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 Castillo, 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 hypothesis that vascular endothelial growth factor (VEGF) and its receptors are relevant elements in this response. Cell viability, VEGF and VEGF receptor (VEGFR1 and VEGFR2) expression, and transcription factor activation were studied on transient exposure of monolayer EC to H2O2. Wild-type and mutant inhibitors of NF-κB (IkBα) constructions were used to further assess the role of NF-κB in the induction of VEGFR2 expression. A concentration of H2O2 ≥60 μM elicited clear-cut damaging effects on 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 by 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 VEGF but also of VEGFR2. VEGFR2 increase by ROS is mainly driven through a NF-κB-dependent pathway.

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 pathological 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 arterial 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 VEGF165 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 51Cr (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 51Cr. Supernatant fractions (200 μl) were sampled and counted at different times, and 51Cr-release was calculated with respect to total 51Cr 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 a transient challenge, 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 CTT CTG TTA G-3′; reverse, 5′-GTG ATA CCT TGC ACA GGA GGA 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 value. Results were expressed as fold changes relative to unstimulated cells, after normalization against housekeeping genes, as occurs in episodes of limited in vivo ischemia-reperfusion, e.g., in revascularization techniques.

**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 bovine. The antibody was affinity purified by a peptide-bound thiopropyl-Sepharose column (Pharmacia Biotech, Uppsala, Sweden). The specificity of the anti-VEGF polyclonal antibody was checked against commercial VEGF (Sigma-Aldrich). Parallel cultures were treated with nonimmune rabbit IgG as negative controls. Cells were incubated with biotinylated swine anti-rabbit IgG (Dako, Glostrup, Denmark, 30 min) sequentially followed by avidin-biotin-peroxidase complex (Dako) and 3,3-diaminobenzidine (Sigma-Aldrich).

**Plasmids and transfections.** Overexpression of NF-κB regulatory subunits was used for blocking NF-κB activation. Because phosphorylation (Ser32 and Ser36) of inhibitors of κB (IκBs) by IκB kinase is necessary for their release from NF-κB and subsequent degradation, two vectors, namely, pcDNA3-IκBα-wt and pcDNA3-IκBα-mt, were transfected and overexpressed. The former leads to competitive blockade of phosphorylation due to excess IκBα; the latter has mutated Ser36 for alanine, therefore impeding phosphorylation and degrading; this leads to a persistent binding of the inhibitory subunit to NF-κB. Both constructions were kindly provided by Dr. J. Alcamí (Instituto Nacional de Microbiología, 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 1X 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 (50X) were performed to assess the specificity of the bands. The usefulness of the antibodies in the present setting had been probed 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.
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 H2O2 (Fig. 1B). However, at a higher H2O2 concentration (250 μM), no further increase in 51Cr-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 H2O2 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 × 10^{-11} M, 10^{-10} M, 5 × 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 H2O2 stimulation. Exposure of EC to H2O2 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 H2O2 concentrations (100–250 μM). In a time-response curve (2 μM H2O2, 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 H2O2 (Fig. 2B; 6 h); however, the immunocytochemical signal for VEGF became faint with 250 μM H2O2 (image not shown).

A screening of VEGFR1 and VEGFR2 was performed by RT-PCR. This experiment showed no changes with H2O2 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...
In the conditions used in the present experiments, exogenously added VEGF (rhVEGF-A165) stimulates VEGFR2 mRNA expression, an effect that was more evident after 24-h incubation (Fig. 4A). 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: I) 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.

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; *NS in H2O2-induced VEGFR2 expression both in the presence or absence of anti-VEGF MAb).
manner (Fig. 6A). 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,
Because in these conditions VEGF-induced NF-κB was not inhibited by PDTC. intracellular ROS, which are scavenged by this agent, e.g., exogenous H2O2 on VEGFR2 expression was mediated by 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 in vivo effects. Because the effects of H2O2 on VEGFR2 expression 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 expression was coincident with 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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