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The Actin Cytoskeleton Reorganization Induced by Rac1 Requires the Production of Superoxide

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ABSTRACT

The small GTPase rac1 controls actin redistribution to membrane ruffles in fibroblasts and other cell types, as well as the activation of the NADPH oxidase in phagocytes. We explored the possibility that these two processes could be related. We used a replication-deficient adenoviral vector to overexpress the constitutively active form of rac1, rac^{V12}, in human and mouse aortic endothelial cells. We show here that, in addition to membrane ruffle formation, rac^{V12} induced an increase in the total amount of F-actin within endothelial cells. Concurrently, rac^{V12}-overexpressing cells produced significantly higher amounts of free radicals, as detected by the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichloro-dihydrofluorescein diacetate, than cells infected with a control virus encoding the bacterial β-galactosidase (Ad-βGal). To assess the specific role of superoxide in rac^{V12}-induced actin reorganization, we co-expressed the human enzyme Cu,Zn-superoxide dismutase (SOD), by means of another adenoviral vector construct. Overexpressed SOD reduced the concentration of superoxide detected in Ad-rac^{V12}-transfected cells and reversed the effects of Ad-rac^{V12} on the content of filamentous actin. MnTMPyP, an SOD mimetic, as well as the antioxidant *N*-acetyl cysteine, had similar effects, in that they reduced not only the free radicals production, but also ruffle formation and the concentration of F-actin within rac^{V12}-overexpressing endothelial cells. Our data support the hypothesis that superoxide is one of the important mediators acting downstream of rac1 on the pathway of actin cytoskeleton remodeling in endothelial cells. Antiox. Redox Signal. 1, 29–43.

INTRODUCTION

THE NATURE OF THE SIGNALING PATHWAYS that control actin reorganization in migrating cells is not characterized. The small GTP-binding proteins of the rho family, of which the best characterized are rhoA, rac1, rac2, and cdc42, were found to coordinate the spatial and temporal changes in the actin cytoskeleton that lead to cellular movements. Microinjection experiments in Swiss 3T3 fibroblasts showed that the sequential activation of cdc42, rac and rho induces, respectively, (i) the formation of filopodia, (ii) a strong pinocytotic and membrane ruffling activity, accompanied by redistribution of actin fibers at the periphery of the cells, and (iii) the assembly of stress fibers and focal adhesions (Ridley and Hall, 1992; Ridley

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et al., 1992; Nobes and Hall, 1995). The involvement of small GTP-binding proteins in actin reorganization has been confirmed in other cell types as well (Norman *et al.*, 1994; Nusrat *et al.*, 1995; Larochelle *et al.*, 1996; Murphy and Montell, 1996; Cox *et al.*, 1997). However, the downstream effectors of the small GTP-binding proteins, and in particular of rac, still represent a controversial issue (Hall, 1998). Several candidates, such as POR1 (Van Aelst *et al.*, 1996), mPAK-3 (Bagrodia *et al.*, 1995), and LIM-kinase 1 (Arber *et al.*, 1998; Yang *et al.*, 1998) were suggested to be the missing links between rac and actin cytoskeleton reorganization.

Rac1 and rac2 proteins are also involved in an apparently unrelated process: recruitment to the plasma membrane of the cytoplasmic components and activation of the phagocytic NADPH oxidase (Abo *et al.*, 1991; Knaus *et al.*, 1991). The enzyme catalyzes the production of superoxide (O_2^-; SO), the reactive oxygen species (ROS) involved in bactericidal activity in phagocytes. Evidence has accumulated that more than one NADPH oxidase system exists within the body, one of which produces SO in nonphagocytic cells (Meier *et al.*, 1991, 1993; Emmendorffer *et al.*, 1993; Zulueta *et al.*, 1995; Griendling and Alexander, 1997; Irani and Goldschmidt-Clermont, 1998). Recently, the presence of cytoplasmic (Jones *et al.*, 1996) and membranous components of the NADPH oxidase (Bayraktutan *et al.*, 1998), was demonstrated in endothelial cells (EC).

Previously, we have studied the effects of a limited period of hypoxia, followed by reoxygenation, on cultured human aortic endothelial cells (HAEC). Interestingly, we observed a pattern of actin reorganization, similar to that observed in cells exposed to growth factors, a process known to involve rac protein: (i) increase of the total filamentous actin, and (ii) translocation of the filaments to the periphery of the cells (Crawford *et al.*, 1996). Reoxygenation after hypoxia is known to be accompanied by the production of ROS, in particular SO (Zweier *et al.*, 1994; Zulueta *et al.*, 1995, 1997). Inhibition of SO accumulation by adenovirus-mediated overexpression of Cu,Zn-superoxide dismutase (SOD) prevented the actin changes observed consequent to hypoxia-reoxygenation of HAEC (Crawford *et al.*, 1996).

The above data suggested that ROS, and specifically SO, could represent important effectors for rac in the control of actin reorganization. The present study was designed to test this hypothesis, and we show that in endothelial cells the regulation of the actin cytoskeleton by a dominant positive mutant of rac requires the production of superoxide within these cells.

MATERIALS AND METHODS

Cells and media

We used human aortic (HAEC) and human coronary (HCEC) endothelial cells (Clonetics, San Diego CA), which were grown in EGM and EGM-2, respectively (Clonetics), and subcultured as recommended by the provider; or a mouse aortic endothelial cell line (MAEC) (Bastaki *et al.*, 1997), grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA).

Adenoviral constructs and endothelial cells infection

The recombinant adenovirus Ad-rac^{V12}, containing the c-*myc*-tagged constitutively activated form of rac (a gift of A. Hall), was constructed by homologous recombination in 293 cells using the adenovirus-based plasmid JM17, as previously described (Sulciner *et al.*, 1996). Replication-incompetent adenoviruses carrying the cDNA for human Cu,Zn-superoxide dismutase (Ad-SOD), and the bacterial β-galactosidase gene (Ad-βGal), were constructed as described (Crawford *et al.*, 1996). Endothelial cell infection was performed by diluting the viral vector in a small amount of culture medium at the desired multiplicity of infection (m.o.i.) (typically, a final volume of 500 μl per well was used in a six-well plate) and kept for 1.5–2 hr on a rocking platform, at 37°C, in 5% CO_2 in air. The cells were then rinsed with medium and maintained in the incubator for 2 more days to allow full expression of the

proteins (Moldovan *et al.*, 1996). For the different biochemical measurements (*vide infra*), adenovirus-infected cells were kept overnight in the appropriate medium containing 0.1% FBS (defined arbitrarily as serum starvation condition).

Actin measurement

For bulk actin concentration, the cells plated on six-well plates were rinsed with buffer (15 m*M* HEPES pH 7.0, 145 m*M* NaCl, 0.1 m*M* $MgCl_2$, 10 m*M* EGTA), supplemented with a mixture of protease inhibitors. The cells were then lysed with the same buffer, now ice-cold, and supplemented with 1% Triton-X 100 (lysis buffer) to which 600 n*M* (final concentration) of rhodamine-phalloidin (Molecular Probes, Eugene, OR) was added. The plates were placed on ice and the cells were scraped and then transferred into a 1.5-ml eppendorf tube. After vortexing for 30 sec, the samples were centrifuged at 50,000 × *g* in a Beckman TL-100 rotor for 30 min at 4°C. The supernatants were removed and used for protein assay. The pellets were gently rinsed with 200 μl of buffer without disrupting them. Then 200 μl of methanol was added and the samples were kept overnight at 4°C. The phalloidin concentration was measured after transferring the samples into a 96-well plate by fluorescence detection in a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA), using a rhodamine-phalloidin standard curve. All determinations were normalized to the protein concentration.

SOD assay

SOD activity was determined with the SOD 525 kit (R&D Systems, Inc., Minneapolis MN), following the manufacturer's recommendations. In brief, cells were washed with phosphate buffered saline (PBS), and harvested with a cell scraper in 200 μl of ice-cold water supplemented with protease inhibitors. Cells were sonicated for 2 × 15 sec on ice, and then the debris was pelleted for 10 min in a microcentrifuge, at maximum speed, at 4°C. To the supernatants, a mixture of ethanol-chloroform (62.5:37.5, vol/vol) was added, vortexed, and microfuged as above. The upper, aqueous phase was used for SOD determination. The change in absorbency was measured at 525 nm, in a Beckman DU 640B spectrophotometer. The initial rate was used to calculate the SOD 525 units, according to the formula:

$$\text{SOD 525 units} = 0.93\ (\text{RsRc}^{-1} - 1)(1.073 - 0.073\ \text{RsRc}^{-1})^{-1}$$

where Rs is the rate of autooxidation of the reagent 5,6,6*a*,11*b*-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorene in the presence of the cell lysate, and Rc is the rate of its autooxidation in the presence of H_2O (control). All the measurements were normalized to the protein concentration.

Fluorescence microscopy and image analysis

Cells grown on coverslips and infected with the adenoviral constructs were rinsed with Dulbecco's PBS (Gibco BRL, Grand Island, NY), then fixed with 3.7% formaldehyde in PBS for 10 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. The rest of the procedure was done away from light. The coverslips were inverted on a 200-μl rhodamine-phalloidin drop for 30 min, and then rinsed with PBS. The slides were incubated afterwards with a mixture of the following first antibodies, for 1 hr: a 1:500 dilution of either rabbit anti-SOD or preimmune rabbit IgG (as control) (Accurate, Westbury, NY), and a 1:100 mouse anti-c-*myc* epitope (9E10, SantaCruz Biotechnology, Inc., Santa Cruz, CA). After washing, the coverslips were incubated with a mixture of the secondary antibodies: a 1:60 FITC-goat anti-mouse (SantaCruz) and a 1:100 Cy5-goat anti-rabbit (Jackson Laboratories, Bar Harbor, ME). The coverslips were then rinsed with PBS and mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL). The cells were examined with a Noran confocal microscope (Noran Instruments, Middleton, WI) or with a Nikon Eclipse 800 microscope.

Fluorescence-activated cell sorting analysis of intracellular antigens

For fluorescence-activated cell sorting (FACS) analysis, the cells were suspended with

0.25% trypsin for 5 min. Then trypsin-neutralizing solution (Clonetics) was added, and the cell suspension was centrifuged at 200 × *g* (1,000 rpm) in a Sorvall RT 7 centrifuge for 5 min at room temperature. The cells were then fixed for 10 min by resuspension in freshly prepared 1% paraformaldehyde in PBS, rinsed in PBS supplemented with 1% bovine serum albumin (BSA-PBS), permeabilized with 0.3% saponin in BSA-PBS for 5 min, and pelleted as above. The incubation with the first antibody anti-c-*myc*, diluted in BSA-PBS with 0.3% saponin, was done for 30 min on ice. Cells were washed two to three times with the same buffer, incubated on ice with the secondary antibody, either fluorescein isothiocyanate (FITC)- or Texas Red-labeled, washed two to three times, resuspended in BSA-PBS, and analyzed immediately with a FACSCalibur flow cytometer (Beckton Dickinson, Mountain View, CA).

Oxidant detection

The production of oxidants was measured by two different assays. The lucigenin assay was done as previously reported (Irani *et al.*, 1997). The fluorescence assay was done by loading the cells with 5-(and-6)-chloromethyl-2',7'dichloro-dihydrofluorescein diacetate (CM-DCF-DA) (Molecular Probes). Cells were rinsed with Hanks' balanced salt solution (HBSS) (Gibco), and incubated with 5 μM CM-DCF-DA in HBSS for 20–30 min at 37°C. The cells were then rinsed with HBSS, and either trypsinized as described and used immediately for FACS analysis, or examined alive with a Nikon Eclipse 800 microscope at an excitation wavelength of 490 nm.

Western blots

The cells grown in six-well plates were lysed in 200 μl of lysis buffer, harvested on ice, sonicated 2 × 15 sec, and the debris pelleted in a microcentrifuge at maximum speed for 10 min at 4°C. A total of 5–10 μg of protein from the supernatants was run on a 5–20% SDS-PAGE for 1 hr at 150 V. The gels were transferred overnight at 5 V to Hybond ECL nitrocellulose membranes (Amersham Corporation, Arlington Heights, IL). The first antibody was an anti-c-*myc* epitope (Santa Cruz) and the secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse (Hyclone Laboratories, Logan UT). The binding was detected by SuperSignal® (Pierce Chemical Co., Rockford, IL), as directed by the manufacturer.

Protein assay

The protein concentration was determined by the bicinconinic acid (BCA) assay (Sigma Chemical Co., St. Louis, MO), following the Sigma procedure No. TPRO-562, and using BSA as standard.

RESULTS

Overexpression of rac^{V12} protein in endothelial cells induces dose-dependent changes in F-actin content

To create a reliable system in which bulk biochemical measurements could be performed on multiple cells, such as quantitation of filamentous actin as well as other parameters, we have engineered replication-deficient adenoviral constructs. The E1A gene of the adenovirus was replaced, by homologous recombination, with the cDNA for the constitutively activated mutant of rac, rac^{V12} (Ad-rac^{V12}), under the control of the cytomegalovirus promoter (Sulciner *et al.*, 1996). Western blot analysis of infected HAEC, using an antibody against the c-*myc* tag epitope as a probe, showed that the rac protein is strongly expressed, and that the amount of protein increases with the m.o.i. (Fig. 1A), whereas no such rac expression is detected in the cells infected with a control virus, in which the bacterial β-galactosidase gene was inserted (Ad-βGal). Likewise, mouse endothelial cells could be infected by the viral construct and expressed heightened levels of rac^{V12}, as determined by FACS analysis of the MAEC infected with increasing concentrations of the adenovirus (not shown). However, in mouse cells, we detected a plateau of rac^{V12} expression at around 250 m.o.i., therefore, this was the maximum m.o.i. used with these cells in the following experiments.

In HAEC which overexpress rac^{V12}, the changes in F-actin distribution and content were dependent upon the level of rac^{V12} protein expression (Fig. 1B). When the rac^{V12} content was low in HAEC exposed to reduced serum conditions (0.1% FBS), as detected by im-

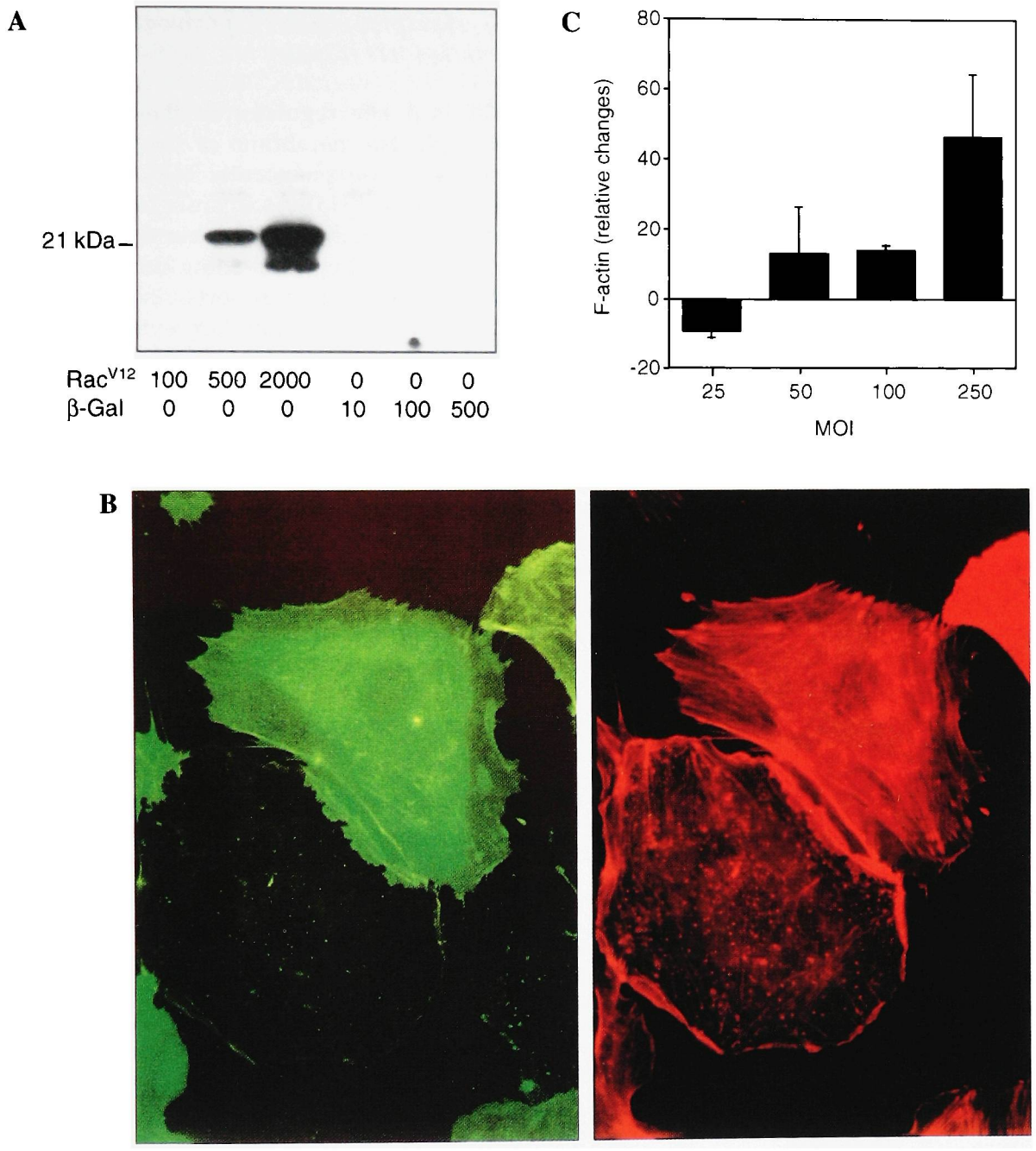

FIG. 1. Rac^{V12} overexpression induces a m.o.i.-dependent increase in F-actin content. A. Human aortic endothelial cells transfected with Ad-rac^{V12} express constitutively activated rac. The expression of rac^{V12} was detected with an antibody directed against the c-*myc* tag of the protein and by Western blot of human aortic endothelial cells. The infections were at the indicated m.o.i., and the assays were performed after 3 days, on overnight serum-starved cells. **B.** The organization of the actin cytoskeleton in Ad-rac^{V12}-infected cells depends upon the amount of expressed rac^{V12} Human aortic endothelial cells were infected with Ad-rac^{V12} at an m.o.i. of 100. The cells were double-stained for rac^{V12} expression (*left panel*) and F-actin (*right panel*). Rac^{V12} was detected by an antibody directed against the c-*myc* tag and an FITC-labeled secondary antibody, and F-actin organization was assessed by staining with rhodamine-phalloidin on the same cells. **C.** The concentration of F-actin in Ad-rac^{V12}-infected cells. Mouse aortic endothelial cells were infected with Ad-rac^{V12} or Ad-βGal, at the indicated m.o.i. After 3 days, cells were serum-starved overnight and lysed, and F-actin content was assayed by rhodamine-phalloidin binding. The amount of F-actin-bound rhodamine-phalloidin was determined fluorimetrically relative to a standard curve and was normalized to the protein content. The data are expressed as percent of change in the amount of rhodamine-phalloidin in Ad-rac^{V12} versus Ad-βGal-infected cells at the same m.o.i. ± SEM. The increase at 100 and 250 m.o.i. is significant ($p < 0.03$) when compared to control cells (Ad-βGal-infected at matching m.o.i.) and to cells infected with lower Ad-rac^{V12} m.o.i. (25). The data are representative of three independent experiments.

munostaining, stress fibers disintegrated and F-actin was mostly present as punctate foci and at the level of peripheral ruffles, where rac^{V12} was detected. In cells where rac^{V12} expression was high, strong rhodamine-phalloidin staining was detected throughout the cells, including staining of actin fibers that were less prominent and thick than typical endothelial cell stress fibers (Fig. 1B).

Because of the high efficiency of transfection provided by the adenoviral method, we could assess bulk changes in F-actin concentration that were associated with the reorganization of the cytoskeleton. When compared to the Ad-βGal-infected cells, both HAEC and MAEC infected with Ad-rac^{V12} were consistently found to contain increased F-actin concentration, which was proportional to the m.o.i. of the recombinant adenovirus added to the cells (Fig. 1C). However, the cells infected with the lowest m.o.i. displayed a reproducible small decrease of F-actin (Fig. 1C, first column), which probably reflected the dissolution of stress fibers (Fig. 1B).

Cells overexpressing rac^{V12} produce increased amounts of free radicals

Rac is a key regulator in the recruitment to the plasma membrane of the cytoplasmic subunits of the phagocytic NADPH oxidase (Dagher *et al.*, 1995). As a consequence, the enzyme is activated and produces the SO necessary for the killing of invading bacteria within phagosomes. Several reports, including our own, showed that in nonphagocytic cells SO is also produced by an NADPH oxidase-like mechanism (Emmendorffer *et al.*, 1993; Zulueta *et al.*, 1995; Crawford *et al.*, 1996; Griendling and Alexander, 1997; Bayraktutan *et al.*, 1998; Irani and Goldschmidt-Clermont, 1998). We tested whether rac^{V12} overexpression in EC could activate NADPH oxidase and induce SO production. We used the fluorescent probe CM-DCF-DA, a cell-permeable fluorescein derivative with a high cellular retention, that fluoresces upon oxidation (Zulueta *et al.*, 1997). This probe detects mainly H_2O_2, resulting from

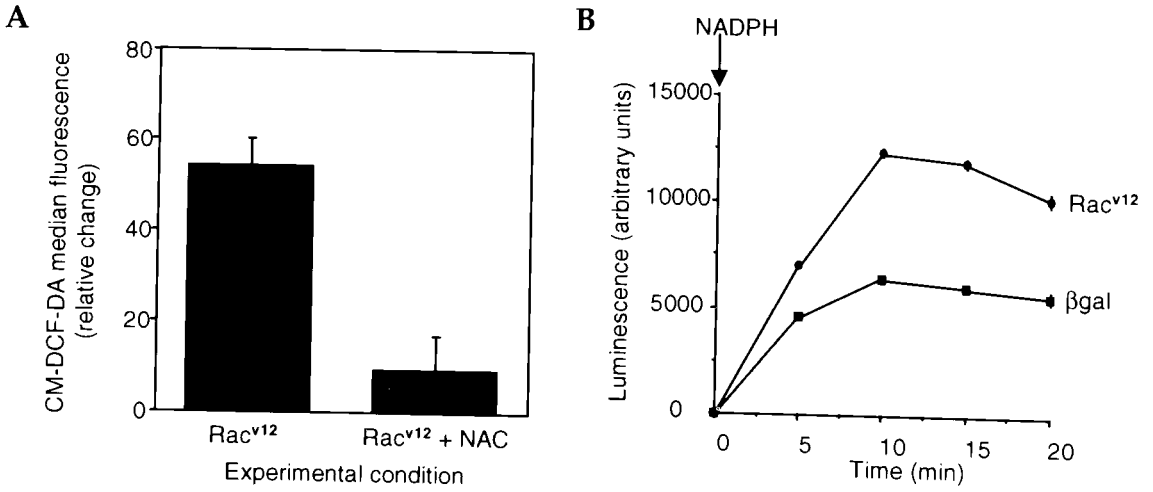

FIG. 2. Oxidant production is increased in aortic endothelial cells that overexpress Rac^{V12}. A. MAEC were infected with Ad-rac^{V12} or Ad-βGal, at an m.o.i. of 250. After 2 days, cells were serum-starved overnight, then loaded with CM-DCF-DA (5 μ*M*) for 30 min, without (Rac^{V12}) or with a 1-hr preincubation with 10 m*M* *N*-acetyl cysteine (Rac^{V12} + NAC). At the end of the incubation period, the cells were trypsinized and analyzed by flow cytometry for CM-DCF-DA fluorescence. Results are expressed as percent change in CM-DCF-DA median fluorescence versus the respective Ad-βGal-infected controls ± SEM. The data are representative of two independent experiments. **B.** HAEC were transfected with Ad-rac^{V12} or Ad-βGal at a m.o.i. of 500. After 2 days, cells were serum-starved overnight, then they were harvested and resuspended in Krebs-HEPES buffer. NADPH (1 m*M*) was added at time 0 and the cells incubated for the indicated times at 37°C. A total of 100,000 cells were then added to lucigenin (0.25 m*M* in the same buffer) and chemiluminescence was measured in a Monolight 2010 luminometer for 60 sec. Values represent mean ± SEM from three independent measurements, and are expressed as relative light units per 100,000 cells.

the scavenging of SO by SOD, and was used as an indicator of the total ROS production by the infected cells. FACS analysis of Ad-rac^{V12}-infected, CM-DCF-DA-loaded cells showed increased production of free radicals, as compared to Ad-βGal-infected control cells. This increase was inhibited by addition of the cell permeable antioxidant *N*-acetyl cysteine (NAC) (Fig. 2A). We also used an assay more specific for SO detection, the luciferase chemiluminescence assay (Irani *et al.*, 1997; Li *et al.*, 1998). HAEC infected with Ad-rac^{V12}, compared with control, Ad-βGal-infected cells, displayed increased production of free radicals, which was proportional to the m.o.i. of the infecting adenovirus (Fig. 2B). We conclude that in endothelial cells overexpressing the constitutively activated mutant of rac, an increased production of ROS is triggered, as it is the case in fibroblasts, probably as a result of the activation of a NADPH oxidase-like enzyme complex by rac^{V12}.

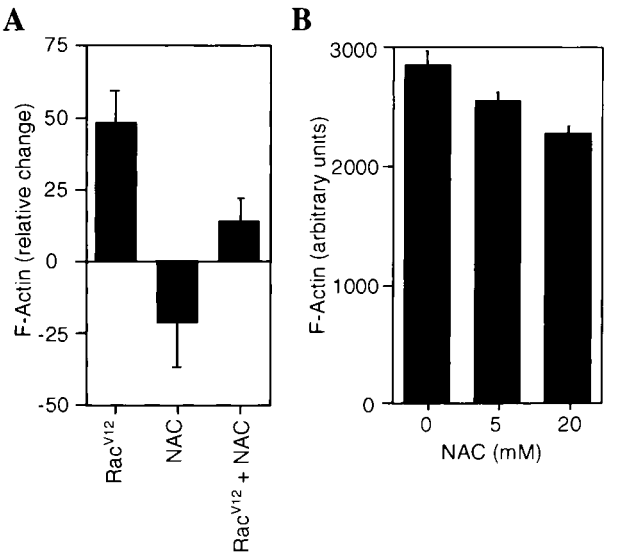

FIG. 3. NAC decreases the F-actin content in both control and Ad-rac^{V12}-infected endothelial cells. A. Human aortic endothelial cells were infected with Ad-rac^{V12} or Ad-βGal, at a m.o.i. of 100. After 3 days, the cells were treated, where indicated, with 20 m*M* NAC for 1 hr prior to F-actin assay by fluorimetry. The results are expressed as percent change in rhodamine-phalloidin fluorescence versus the Ad-βGal infected (matching m.o.i.) control cells ± SEM. The differences between NAC treated and untreated rac-overexpressing cells are significant ($p < 0.05$). **B.** Uninfected human aortic endothelial cells were treated for 1 hr with the indicated concentrations of NAC, and F-actin was assayed as described. The differences between treated versus nontreated cells are significant ($p < 0.05$).

NAC and a SOD mimetic reverse the effects of rac^{V12} overexpression on actin organization

If superoxide produced by rac^{V12}-mediated mechanism(s) were contributing to actin cytoskeleton reorganization, then we expected that superoxide scavengers could reverse this process. We used several antioxidants/ROS scavengers to define the contribution of SO and other ROS produced within endothelial cells, to the actin changes induced as a result of rac^{V12} overexpression. In one set of experiments we incubated the Ad-rac^{V12}-infected HAEC with NAC. We found that NAC reduced the increase in F-actin resulting from overexpressing rac^{V12} (Fig. 3A, column 3). Interestingly, it also decreased slightly, but significantly, the amount of actin filaments in control Ad-βGal-infected cells (Fig. 3A, column 2) and in uninfected EC (Fig. 3B). Hence, it is likely that in endothelial cells, the dynamic generation of free radicals contributes to the maintenance of F-actin content.

In another set of experiments we incubated Ad-rac^{V12}-infected MAEC and HCEC with the SOD mimetic, MnTNPyP (Faulkner *et al.*, 1994). F-actin was stained by rhodamine-phalloidin and analyzed either by FACS or by fluorescence microscopy. The organization of actin in these cells was clearly affected by SO scavenging: the ruffles were no longer visible, while we observed a more prominent bundling of F-actin in long, parallel fibers (Fig. 4A). In the presence of this more specific SO scavenger, the amount of actin filaments in rac^{V12}-overexpressing cells was significantly reduced as determined by bulk F-actin measurement (Fig. 4B).

SOD co-expressed with rac^{V12} reduces the effect of the constitutively active form of rac on F-actin organization

The experiments with the SOD mimetic suggested that SO might be an important mediator of the actin changes induced by rac^{V12}. To confirm this hypothesis, we used another specific scavenger of the SO radical, the enzyme Cu,Zn-superoxide dismutase, to decrease the intracellular level of SO, as we had done to

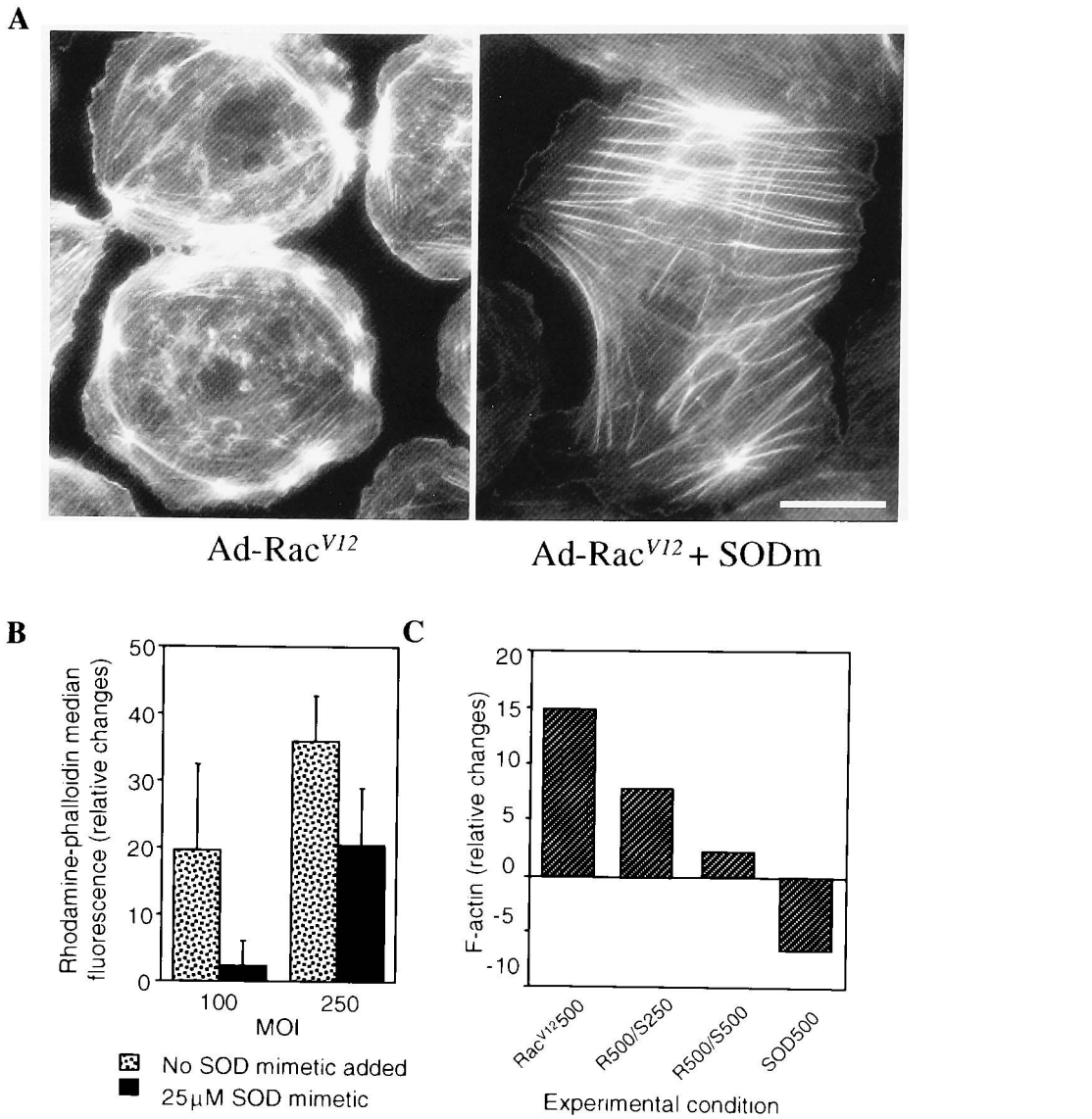

FIG. 4. MnTMPyP and superoxide dismutase reverse the effects of constitutively activated Rac on F-actin content. A. HCEC were infected with Ad-rac^{V12} at a m.o.i. of 500. After 3 days, overnight serum-starved cells were incubated with MnTMPyP (25 μM) or vehicle (PBS) for 1 hr, then cells were fixed, stained for F-actin with rhodamine-phalloidin, and examined with a Nikon Eclipse 800 fluorescence microscope. Bar, 50 μM. **B.** Mouse aortic endothelial cells were infected with Ad-rac^{V12} or Ad-βGal at the indicated m.o.i. After 3 days and overnight serum starvation, cells were incubated for 1 hr with the SOD mimetic MnTMPyP (25 μM), and F-actin assayed by rhodamine-phalloidin binding and flow cytometry. Results are expressed as percent change in median fluorescence of bound rhodamine-phalloidin versus control cells (Ad-βgal-infected at matching m.o.i.) ± SEM. **C.** HAEC were infected concomitantly with Ad-rac^{V12} and/or Ad-SOD. F-actin was assayed fluorimetrically as described. The results are expressed as relative changes compared to cells infected with Ad-βgal (matching m.o.i.), and are representative of two independent experiments.

study endothelial cells exposed to hypoxia/reoxygenation (Crawford *et al.*, 1996), and to determine the consequent changes in actin cytoskeleton in the presence or the absence of rac^{V12} overexpression. Because neither exogenously added SOD nor the intracellular SO anion are membrane permeable, we chose to transfect HAEC with an adenoviral vector that contains the cDNA of human SOD (Ad-SOD) (Crawford *et al.*, 1996). In cells overexpressing SOD, the activity of the enzyme is increased, and the increase is proportional to the m.o.i. (Fig. 5A). When we co-infected HAEC with Ad-rac^{V12}, Ad-SOD, or Ad-βGal and then studied the SOD activity, a significant increase in SOD activity was detected in Ad-SOD-infected cells,

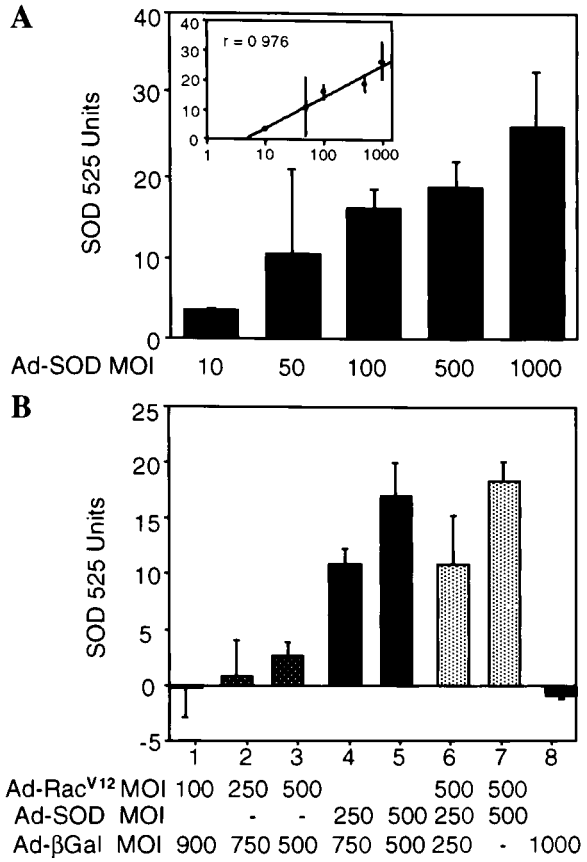

FIG. 5. Detection of SOD in HAEC infected with Ad-SOD. A. HAEC were infected with Ad-SOD at the indicated m.o.i. and the SOD activity was assayed after 3 days. Results are expressed as SOD 525 units, normalized to the protein content of each sample. *Inset:* Dependency of SOD activity upon viral m.o.i. **B.** SOD activity assayed in HAEC infected concomitantly with Ad-SOD, Ad-rac^{V12}, and Ad-βGal, at the indicated m.o.i.

and this effect was unaltered by the co-transfection of the cells by Ad-rac^{V12} or Ad-βGal (Fig. 5B). We maintained the mix of m.o.i. constant by varying the m.o.i. of control Ad-βGal, to account for the unspecific effect of the replication-deficient virus.

We first analyzed the actin changes by bulk measurements in a similar co-infection experiment. When F-actin is measured by rhodamine-phalloidin staining, an increase was detected in rac^{V12}-overexpressing cells compared to the level detected in Ad-βGal control cells (Fig. 4C, column 1). In cells overexpressing SOD alone, F-actin decreased (Fig. 4C, column 4) consistent with the findings obtained with cells exposed to NAC and MnTMPyP, again suggesting that the baseline F-actin content in these cells might be sensitive to the redox state. When both rac^{V12} and SOD were co-expressed, F-actin level was heightened by rac^{V12}, but this increase was reduced in a dose-dependent fashion by the overexpression of SOD (Fig. 4C, columns 2 and 3).

To confirm these biochemical findings with morphological data, we used a triple staining of HAEC infected with Ad-rac^{V12}, Ad-βGal, and Ad-SOD to maintain a final m.o.i. of 1,000, but where the relative m.o.i. of each adenovirus was varied. We detected F-actin by staining the cells with rhodamine-phalloidin, and the expression of the two proteins rac^{V12} and SOD by immunostaining. Rac^{V12} protein was detected by a primary monoclonal antibody directed to the c-*myc* tag, and a fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody. SOD was detected by a rabbit anti-human SOD polyclonal antibody and a Cy5-labeled anti-rabbit secondary antibody. Confocal microscopic analysis revealed specific patterns of actin organization that were strongly dependent upon the levels of overexpressed proteins. The control endothelial cells, infected with Ad-βGal only, displayed a typical actin network rich in stress fibers across the entire cell. No rac^{V12} (c-*myc* tag) or SOD are immunologically detectable within these cells (Fig. 6a–c). In cells containing high amounts of immunologically reactive rac^{V12} (Fig. 6d), we observed a profound reorganization of the actin cytoskeleton (Fig. 6f). The stress fibers were no longer present, and prominent ruffles formed at the cell edges, containing high amounts of F-actin. The appearance of F-actin in SOD-overexpressing cells was not markedly different from control cells, except for the fact that some of these cells acquired an elongated shape, different from the cobblestone appearance of cultured aortic endothelial cells, and the stress fibers were thicker and more parallel, similar to the rac^{V12}-overexpressing cells treated with the SOD mimetic (Fig. 6g–i). When both rac^{V12} and SOD were expressed at high level within the same cell (Fig. 6j–k), the F-actin appearance was intermediate between that present in Ad-rac^{V12}- and Ad-SOD-infected cells (Fig. 6l): the membrane ruffles were still present, but smaller than in rac^{V12}-overexpressing cells, and actin stress fibers, slightly

thinner than in SOD-expressing cells, were present within the cell body.

DISCUSSION

We show in the present study that rac^{V12} overexpression, in either human or mouse aortic endothelial cells, induces actin reorganization and production of free radicals and that the two processes are linked. The last decade has brought an important change in the framework wherein free radicals were considered solely as harmful molecular species, which, through their oxidizing activity, impair normal cellular functioning. Previous reports indicated that the effect of ROS might be more complex (Halliwell, 1986). It was more recently established that ROS can be important mediators for multiple processes that include the regulation of transcription (Sen and Packer, 1996; Suzuki *et al.*, 1997), cell proliferation and apoptosis (Burdon, 1995; Irani *et al.*, 1997; Polyak *et al.*, 1997; Kim *et al.*, 1998), and cellular activation (Goldstone *et al.*, 1995, 1996). In this context, considering the controlling role of rac in the activation of the phagocytic NADPH oxidase and actin cytoskeleton organization, the next logical step was to ask whether rac-induced formation of free radicals, in particular SO, was involved in the reorganization of the actin cytoskeleton. This possibility was also suggested by the fact that an NADPH oxidase-like enzyme has been demonstrated in cells other than the phagocytic lineage (Meier *et al.*, 1991, 1993; Zulueta *et al.*, 1995; Bayraktutan *et al.*, 1998), as well as by the recent finding that rac1-induced production of free radicals is involved in collagenase-1 expression (Kheradmand *et al.*, 1998). In addition, previous findings from this laboratory supported a role for SO in actin regulation. We showed that rac1 is a downstream effector of ras on the pathway of SO production in nonphagocytic cells (Sundaresan *et al.*, 1996; Irani *et al.*, 1997). Moreover, conditions known to increase intracellular SO and other free radicals in these cells were usually accompanied by a process of actin reorganization that was similar to that described in cells microinjected with rac (Crawford *et al.*, 1996; Heldman *et al.*, 1996; Ridley *et al.*, 1992).

To test our hypothesis, we designed a model system that allowed us to perform quantitative biochemical measurements to correlate the level of activated rac expression to the production of free radicals and to the changes in actin organization. We used the constitutively activated form of rac1, rac^{V12}, in which the mutation of the amino acid in position 12 from glycine to valine results in the abolishment of the GTPase activity of rac (Dieckmann *et al.*, 1991). We and others have used replication-incompetent (E1/E3-deleted) adenoviruses as vectors to introduce new genes into cells, which express on their surface the glycoproteins required for virus adhesion and uptake (Moldovan *et al.*, 1996; Wilson, 1996; Hashimoto *et al.*, 1997), and we could thus study the regulation of the actin cytoskeleton in cells infected with replication-incompetent adenoviruses containing the gene of either an active rac^{V12} or control β-Gal. Because endothelial cells are susceptible to adenoviral infection, the introduction within these cells of the rac^{V12}-encoding gene mediated by our adenovirus construct was remarkably efficient. We could readily detect by Western blots, FACS analysis, and immunohistochemistry that HAEC and, to a lesser extent, MAEC expressed rac^{V12} and that the level of expression was directly related to the number of infectious viral particles added per cell (Fig. 1).

The organization of F-actin within endothelial cells *in situ* and in culture comprises prominent stress fibers, both at the periphery of the cells and crossing the entire cell body, attached at one or both ends to focal adhesions (Wong *et al.*, 1982; Gabbiani *et al.*, 1983). When a discontinuity within the monolayer appears, a profound reorganization of the actin cytoskeleton takes place before the cells start migrating to cover the gap. The various steps of this process are documented from both *in vivo* and culture systems: a typical time-course comprises the formation of microspikes within minutes after wounding, and the appearance of membrane ruffles within a couple of hours and of stress fibers within a day from wounding (Vyalov *et al.*, 1996; Aepfelbacher *et al.*, 1997). The spatial and temporal control of the actin cytoskeleton organization in Swiss 3T3 fibroblasts was ascribed to the sequential activation of cdc42, rac, and rho (Nobes and Hall, 1995). In our hands, dissolution of stress fibers,

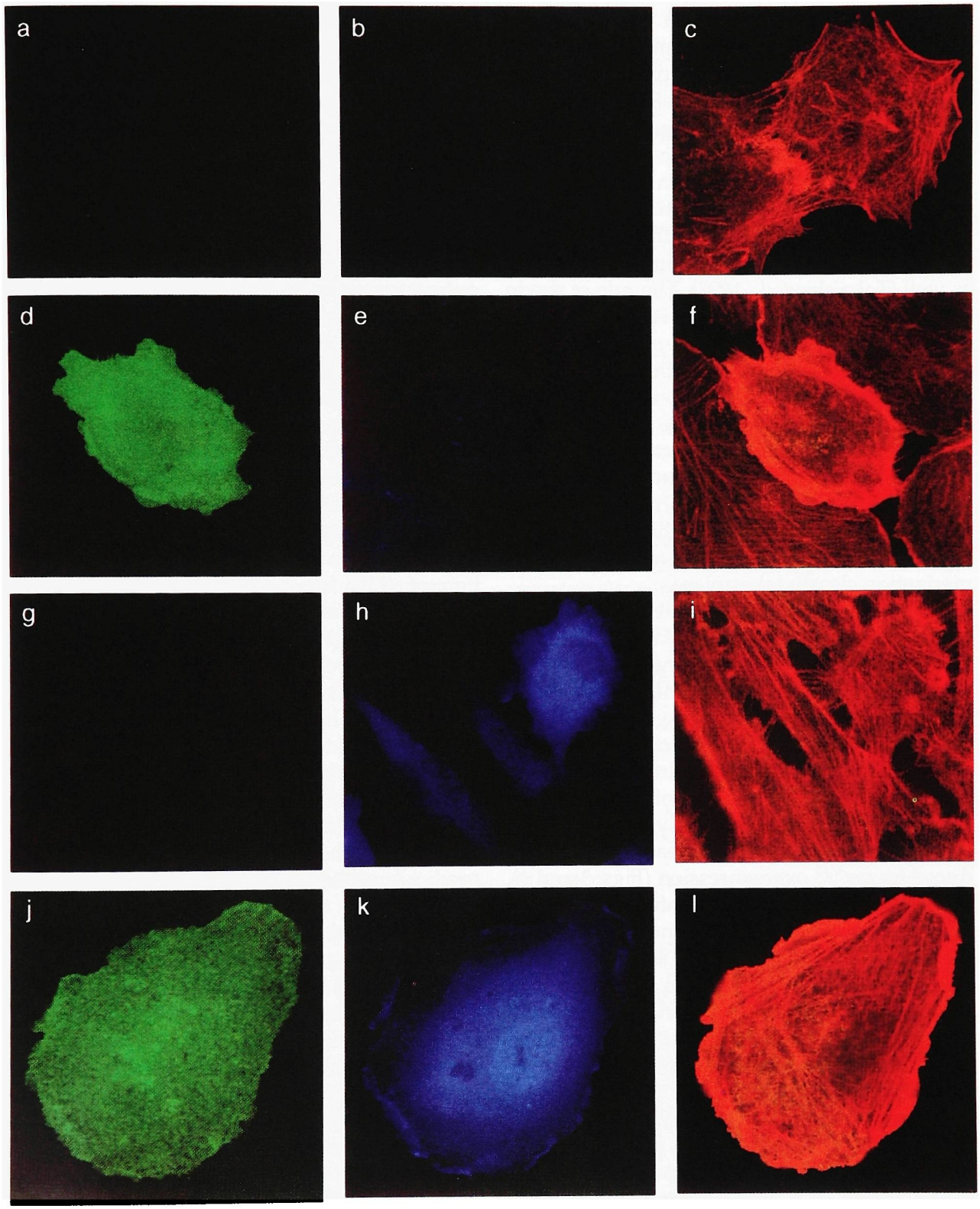

FIG. 6. SOD overexpression in HAEC reduces the effects of Rac^{V12} on F-actin organization. Cells were transfected with Ad-βGal at a m.o.i. of 1,000 **(a–c)**, Ad-rac^{V12} + Ad-βGal at m.o.i. of 500 each **(d–f)**, Ad-SOD + Ad-βGal at m.o.i. 500 each **(g–i)**, and Ad-rac^{V12} + Ad-SOD at m.o.i. 500 each **(j–l)**, for 2 days. Cells were then starved of serum overnight and were processed for immunocytochemistry as follows: rac^{V12} was detected by a mouse monoclonal antibody against the c-*myc* tag and a secondary FITC-labeled antibody **(a, d, g, j)**; SOD was detected by a rabbit anti-human SOD polyclonal antibody, and a secondary Cy5-labeled antibody **(b, e, h, k)**; F-actin was detected by staining with rhodamine phalloidin **(c, f, i, l)**. Cells were examined with a Noran confocal microscope. Original magnification, 63×.

the punctate aspect of F-actin within the cell body, and presence of high amounts of fibers at the membrane ruffles, were observed in cells deprived of serum, but expressing low amount of rac^{V12}, similar to the changes described in Swiss 3T3 fibroblasts upon rac microinjection. We detected an elevated F-actin content in cells that expressed high concentrations of rac^{V12} in both human and mouse endothelial cells (Fig. 1), which has been observed in other systems as well (Takaishi *et al.*, 1997). The role of rho in inducing actin stress fibers in endothelial cells was recently characterized (Aepfelbacher *et al.*, 1997). It is conceivable that the intrinsic rho contributes to F-actin generation in rac^{V12}-overexpressing cells. However, we cannot ascribe the increased formation of F-actin solely to rho activity, because rho activation reportedly induces the assembly of preformed actin filaments into stress fibers (Machesky and Hall, 1997), whereas in our cells *de novo* actin polymerization clearly takes place.

The Ad-rac^{V12}-infected cells produce increased amounts of free radicals (Fig. 2 and Irani *et al.*, 1997). By using either a nonspecific yet powerful cell-permeable antioxidant, NAC, or the more specific SO scavengers, MnTMPyP (Faulkner *et al.*, 1994; Weiss *et al.*, 1996) and SOD expression with adenovirus transfection, we decreased the amount of ROS present within these cells. Remarkably, each of these antioxidants reversed the increase in F-actin induced by rac^{V12} overexpression (Figs. 3 and 4). Moreover, when cells were infected with Ad-SOD only, or when control or noninfected cells were treated with NAC, we also observed a slight decrease in the amount of filamentous actin relative to control cells. We consider that these findings support the interpretation that a minimal level of SO within the cytoplasm is required for the maintenance of the F-actin level in quiescent endothelial cells, while an increase in the level of free radicals in stimulated cells is necessary for the breakdown of existing stress fibers and repolymerization of actin fibers at the membrane ruffles. It is important to realize that the steady state reached by cells in terms of F-actin is by no means a rigid state: G- and F-actin are in a dynamic equilibrium (Mitchison and Cramer, 1996), and we propose that free radicals are, in fact, accelerating this turnover.

Joneson and Bar-Sagi have recently studied the role of superoxide and free radicals as signaling mediators for mitogenesis and oncogenesis (Joneson and Bar-Sagi, 1998). Using microinjection of cells, they have increased the cellular concentration of rac or mutants thereof. Using this approach, they have confirmed our previous observation that superoxide is required for the mitogenic activity and transformation of Rac-overexpressing cells (Sundaresan *et al.*, 1996; Irani *et al.*, 1997). However, they have found that oxidants were not needed to induce the actin cytoskeleton changes. Several important differences between the two studies are noteworthy. Joneson and Bar-Sagi used fibroblastoid cells, whereas our studies involved endothelial cells exclusively. Moreover, the approach used to overexpress Rac is different between the two studies. They used microinjection of cells for most of their experiments, whereas we have used a very efficient adenovirus-mediated cell transfection technique, allowing for expression of rac in >95% of the infected cells. Therefore, we have been able to perform bulk biochemical assays to quantitate filamentous actin which the study of Joneson and Bar-Sagi did not permit. Moreover, to inhibit superoxide-mediated pathways, they used antagonists such as SOD protein added externally to the cells. Although it is difficult to predict how an effect of SOD added outside the cells might be transmitted, because SOD does not seem to be cell permeant, and superoxide does not cross membranes either, we have overexpressed SOD directly in cells using a replication-incompetent adenovirus coding for SOD.

We consider that the differences between these two studies might reflect at least two important characteristics of rac signaling. (i) As it is true with many other signal transduction pathways, the messenger molecules involved are strongly cell type-dependent. Our findings were similar in EC from two different species and with different genomic programming (primary culture vs. immortalized cell line), which supports the role of rac-induced formation of ROS in actin reorganization in EC. (ii) These re-

sults underscore the multifaceted character of rac as a signaling molecule. Multiple rac-ligand mediated molecular reactions are needed to transduce rac signals fully (Freeman *et al.*, 1996; Joneson *et al.*, 1996; Westwick *et al.*, 1997). Our results are consistent with the concept that rac controls multiple and probably redundant pathways to induce a specific effect upon the actin cytoskeleton.

In conclusion, we propose that a key mediator for rac downstream effects, such as actin reorganization in endothelial cells, is superoxide, because antioxidants—and in particular superoxide dismutase—reverse the effects of constitutive activation of rac upon actin. We also propose a major role for oxidants in actin reorganization through the modulation of interactions between actin and actin-binding proteins. For example, SO could alter the activity of capping proteins near the plasma membrane, promoting the addition of monomeric actin subunits at the barbed end of filaments. H_2O_2, which diffuses through cells more readily, could potentiate the activity of severing proteins at the level of stress fibers, which in the presence of monomeric actin concentrations that are supracritical, could induce actin polymerization and the thickening of stress fibers (Arcaro, 1998; Goldschmidt-Clermont and Moldovan, 1999).

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REFERENCES

AEPFELBACHER, M., ESSLER, M., HUBER, A., SUGAI, M., and WEBER, P.C. (1997). Bacterial toxins block endothelial wound repair. Evidence that rho GTPases control cytoskeletal rearrangements in migrating endothelial cells. Arterioscler. Thromb. Vasc. Biol. **17,** 1623–1629.

ABO, A., PICK, E., HALL, A., TOTTY, N., TEAHAN, C.G., and SEGAL, A.W. (1991). Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature **353,** 668–670.

ARBER, S., BARBAYANNIS, F.A., HANSER, H., SCHNEIDER, C., STANYON, C.A., BERNARD, O., and CARONI, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature **393,** 805–809.

ARCARO, A. (1998). The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. J. Biol. Chem. **273,** 805–813.

BAGRODIA, S., TAYLOR, S.J., CREASY, C.L., CHERNOFF, J., and CERIONE, R.A. (1995). Identification of a mouse p21Cdc42/rac activated kinase. J. Biol. Chem. **270,** 22731–22737.

BASTAKI, M., NELLI, E.E., DELL'ERA, P., RUSNATI, M., MOLINARI-TOSATTI, M.P., PAROLINI, S., AUERBACH, R., RUCO, L.P., POSSATI, L., and PRESTA, M. (1997). Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. Arterioscler. Thromb. Vasc. Biol. **17,** 454–464.

BAYRAKTUTAN, U., DRAPER, N., LANG, D., and SHAH, A.M. (1998). Expression of functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. Cardiovasc. Res. **38,** 256–262.

BURDON, R.H. (1995). Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Rad. Biol. Med. **18,** 775–794.

COX, D., CHANG, P., ZHANG, Q., REDDY, P.G., BOKOCH, G.M., and GREENBERG, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J. Exp. Med. **186,** 1487–1494.

CRAWFORD, L.E., MILLIKEN, E., IRANI, K., ZWIER, J.L., BECKER, L.C., JOHNSON, T.M., EISSA, N.T., CRYSTAL, R.G., FINKEL, T., and GOLDSCHMIDT-CLERMONT, P.J. (1996). Superoxide-mediated actin response in post-hypoxic endothelial cells. J. Biol. Chem. **271,** 26863–26867.

DAGHER, M.C., FUCHS, A., BOURMEYSTER, N., JOUAN, A., and VIGNAIS, P.V. (1995). Small G proteins and the neutrophil NADPH oxidase. Biochimie **77,** 651–660.

DIECKMANN, D., BRILL, S., GARRETT, M.D., TOTTY, N., HSUAN, J., MONFRIES, C., HALL, C., LIM, L., and HALL, A. (1991). BCR encodes a GTPase activating protein for p21rac. Nature **351,** 400–402.

EMMENDORFFER, A., ROESLER, J., ELSNER, J., RAEDER, E., LOHMANN-MATTHES, M.L., and MEIER, B. (1993). Production of oxygen radicals by fibroblasts and neutrophils from a patient with X-linked chronic granulomatous disease. Eur. J. Haematol. **51,** 223–227.

FAULKNER, K.M., LIOCHEV, S.I., and FRIDOVICH, I. (1994). Stable Mn(III) porphyrins mimic superoxide dismutase in vitro and substitute for it in vivo. J. Biol. Chem. **269,** 23471–23476.

FREEMAN, J.L., ABO, A., and LAMBETH, J.D. (1996). Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J. Biol. Chem. **271,** 19794–19801.

GABBIANI, G., GABBIANI, F., LOMBARDI, D., and SCHWARTZ, S.M. (1983). Organization of actin cytoskeleton in normal and regenerating arterial endothelial cells. Proc. Natl. Acad. Sci. USA **80,** 2361–2364.

GOLDSCHMIDT-CLERMONT, P.J., and MOLDOVAN, L. (1999). Stress, superoxide and signal transduction. Gene Expression (In press).

GOLDSTONE, S.D., FRAGONAS, J.C., JEITNER, T.M., and HUNT, N.H. (1995). Transcription factors as targets for oxidative signalling during lymphocyte activation. Biochim. Biophys. Acta **1263,** 114–122.

GOLDSTONE, S.D., MILLIGAN, A., and HUNT, N.H. (1996). Oxidative signalling and gene expression during lymphocyte activation. Biochim. Biophys. Acta **1314,** 175–182.

GRIENDLING, K.K., and ALEXANDER, R.W. (1997). Oxidative stress and cardiovascular disease. Circulation **96,** 3264–3265.

HALL, A. (1998). Rho GTPases and the actin cytoskeleton. Science **279,** 509–514.

HALLIWELL, B. (1986). Superoxide dismutase and the superoxide theory of oxygen toxicity. A critical appraisal. In *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine.* G. Rotilio, ed. (Elsevier Publ. Co., New York) pp. 63–102.

HASHIMOTO, Y., KOHRI, K., AKITA, H., MITANI, K., IKEDA, K., and NAKANISHI, M. (1997). Efficient transfer of genes into senescent cells by adenovirus vectors via highly expressed avb5 integrin. Biochem. Biophys. Res. Commun. **240,** 88–92.

HELDMAN, A.W., KANDZARI, D.E., TUCKER, R.W., CRAWFORD, L.E., FEARON, E.R., KOBLAN, K.S., and GOLDSCHMIDT-CLERMONT, P.J. (1996). EJ-ras inhibits phospholipase Cg1 but not actin polymerization induced by platelet-derived growth factor-β via phosphatidylinositol 3-kinase. Circ. Res. **78,** 312–321.

IRANI, K., and GOLDSCHMIDT-CLERMONT, P.J. (1998). Ras, superoxide, and signal transduction. Biochem. Pharmacol. **55,** 1339–1346.

IRANI, K., XIA, Y., ZWIER, J.L., SOLOTT, S.J., DER, C.J., FEARON, E.R., SUNDARESAN, M., FINKEL, T., and GOLDSCHMIDT-CLERMONT, P.J. (1997). Mitogenic signalling mediated by oxidants in ras-transformed fibroblasts. Science **275,** 1649–1652.

JONES, S.A., O'DONNELL, V.B., WOOD, J.D., BROUGHTON, J.P., HUGHES, E.J., and JONES, O.T. (1996). Expression of phagocyte NADPH oxidase components in human endothelial cells. Am. J. Physiol. **271,** H1626–H1634.

JONESON, T., and BAR-SAGI, D. (1998). A rac1 effector site controlling mitogenesis through superoxide production. J. Biol. Chem **273,** 17991–17994.

JONESON, T., MCDONOUGH, M., BAR-SAGI, D., and VAN AELST, L. (1996). Rac regulation of actin polymerization and proliferation by a pathway distinct from jun kinase. Science **274,** 1374–1376.

KHERADMAND, F., WERNER, E., TREMBLE, P., SYMONS, M., and WERB, Z. (1998). Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. Science **280,** 898–902.

KIM, K.S., TAKEDA, K., SETHI, R., PRACYK, J.B., TANAKA, K., ZHOU, Y.F., YU, Z.X., FERRANS, V.J., BRUDER, J.T., KOVESDI, I., IRANI, K., GOLDSCHMIDT-CLERMONT, P.J., and FINKEL., T. (1998). Protection from reoxygenation injury by inhibition of rac1. J. Clin. Invest. **101,** 1821–1826.

KNAUS, U.G., HEYWORTH, P.G., EVANS, T., CURNUTTE, J.T., and BOKOCH, G.M. (1991). Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. Science **254,** 1512–1515.

LAROCHELLE, D.A., VITHALANI, K.K., and DE LOZANNE A. (1996). A novel member of the rho family of small GTP-binding proteins is specifically required for cytokinesis. J. Cell Biol. **133,** 1321–1329.

LI, Y., ZHU, H., KUPPUSAMY, P., ROUBAUD, V., ZWEIER, J.L., and TRUSH, M.A. (1998). Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. J. Biol. Chem. **273,** 2015–2023.

MACHESKY, L.M., and HALL, A. (1997). Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. J. Cell Biol. **138,** 913–926.

MEIER, B., CROSS, A.R., HANCOCK, J.T., KAUP, F.J., and JONES, O.T.G. (1991). Identification of a superoxide-generating NADPH oxidase system in human fibroblasts. Biochem. J. **275,** 241–245.

MEIER, B., JESAITIS, A.J., EMMENDORFER, A., ROESLER, J., and QUINN, M.T. (1993). The cytochrome b-558 molecules involved in the fibroblast and polymorphonuclear leukocyte superoxide-generating NADPH oxidase systems are structurally and genetically distinct. Biochem. J. **289,** 481–486.

MITCHISON, T.J., and CRAMER, L.P. (1996). Actin-based cell motility and cell locomotion. Cell **84,** 371–379.

MOLDOVAN, N.I., MILLIKEN, E.E., IRANI, K., CHEN, J., SOHN, R.H., FINKEL, T., and GOLDSCHMIDT-CLERMONT, P.J. (1996). Regulation of endothelial cell adhesion by profillin. Curr. Biol. **7,** 24–30.

MURPHY, A.M., and MONTELL, D.J. (1996). Cell type-specific roles for Cdc42, Rac, and RhoL in Drosophila oogenesis. J. Cell Biol. **133,** 617–630.

NOBES, C.D., and HALL, A. (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular complexes associated with actin stress fibers, lamellipodia, and fillopodia. Cell **81,** 53–62.

NORMAN, J.C., PRICE, L.S., RIDLEY, A.J., HALL, A., and KOFFER, A. (1994). Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. J. Cell Biol. **126,** 1005–1015.

NUSRAT, A., GIRY, M., TURNER, J.R., COLGAN, S.P., PARKOS, C.A., CARNES, D., LEMICHEZ, E., BOUQUET, P., and MADARA, J.L. (1995). Rho protein regulates tight junction and prejunctional actin organization in polarized epithelia. Proc. Natl. Acad. Sci. USA **92,** 10629–10633.

POLYAK, K., XIA, Y., ZWIER, J.L., KINZLER, K.W., and

VOGELSTEIN, B. (1997). A model for p53-induced apoptosis. Nature **389,** 300–305.

RIDLEY, A.J., and HALL, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70,** 389–399.

RIDLEY, A.J., PATERSON, H.F., JOHNSTON, C.L., DIEKMANN, D., and HALL, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70,** 401–410.

SEN, C.K., and PACKER, L. (1996). Antioxidant and redox regulation of gene transcription. FASEB J **10,** 709–720.

SULCINER, D.J., IRANI, K., YU, Z.X., FERRANS, V.J., GOLDSCHMIDT-CLERMONT, P.J., and FINKEL, T. (1996). rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kB activation. Mol. Cell. Biol. **16,** 7115–7121.

SUNDARESAN, M., YU, Z.X., FERRANS, V.J., SULCINER, D.J., GUTKIND, J.S., IRANI, K., GOLDSCHMIDT-CLERMONT, P.J., and FINKEL, T. (1996). Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. Biochem. J. **318,** 379–382.

SUZUKI, Y.J., FORMAN, H.J., and SEVANIAN, A. (1997). Oxidants as stimulators of signal transduction. Free Rad. Biol. Med. **22,** 269–285.

TAKAISHI, K., SASAKI, T., KOTANI, H., NISHIOKA, H., and TAKAI, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. J. Cell Biol. **139,** 1047–1059.

VAN AELST, L., JONESON, T., and BAR-SAGI, D. (1996). Identification of a novel Rac1-interacting protein involved in membrane ruffling. EMBO J. **15,** 3778–3786.

VYALOV, S., LANGILLE, B.L., and GOTTLIEB, A.I. (1996). Decreased blood flow rate disrupts endothelial repair in vivo. Am. J. Pathol. **149,** 2107–2118.

WEISS, R.H., FRETLAND, D.J., BARON, D.A., RYAN, U.S., and RILEY, D.P. (1996). Manganese-based superoxide dismutase mimetics inhibit neutrophil infiltration in vivo. J. Biol. Chem. **271,** 26149–26156.

WESTWICK, J.K., LAMBERT, Q.T., CLARK, G.J., SYMONS, M., VAN AELST, L., PESTELL, R.G., and DER, C.J. (1997). Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol. Cell. Biol. **17,** 1324–1335.

WILSON, J.M. (1996). Adenoviruses as gene-delivery vehicles. Mol. Med. **334,** 1185–1187.

WONG, A.J., POLLARD, T.D., and HERMAN, I.M. (1982). Actin filament stress fibers in vascular endothelial cells in vivo. Science **219,** 867–869.

YANG, N., HIGUCHI, O., OHASHI, K., NAGATA, K., WADA, A., KANGAWA, K., NISHIDA, E., and MIZUNO, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature **393,** 809–812.

ZULUETA, J.J., YU, F.S., HERTIG, I.A., THANNICKAL, V.J., and HASSOUN, P.M. (1995). Release of hydrogen peroxide in response to hypoxia-reoxygenation: role of an NAD(P)H oxidase-like enzyme in endothelial cells plasma membrane. Am. J. Respir. Cell Mol. Biol. **12,** 41–49.

ZULUETA, J.J., SHAWNWY, R., YU, F.S., COTE, C.C., and HASSOUN, P.M. (1997). Intracellular generation of reactive oxygen species in endothelial cells exposed to anoxia-reoxygenation. Am. J. Physiol. **272,** L897–L902.

ZWEIER, J.L., BRODERICK, R., KUPPUSAMY, P., THOMPSON-GORMAN, S., and LUTTY, G.A. (1994). Determination of the mechanism of free radical generation of human aortic endothelial cells exposed to anoxia and reoxygenation. J. Biol. Chem. **269,** 24156–24162.

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1. Vijayalakshmi Thamilselvan, Mani Menon, Sivagnanam Thamilselvan. 2012. Selective Rac1 inhibition protects renal tubular epithelial cells from oxalate-induced NADPH oxidase-mediated oxidative cell injury. *Urological Research* **40**:4, 415-423. [CrossRef]
2. Lalchhandami Tochhawng, Deng Shuo, Shazib Pervaiz, Celestial T. Yap. 2012. Redox regulation of cancer cell migration and invasion. *Mitochondrion* . [CrossRef]
3. Sanja Coso , Ian Harrison , Craig B. Harrison , Antony Vinh , Christopher G. Sobey , Grant R. Drummond , Elizabeth D. Williams , Stavros Selemidis . 2012. NADPH Oxidases as Regulators of Tumor Angiogenesis: Current and Emerging Concepts. *Antioxidants & Redox Signaling* **16**:11, 1229-1247. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
4. John W. Fuseler, Mani T. Valarmathi. 2011. Modulation of the migration and differentiation potential of adult bone marrow stromal stem cells by nitric oxide. *Biomaterials* . [CrossRef]
5. Xiong-Ze Zhang, Xi Huang, Ji-Hong Qiao, Jun-Jun Zhang, Mei-Xia Zhang. 2011. Inhibition of hypoxia-induced retinal neovascularization in mice with short hairpin RNA targeting Rac1, possibly via blockading redox signaling. *Experimental Eye Research* **92**:6, 473-481. [CrossRef]
6. J Du, J Liu, B J Smith, M S Tsao, J J Cullen. 2011. Role of Rac1-dependent NADPH oxidase in the growth of pancreatic cancer. *Cancer Gene Therapy* **18**:2, 135-143. [CrossRef]
7. Ruei-Jiun Hung, Jonathan R. Terman. 2011. Extracellular inhibitors, repellents, and semaphorin/plexin/MICAL-mediated actin filament disassembly. *Cytoskeleton* n/a-n/a. [CrossRef]
8. Giovambattista Pani, Tommaso Galeotti, Paola Chiarugi. 2010. Metastasis: cancer cell's escape from oxidative stress. *Cancer and Metastasis Reviews* **29**:2, 351-378. [CrossRef]
9. Sadashib Ghosh, Richa Tewari, Deobrat Dixit, Ellora Sen. 2010. TNF# induced oxidative stress dependent Akt signaling affects actin cytoskeletal organization in glioma cells. *Neurochemistry International* **56**:1, 194-201. [CrossRef]
10. Masuko Ushio-Fukai Approaches for Studying Redox Signaling in Angiogenesis 191-200. [Abstract] [Summary] [Full Text PDF] [Full Text PDF with Links]
11. Masuko Ushio-Fukai , Norifumi Urao . 2009. Novel Role of NADPH Oxidase in Angiogenesis and Stem/Progenitor Cell Function. *Antioxidants & Redox Signaling* **11**:10, 2517-2533. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
12. Brian M. Barth, Shelli Stewart-Smeets, Thomas B. Kuhn. 2009. Proinflammatory cytokines provoke oxidative damage to actin in neuronal cells mediated by Rac1 and NADPH oxidase. *Molecular and Cellular Neuroscience* **41**:2, 274-285. [CrossRef]
13. Masuko Ushio-Fukai, Yoshimasa Nakamura. 2008. Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Letters* **266**:1, 37-52. [CrossRef]
14. Balakrishnan Selvakumar, Douglas T. Hess, Pascal J. Goldschmidt-Clermont, Jonathan S. Stamler. 2008. Co-regulation of constitutive nitric oxide synthases and NADPH oxidase by the small GTPase Rac. *FEBS Letters* **582**:15, 2195-2202. [CrossRef]
15. Uno Lindberg, Roger Karlsson, Ingrid Lassing, Clarence E. Schutt, Anna-Stina Höglund. 2008. The microfilament system and malignancy. *Seminars in Cancer Biology* **18**:1, 2-11. [CrossRef]
16. Gary B. Dunphy, Genhui Chen, John M. Webster. 2007. The antioxidants dimethylsulfoxide and dimethylthiourea affect the immediate adhesion responses of larval haemocytes from 3 lepidopteran insect species. *Canadian Journal of Microbiology* **53**:12, 1330-1347. [CrossRef]
17. Ingrid Lassing, Florian Schmitzberger, Mikael Björnstedt, Arne Holmgren, Pär Nordlund, Clarence E. Schutt, Uno Lindberg. 2007. Molecular and Structural Basis for Redox Regulation of #-Actin. *Journal of Molecular Biology* **370**:2, 331-348. [CrossRef]
18. Nicanor I. Moldovan . 2005. Emerging Roles of Reactive Oxygen and Nitrogen Species in Stem/Progenitor Cells. *Antioxidants & Redox Signaling* **7**:11-12, 1409-1412. [Citation] [Full Text PDF] [Full Text PDF with Links]

19. Hamdy H. Hassanain, Fawzi Irshaid, Sheik Wisel, John Sheridan, Robert E. Michler, Pascal J. Goldschmidt-Clermont. 2005. Smooth muscle cell expression of a constitutive active form of human Rac 1 accelerates cutaneous wound repair. *Surgery* **137**:1, 92-101. [CrossRef]
20. Leni Moldovan, Nicanor I. Moldovan. 2004. Oxygen free radicals and redox biology of organelles. *Histochemistry and Cell Biology* **122**:4, 395-412. [CrossRef]
21. Johannes Boonstra, Jan Andries Post. 2004. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* **337**, 1-13. [CrossRef]
22. David Gregg , Daniela D. de Carvalho , Hervé Kovacic . 2004. Integrins and Coagulation: A Role for ROS/Redox Signaling?. *Antioxidants & Redox Signaling* **6**:4, 757-764. [Abstract] [Full Text PDF] [Full Text PDF with Links]
23. Dipak K. Das Methods in Redox Signaling . [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]