–125.

Tang FY, Nguyen N, Meydani M (2003) Green tea catechins inhibit VEGF-induced angiogenesis *in vitro* through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule. Int J Cancer 106: 871–878.

Terada LS (1996) Hypoxia-reoxygenation increases O_2^- efflux which injures endothelial cells by an extracellular mechamism. Am J Physiol 270: H945–950.

Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, Quinn MT, Pagano PJ, Johnson C, Alexander RW (2002) Novel role of $gp91^{phox}$-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. Circ Res 91: 1160–1167.

Vailhe B, Ronot X, Lecomte M, Wiernsperger N, Tranqui L (1996) Description of an *in vitro* angiogenesis model designed to test antiangiogenic molecules. Cell Biol Toxicol 12: 341–344.

Vailhe B, Ronot X, Tracqui P, Usson Y, Tranqui L (1997) *In vitro* angiogenesis is modulated by the mechanical properties of fibrin gels and is related to $\alpha_v\beta_3$ integrin localization. In Vitro Cell Dev Biol - Animal 33: 763–773.

Weis M, Heeschen C, Glassford AJ, Cooke JP (2002) Statins have biphasic effects on angiogenesis. Circulation 105: 739–745.

Yasuda M, Shunichi S, Tokuyama S, Watanabe T, Kiuchi Y, Yamamoto T (1999) Stimulation of *in vitro* angiogenesis by hydrogen peroxide and the relation with ETS-1 in endothelial cells. Life Sci 64: 249–258.

Yasuda M, Shunichi S, Tokuyama S, Watanabe T, Kiuchi Y, Yamamoto T (2000) A novel effect of polymorphonucleukocytes in the facilitation of angigoenesis. Life Sci 66: 2113–2121.

Zweier JL, Broderick R, Kuppusamy P, Thompson GS, Lutty GA (1994) Determination of the mechanism of free radical generation in reoxygenated human aortic endothelial cells exposed to anoxia and reoxygenation. J Biol Chem 269: 24156–24162.