Tyrosine phosphorylation of cortactin is required for H$_2$O$_2$-mediated injury of human endothelial cells

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The injury of endothelial cells underlying the lumen of blood vessels contributes significantly to the development of atherosclerosis and hypertension. Numerous studies have shown that exposure to local reactive oxygen species is one of the main causes for the injury of endothelial cells. Reactive oxygen species can be derived from the dismutation of superoxide anion or from the products of activated neutrophils or macrophages that accumulate in the blood vessel wall as a consequence of ischemia and reperfusion injury (1–3). Cancer cells also produce reactive oxygen species in vivo, which may contribute to the damage of endothelium during the metastatic process (4). Hydrogen peroxide (H$_2$O$_2$) is known to be one of the common forms of reactive oxygen species and can easily penetrate the plasma membrane and affect neighboring cells as well as H$_2$O$_2$-producing cells (5). One of the prominent events within endothelial cells upon exposure to H$_2$O$_2$ is the formation of plasma membrane blebs (6). The surface blebbing of endothelial cells promotes platelets to adhere to the injured endothelium as well as leukocytes to block capillary lumens, which would lead ultimately to cardiac, brain, lung, kidney, and liver failure. Although the mechanism of the formation of membrane blebs is not fully understood, it appears to be intimately associated with the reorganization of the actin cytoskeleton that are mediated by cytoskeleton binding or cross-linking proteins. For example, cells deficient in an actin filament cross-linking protein, ABP-280, show prolonged blebbing (7). Similarly, redistribution of filamin, another F-actin cross-linking protein, is one of the early events in H$_2$O$_2$-mediated endothelial cell injury (8).

H$_2$O$_2$ triggers signal transduction within cells in a similar manner as growth factors (9, 10). Exposure to H$_2$O$_2$ induces a rapid increase in tyrosine phosphorylation of various proteins, including Src- and Syk-related non-receptor protein-tyrosine kinases (11), and enhances the kinase activity of Src in endothelial cells (12). The implication of protein-tyrosine phosphorylation in H$_2$O$_2$-mediated signal transduction is further strengthened by the findings that non-selective tyrosine kinase inhibitors such as genistein and herbimycin A can abolish the response of cells to H$_2$O$_2$ (13, 14). In addition to protein-tyrosine kinases, H$_2$O$_2$ also induces the activity of extracellular signal-regulated kinase (Erk), a member of the mitogen-activated protein kinase family (15). Induction of Erk may represent a surviving pathway because selective inhibition of Erk by PD98059, can increase apoptosis induced by H$_2$O$_2$ (9, 15).

Cortactin, a cortical actin-associated protein that is widely expressed in most adherent cells (17), is a prominent substrate of protein-tyrosine kinase Src in vivo and in vitro (18, 19). The protein sequence of cortactin is featured by six and a half a-helical structures for the SH3 domain at the carboxyl terminus. Between the repeat and the SH3 domain, there is an a-helical structure.

Injury of endothelial cells induced by reactive oxygen species plays an important role in the development of early stages of vascular diseases such as hypertension and atherosclerosis. Exposure of human umbilical vein endothelial cells to hydrogen peroxide (H$_2$O$_2$), a common form of reaction oxygen species, triggers a series of intracellular events, including actin cytoskeletal reorganization, cytoplasm shrinkage, membrane blebbing and protein-tyrosine phosphorylation. The effect of H$_2$O$_2$ on endothelial cells is dramatically enhanced when a survival pathway involving extracellular signal-regulated kinase is blocked by PD098059. In contrast, when a survival pathway involving extracellular signal-regulated kinase is blocked by PD098059 or H$_2$O$_2$ alone at 200 μm exhibited a dramatic shape change characterized by rounding up or aggregation. However, the similar changes were not detected with cells overexpressing a cortactin mutant deficient in tyrosine phosphorylation. These data demonstrate an important role of the Src/cortactin-dependent actin reorganization in the injury of endothelial cells mediated by reactive oxygen species.

The abbreviations used are: Erk, extracellular signal-regulated kinase; F-actin, filamentous actin; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HIVE, human umbilical vein endothelial; PCR, polymerase chain reaction; SH3, Src homology 3; wt, wild type; PBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.
followed by a proline-rich region. Our previous studies have determined that Src targets cortactin primarily at three residues (Tyr-421, Tyr-466, and Tyr-482) between the proline-rich and the SH3 domain (20). In vitro, cortactin binds to and cross-links F-actin into meshworks (18). The F-actin cross-linking activity of cortactin can be dramatically reduced upon tyrosine phosphorylation mediated by Src (18). The role of Src in the function of cortactin was also appreciated during the study of Src-deficient cells (Src−/−) in which cortactin fails to respond to FGF-1 for tyrosine phosphorylation and shows less association with polarized lamellipodia. Concomitant with low levels of tyrosine phosphorylation of cortactin, Src−/− cells are less motile compared with normal cells and resistant to FGF-1-mediated shape changes (19). These data suggest that tyrosine phosphorylation of cortactin plays an important role in the dynamic change of the actin cytoskeleton induced by growth factors. In the study presented here, we examined the role of cortactin and Src in the injury of endothelial cells induced by H2O2. We found that H2O2 induces a significant increase in tyrosine phosphorylation of human endothelial cortactin in a manner dependent on the activity of Src. Furthermore, overexpression of wild-type cortactin enhances the response of endothelial cells to H2O2, whereas overexpression of a cortactin mutant deficient in tyrosine phosphorylation significantly reduces the H2O2-mediated injury of endothelial cells. Thus, this study demonstrates that the signal pathway involving Src and cortactin is implicated in the injury of endothelial cells.  

EXPERIMENTAL PROCEDURES  
Reagents—H2O2, dimethyl sulfide (MeSO), and heparin were purchased from Sigma. PP2, SB203580, and PD098059 were from Calbiochem. LipofectAMINE and G418 were purchased from Life Technologies, Inc. Protein A-Sepharose CL-4B and ECL Western blotting kits were from Amersham Pharmacia Biotech. Antibodies—Monoclonal anti-phosphotyrosine antibody (4G10) and monoclonal anti-cortactin antibody 4F11 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against Erk (both type 1 and type 2 forms) were from Promega (Madison, WI). Polyclonal cortactin antibodies were purchased as described previously (21).  
Cell Culture—Human umbilical endothelial (HUVE) cells were purchased from Clonetics (Walkersville, MD). HUVE cells were grown in medium M199 supplemented with 10% (v/v) fetal bovine serum and antibiotics. DMEM supplemented with 10% FBS, 1 mM minimal essential medium, sodium pyruvate, 2 mM L-glutamine, antibiotics, 1 μg/ml tetracycline, 2 μg/ml puromycin, and 0.3 mg/ml G418. Packaging cells (1 × 106) were transfected with MGIn viruses using Superfect transfection reagent (Qiagen Inc., Valencia, CA). To harvest viruses, the transfectants were grown in DMEM containing 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine. The medium of the transfectants was collected at 48, 72, and 96 h after transfection and filtered through a 0.45-μm filter (Gelman Sciences, Ann Harbor, MI). The virus medium was stored at −70 °C.  
Viral Infection—HUVE cells were plated at density of 1 × 105 in 35-mm dishes. On the next day, the medium was replaced with 1 ml of viral supernatant containing 8 μg/ml Polybrene. After 24 h of incubation, the medium was exchanged to M199 containing 10% FBS, 10 ng/ml FGF-1, and 10 μg/ml heparin. Expression of GFP proteins was monitored by fluorescent microscopy. To increase the efficiency of infection, the cells were infected with viruses for two or three times. Fluorescence-activated Cell Sorting Analysis and Sorting—Infected HUVE cells were trypsinized. The suspended cells (2 × 106) were washed one time with PBS supplemented with 2% FBS. The washed cells were then resuspended in PBS plus 2% FBS and sorted in a fluorescence-activated cell sorting system (Beckton Dickinson, Franklin Lakes, NJ) according to light scatter and fluorescence intensity.  
Phosphotyrosine Immunoblot Analysis—Cells were extracted in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml Na3VO4, and 1 mM NaF). The extracts were centrifuged at 14,000 × g for 10 min at 4 °C. The clarified supernatants were immunoprecipitated with polyclonal cortactin antisera 2719 (21). The immunoprecipitates were resolved in a SDS-polyacrylamide gel electrophoresis (7.5%, w/v), transferred to a nitrocellulose membrane, and further blotted with a monoclonal phosphotyrosine antibody (4G10). In some experiments, the blot membrane was stripped and re-blotted with monoclonal cortactin antibody 4F11 as described previously (22).  
Analysis of Activated Mitogen-activated Protein Kinases—Cells were lysed in 0.5 ml of 2× SDS sample buffer. The cell lysates were analyzed in 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blotted with either active Erk antibody or Erk antibody.  
Immunofluorescent Microscopy—Cells were plated on glass coverslips pre-coated with fibronectin. After treatment, the cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS for 5 min. The permeabilized cells were incubated with a monoclonal cortactin antibody 4F11 at the concentration of 0.2 μg/ml in PBS containing 5% bovine serum albumin for 1 h. The cells were then incubated for 1 h with rhodamine-conjugated goat anti-mouse IgG (Pierce) at the concentration of 5 μg/ml and fluorescein isothiocyanate (FITC)-labeled phallolidin at the concentration of 1 μM. Between each step, three washes with PBS were applied. After staining with antibodies, the cells were mounted on a glass slide and inspected under a laser confocal scanning or fluorescent microscope. RESULTS  
H2O2 Induces Tyrosine Phosphorylation of Cortactin—In an attempt to study the mechanism by which oxygen radicals induce the injury of endothelial cells, we examined tyrosine phosphorylation of cortactin in HUVE cells upon exposure to H2O2. Cortactin was immunoprecipitated from the lysates of HUVE cells treated with H2O2 for 1 h at concentrations up to 2 mM. The precipitates were further immunoblotted with a phosphotyrosine antibody. As shown in Fig. 1A, H2O2 induced tyrosine phosphorylation of cortactin in a dose-dependent manner. Significant increase in the tyrosine phosphorylation of cortactin was apparent in the presence of 1.4 mM H2O2, and reached a maximal level at 2 mM. To evaluate the function of tyrosine phosphorylation of cortactin induced by H2O2, we examined morphological changes of endothelial cells treated with H2O2 at different concentrations by analyzing actin filament staining, that with FITC-labeled phallolidin. As shown in Fig. 1B, cells treated with H2O2 exhibited significant cytoskeletal reorganization and shape changes characterized by formation of membrane blebbing and cytoplasmic shrinkage. However, these changes only occurred when cells were treated with H2O2 at high doses (greater than 1.6 mM) when phosphorylation of cortactin was apparent. Thus, the apparent morphological...
Changes were correlated with increased in the levels of tyrosine phosphorylation of cortactin.

Although high doses of H$_2$O$_2$ were required to induce morphological changes that correlate with cortactin tyrosine phosphorylation, the conditions also exerted a toxic effect on cells, as indicated by increased cell detachment from culture dishes (data not shown). To reduce the cytotoxicity of H$_2$O$_2$, we attempted to search for less harsh conditions using lower concentrations of H$_2$O$_2$. It has been reported that H$_2$O$_2$ induced a protective pathway involving Erk, and inhibition of Erk can enhance the response of cells to apoptotic signals (23–27). Thus, we examined the response of cells to H$_2$O$_2$ in the presence of PD098059, a specific inhibitor for the activation of Erk (28). As shown in Fig. 2A, the presence of 100 $\mu$M PD098059 enhanced dramatically the increase in tyrosine phosphorylation of cortactin even at 50 $\mu$M H$_2$O$_2$. Similarly, titration of different amounts of PD098059 indicated that the inhibitor at 50 $\mu$M is sufficient to induce tyrosine phosphorylation of cortactin in the presence of both H$_2$O$_2$ and PD098059 was readily detected at 30 min after treatment and reached a maximal level at approximately 45 min (Fig. 2C).

Concomitant with increased cortactin tyrosine phosphorylation, inhibition of ERK also potentiated the morphologic response of cells to H$_2$O$_2$. There were no apparent shape changes when cells were exposed to either H$_2$O$_2$ alone at 100 $\mu$M (Fig. 3A, c) or PD098059 alone (data not shown), although careful examination revealed a slight increase in the formation of stress fibers in the cells treated with H$_2$O$_2$ alone (Fig. 3A, d). In contrast, cells treated with H$_2$O$_2$ plus PD098059 showed dramatic shape changes characterized by formation of membrane blebbings. (Fig. 3A, e and f). The H$_2$O$_2$-induced shape changes in the presence of PD098059 appeared to require the activity of Src because these changes did not occur when cells were pretreated with PP2, a selective inhibitor of Src (Fig. 3A, g and h). PP2 also inhibited the shape changes induced by H$_2$O$_2$ alone at 2 mM (data not shown). The effect of H$_2$O$_2$ on the shape changes of HUVE cells was also quantified by measuring the numbers of cells forming membrane blebs and cytoplasm shrinkage (Fig. 3B). Based on these criteria, H$_2$O$_2$ alone at 100 $\mu$M induced shape changes in 18% of cells, whereas H$_2$O$_2$ plus PD098059 was able to induce changes in nearly 45% of cells. The shape changes were significantly reduced by more than 50% in the presence of PP2.

PD098059 enhances the effect of H$_2$O$_2$ at the concentrations that also inhibit activation of Erk, as determined by immunoblot with an antibody specifically against active Erk (Fig. 4, lanes 3 and 4; data not shown). However, the inhibition of Erk does not appear to be necessary for the H$_2$O$_2$-induced injury of endothelial cells. First, shape changes of HUVE cells could...
be also induced when cells treated with H$_2$O$_2$ at high doses (greater than 1.6 mM) in the absence of PD098059 (Fig. 1B), the condition under which Erk was also activated (Fig. 4, lane 6). Second, when cells were treated with H$_2$O$_2$ at a low concentration (100 mM) plus vanadate, a mixture (peroxide vanadate) that can efficiently increase the tyrosine phosphorylation of cortactin and induce shape changes of HUVE cells (data not shown) was also able to induce efficiently the activation of Erk (Fig. 4, lane 6). Thus, inhibition of Erk potentiates the effect of H$_2$O$_2$ but is not required for shape changes. In contrast, H$_2$O$_2$-induced shape changes of endothelial cells are more intimately associated with the increase in tyrosine phosphorylation of cortactin. Because the condition where cells are exposed to PD098059 and H$_2$O$_2$ at low concentrations appeared to be milder and causes less cell detachment compared with high concentrations of H$_2$O$_2$, it was used in the rest of experiments described below.

Expression of Cortactin Mutant Confers Cells Resistant to H$_2$O$_2$-mediated Shape Change—To further evaluate the role of tyrosine phosphorylation of cortactin in the injury of endothelial cells, we constructed several retroviruses encoding a green fluorescent protein (GFP)-tagged wild-type cortactin and a cortactin mutant CortF421F466F482, which is deficient in tyrosine phosphorylation (20). To ensure that most cells to be analyzed express GFP-cortactin proteins, infected cells were sorted based on GFP by a flow cytometry system. After sorting, 80% cells exhibited green under a fluorescent microscope. GFP-cortactin proteins were also evaluated by immunoblot, which demonstrated comparative levels of GFP-cortactin proteins with endogenous cortactin (Fig. 5). Furthermore, the GFP-wt-cortactin behaved similarly to endogenous cortactin because it could be phosphorylated in response to H$_2$O$_2$ as efficiently as...
the endogenous cortactin. In contrast, the mutant GFP-CortF421F466F482 was unable to be phosphorylated under the same conditions, which confirmed our previous conclusion that Tyr-421, Tyr-466, and Tyr-482 are the primary sites for Src in vivo (20).

The cells expressing different forms of GFP-cortactin variants respond differentially to H$_2$O$_2$. The GFP-wt-cortactin expressors developed significant shape changes as early as 30 min after treatment (Fig. 6A, e and h). Because the similar changes in control cells expressing GFP only were not observed until 1 h after treatment, the susceptibility of cells to H$_2$O$_2$ appeared to be enhanced by overexpression of GFP-wt-cortactin. In contrast, cells expressing CortF421F466F482 showed little difference in morphology compared with untreated cells either by 30 min or by 1 h of treatment (Fig. 6A, f and i), indicating that the mutant acts in a dominant negative fashion.

The activities of cortactin variants were also evaluated in NIH 3T3 cells stably transfected with Myc-tagged cortactin proteins (20). Like endothelial cells, overexpression of Myc-wt-cortactin enhanced the response of NIH 3T3 cells to H$_2$O$_2$, the effectors could develop a dramatic shape change as characterized by rounding up and aggregation either in the presence of H$_2$O$_2$ plus PD098059 or H$_2$O$_2$ alone at 200 μM. Under the same conditions, no apparent changes were observed with control cells expressing vector only. In contrast to cells expressing Myc-wt-cortactin, no significant changes were observed with cells overexpressing Myc-CortF421F466F482 in the presence of either H$_2$O$_2$ alone or H$_2$O$_2$ plus PD098059. These data confirm that overexpression of cortactin enhances H$_2$O$_2$-induced shape changes in a tyrosine phosphorylation-dependent manner.

**DISCUSSION**

In this study, we provide evidence that tyrosine phosphorylation of cortactin is required for H$_2$O$_2$-induced shape changes in human endothelial cells. First, either H$_2$O$_2$ alone at high concentrations (more than 1.6 mM) or H$_2$O$_2$ at a low concentrations (100 μM) in the presence of PD098059, a selective inhibitor for Erk, induces a significant increase in tyrosine phosphorylation of cortactin. Tyrosine phosphorylation of cortactin appears to be one of the major events induced by H$_2$O$_2$, as indicated by analyzing total phosphotyrosyl proteins (data not shown). Second, the level of tyrosine phosphorylation of cortactin is correlated with the shape changes induced by H$_2$O$_2$. The conditions that induce high levels of tyrosine phosphorylation of cortactin are also able to induce shape changes of endothelial cells. These conditions include high concentrations of H$_2$O$_2$, H$_2$O$_2$ plus PD098059, and H$_2$O$_2$ plus vanadate. In addition, tyrosine phosphorylation induced by H$_2$O$_2$ is time-dependent and plateaus at 45 min (Fig. 2C). This kinetics of tyrosine phosphorylation of cortactin is correlated with shape changes induced by H$_2$O$_2$ (data not shown). Third, tyrosine phosphorylation of cortactin is dependent upon the activity of Src. Treatment of cells with a selective Src inhibitor PP2 can abrogate tyrosine phosphorylation of cortactin (data not shown) as well as the shape changes induced by H$_2$O$_2$ (Fig. 3). Finally, overexpression of a cortactin mutant deficient in tyrosine phosphorylation can significantly inhibit shape changes induced by H$_2$O$_2$ either in endothelial cells or in NIH 3T3 cells (Figs. 6 and 7).

Cortactin, a prominent substrate of Src, is a potent filament actin-binding protein. *In vitro*, cortactin also exhibits a potent activity to cross-link actin filaments into a filamentous meshwork (21). Importantly, this F-actin cross-linking activity can be downregulated upon tyrosine phosphorylation mediated by Src. Thus, tyrosine phosphorylation of cortactin likely constitutes an important mechanism by which Src or its-related protein-tyrosine kinases regulate the dynamics of the actin cytoskeleton. Consistent with the role of tyrosine phosphorylation of cortactin in cell shape changes, cells such as Src−/− cells in which cortactin is deficient in tyrosine phosphorylation are more resistant to shape changes induced by extracellular stimuli compared with cells with elevated tyrosine phosphorylation of cortactin (19). Similarly, non-phosphorylated cortactin tend to accumulate within the cytoplasm. Thus, the cortactin mutant deficient in tyrosine phosphorylation likely acts as a dominant negative fashion (20). Conversely, cells overexpressing wild-type cortactin increase susceptibility to H$_2$O$_2$ (Figs. 6 and 7).

Upon exposure to H$_2$O$_2$, many endothelial cells develop extensive membrane blebs. Similar morphological changes were also observed in cells expressing high levels of cortactin. For example, tumor cells with high levels of cortactin expression due to gene amplification often develop large spherical membrane protrusions. Furthermore, transient transfection of GFP-wt-cortactin, which led to expression of extreme high levels of expression, can also result in apoptosis-like membrane blebbing. Membrane blebbing may involve a mechanism similar to the formation of membrane protrusions, lamellipodia, and filopodia (29). In normal cells, cortactin is mainly associated with cell cortical structures, including lamellipodia, membrane ruffles, and punctate-like protrusions. Within these

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2 E. Schuuring, personal communication.
3 X. Zhan, unpublished result.
structures, cortactin co-localizes with F-actin (20, 30). We found that H_2O_2-induced blebs are rich in cortactin (Fig. 3), suggesting that locally concentrated cortactin may be implicated in the formation of membrane blebs. The primary driving force to form membrane protrusions is the actin polymerization occurring underneath the plasma membrane (31). One possibility is that cortactin could be directly implicated in actin polymerization. Evidence to support this possibility is our recent finding that cortactin is a potent activator of Arp2/3, a protein complex that is responsible for the nucleation of actin polymerization. Cortactin may also contribute to membrane blebbing through its regulation of F-actin cross-linking. It has been postulated that the flow of the cortical actin gel is a determining factor in the formation of membrane blebs (7). The rate of flow of the actin gel is inversely regulated by the activity of F-actin cross-linking proteins. Thus, tyrosine-phosphorylated cortactin, which has a significant lower F-actin cross-linking activity than non-phosphorylated cortactin (18), would increase the flow of the actin gel and eventually favor a tuned balance toward the formation of membrane blebs. Indeed, human melanoma cell lines deficient in an actin filament cross-linking protein, ABP-280, show prolonged and extensive membrane blebbing (7).

The effect of H_2O_2 on the shape changes in endothelial cells can be dramatically enhanced by PD098059, a chemical that specifically inhibits the activity of Erk 1/2. However, the role of activation of Erk in the oxidant-mediated injury of endothelial cells is still not clear. Because the activation of Erk is implicated in the signal pathways of growth factors, Erk may represent a survival factor for cells to antagonize the effect of H_2O_2 (15). Although our data appear to be consistent with this view, we did not find an intimate correlation of the activity of Erk either with phosphorylation of cortactin nor with shape changes. Exposure to H_2O_2 at high concentrations or H_2O_2 at low concentrations in the presence of vanadate can increase tyrosine phosphorylation of cortactin and shape changes as well (Fig. 4). These treatments also induce significant increase in the activity of Erk (Fig. 4). Thus, inhibition of Erk by PD098059 is not necessary either for the H_2O_2-mediated tyrosine phosphorylation of cortactin or for cell shape changes. It appears that inhibition of Erk only affects tyrosine phosphorylation of cortactin when cells expose to low concentrations of H_2O_2. One explanation is that inhibition of Erk may potentiate the activation of Src related kinases by H_2O_2. It has been shown that extracellular H_2O_2 can activate the intracellular Ras pathway (32). Recent studies also demonstrated that Ras can further activate both Src via Raf and Erk via Raf independently (16, 33). Thus, it is possible that inhibition of the activation of Erk pathway would favor the pathway by which Ras/Ral

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4 T. Uruno, J. Liu, B. Zhang, X. Fan, and X. Zhan, personal communication.
5 Y. Li, unpublished data.
activates Src and leads to apparent increase in the tyrosine phosphorylation of cortactin.

In summary, the data presented in this study demonstrate an important role of Src and cortactin in H$_2$O$_2$-induced shape changes of endothelial cells. The future studies using small antagonists for cortactin may reveal a novel approach to target specifically at the actin cytoskeleton and protect endothelium from reactive oxygen species.

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REFERENCES
1. Hull, D. S., Green, K., Thomas, L., and Alderman, N. (1984) Invest. Ophthalmol. Vis. Sci. 25, 1246–1253
2. Schror, K., Thiennemann, C., and Ney, P. (1988) Naunyn Schmiedebergs Arch. Pharmacol. 338, 268–274
3. Sacks, T., Moldow, C. F., Craddock, P. R., Bowers, T. K., and Jacob, H. S. (1978) J. Clin. Invest. 61, 1161–1167
4. Orr, F. W., Wang, H. H., Lafrenie, R. M., Scherbarth, S., and Nance, D. M. (1990) J. Pathol. 160, 310–329
5. Nakamura, H., Nakamura, K., and Yodoi, J. (1997) Annu. Rev. Immunol. 15, 351–369
6. Hinchaw, D. B., Sklar, L. A., Bohl, B., Schraufstatter, I., Hyslop, P. A., Rossi, M. W., Spragg, R. G., and Cochrane, C. G. (1986) Am. J. Pathol. 125, 454–464
7. Cunningham, C. C. (1995) J. Cell Biol. 129, 1589–1599
8. Hastie, L. E., Patton, W., Hechtman, H. B., and Shepro, D. (1998) J. Cell. Biochem. 68, 511–524
9. Peus, D., Vasa, R. A., Meves, A., Pott, M., Beyerle, A., Squillace, K., and Pittelkow, M. R. (1998) J. Invest Dermatol. 110, 966–971
10. Berk, B. C. (1999) Thromb. Haemost. 82, 810–817
11. Natarajan, V., Scribner, W. M., Al Hassani, M., and Vepa, S. (1998) Environ. Health Perspect. 106, 1205–1212
12. Abe, J., Takahashi, M., Ishida, M., Lee, J. D., and Berk, B. C. (1997) J. Biol. Chem. 272, 20389–20394
13. Carbajal, J. M., and Schaeffer, R. C., Jr. (1998) Biochem. Cell Biol. 249, 461–466
14. Barchowsky, A., Munro, S. R., Morana, S. J., Vincenti, M. P., and Treadwell, M. (1995) Am. J. Physiol. 269, L829–L836
15. Guyot, K. Z., Liu, Y., Gerope, M., Xu, Q., and Holbrook, N. J. (1996) J. Biol. Chem. 271, 4138–4142
16. Goi, T., Shipton, M., Lu, Z., Foster, D. A., Kline, S. G., and Feig, L. A. (2000) EMBO J. 19, 623–630
17. Zhan, X., Haudenschild, C. C., Ni, Y., Smith, E., and Huang, C. (1997) Blood 89, 457–464
18. Huang, C., Ni, Y., Gao, Y., Wang, T., Haudenschild, C. C., and Zhan, X. (1997) J. Biol. Chem. 272, 13911–13915
19. Liu, J., Huang, C., and Zhan, X. (1999) Oncogene 18, 6700–6706
20. Huang, C., Liu, J., Haudenschild, C. C., and Zhan, X. (1998) J. Biol. Chem. 273, 25770–25776
21. Zhan, X., Hu, X., Hampton, B., Burgess, W. H., Friesel, R., and Maciag, T. (1993) J. Biol. Chem. 268, 24427–24431
22. Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R., and Parsons, J. T. (1991) Mol. Cell. Biol. 11, 5113–5124
23. Levy-Toledano, S., Grelac, F., Caen, J. P., and Machouf, J. (1995) Thromb. Haemost. 73, 857–861
24. Kuroda, K., Ozaki, Y., Qi, R., Asazuma, N., Yatomi, Y., Satoh, K., Nomura, S., Suzuki, M., and Kume, S. (1995) J. Immunol. 155, 4427–4436
25. Yanaga, P., Posie, A., Asselin, J., Blake, R., Schievien, G. L., Clark, E. A., Law, C. L., and Watson, S. P. (1995) Biochem. J. 311, 471–478
26. Aikawa, R., Komuro, I., Yamaraki, T., Zou, Y., Kodoh, S., Tanaka, M., Shiojima, I., Hiroi, Y., and Yazaki, Y. (1997) J. Clin. Invest. 100, 1813–1821
27. Houst, J., Houle, P., Rouxseau, S., Deschesnes, R. G., Shah, G. M., and Landry, J. (1998) J. Cell Biol. 143, 1361–1373
28. Alesci, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494
29. Hagmann, J., Burger, M. M., and Dagan, D. (1999) J. Cell. Biochem. 73, 488–499
30. Wu, H., and Parsons, J. T. (1993) J. Cell Biol. 120, 1417–1426
31. Mitchison, T. J., and Cranner, L. P. (1996) Cell 84, 371–379
32. Lande, H. M., Ogiste, J. S., Teng, K. K., and Novogrodsky, A. (1995) J. Biol. Chem. 270, 21195–21198
33. Spaargaren, M., and Bischoff, J. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12609–12613