

Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release

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Received 14 December 1999; accepted in final form 16 January 2001

Cho, Michael, Thomas K. Hunt, and M. Zamirul Hussain. Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. *Am J Physiol Heart Circ Physiol* 280: H2357–H2363, 2001.—Neutrophils gather at the wound site shortly after trauma and release bactericidal reactive oxygen species (ROS) and H₂O₂ to kill bacteria and prevent infection. Macrophages arrive at the wound in response to environmental stimuli, phagocytose foreign particles, and release vascular endothelial growth factor (VEGF), an angiogenic factor crucial for wound healing. Because oxidants are released early in inflammation and have been found to regulate transcription factors, we investigated a possible role of H₂O₂ in VEGF stimulation. Human U937 macrophages exposed to H₂O₂ and allowed to recover in H₂O₂-free medium rapidly showed an increase in VEGF mRNA. The H₂O₂-mediated mRNA increase was dose dependent, blocked by catalase, and associated with elevated VEGF in conditioned media. The increase in VEGF was also found in primary rat peritoneal macrophages and the RAW 264.7 murine macrophage cell line. Transcriptional inhibition with actinomycin D revealed no significant difference in mRNA half-life. Transient transfections with a 1.6-kb VEGF promoter-luciferase construct (Shima DT, Kuroki M, Deutsch U, Ng YS, Adamis AP, and D'Amore PA. *J Biol Chem* 271: 3877–3883, 1996) showed a ninefold stimulation of VEGF gene promoter activity. We concluded that H₂O₂ increases macrophage VEGF through an oxidant induction of VEGF promoter. This oxidant stimulation can be mediated by activated neutrophils.

angiogenesis; neutrophil; oxidative stress; wound healing; antioxidant

SHORTLY AFTER THE INFLICTION of a wound, coagulation occurs, and neutrophils gather at the wound site to release bactericidal reactive oxygen species (ROS) and H₂O₂ in an oxygen-consuming respiratory burst. It is commonly understood that in this early phase of wound healing, oxidants serve mainly to kill bacteria and prevent infection (39). Oxidants also damage surrounding host cells, including macrophages, by creating DNA strand breaks and depleting NAD stores (30, 32, 39). Recent studies, however, have revealed that oxidants serve as redox regulators of transcription factors such as p53, AP-1, nuclear factor (NF)- κ b (37), and SP-1 (41).

Macrophages, which infiltrate the wound after neutrophils, phagocytose debris and predominantly modulate wound angiogenesis by releasing angiogenic factors such as fibroblast growth factor, transforming growth factor- β , platelet-derived growth factor, and the endothelial cell-specific vascular endothelial growth factor (VEGF). Low tissue oxygen tensions (17) and high lactate levels (15) are believed to stimulate the release of VEGF by macrophages, along with many other positive and negative regulators of angiogenesis such as interferon- γ (8), tumor necrosis factor- α (29), and interleukins (18). ROS elicit VEGF release in cultured retinal keratinocytes (4), vascular smooth muscle cells (27), and retinal epithelial cells (20). They have been shown to stimulate VEGF in reperfusion injury of diabetic retinopathy (20) and atherosclerosis (27). Because oxidants are released early during inflammation, we investigated a possible role of H₂O₂ as a signaling molecule for the release of macrophage VEGF.

Three recent findings in our laboratory suggest that ROS stimulate macrophages to release higher levels of VEGF and can thereby drive angiogenesis in wounds. First, neutrophils exposed to hyperoxia in vitro produce elevated levels of ROS (1). Second, hyperoxia stimulates VEGF in wound cylinders. Third, hyperoxia elicits higher angiogenic scores in the in vitro matrigel angiogenesis assay (10). Thus the idea that oxidants participate in a physiological pathway of VEGF release deserves attention.

Until now, ROS have been implicated in causing cell membrane and DNA damage. Consequently, they have been suspected of being detrimental to wound healing (39). We investigated an oxidant-mediated stimulation of VEGF that outlines the importance of maintaining the physiological redox environment of wounds. In this study, we present evidence that VEGF mRNA and VEGF protein release are induced by H₂O₂ in human differentiated U937 macrophages and in primary cultures of rat peritoneal macrophages. In addition, we report that this stimulation can occur by neutrophil-derived oxidants in coculture mimicking the in vivo inflammatory setting. Finally, we show that H₂O₂ in-

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creases VEGF mRNA by upregulating VEGF promoter activity.

MATERIALS AND METHODS

Reagents. Catalase, superoxide dismutase (SOD), phorbol 12-myristate 13-acetate (PMA), and oyster glycogen type 2 were obtained from Sigma (St. Louis, MO). H_2O_2 and actinomycin D were obtained from Fisher (Santa Clara, CA). U937 and RAW 264.7 murine macrophages were obtained from University of California San Francisco Cell Culture Facility (San Francisco, CA).

RPMI 1640 complete media (Roswell Park Memorial Institute), Dulbecco's modified Eagle's media (DMEM), and 0.25% trypsin with 0.1% EDTA were obtained from Mediatech (Herndon, VA). Glutamine (200 mM), penicillin-streptomycin, and fetal calf serum were obtained from Atlanta Biologic (Norcross, GA). Plasticware was from Falcon Labware, Becton-Dickinson (Franklin Lakes, NJ). Human and murine VEGF ELISA kits were obtained from R&D Systems (Minneapolis, MN).

A 1.6-kb mouse VEGF promoter-luciferase construct and deletion mutants were generously donated by Dr. Patricia D'Amore (Schepens Eye Research Institute, Boston, MA) (36). The 1.6-kb construct contains 1.2 kb of the 5'-flanking sequence, the transcription start site, and 0.4 kb of corresponding 5'-UTR ligated upstream of a promoterless luciferase gene in the pGL2-basic plasmid (Promega). The -772 deletion mutant has a deletion of base pairs -1,217 to -772, which contains two AP-1 binding sites. The -449 deletion mutant has further deletion of base pairs -772 to -449, which contains an AP-2 binding site. Lastly, the +126 deletion mutant has a further deletion of base pairs -449 to +126, which includes a NF- κ B binding site, a SP-1 binding site, and the transcription initiation site. The β -galactosidase control plasmid, under the control of a cytomegalovirus promoter, was graciously provided by Dr. Keith Yamamoto (University of California, San Francisco, CA).

Cell cultures. U937 cells were grown in standard RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine at 7.5×10^5 cells/ml in 2 ml. Cells were exposed to 12.5 ng/ μ l PMA for 2 h at 37°C and then washed with the above media. They were then plated into six-well dishes at 0.5×10^6 cell/ml and allowed to adhere and differentiate into macrophage-like cells (12, 19) for 4 days (25). Macrophages were exposed to a range of concentrations of H_2O_2 from 0 to 1 mM in RPMI 1640 containing glutamine-penicillin-streptomycin and 2% heat-inactivated fetal calf serum for 30 min. They were then allowed to recover in H_2O_2 -free standard media for 14 h. The conditioned media were assayed for VEGF by ELISA. Plates were gently trypsinized in 0.25% trypsin, and cells were counted in a Coulter Counter (Coulter Counter Electronics; Hialeah, FL).

Five-month-old Sprague-Dawley rats (Retired Breeders, Simonsen; Gilroy, CA) were injected intraperitoneally with 10 ml of sterile 2.5% oyster glycogen type 2 in Ca/Mg-free PBS (3, 6). Two days later, the rats were euthanized, and their peritoneum were lavaged with 150 ml of lactated Ringer and 5% dextrose solution (Baxter; Deerfield, IL). Lavaged cells were spun down and resuspended in standard RPMI and plated at 5×10^5 cells/ml in 2 ml. Peritoneal macrophages were maintained in culture for 4 days and then treated with H_2O_2 as above.

RAW 264.7 mouse macrophages were grown in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Cells were

plated to 40–60% confluency at 5×10^5 cells/ml in 2-ml volumes and used the next day as above.

Neutrophils were isolated from heparinized human peripheral blood by spinning on a 1077/1119 Histopaque gradient (Sigma) and collecting the buffy coat layer. Cells were washed in PBS, and red blood cells were lysed in sterile water. The remaining neutrophils were washed and resuspended in standard RPMI. Coculture experiments with macrophages and neutrophils were done in six-well chamber plates in which a 0.45- μ m polypropylene filter separates macrophage cultures in the lower compartment from neutrophils placed on the upper compartment. Serum was used to opsonize zymosan particles for 30 min at 37°C and then added to the upper compartment. Cocultures were maintained for 16 h, after which media and cell counts were assessed as above. Neutrophils (3×10^6) in 2 ml of media were incubated with opsonized zymosan (as described above), 1.1 mM *p*-hydroxy-phenylacetate, 50 μ g/ml SOD, and 50 μ g/ml horseradish peroxidase in PBS. H_2O_2 was quantified by fluorescence measurement with excitation at 323 nm and emission at 440 nm at 37°C as described by Hyslop and Sklar (14).

Northern blots. Total RNA was isolated using Qiashredders and RNeasy kits (Qiagen; Valencia, CA). Northern blots were prepared using Northern Max kits (Ambion; Austin, TX). Ten micrograms of total RNA were run in each well and then transferred to BrightStar-plus positively charged nylon membranes (Ambion). Full-length human VEGF cDNA probes (Dr. Napoleone Ferrara, Genentech, San Francisco, CA) were labeled with Redivue [α - 32 P]dCTP using the Rediprime II random prime labeling system (Amersham Life Sciences; Piscataway, NJ) and purified on a G-50 sephadex column (Boehringer Mannheim; Indianapolis, IN). Membranes were hybridized in ExpressHyb (Clontech Laboratories; Palo Alto, CA) for 2 h and washed with $2\times$ saline sodium citrate (SSC)-0.05% SDS and 0.1% SSC-0.1% SDS. Blots were exposed with Kodak (Rochester, NY) Biomax MS with an intensifying screen at -70°C for 12–72 h. To control for total RNA content, the blots were stripped in boiling 0.1% SDS and subsequently hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Ambion). Densitometry was performed on all blots and normalized to the corresponding GAPDH signal for each lane.

VEGF mRNA stability assay. Differentiated U937 macrophages were treated with H_2O_2 for 30 min and allowed to recover for 1 h, and actinomycin D (5 μ g/ml) was then added. At different time intervals, total RNA was isolated, and Northern blot analysis was performed as above.

DNA transfection, luciferase, and β -galactosidase assays. RAW 264.7 macrophages were plated at 7.5×10^5 cells/ml in 2 ml per well overnight. Transient transfection was performed with 1 μ g of test plasmid and 1 μ g of control plasmid using Superfect transfection reagent (Qiagen) for 3 h at 40–60% confluency. Cells were allowed to recover for 3.5 h and treated with H_2O_2 for 30 min in DMEM. Macrophages were allowed to recover in H_2O_2 -free media for 5 h before lysis, and luciferase activity was measured by Luciferase ELISA (PharMingen; San Francisco, CA). β -Galactosidase activity was determined using CPRG color reagent (Boehringer Mannheim), and the value was used to normalize luciferase values.

Statistics. All data were expressed as means \pm SE of three or more experiments. Statistical analysis was performed with ANOVA comparing differences between groups, with $P \leq 0.05$ considered significant. Post hoc tests were performed using the Scheffe's post hoc test on Statview 4.5 (Abacus Concepts; Berkeley, CA).

RESULTS

H₂O₂ stimulates VEGF release by U937 and primary rat peritoneal macrophages. Treatment of U937 macrophages with 0.5 mM H₂O₂ stimulated a 197 ± 15% increase in VEGF in conditioned media. This stimulation was attenuated to the control VEGF level when H₂O₂-treated cultures were coincubated with catalase, indicating that the VEGF stimulation was H₂O₂ specific. Macrophages treated with catalase alone yielded no significant difference in VEGF production compared with untreated macrophages. The addition of actinomycin D to the H₂O₂-treated cultures completely abrogated the increase and reduced the VEGF release to 68 ± 8% below the control value, suggesting that the stimulation was transcriptionally regulated (Fig. 1).

When experiments were repeated with primary cultures of rat peritoneal macrophages, similar results were found. H₂O₂-exposed macrophages secreted 170 ± 14% more VEGF compared with untreated cultures, whereas the addition of catalase completely abolished the increase, and actinomycin D inhibited VEGF release by 75 ± 9% (Fig. 2).

U937 cocultured with activated neutrophils releases VEGF. VEGF levels increased by 250 ± 27% when cultures of U937 macrophages were cocultured with activated neutrophils in a one-to-two ratio. The addition of catalase to the culture diminished the increase in VEGF to the control level, suggesting that the neutrophil-mediated ROS was the stimulant (Fig. 3). To confirm the production of H₂O₂ by neutrophils, we stimulated neutrophils with zymosan and measured H₂O₂ fluorometrically (14). We found a gradual release of H₂O₂ over a period of 3.5 h to a concentration of 170 ± 50 μM after initial zymosan treatment (28).

H₂O₂ upregulates VEGF mRNA in U937. H₂O₂ treatment rapidly increased VEGF mRNA in U937 macro-

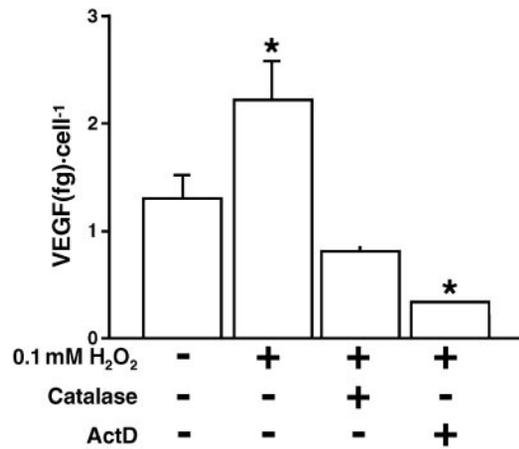


Fig. 2. H₂O₂ stimulates VEGF release by primary peritoneal macrophages. Cultured peritoneal macrophages were treated with 0 and 0.1 mM H₂O₂ in the presence and absence of catalase (1 unit) or actinomycin D (5 μg/ml) for 30 min in RPMI medium containing 2% heat-inactivated fetal calf serum. Cultures were allowed to recover for 11 h in standard RPMI medium with 10% fetal calf serum, and VEGF was assayed in the conditioned media by ELISA. Each value represents mean ± SE. *Significantly different from control (*P* < 0.05, ANOVA).

phages. The increase was time and dose dependent. When cultures were treated with 0.5 mM H₂O₂, the VEGF mRNA stimulation was maximum (3.5-fold) at 60 min (Fig. 4). A dose response was evident with increasing concentrations of oxidant exposure for 30 min. Maximal effect was seen with 1 mM H₂O₂ (Fig. 5).

H₂O₂ does not increase VEGF mRNA stability. The stability of VEGF mRNA induced in cultures of U937 exposed to H₂O₂ was studied by measuring the remaining mRNA at various time intervals after induction. The new mRNA synthesis in these and control cultures

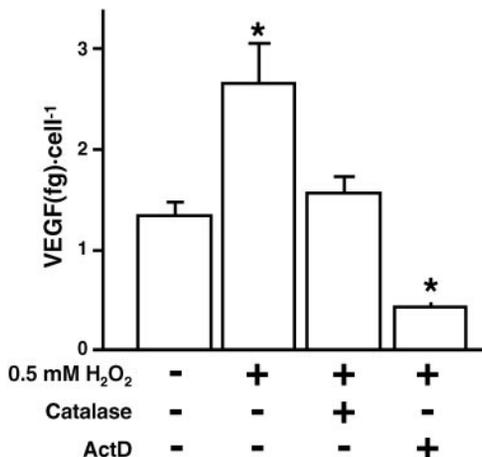


Fig. 1. H₂O₂ stimulates vascular endothelial growth factor (VEGF) release by U937 macrophages. Cultures of U937 were treated with 0 and 0.5 mM H₂O₂ in the presence (+) and absence (-) of catalase (1 unit) or actinomycin D (ActD; 5 μg/ml) for 30 min in RPMI with 2% heat-inactivated fetal calf serum. Cultures were allowed to recover for 14 h in standard RPMI medium containing 10% fetal calf serum. VEGF was measured in the conditioned media by ELISA. Each value represents mean ± SE. *Significantly different from control (*P* < 0.05, ANOVA).

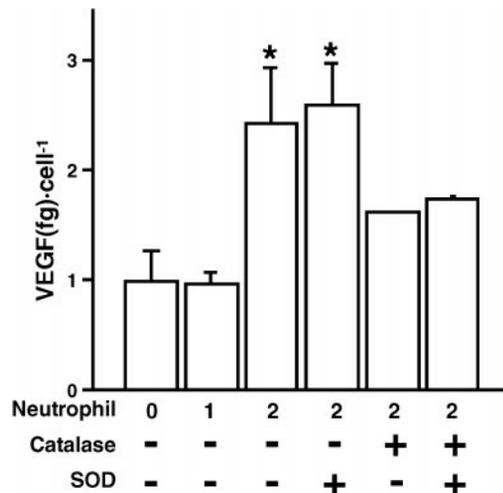


Fig. 3. H₂O₂ produced by activated neutrophils stimulates VEGF in U937 macrophages. U937 macrophages were cocultured across a 0.45-μm membrane with activated neutrophils in a 1:1 or 1:2 ratio, respectively. Catalase (1 unit), superoxide dismutase (SOD; 100 milliunits), or both were added to the cultures. Cocultures were maintained for 16 h in standard RPMI media with 10% fetal calf serum, and VEGF was measured as in Fig. 2. Each value is mean ± SE. *Significantly different from control (*P* < 0.05, ANOVA).

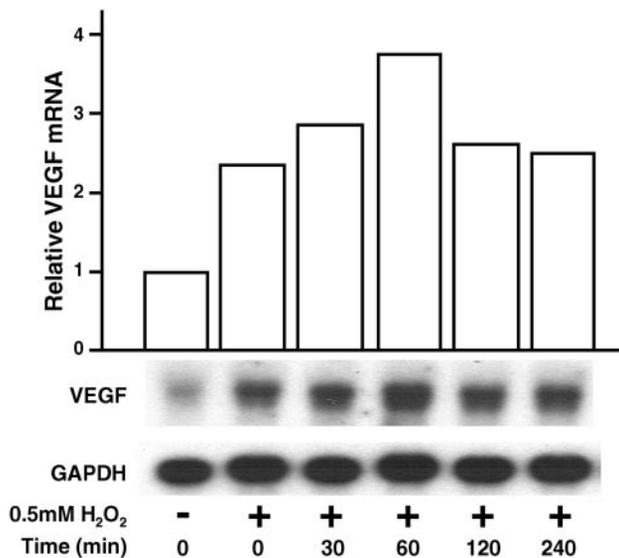


Fig. 4. H₂O₂ stimulates a time-dependent increase in VEGF mRNA accumulation. Cultures of U937 were exposed to 0.5 mM H₂O₂ for 30 min in RPMI with 2% heat-inactivated fetal calf serum. Cultures were allowed to recover for indicated times, and total RNA was isolated and analyzed by Northern blot with a human VEGF cDNA probe. For comparison, the same blot was stripped and hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

were concurrently blocked by the addition of actinomycin D. As shown in Fig. 6, the decay curves of VEGF mRNA of control and H₂O₂-treated cultures were not significantly different in mRNA stability. The half-life of VEGF mRNA in control cells is consistent with previous report (35) of 60–90 min.

H₂O₂ increases VEGF promoter activity in RAW 264.7. To examine whether the increase in macrophage VEGF mRNA is regulated at the transcription level,

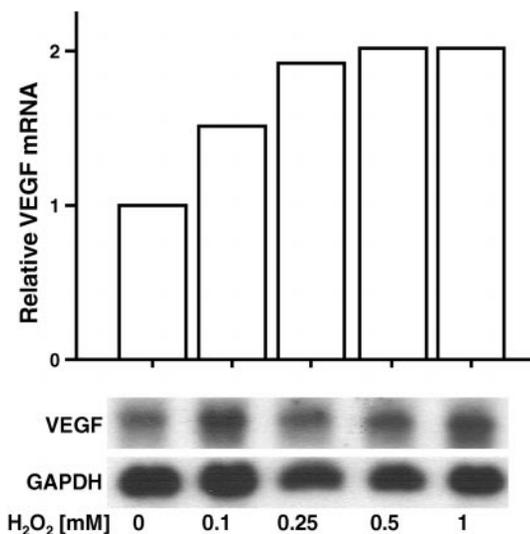


Fig. 5. H₂O₂ stimulates a dose-dependent increase in VEGF mRNA accumulation. Cultures of U937 were exposed to increasing concentrations of H₂O₂ for 30 min in RPMI with 2% heat-inactivated fetal calf serum. Cultures were allowed to recover for indicated times, and total RNA was isolated and analyzed by Northern blot with a human VEGF cDNA probe. For comparison, the same blot was stripped and hybridized with a GAPDH probe.

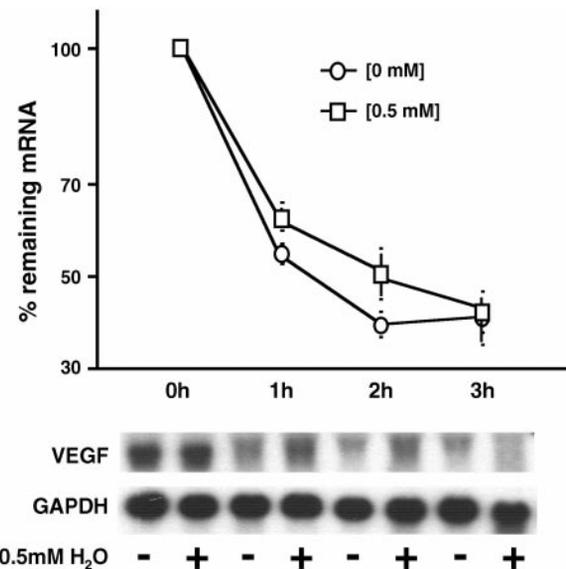


Fig. 6. H₂O₂ does not significantly alter VEGF mRNA stability. Cultures of U937 were treated with 0.5 mM H₂O₂ for 30 min and allowed to recover for 60 min in H₂O₂-free standard RPMI before 5 μg/ml of actinomycin D was added. Total RNA was isolated at the indicated times after the addition of actinomycin D and analyzed by Northern blot with a human VEGF cDNA probe. For comparison, the same blot was stripped and hybridized with a GAPDH probe. Graphic representation of the decay rates for VEGF mRNA for control and H₂O₂-treated cultures are shown.

we used a reporter assay using the immortalized RAW 264.7 murine macrophages transfected with a plasmid containing a VEGF promoter sequence fused to a promoterless reporter luciferase gene. Transfected macrophages were exposed to 1 mM H₂O₂ for promoter activation because prior experiments demonstrated optimum stimulation of VEGF at this concentration in RAW 264.7 cultures (Fig. 7). Luciferase expression by transfected cultures was standardized by cotransfection with a control pCMV-β-gal plasmid, and luciferase

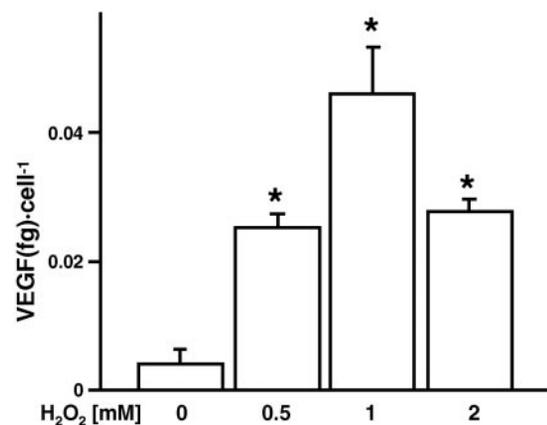


Fig. 7. H₂O₂ increases VEGF release by RAW 264.7 macrophages. Cultured RAW 264.7 murine macrophages were exposed to increasing concentrations of H₂O₂ for 30 min in DMEM with 2% inactivated fetal calf serum and allowed to recover for 16 h in DMEM containing 10% fetal calf serum. VEGF was measured in the conditioned media by ELISA. Each value represents mean ± SE. *Significantly different from control ($P < 0.05$, ANOVA).

activity was normalized against that of β-galactosidase. Fig. 8 shows the relative luciferase activity obtained in the presence of 1 mM H₂O₂ when VEGF promoters of different size sequences were transfected. An 8.9-fold luciferase induction was observed with the -1,217 promoter. Deletions of promoter sequence did not significantly affect H₂O₂ stimulation. H₂O₂ inducibility was retained until the deletions were extended to +126 [plasmid pVEGF(+126)/Luc], at which point the basal activity was lost.

DISCUSSION

Oxidants are prevalent in the inflammatory stage of wounds and are produced in elevated amounts by neutrophils during respiratory burst (7). The cells utilize NADPH oxidase and O₂ to generate superoxide, which forms H₂O₂ spontaneously and through the action of SOD. H₂O₂ is a relatively stable oxidant but is also converted by neutrophils and macrophages to the more reactive species such as superoxide and hydroxyl radicals. Because these oxidants are usually considered detrimental to the surrounding cells (30, 31, 38, 39), many studies (33, 40) have used antioxidants to ameliorate tissue destruction and improve wound healing, especially under ischemic conditions. The results of the present study do not address the validity of antioxidants in cases of oxidant overproduction such as ischemic colonic anastomoses (2, 13, 34), myocardial ischemia (9), ischemic skin flaps (33), burns (24), and inflammatory bowel diseases (26). Instead, we report a physiological role for oxidants in stimulating the release of VEGF in wound angiogenesis. The use of antioxidants in acute wounds may, therefore, reverse the oxidative environment and actually suppress the endogenous signaling for angiogenesis. We propose that the oxidant-mediated angiogenesis is an important component of the healing process.

After wound infliction, cellular processes result in both early and late activation products (11). There is

great merit in understanding how the two phases of wound healing, namely, inflammation and angiogenesis, are linked by early activation products, such as oxygen free radicals and H₂O₂. These products may regulate the release of late activation products involved in wound healing. To illustrate this concept, we investigated whether H₂O₂ can mediate VEGF release.

The results of our studies demonstrate that H₂O₂ significantly stimulates the synthesis and release of VEGF by both U937 and primary cultures of macrophages. The stimulation is H₂O₂ specific because it is abolished by catalase. The concentration of H₂O₂ needed to mediate this stimulation is within physiological range. We also found that the effective concentration of H₂O₂ for primary macrophages was lower compared with that for U937 macrophages. This finding is expected because primary cells are more sensitive to external stimuli than transformed cells. In a separate study, we observed that human wounds contain higher levels of oxidants than rat wounds. This is supported by the finding that human neutrophils express higher levels of oxidants than rat neutrophils (16). In the present study, however, exogenous H₂O₂ stimulates parallel VEGF release in both human U937 cell line and rat peritoneal macrophage populations.

The low level of VEGF release, when transcription is inhibited by actinomycin D, suggests that the VEGF measured is newly synthesized and that the stimulation seen in our experiments is primarily through up-regulation of VEGF transcription. More importantly, the H₂O₂-mediated macrophage VEGF stimulation is replicated by activated neutrophils. This observation implies that the oxidant pathway could occur during healing of wounds.

We studied VEGF mRNA production by Northern blot analysis. These experiments showed an optimum increase in VEGF mRNA production at 60 min post-H₂O₂ exposure. This is consistent with previous reports in other cell types. The increase in VEGF mRNA

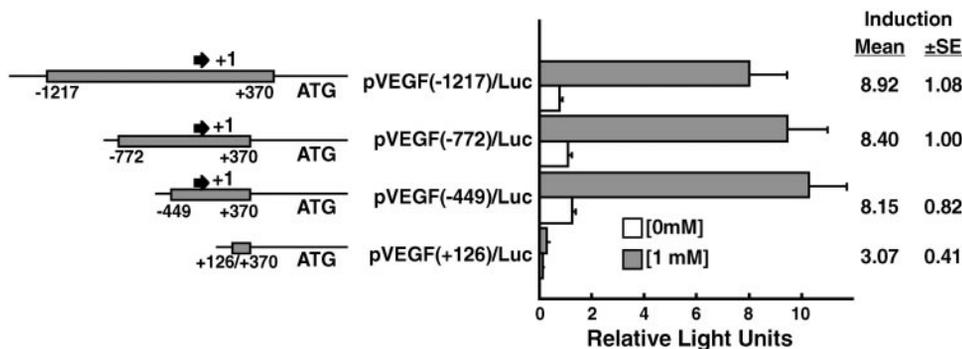


Fig. 8. H₂O₂ increases VEGF promoter activity in RAW 264.7 macrophages. pGL2-basic plasmids containing full-length or deletion mutant VEGF promoters, with 5'-flanking sequences fused to a promoterless luciferase gene, were cotransfected into RAW 264.7 macrophages with pCMV-β-gal control plasmids. Transfected cultures were exposed to 0 mM H₂O₂ (control) and 1 mM H₂O₂ for 30 min. Cultures were then allowed to recover for 5 h in H₂O₂-free DMEM containing 10% fetal calf serum. Luciferase and β-galactosidase activities were measured as described in MATERIALS AND METHODS. Luciferase values were normalized to β-galactosidase activity. The magnitudes of induction of luciferase and the standard errors are shown on the right. The shaded boxes on the left represents sequences from the VEGF promoter region, and the nucleotides at the ends of the inserted VEGF sequences are numbered relative to the transcription initiation site (arrows, designated +1). The luciferase gene translation start codon ATG is marked.

may be attributed to increased mRNA stability (35) and/or increased transcription of the VEGF gene (21, 23). Hypoxia-induced increases in VEGF mRNA is associated with enhanced mRNA stability mediated by proteins HuR and 5' UTRs (5, 22). However, our VEGF mRNA stability experiments demonstrated no significant difference in half-lives between the control and H₂O₂-treated cells. On the other hand, the VEGF promoter activity was largely induced by H₂O₂ at the same concentration that elicited enhanced VEGF release. These findings suggest that the oxidant-stimulated VEGF release is mediated by transcriptional upregulation of VEGF mRNA.

We postulate that the mechanism by which H₂O₂ activate VEGF transcription may involve oxidant sensitive proteins such as NF- κ B, AP-1, and SP-1. These transcription factors are known to have binding sites on the mouse VEGF promoter (36). Figure 7 shows a significant difference in luciferase induction between the pVEGF(-449) and pVEGF(+126) deletion mutants, indicating that there may be a sequence between -449 and the transcription initiation site that regulates oxidant-mediated VEGF transcription. It is noteworthy that there are two consensus binding sites for NF- κ B and SP-1 in this -449 to +1 region that could be responsible for VEGF upregulation (36). Further transcription factor studies are needed to delineate the transcriptional mechanism.

In summary, our studies demonstrate that H₂O₂ stimulates VEGF gene promoter activity, mRNA levels, and release by macrophages. Also, this stimulation can occur through neutrophil-derived ROS. This new regulatory mechanism delineates another role of oxidants in physiological wound healing.

We thank Dr. Chandan Sen for helpful discussions. We also thank Heinz Scheuenstuhl, Yu Long Hu, Michael Cronin, Dong Fong, and You Ping for generous teaching.

This work was supported by a Howard Hughes Medical Student Fellowship (to M. Cho) and by National Institute of General Medical Sciences Grant GM-27345.

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