Mechanisms of endothelial response to oxidative aggression: protective role of autologous VEGF and induction of VEGFR2 by H2O2

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González-Pacheco, Francisco R., Juan J. P. Deudero, María C. Castellanos, María Ángeles Castilla, María Victoria Álvarez-Arroyo, Susana Yagüe, and Carlos Caramelo. Mechanisms of endothelial response to oxidative aggression: protective role of autologous VEGF and induction of VEGFR2 by H2O2. Am J Physiol Heart Circ Physiol 291: H1395–H1401, 2006; doi:10.1152/ajpheart.01277.2005.—The defense mechanisms of endothelial cells (EC) against reactive oxygen species (ROS) are insufficiently characterized. We have addressed the need for the role of NF-κB in EC, without affecting VEGFR1 expression. Also, H2O2 challenge was accompanied by increased NF-κB in EC, whereas lower concentrations (2–4 μM) were cytoprotective. The cytoprotective effect was shifted to an EC-damaging pattern by means of specific VEGF blockade, therefore revealing a major role of autologous VEGF. Exposure to H2O2 increased VEGF and VEGFR2 mRNA and protein in EC, without affecting VEGFR1 expression. Also, H2O2 challenge was accompanied by increased NF-κB, activator protein-1, and specific protein-1 nuclear binding. A role of NF-κB as the mediator of the H2O2 induction of VEGFR2 mRNA expression was supported by inhibition of the ROS scavenger pyrrolidine dithiocarbamate and by the blocking effect of transfected IkBα. Exposure to exogenous VEGF also increased VEGFR2 and induced NF-κB in EC. In summary, autologous VEGF is instrumental for EC protection induced by low concentrations of ROS. ROS induce expression not only of NF-κB but also of VEGFR2. VEGFR2 increase by ROS is mainly driven through a NF-κB-dependent pathway.

cytoprotection; reactive oxygen species; vascular endothelial growth factor receptor; nuclear factor-κB

OXIDATIVE MECHANISMS are involved in numerous conditions of vascular injury, e.g., ischemia-reperfusion, hypertension, vasculitis, and atherosclerosis, or diabetes mellitus. In these processes, the endothelium is a direct target for reactive oxygen species (ROS)-induced damage.

Most of the studies dealing with the effects of ROS on endothelial cells (EC) have been devoted to examining their injuring capability (18). On the other hand, the mechanisms of vascular defense against ROS have received considerably less attention. In fact, at moderate, nontoxic concentrations, ROS act as physiological signal transduction messengers (18). In the same line of evidence, a variety of natural stimuli works by changing the cellular redox state through ROS formation as a part of the normal intracellular signaling network (18). In practical terms, the characterization of the factors involved in the ability of the vessel wall to mount an efficient protective response against ROS-mediated injury has outstanding pathophysiological and therapeutic implications.

Among the factors putatively involved in the response of the endothelium to oxidative challenge is VEGF, the importance of which is emphasized by recent data. The VEGFs and, in particular, VEGF-A (hereinafter referred to as VEGF) have a critical role in angiogenesis and vascular permeability. VEGF signal in arteriolar and venous EC is transduced through two main receptors with tyrosine kinase activity, namely, VEGFR1 and VEGFR2 (6). Although the initial investigations on the role of VEGF were mainly focused on its angiogenic and permeabilizing properties, a number of studies, including ours, have given support to outstanding properties of VEGF as a mediator of EC survival (3, 7).

Endothelial and vascular smooth muscle cells are both capable of producing VEGF under diverse stimuli (3, 5, 6, 8, 15). More specifically for the present study, a stimulatory effect of H2O2 on VEGF expression by EC and vascular smooth muscle cells has been described, although without characterizing other VEGF-related responses, e.g., VEGF receptor expression or the actual functional role of autologous VEGF in the cellular response against oxidation (5, 15). The fact that the expression of VEGF receptors, particularly VEGFR2, is stimulated in conditions coincident with VEGF induction, e.g., tumor growth, suggests that the exposure to H2O2 may induce changes not only on VEGF but also on its receptors, which can be putatively related to EC defense mechanisms. Moreover, the possibility that endogenous VEGF is involved in endothelial protective actions during oxidative stress has not been specifically addressed. The present study, therefore, has been aimed at examining the effects of ROS challenge on the survival properties of EC and in the regulation of VEGF and VEGF receptor expression. Experiments on mechanisms were more specifically focused on the role of the transcription factor NF-κB, for its particular importance in the response to oxidative challenge and its critical role on VEGFR2 expression.

MATERIALS AND METHODS

Cell culture and experimental maneuvers. Bovine aorta EC were cultured and characterized as previously described (1, 3). Challenge with H2O2 (30 min) used a range of concentrations involving those...
reported in normal in vivo conditions (12). A specific anti-VEGF MAB (1 μg/ml; Sigma-Aldrich, Madrid, Spain) was used to inhibit VEGF effects. This antibody has been extensively tested in our laboratory and blocks the effects of VEGF within a range of physiological and supraphysiological concentrations, i.e., up to 10^{-9} M (1, 3). Phorbol myristate acetate (200 nM, Sigma-Aldrich) was employed as positive control for the stimulation of expression of both VEGF and VEGF receptors, and pyrrolidine dithiocarbamate (PDTC) (Sigma-Aldrich) was used as ROS scavenger and inhibitor of NF-κB activation.

Citotoxicity assay. Confluent EC were preincubated as previously described (3) for 18 h in MEM d-Val containing 20% FBS and 0.5 μCi/ml of sodium ^{51}Cr (Amersham Biosciences, Barcelona, Spain). EC were exposed to ROS or vehicle and incubated for an additional 24 h in MEM d-Val with 0.5% FBS and without ^{51}Cr. Supernatant fractions (200 μl) were sampled and counted at different times, and ^{51}Cr-release was calculated with respect to total ^{51}Cr content. Flow cytometry was used as a confirmatory method, as previously described (1, 3, 4). The length of ROS exposure (30 min) was designed to resemble an ischemic event, as occurs in episodes of limited in vivo ischemia-reperfusion, e.g., in revascularization techniques.

Northern blot analysis, RT-PCR, and real-time quantitative PCR. Total RNA was extracted from confluent EC at 0, 6, and 24 h after different treatments. Northern blot analysis and routine RT-PCR were carried out with the use of a probe and specific primers of bovine VEGF, VEGFR1, and VEGFR2, based on previously described sequences [National Center for Biotechnology Information GenBank (1, 3, 4)]. For real-time quantitative PCR, primers and TaqMan MGB probe were designed using the Primer Express software (Applied Biosystems, Foster City, CA) from VEGFR2 sequence: forward, 5’-TCT CCG TTA TTG CCT CTG TTA G-3’; reverse, 5’-GTG ATA CCT TGC ACA GAG TGA CAC-3’; and TaqMan MGB probe, 5’-ACA AAA AAC AAA ACT GAC AC-3’ were used. cDNA was synthesized from 2 μg of total RNA, and real-time quantitative PCR was carried out with the ABI PRISM 7700 Systems (Applied Biosystems), following the manufacturer’s instructions. PCR amplification of 18S RNA was done for each sample as loading control and to allow normalization between samples. The mRNA fold changes were calculated on four triplicate experiments by using the comparative CT method; this leads to a persistent binding of the inhibitory subunit to NF-κB, the latter has mutated Ser36 for alanine, therefore impeding phosphorylation and degradation; this leads to a persistent binding of the inhibitory subunit to NF-κB. Both constructions were kindly provided by Dr. J. Alcami (Instituto de Investigaciones Biomédicas, Instituto de Salud Carlos III).

Transient transfection of EC was carried out with the use of FuGENE 6 transfection reagent (Roche Diagnostics, Barcelona, Spain) according to the manufacturer’s protocol. The optimal transfection of plasmid DNA was achieved at a 6:2 (vol/wt) ratio of transfection reagent to DNA complex, with a transfection efficiency of 36.9 ± 2%. Flow cytometry was used to evaluate the transfection efficiency with pEGFP-N1 vector (BD, Madrid, Spain). A pcDNA3-empty vector was used as control of nonspecific effects of the transfection (data not shown).

Nuclear extraction and electrophoretic mobility shift assay. Nuclear proteins were extracted as previously described (1) at 0, 1, 5, 10, and 180 min after H2O2 challenge (2 and 250 μM, 30 min). For the case of NF-κB, a specific concentration-response curve was also performed 6 h after exposure to H2O2. Nuclear proteins of untreated EC were used as controls. Electrophoretic mobility shift assay was performed by using nuclear proteins (6 μg) and different consensus oligonucleotide sequences (dsDNA, 0.5 ng) labeled with [γ-^32P]dATP in 1× binding buffer. Poly(dI-dC)-Poly(dI-dC) was included as a competitive DNA. After incubation, samples were separated by electrophoresis in 6% acrylamide-bisacrylamide gels and exposed to X-Omat films. Commercial oligonucleotides (Santa Cruz Biotechnology) were used for activator protein-1 (AP-1), specific protein-1 (Sp-1), and NF-κB electrophoretic mobility shift assays. Supershift with specific antibodies (anti-c-Fos, anti-c-Jun, anti-p65, and anti-p50) and competitive assays in the presence of consensus cold oligonucleotides (50×) were performed to assess the specificity of the bands. The usefulness of the antibodies in the present setting had been proved in previous studies.

Statistics. Data are shown as means (SD) and, unless stated otherwise, correspond to at least four triplicate experiments. Paired and unpaired Student’s t-tests were used when appropriate. Multiple comparisons were done by one-way ANOVA and Scheffe’s tests (StatView and SPPS 10.0 packages, Jandel, San Rafael, CA; and Windows). A P value <0.05 was considered significant.

RESULTS

Role of autocrine VEGF on EC viability under oxidative stress. Exposure to H2O2 elicited a dual response on EC; namely, at concentrations from 60 μM to 1 mM, a cytotoxic effect was evident. On the contrary, at lower concentrations (2–4 μM), cytotoxicity was no longer evident; instead, a moderate, albeit consistent, cytoprotective effect was observed (significant decrease with respect to basal conditions; Fig. 1A). As a confirmation of these results, a similar concentration-response pattern was observed by means of flow cytometry analysis of cell death (data not shown). To examine the role of...
VEGF in the observed responses, similar experiments were conducted in the presence of a specific blocking anti-VEGF MAb. These experiments resulted in a clear-cut shifting from protective or neutral effects to cell-damaging effects of H$_2$O$_2$ (Fig. 1B). However, at a higher H$_2$O$_2$ concentration (250 μM), no further increase in $^{51}$Cr-release was induced in the presence of anti-VEGF MAb.

Further studies were performed as methodological controls, which yielded the following results: 1) the protective and damaging effects of H$_2$O$_2$ were almost completely blocked in the presence of catalase (0.5 μg/ml, data not shown); and 2) the efficacy of the anti-VEGF MAb was assessed by analyzing its effect on the VEGF-induced EC proliferation. A titration curve of VEGF anti-VEGF MAb was performed by EC count in a Neubauer chamber. The anti-VEGF antibody inhibited the proliferative effect of $5 \times 10^{-11}$ M, $10^{-10}$ M, $5 \times 10^{-10}$ M, and $10^{-9}$ M VEGF by 97 (SD 3.0), 96 (SD 2.7), 93 (SD 5.7), and 85% (SD 7.9), respectively, all $P < 0.001$ with respect to the controls in the presence of a nonspecific IgG.

Induction of VEGF and VEGFR2 mRNA and protein expression on H$_2$O$_2$ stimulation. Exposure of EC to H$_2$O$_2$ increased VEGF mRNA expression in a concentration-dependent manner between 0.5 and 10 μM (Fig. 2A); this stimulatory effect was not evident at higher H$_2$O$_2$ concentrations (100–250 μM). In a time-response curve (2 μM H$_2$O$_2$, n = 2), the expression of VEGF was significantly increased from 3 h ($P < 0.05$), was maximal at 6 h ($P < 0.01$), and returned to baseline ($P = $ not significant (NS) with respect to time 0) at 24 h (data not shown). VEGF protein markedly increased with 2 μM H$_2$O$_2$ (Fig. 2B, 6 h); however, the immunocytochemical signal for VEGF became faint with 250 μM H$_2$O$_2$ (image not shown).

A screening of VEGFR1 and VEGFR2 was performed by RT-PCR. This experiment showed no changes with H$_2$O$_2$ in the mRNA expression of VEGFR1 and an increase in VEGFR2 mRNA (data not shown). As a control of the reliability of the response, VEGFR1 expression increased significantly with phorbol myristate acetate. Thence, only the increase in VEGFR2 mRNA was quantified by real-time quantitative

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Fig. 1. A: effect of different concentrations of H$_2$O$_2$ on $^{51}$Cr release by endothelial cells (EC). $^{51}$Cr release (24 h) was used for evaluation of cytotoxicity, as previously described (3). Concentration-response curve extends from 0 (basal conditions, no exogenous H$_2$O$_2$ added) to 1 mM H$_2$O$_2$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ with respect to basal conditions. Data are expressed as means (SD) of percentage of $^{51}$Cr release with respect to basal conditions. Note that the bars are represented with respect to baseline $^{51}$Cr release, which is situated at the x-axis; therefore, values above or below baseline are consistent with increased or decreased cell damage, respectively. B: effect of VEGF blockade on cytoprotection by H$_2$O$_2$: changes in percentage of $^{51}$Cr release in the presence of anti-VEGF MAb (1 μg/ml). *$P < 0.05$ and **$P < 0.01$ between cells incubated with (solid bars) or without (shaded bars) anti-VEGF MAb. Controls used identical conditions in the presence of a nonspecific IgG antibody (1 μg/ml).

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Fig. 2. Effects of H$_2$O$_2$ on mRNA expression of VEGF. A: Northern blot analysis. Induction of the expression (6 h) of VEGF (top lane) by H$_2$O$_2$ is shown (0.5–250 μM, 30 min); housekeeping 28S was used for comparison (bottom lane). This experiment is representative of 3 experiments, giving similar results. Mean values of the 3 experiments are shown in bar diagram. *$P < 0.05$, **$P < 0.01$ with respect to baseline. B: VEGF immunocytochemistry (magnification ×100). Increased VEGF immune signal (6 h) as induced by 30 min exposure to 2 μM H$_2$O$_2$. Inset: nonstimulated cells.
In the conditions used in the present experiments, exogenuously added VEGF (rhVEGF-A165) stimulates VEGFR2 mRNA expression, an effect that was more evident after 24-h incubation (Fig. 4A) (4). This effect involved also increased VEGFR2 protein (Fig. 4B). Of interest, the latter was not inhibited in the presence of PDTC (25 μM), therefore indicating a difference with the H₂O₂-induced VEGFR2 expression (Fig. 4B) (see below for more related results).

**Activation of transcription factors: role of NF-κB.** The transcription factors analyzed were selected on the basis of previously available information on the regulation of VEGF and VEGFR2 expression (6). The electrophoretic mobility shift assays of the transcription factors analyzed revealed different individual responses (Fig. 5) as follows: 1) a brief AP-1 activation appeared with both H₂O₂ concentrations; this increase was detected at 10 min of incubation but was no longer evident at 3 h (Fig. 5A); 2) Sp-1 was rapidly (1 min) activated by the smaller (2 μM), but not by the higher (250 μM), H₂O₂ concentration, and this activation persisted at a high level until 10 min and at a lower but persistent level until 3 h (Fig. 5B); and 3) NF-κB has a short-lived, bimodal (1 and 10 min) activation with 2 μM H₂O₂, which did not persist at 3 h. However, due to the potential importance of this factor, additional experiments were done to further trace activation up to 6 h after exposure to H₂O₂; at this time, no changes were observed with 2 μM H₂O₂, but a significant activation of NF-κB was present in EC treated with the higher H₂O₂ concentration (250 μM) (Fig. 5C). A consistent activation of NF-κB was also obtained by exposure of the EC to VEGF, which increased nuclear NF-κB in a concentration-dependent manner (Fig. 5D).

**PCR.** This experiment showed a significant increase of VEGFR2 expression with exposure to 2 μM H₂O₂; the increase was, however, significantly higher at 250 μM H₂O₂ (Fig. 3A). To ascertain the consequences of the changes in VEGFR2 mRNA at the level of the gene product, VEGFR2 protein was studied by Western blot analysis. Data of VEGFR2 protein are shown in Fig. 3B. As can be seen, both 2 and 250 μM H₂O₂ stimulated the increase of VEGFR2 protein in a similar proportion. Furthermore, additional experiments were performed to analyze the role of the autocrine effects of autologous VEGF generated by the EC in the setting of H₂O₂ challenge. This question is indeed more relevant in situations of increased VEGF production, as actually occurs under exposure to H₂O₂, and was examined by challenging the EC with H₂O₂ in the presence of a blocking anti-VEGF MAb (n = 3). No effect on the increased VEGFR2 expression by H₂O₂ occurred with the anti-VEGF MAb, as assessed by RT-PCR (Fig. 3C). This was also determined by Western blot analysis (see Fig. 4B; P = NS in H₂O₂-induced VEGFR2 expression both in the presence or absence of anti-VEGF MAb).
The effect of VEGF was already detected at 3 h and persisted up to the longest incubation time (24 h, Fig. 6B).

To assess the functional importance of these effects on NF-κB, further experiments were conducted. The results revealed that the effects of H2O2 on VEGFR2 expression were inhibited in the presence of the intracellular ROS scavenger PDTC (Fig. 7A). This result could be interpreted either as a direct effect on ROS or by the NF-κB inhibitory effect of PDTC. To further clarify this issue, the EC were transfected to overexpress the wild-type and mutant IκBα, with the purpose of blocking NF-κB activation. As shown in Fig. 7B, in the transfected cells, the stimulatory effect of H2O2 on VEGFR2 mRNA expression was markedly inhibited. The absence of effects of a null transfection control ruled out any effect of the transfection procedure itself (data not shown). The VEGF-induced increase in NF-κB activation was not inhibited by PDTC (P = NS in the presence with respect to the absence of PDTC; data not shown). This is an important difference with respect to the findings in the experiments with H2O2 and may help explain why PDTC did not affect the VEGF-induced increase in VEGFR2.

**DISCUSSION**

The present results show, for the first time, the functional significance of autologous VEGF in endothelial cytoprotection against oxidative challenge. Furthermore, in terms of mechanism, the study demonstrates that besides the known induction of VEGF by H2O2 (5, 15–17), there is also a significant VEGFR2 induction. Furthermore, VEGF production is most frequently a paracrine phenomenon, and only a few conditions or agents have been shown to elicit autologous VEGF production by the endothelium, e.g., hypoxia, cobalt, cytochalasin D, ANG II, or H2O2 (11). Our results add new information on this point by revealing that H2O2 challenge increases VEGFR2 mRNA expression. To the best of our knowledge, no previous data are available analyzing VEGFR2 expression under exposure to exogenous ROS. Collectively, the information obtained herein has direct projections in vascular biology in oxidative conditions.

In terms of mechanism, even though the induction of the expression of its own VEGFR2 receptor by VEGF has been described in different circumstances (4, 19), our results with a blocking anti-VEGF antibody reveal that this type of regulation is not relevant in the case of challenge with H2O2. Therefore, in the present experimental conditions, VEGFR2 upregulation appears to be mostly dependent on a direct effect of H2O2. Accordingly, the possibility was raised, as previously described (2), of a stimulation of transcriptional elements by redox changes.

The data on transcription factors activation add some clues to the interpretation of the results. The factors activated correspond to those involved in VEGF and/or VEGFR2 transactivation (9, 10, 14). At least three transcription factors, namely, AP-1, Sp-1, and NF-κB, were activated by oxidative challenge,
as predicted according to previous descriptions (16, 17, 21). Because of its particular importance in the response to oxidative challenge and its critical role in VEGFR2 expression, major interest was focused on NF-κB. The pattern of activation of NF-κB demonstrated a short-lived peak with the smaller (2 μM) concentration and a longer activation with the higher (250 μM) concentration. The latter was coincident with stimulation of VEGFR2 mRNA and protein expression. Of additional interest, we have found that VEGF per se activates NF-κB in a time- and concentration-dependent manner. This result adds further information to data previously published by other groups (13).

To further ascertain the mechanisms of the effect of H2O2, additional experiments were carried out by using the cell-permeable ROS scavenger PDTC (2). The inhibition of VEGFR2 expression by PDTC suggested that the effect of exogenous H2O2 on VEGFR2 expression was mediated by intracellular ROS, which are scavenged by this agent, e.g., ·OH radical. This result could be interpreted either as a direct effect on ROS or by the NF-κB inhibitory effect of PDTC. This specific issue was addressed by transfecting the EC with IκBα (2). These studies demonstrated an inhibition of the VEGFR2 stimulation by IκBα, therefore supporting the significance of the role of NF-κB in the effect of H2O2. Of interest, the effect of VEGF on VEGFR2 expression was not inhibited by PDTC. Because in these conditions VEGF-induced NF-κB was not blocked by PDTC, this indicates that relevant differences do exist between the mechanisms involved in NF-κB activation by H2O2 and VEGF. Albeit of considerable interest, the complete analysis of the molecular basis of these differences is beyond the scope of the present study.

As a complementary remark, AP-1 and Sp-1 activation could have a role in the induction of VEGFR2 expression, but further studies need to be performed to clarify this point. No experiments were done to examine the role of NF-κB in the induction of VEGF, due to the existence of previous studies demonstrating such a relationship (6). In the same regard, no in-depth studies were performed on VEGFR1, due to the absence of change in its expression with the experimental maneuvers, with the exception of the previously known stimulation by phorbol myristate acetate (6).

Beyond the description of the mechanisms of combined increase of VEGF and VEGFR2, our results illustrate its functional significance in terms of EC damage and protection. In particular, the present results demonstrate that autologous VEGF has a significant role in the survival of EC in conditions of oxidative aggression; the H2O2 concentrations affording EC protection were within a relevant in vivo range. Of importance, the protective concentrations of H2O2 were coincident with those eliciting VEGF expression. This finding supports the role of VEGF as a principal component of a protective loop. Even though the action of VEGF in endothelial cytoprotection is known (3), no specific information was available up to date to indicate such a role during oxidative injury. Of particular interest, our main data were obtained in the absence of exogenously added VEGF and are, therefore, more directly related to in vivo effects. Because the effects of H2O2 on VEGFR2 span within the damaging range of H2O2 concentrations, the increased expression of VEGFR2 might be traced to extreme, albeit functionally ineffective, stimulation of defense mechanisms in a background of severe cellular injury. The results on VEGFR2 protein are further revealing of the actual functioning of this mechanism; i.e., the marked increase in VEGFR2 mRNA with high H2O2 concentrations did not result in a proportional increase in VEGFR2 protein. This can be either due to a submaximal stimulation of protein translation or increased protein catabolism, due to oxidative activation of proteases.

In summary, our results add elements of interpretation to the effect of mild oxidative stress as a protective mechanism in EC, indicating that autologous VEGF-related mechanisms are implicated. In other words, our data show that autocrine VEGF makes EC more resistant to injury by oxidative agents; on the contrary, VEGF blockade favors injuring effects by H2O2. These effects involve significant changes in VEGF and VEGFR2 gene expression. The latter is first described on oxidative challenge and appears to involve a NF-κB-dependent mechanism.

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REFERENCE


