SAPK2/p38-dependent F-Actin Reorganization Regulates Early Membrane Blebbing during Stress-induced Apoptosis

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Abstract. In endothelial cells, H₂O₂ induces the rapid formation of focal adhesion complexes at the ventral face of the cells and a major reorganization of the actin cytoskeleton into dense transcytoplasmic stress fibers. This change in actin dynamics results from the activation of the mitogen-activated protein (MAP) kinase stress-activated protein kinase-2/p38 (SAPK2/p38), which, via MAP kinase-activated protein (MAPKAP) kinase-2/3, leads to the phosphorylation of the actin polymerization modulator heat shock protein of 27 kD (HSP27). Here we show that the concomitant activation of the extracellular signal-regulated kinase (ERK) MAP kinase pathway by H₂O₂ accomplishes an essential survival function during this process. When the activation of ERK was blocked with PD098059, the focal adhesion complexes formed under the plasma membrane, and the actin polymerization activity led to a rapid and intense membrane blebbing. The blebs were delimited by a thin F-actin ring and contained enhanced levels of HSP27. Later, the cells displayed hallmarks of apoptosis, such as DEVD protease activities and internucleosomal DNA fragmentation. Bleb formation but not apoptosis was blocked by extremely low concentrations of the actin polymerization inhibitor cytochalasin D or by the SAPK2 inhibitor SB203580, indicating that the two processes are not in the same linear cascade. The role of HSP27 in mediating membrane blebbing was assessed in fibroblastic cells. In control fibroblasts expressing a low level of endogenous HSP27 or in fibroblasts expressing a high level of a nonphosphorylatable HSP27, H₂O₂ did not induce F-actin accumulation, nor did it generate membrane blebbing activity in the presence or absence of PD098059. In contrast, in fibroblasts that expressed wild-type HSP27 to a level similar to that found in endothelial cells, H₂O₂ induced accumulation of F-actin and caused bleb formation when the ERK pathway was inhibited. Cisplatinum, which activated SAPK2 but induced little ERK activity, also induced membrane blebbing that was dependent on the expression of HSP27. In these cells, membrane blebbing was not followed by caspase activation or DNA fragmentation. We conclude that the HSP27-dependent actin polymerization–generating activity of SAPK2 associated with a misassembly of the focal adhesions is responsible for induction of membrane blebbing by stressing agents.

Key words: SAPK2/p38 • HSP27 • F-actin • blebbing • apoptosis

Membrane blebbing is an early manifestation of toxicity. In vivo, it has been observed after cell injury in liver, brain, heart, and kidney. In vitro, it is frequently found after exposure of cells to stressful stimuli (for review see Gores et al., 1990). The physiopathological consequence of bleb formation in vivo may be dramatic. Surface blebbing of endothelial cells results in a narrowing of the vascular lumen, which would increase the vascular resistance, leading ultimately to cardiac failure (Becker and Ambrosio, 1987). Bleb shedding from ischemic renal tubule cells may lead to tubular obstruction (Phelps et al., 1989). In liver, bleb shedding from hepatocytes seems to be responsible for the release in the blood stream of viral antigens, such as hepatitis B, as well as the hepatic enzymes that are routinely used clinically as an indication of hepatic injury (Gores et al., 1990). The mechanisms of blebbing are not well understood. Membrane blebbing has been associated with various biochemical alterations (including a decrease in ATP levels, increase in cellular calcium, activation of nonlysosomal

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The proteolytic system, and activation of phospholipase A₂ as well as with cytoskeletal alterations affecting the dynamics of actin and myosin and the formation of focal adhesion complexes (Fishkind et al., 1991; Cunningham, 1995; Martin et al., 1995; Miyoshi et al., 1996; Mills et al., 1998).

Apoptosis defines a type of regulated cell death associated with various morphological features that include cell shrinkage, chromatin condensation, nuclear/cytoplasmic fragmentation, and formation of dense bodies that are quickly removed via phagocytosis by neighboring cells (Martin et al., 1994). Apoptosis can result from a genetic program aimed at regulating the cell number in different physiological and pathological conditions. Apoptosis is also induced in response to environmental changes, namely growth factor withdrawal (Xia et al., 1995), loss of normal adhesion to extracellular matrix during anoikis (Frisch and Francis, 1994; Re et al., 1994), or exposures to a variety of stresses and cytotoxic drugs (Verheij et al., 1996; Zanke et al., 1996; Lee et al., 1997). Once initiated, the apoptotic program involves activation of a series of biochemical events that underlie the morphological hallmarks of apoptosis. This includes increased intracellular Ca²⁺ concentrations, uncoupling of mitochondrial electrical potential, internucleosomal DNA fragmentation, and proteolytic degradation of specific substrates, such as poly-(ADP-ribose)-polymerase (PARP) and lamin. Proteolysis is achieved through activation of cysteine proteases called caspasas, a family of enzymes related to the interleukin 1β-converting enzyme (ICE) or ICE/CED3 proteases (Juo et al., 1997). In vitro and in vivo studies showed that caspasas act in a cascade to convey the apoptotic signal (Darmon et al., 1995; Na et al., 1996).

In most cases, the signaling pathways that lead to membrane blebbing as well as those that link the apoptotic signal to activation of the death execution programs remains to be clarified. The mitogen-activated protein (MAP) kinase ERK1/2 (extracellular signal-regulated kinase) and the stress-activated protein kinases-1 (SAPK1/JNK) and -2 (SAPK2/p38) are central elements of homologous pathways used by mammalian cells to orchestrate the signals generated by environmental stimuli (Waskiewicz and Cooper, 1995; Cohen, 1997). These MAP kinases are activated by many agents that induce apoptosis, such as oxidative stress, inflammatory cytokines, UV light, and anticancer drugs. SAPK1 and SAPK2 appear as important modulators of the apoptotic program. For example, SAPK1 modulates apoptosis initiated by Fas/APO, tumor necrosis factor α (TNFα), oxidative stress, heat shock, and the anticancer drug cis-platinum (Wei et al., 1995; Caihil et al., 1996; Verheij et al., 1996; Zanke et al., 1996). In some cell lines and in certain contexts, activation of SAPK1 lies upstream of caspase-3-mediated apoptosis (Seimiya et al., 1997), while in others, activation of SAPK1/JNK is downstream of ICE-like protease (Mosser et al., 1997). In anoikis, for example, caspasas activate MEKK-1, an activator of SAPK1, suggesting that SAPK1 lies downstream of caspase (Cardone et al., 1997). SAPK2 modulates apoptosis induced by sodium salicylate in Rat-1 cells (Schwenger et al., 1997), glutamate in rat cerebellar granule cells (Kawasaki et al., 1997), withdrawal of trophic factors in Rat-1 fibroblasts and differentiated PC12 cells (Kummer et al., 1997), and Fas activation in Jurkat clone A3 (Brenner et al., 1997; Jou et al., 1997). Fas induction of SAPK2 activity requires the action of ICE/CED3 in Jurkat clone A3, whereas etoposide activation of SAPK2-mediated apoptosis relies on protease-independent mechanisms or on proteases unrelated to caspases-3 (Juo et al., 1997). Recently, apoptosis signal-regulating kinase (ASK) 1 has been identified as a redox-sensitive MAP kinase kinase kinase that activates SAPK1 and SAPK2 during apoptosis induced by TNFα (Saitoh et al., 1998). Whereas SAPK1 and SAPK2 appear to be involved in modulating apoptosis, ERK is generally considered as a survival factor (Xia et al., 1995). In fact, growth factor withdrawal down-regulates ERK and induces apoptosis in several cell systems, whereas activation of ERK during stress can be protective (Xia et al., 1995; Guyton et al., 1996). Apoptosis has been reported to be regulated by a balance between activation of SAPKs and ERK (Xia et al., 1995).

Vascular endothelial cells are in heavy contact with circulating oxyradicals produced or released from normal oxygen metabolism, oxidizable xenobiotics, or the respiratory burst of activated phagocytic cells, monocytes, macrophages, and neutrophils (Hardy et al., 1994). Endothelial cells themselves produced oxyradicals after abnormal oxygenation, such as intermittent hypoxia or hypoxia alternating with hypoxia (Zuluet et al., 1997). Under these circumstances, they produce large amounts of superoxide anions, at least partially because of the conversion of xanthine dehydrogenase to xanthine oxidase during hypoxia (Michiels et al., 1992). Nitric oxide, another type of oxyradical, is released from endothelial cells in response to various agonists, including histamine and VEGF (Van de Voorde et al., 1987; van der Zee et al., 1997). We have previously shown that exposure to H₂O₂ induced in endothelial cells a SAPK2-dependent physiological-like reorganization of the F-actin cytoskeleton that rearranges from cortical ruffles into trans-cytoplasmic stress fibers (Huot et al., 1997). This process was shown to be dependent on the SAPK2/MAPKAPK-2/3–induced phosphorylation of the actin polymerization modulator HSP27. Exposures to similar oxidative stress resulted in deleterious fragmentation of F-actin in fibroblasts and to intense surface blebbing in hepatocytes (Huot et al., 1996; Mirabelli et al., 1988). This illustrates the high level of intrinsic resistance of endothelial cells to oxidative stress.

In the present study, we show that membrane blebbing is induced when the ERK pathway is not appropriately co-activated with SAPK2 in endothelial cells exposed to oxidative stress. We provide several lines of evidence supporting that membrane blebbing results from the strong F-actin polymerization activity generated by activation of SAPK2 and from a defect in the assembly of focal adhe-
sions. A similar SAPK2 and HSP27 phosphorylation–dependent cell blebbing was observed in fibroblastic cells treated with the antineoplastic drug cis-platinum, suggesting that activation of the SAPK2/MAPKAPK-2/HSP27 pathway represents, at least in some cellular context, a mechanism for the early formation of blebs. We also show that membrane blebbing can be dissociated from caspase activation and internucleosomal DNA fragmentation.

**Materials and Methods**

**Materials**

[y$_{32}$P]ATP (3,000 Ci/mmol) was purchased from NEN$^{\text{TM}}$ Life Science Products, Boston, MA. H$_{2}$O$_{2}$, VEGF$_{mu}$ and endothelial cell growth supplement (ECGS) were from Sigma Chemical Co. (St. Louis, MO). Recombinant HSP27 was purified from Escherichia coli transformed with a plasmid containing the coding sequence for Chinese hamster HSP27. SB203580 and PD098059 were purchased from Calbiochem (La Jolla, CA) and Research Biochemical International (Natick, MA). These inhibitors were diluted in DMSO to make stock solutions of 40 and 20 mM, respectively. Cytochalasin D was purchased from Sigma Chemical Co., zVAD-fmk was purchased from Enzyme System Products (Livermore, CA), and DEVDase was purchased from BIORAD Research Laboratories (Plymouth Meeting, PA). Chemicals for electrophoresis were obtained from Bio-Rad Labs (Hercules, CA) and Fisher Scientific (Pittsburgh, PA).

**Antibodies**

Hu27ab is a rabbit antiserum that specifically recognizes human HSP27 (Landry et al., 1989). Anti–GST-MAPKAP kinase-2/3 is a rabbit polyclonal antibody raised against a synthetic peptide that corresponds to the 14 COOH-terminal amino acids of rat ERK2 (Huot et al., 1995). This antibody was obtained from Dr. D. Nicholson (Merck Centre for Therapeutics Research, Pointe Claire, Quebec, Canada). Anti–GST-MAPKAP kinase-2/3 was used to detect the p17 subunit of caspase-3. Anti–rabbit IgG-HRP was used to detect the selection gene, Vc protein. The transfectants were maintained in DME containing 1% Triton X-100, 1 mM benzamidine, 100 mM DTT, and 1 mM PMSF. The extracts were vortexed and centrifuged at 17,000 g for 12 min at 4°C. The clarified supernatants were either immediately used for immunoprecipitation or stored at −80°C. The further steps were carried out at 4°C. The clarified supernatant was diluted four times in buffer I (0.2 mM Tris-HCl, pH 7.5, 130 mM NaCl, 0.1 mM EDTA, 1 mM MgCl$_{2}$, 1 mM Na$_{2}$VO$_{4}$, 1% Triton X-100, 1 mM PMSF). Unlabeled antibodies were added in limiting concentrations, and the mixtures were incubated for 1 h. 10–15 μl of protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ) 50% vol/vol in buffer I was added, and the mixtures were incubated for 30 min. Samples were centrifuged for 15 s, and washed three times with 300 μl of buffer I. Immunoprecipitates were directly used for the kinase assays.

**Immunocomplex Kinase Assays**

Kinase activities were assessed in immune complexes. SAPK2 activation was measured by assessing the activity of its substrate MAPKAP kinase-2. The activity of immunoprecipitated MAPKAP kinase-2 was measured using recombinant HSP27 (Huot et al., 1995). The assays were carried out in 25 μl of kinase buffer K (100 μM ATP, 3 μCi of [γ$_{32}$P]ATP [3,000 Ci/ mmol], 40 mM p-nitrophenyl phosphate, 20 μM MOPS pH 7: 10% glycerol, 15 mM MgCl$_{2}$, 0.05% Triton X-100, 1 mM dithiothreitol, 1 mM leupeptin, 0.1 mM PMSF, and 0.3 μg of protein kinase A inhibitor. The kinase activity was assayed for 30 min at 30°C and was stopped by the addition of 10 μl SDS-PAGE loading buffer. Immunoprecipitated ERK2 was assayed analogously using myelin basic protein as substrate (Huot et al., 1997) in buffer K containing 10 mM MgCl$_{2}$. Kinase activities were evaluated by measuring incorporation of the radioactivity into the specific substrates after resolution by SDS-PAGE and quantification using PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**PARP Cleavage Assay**

After treatments, floating cells (F1) were collected in PBS and centrifuged, and the pellet was frozen on dry ice. Adherent cells were incubated for an additional 6 h. The floating cells (F2) were collected and pelleted. The remaining adherent cells were scraped in PARP extraction buffer (62.5 mM Tris, pH 6.8, 2% SDS, 6 M Urea, 10% glycerol, 0.00125% bromophenol blue, 720 mM β-mercaptoethanol) and pooled with F1 and F2. Proteins were separated on 8.5% SDS-PAGE and transferred onto nitrocellulose membrane. PARP was immunodetected by standard Western blotting procedure with the C2-10 antibody (Shah et al., 1995, 1996).

**DEV/Dase Cleavage Activity**

DEV/Dase cleavage activity was determined according to Enari et al. (1996) with some modifications. In brief, after treatments or incubation periods, floating and adherent cells were pooled, and cytosolic extracts were prepared by eight repeated cycles of freezing and thawing in 100 μl of DEV/Dase extraction buffer (50 mM MOPS, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_{2}$, 1 mM DTT, 20 μM cytochalasin D, 1 mM PMSF, 1 μg/l leupeptin, 1 μg/l pepstatin A, 50 μg/l antipain). Extracts were clarified by centrifugation for 12 min in a microfuge at 4°C. Protein extracts (or buffer only for blank) were mixed with 500 μl of ICE standard buffer (100 mM Hepes-KOH, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin) containing 1 μM DEV/Dase-AMC and incubated at 30°C for 30 min. The fluorogenic product substrate AMC was detected by excitation at 380 nm and emission at 460 nm with a luminescence spectrometer (model LS50B; Perkin-Elmer Corp., Norwalk, CT). Caspase activities were normalized for protein concentrations of individual extracts and then were calculated relatively to the activity of the control samples. Caspase-3 activity was evaluated by Western blotting on cell extracts prepared as for PARP cleavage. After extraction, samples containing 35 μg of proteins were separated by SDS-PAGE and transferred on nitrocellulose membrane. Rabbit polyclonal anti–caspase-3 R#MF393 antibody was used to detect the p17 subunit of caspase-3. Anti–rabbit IgG-HRP was used to reveal the antigen–antibody complex.

**Internucleosomal DNA Fragmentation**

Cells prepared as for PARP cleavage assay were lysed in TENS–proteinase K buffer (0.1 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% SDS, 0.2 mg/ml proteinase K) at 37°C for 16 h. After phenol-chloroform extrac-
tion, DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mg/ml RNAse). The DNA was separated into an 1.0% agarose gel.

**Confocal Fluorescence Microscopy**

Confocal microscopy was used for immunofluorescent visualization of F-actin, vinculin, and HSP27 (Huot et al., 1995). The cells were plated on gelatin-coated LabTek dishes (Naperville, IL). After treatment, they were fixed with 3.7% formaldehyde and permeabilized with 0.1% saponin in PBS, pH 7.5. F-actin was detected using FITC-conjugated phalloidin (33.3μg/ml) diluted 1:50 in phosphate buffer. bVNI-1 monoclonal antibody and HU27 antibody were used to detect vinculin and human HSP27, respectively. Vinculin and HSP27 antigen antibody complexes were detected with biotin-labeled anti–mouse IgG (vinculin) or anti–rabbit IgG (HSP27) and were revealed with Texas red–conjugated streptavidin. The cells were examined as previously reported by confocal microscopy with a Bio-Rad MRC-1024 imaging system mounted on a Nikon Diaphot-TDM (Melville, NY) equipped with a 60× objective lens with a 1.4 numerical aperture (Huot et al., 1997).

**Results**

**Activation of ERK and SAPK2 by H2O2**

In primary cultures of exponentially growing HUVECs, oxidants induced a 10-12- and 4-fold activation of SAPK2 and ERK2, respectively (Fig. 1 and Huot et al., 1997). Maximal activation was observed after 5 min in both cases. In contrast, SAPK1/JNK was not activated during the first hour of exposure to H2O2. Some activity was induced after 6 h, suggesting an indirect mechanism of activation (data not shown). Exposure of cells to 10μM SB203580 inhibited H2O2-induced SAPK2 activity as reflected by the inhibition of MAPKAP kinase-2/3 activation, an immediate downstream physiological substrate of SAPK2 (Fig. 1 and Huot et al., 1997). However, inhibition of SAPK2 by SB203580 was not modified by PD098059 (Fig. 1a), nor was the inhibition of ERK by PD098059 modified by SB203580 (Fig. 1b). These findings confirm the high specificity of SB203580 and PD098059 as inhibitors of SAPK2 activity and of ERK activation, respectively (Cuenda et al., 1995; Dudley et al., 1995; Kumar et al., 1996; Rousseau et al., 1997).

**ERK Activation Prevents H2O2-induced Apoptosis**

HUVECs in primary cultures are extremely resistant to cytotoxicity and apoptosis induced by oxidative stress. Exposures to 0.5 mM H2O2 for 1 h induce little cell blebbing, no DEVD cleavage activities, and no DNA fragmentation (Fig. 2). In several cell systems, activation of ERK during stress confers survival advantages. Treatment of cells with activators of the ERK pathways induces protection against stress, whereas inhibiting activation of ERK enhances toxicity (Xia et al., 1995; Guyton et al., 1996; Katoh et al., 1995). Consistent with the notion that ERK activation has protective functions and antiapoptotic properties, we found that blocking the H2O2-induced ERK activation with PD098059 resulted in an intense blebbing activity in up to 60% of the cells immediately after treatment (Figs. 2a and 3c), a progressive increase in zVAD-fmk–sensitive caspase activity (Fig. 2b), PARP cleavage that started around 3 h (Fig. 2e), and after 6 h, a major zVAD-fmk–inhibitable internucleosomal DNA fragmentation (Fig. 2d). PD098059 by itself did not induce any of these manifestations of cytotoxicity. Because activation of SAPK2 has often been associated with activation of apoptosis, we analyzed the contribution of H2O2-induced SAPK2 activity on apoptotic events. In the presence of SB203580, membrane blebbing was inhibited and found in less than 10% of the cells (Fig. 2a). The inhibition occurred without any significant modification in the number of adherent cells (data not shown). SB203580 had no effect on DEVDase and caspase-3 activities (Fig. 2, b and c) or on DNA fragmentation (Fig. 2, d), but it did inhibit the cleavage of PARP (Fig. 2e). Overall, these results indicate that activation of ERK contributed to prevent induction of several manifestations of H2O2-induced cytotoxicity and apoptosis, among which, some were dependent on SAPK2 activity, like cell blebbing. Subsequent investigations reported in this manuscript focused on SAPK2-dependent mechanisms of cell blebbing.

**Membrane Blebbing Is Modulated by the SAPK2/HSP27-dependent Regulation of F-Actin Dynamics and Involves Misassembly of Focal Adhesions**

One major consequence of SAPK2 activation in HUVECs is a reorganization of the actin filaments. In control HUVECs, actin microfilaments are found mostly in cortical structures forming characteristic ruffles (Fig. 3a). Exposures to 0.5 mM H2O2 for 1 h induced a dramatic SAPK2-dependent reorganization of F-actin, which rearranged from cortical microfilaments into long stress fibers that traversed the cells (Fig. 3b). This reorganization was totally inhibited by SB203580 (data not shown and Huot et al., 1997). Intriguingly, inhibiting ERK activation was associated with dramatic changes in the H2O2-induced microfilament organization. Instead of forming transcyto-
plasmic stress fibers, F-actin accumulated at the periphery of the cell, where actin filaments formed a dense spherical network separating the blebs from the cytoplasm. F-actin also accumulated at the perimeter of the blebs (Figs. 3 c and 6 c). In presence of SB203580, the inhibition of membrane blebbing was associated with an almost total reversion of the F-actin reorganization toward control cells (Fig. 3 d). To determine whether membrane blebbing was a direct consequence of the SAPK2-mediated F-actin polymerization activities, we looked at the effect of the actin polymerization inhibitor cytochalasin D using a very low concentration (10 nM), just sufficient to inhibit microfilament reorganization without significantly affecting the filament organization of uninduced cells. At this concentration, cytochalasin D blocked the formation of blebs induced by the combination of PD098059 and H$_2$O$_2$ and partially restored the normal pattern of F-actin as seen in untreated cells (Fig. 3 e). Conversely, membrane blebbing was not blocked by treating the cells with zVAD-fmk, which totally inhibited caspase activities and DNA fragmentation (Figs. 3 f and 2, b and d). Furthermore, cytochalasin D, like SB203580, had no influence on the induction of DEVDase activities and internucleosomal DNA fragmentation (data not shown and Fig. 2, b and d). We next verified whether inhibition of ERK was a sufficient condition to induce membrane blebbing. HUVECs pretreated (Fig. 4, b, d, and f) or not (Fig. 4, a, c, and e) with PD098059 were treated with FGF (Fig. 4, c and d) to activate ERK but not SAPK2 or VEGF (Fig. 4, e and f) to activate both ERK and SAPK2 (Huot et al., 1997; Rousseau et al., 1997). Consistent with a role of SAPK2 in actin reorganization, we found that VEGF but not FGF induced stress fiber formation in HUVECs (Fig. 4, c and e). However, neither FGF (Fig. 4, c and d) nor VEGF (Fig. 4, e and f) induced membrane blebbing in presence of PD098059. Moreover, stress fiber formation after VEGF was maintained even in presence of PD098059.

The observation that the SAPK2-mediated actin reorganization in presence of PD098059 plus H$_2$O$_2$ results in membrane blebbing, whereas it still drives stress fiber formation in presence of PD098059/VEGF, suggests that oxidative stress induced some specific alterations that caused a misassembly of actin into transcytoplasmic stress fibers. Accordingly, we investigated the possibility that inhibiting ERK activation in the presence of oxidative stress but not VEGF was associated with an early defect in the assembly of the focal adhesion plaques to which stress fibers anchor. We found, within the first 5 min of treatments, a marked alteration in the distribution of focal adhesion plaques in cells treated with PD098059 and H$_2$O$_2$. In these cells, vinculin accumulated at the periphery of the cells instead of forming the typical elongated pattern found at the ventral face in cells treated with H$_2$O$_2$ only (Fig. 5, a and b). In contrast, pretreating the cells with PD098059 did not affect the VEGF-induced ventral distribution of vinculin into elongated focal adhesions (Fig. 5, c and d). The rapidity at which the redistribution of focal adhesion occurs after treatment with PD098059/H$_2$O$_2$ suggests that it may underlie the accumulation of F-actin required at the periphery of the cells to trigger bleb formation.

Figure 2. Inhibition of ERK activation induces membrane blebbing and apoptosis in the presence of H$_2$O$_2$. (a) HUVECs plated in gelatin-coated LabTek were pretreated for 60 min with vehicle (DMSO 0.5%) or PD098059 (50 μM) alone (PD), or PD098059 (50 μM) plus SB203580 (SB, 10 μM) and then treated or not with H$_2$O$_2$ for 60 min (500 μM). Actin was stained as described in Materials and Methods using FITC-phalloidin and examined by confocal microscopy. Blebbing cells were counted, and means ± standard deviation from three separate experiments are shown. (b) Kinetics of DEVDase activities in HUVECs pretreated for 60 min with vehicle (DMSO 0.5%, open and filled squares) or PD098059 (25 μM, filled circles), PD098059 (25 μM) plus SB203580 (10 μM, open circles), or PD098059 (25 μM) plus zVAD-fmk (50 μM, filled triangles) and then treated or not (filled squares) with 500 μM H$_2$O$_2$ for 30 min (open squares, filled circles, open circles, filled triangles). At different times after treatment, adherent cells were pooled with floating cells and processed for DEVDase activity assay as described in Materials and Methods. (c) HUVECs plated in gelatin-coated Petri dishes were pretreated for 60 min with vehicle (DMSO 0.15%) or PD098059 (25 μM) alone (PD), or PD098059 (25 μM) plus SB203580 (SB, 10 μM) and then treated or not with H$_2$O$_2$ for 30 min (500 μM). 6 h later, proteins were extracted. Caspase-3 and caspase-3 active fragment p17 (arrowhead) were detected as described in Materials and Methods. (d) HUVECs pretreated for 60 min with vehicle (DMSO 0.15%, left; 0.4%, right) or PD098059 (25 μM), PD098059 (25 μM) plus SB203580 (10 μM), or PD098059 (25 μM) plus zVAD-fmk (50 μM) and then treated or not with 500 μM H$_2$O$_2$ for 30 min. DNA was extracted and prepared as described in Materials and Methods and run into 1% agarose gel. DNA marker is shown (M). (e) HUVECs plated in gelatin-coated Petri dishes were pretreated for 60 min with vehicle (DMSO 0.15%) or PD098059 (25 μM) alone (PD) or PD098059 (25 μM) plus SB203580 (SB, 10 μM) and then treated or not with H$_2$O$_2$ for 30 min (500 μM). 6 h later, proteins were extracted, and PARP was detected by Western blotting as described in Materials and Methods. The arrowhead shows intact PARP.
merization factor that has a general cytoplasmic localization but is found at particularly high level at the leading edge of lamellipodia and membrane ruffles where actin polymerization is very intense (Lavoie et al., 1993; Pietrowicz and Levin, 1997; Fig. 6, a and b). Intriguingly, we found that membrane blebs, which were delimited by a thin ring of F-actin, contained an enhanced concentration of HSP27 (Fig. 6, c and d). Since inhibition of bleb formation by SB203580 was accompanied by an inhibition of HSP27 phosphorylation, this suggested that HSP27 may be involved in modulating the local polymerization of F-actin that was required for bleb formation. Endothelial cells constitutively express high levels of HSP27 (6 ng/mg total protein). To verify the role of HSP27 in bleb formation, we used three distinct clonal cell lines derived from the CCL39 Chinese hamster fibroblasts. They express, in addition to a low level of endogenous Chinese hamster HSP27 (1.5 ng HSP27/μg total proteins), either a wild-type human HSP27 (clone B12, 4.8 ng HSP27/μg total proteins), a nonphosphorylatable HSP27 (clone V, 3.3 ng HSP27/μg total proteins), or the neo gene only (clone 3) (Fig. 7, a–i). Clone 3 and clone V respond similarly to H2O2. In both cases, the oxidant induced a severe fragmentation of F-actin, but no bleb was formed whether PD098059 was present or not (Fig. 7, a, d, and g, and Fig. 7, c, f, and i). In contrast, the clone B12, similarly to endothelial cells, formed stress fibers when exposed to H2O2 (Fig. 7, b and e) and membrane blebbing when H2O2 was added together with PD098059 (Fig. 7, b and h). In addition, as in HUVECs, the blebbing activity was inhibited by treating B12 cells with SB203580 (data not shown). ERK inhibition in the presence of H2O2 was not associated (even after 10 h) with DEVDase activation or, after 24 h, with internucleosomal DNA fragmentation (Fig. 8, a and b) in any of these three cell lines. These results identify HSP27 phosphorylation as being involved in coupling SAPK2 activity to bleb formation, and they clearly show that surface blebbing can be dissociated from apoptosis.

Cis-platinum–induced SAPK2-dependent Membrane Blebbing

The ERK pathway is mostly activated by physiological agonists that induce growth or differentiation responses, and in contrast to H2O2, most toxic agents induce little ERK activity. This raises the possibility that SAPK2-dependent membrane blebbing may be a general phenomenon that could be associated with the stress response generated by
anticancer drug treatment. To test this possibility, the CCL39 clones were treated with 100 μM cis-platinum, a concentration that weakly activates ERK but increases SAPK2 activity up to five times (data not shown). Cis-platinum treatments of clone B12, which overexpresses the wild-type form of HSP27, resulted in the relocalization of F-actin at the cell cortex and a marked cell blebbing activity (Fig. 9, c and d). Again, this blebbing activity was inhibited by inhibiting SAPK2 with SB203580 (Fig. 9, e and f). In comparison, very little cell blebbing was observed in the control clone 3 (Fig. 9, a and b) and in the clone V that expresses the nonphosphorylatable form of HSP27 (data not shown).

Discussion
Both SAPK2/p38 and ERK, two members of the MAP kinase family, are activated by oxidative stress in vascular endothelial cells. Activation of SAPK2 leads to phosphorylation of the actin polymerization factor HSP27, which in turn modulates stress fiber formation and recruitment of vinculin to focal adhesions (Guay et al., 1997; Huot et al., 1997). This cytoskeleton reorganization is involved in changing the shape of the endothelial cell and in modulating properties of the endothelium, such as permeability to fluids, macromolecules, and inflammatory cells. In the present study, we found that activation of the ERK pathway also had a major effect on the cell response to H₂O₂. In the presence of PD098059, actin filaments, instead of bundling into stress fibers, rather accumulated under the plasma membrane, generating within 1 h intense membrane blebbing activities. Several hallmarks of apoptotic cell death also became apparent at later times, namely caspase activation, cleavage of PARP, and internucleosomal DNA fragmentation.

The rapidity and synchrony at which membrane blebbing occurred suggested a direct mechanism of induction. Our results indicated that membrane blebbing lies in a pathway distinct from apoptosis and can even be dissociated from it. Membrane blebbing was not downstream of any of the apoptotic events measured in HUVECs. It occurred very early, well before DEVDase activation, PARP cleavage, and internucleosomal DNA fragmentation, and it occurred independently of zVAD-fmk–sensitive caspase activation. Membrane blebbing was not upstream of these events since cytochalasin D and SB203580 inhibited bleb formation but did not affect DEVDase activities or internucleosomal DNA fragmentation. Moreover, in CCL39 cells expressing high levels of HSP27, membrane blebbing...
was induced without detectable caspase activation or DNA fragmentation. In addition to membrane blebbing, PARP cleavage was also inhibited by SB203580. However, PARP cleavage did not result from membrane blebbing since it was not inhibited by cytochalasin D. This suggests that PARP cleavage and membrane blebbing are not required in the process of apoptosis. The existence of distinct independent pathways mediating the execution of apoptosis has been suggested in several recent studies. In KB cells, it was shown that Fas-mediated chromatin condensation and DNA fragmentation occur independently of SAPK1- and SAPK2-mediated membrane blebbing.

Figure 5. Inhibition of ERK activation in the presence of H2O2 but not VEGF is associated with redistribution of focal adhesions at the periphery of the cells. HUVECs were pretreated or not (a and c) with PD098059 (50 μM, b and d) for 60 min and then for 5 min with H2O2 (500 μM, a and b) or VEGF (5 ng/ml, c and d). Vinculin was detected using the hVIN-1 monoclonal antibody and the vinculin antigen–antibody complexes were detected with biotin-labeled anti-mouse IgG and were revealed with Texas red–conjugated streptavidin. Cells were examined by confocal microscopy. Representative fields are shown. Bar, 25 μm.

Figure 6. Blebs contain enhanced expression of the actin polymerization factor, HSP27. HUVECs plated in gelatin-coated LabTek were pretreated for 60 min with vehicle (DMSO 0.4%, a and b) or PD098059 (50 μM, c and d) and then treated or not (a and b) with H2O2 for 60 min (500 μM, c and d). Insets show a zoom section with actin at the periphery of the blebs (c) and HSP27 in the core (d). Cells were stained for actin (a and c) and HSP27 (b and d). Cells were examined by confocal microscopy. White arrowheads show ruffles, and black arrowheads show blebs. Representative fields are shown. Bar, 25 μm.
cell shrinkage, and induction of Apo2.7 staining in mitochondria (Toyoshima et al., 1997). Moreover, expression of a constitutively active Rac mutant induces membrane blebbing but not apoptosis in NIH-3T3 cells (Alexander et al., 1998). In other studies, it was demonstrated that cells in which apoptosis is induced by serum deprivation, c-myc, etoposide, or Bak accumulate in a doomed blebbing state when caspases are inhibited with zVAD-fmk (McCarthy et al., 1997; Mills et al., 1997). It was proposed that membrane blebbing is a discrete subprogram operating during mammalian apoptosis that can lead to cell death via caspase-independent mechanism (McCarthy et al., 1997). Our data further suggest the corollary that surface blebbing is not essential for apoptosis completion and can even be dissociated from it. Membrane blebbing is thus a primary manifestation of cell toxicity that is not necessarily linked to apoptosis.

In various cell types, caspase-3 is the major caspase involved in PARP cleavage. Intriguingly, we found that inhibiting SAPK2 activity with SB203580 inhibited PARP

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**Figure 7.** Overexpression of wild-type form but not of a nonphosphorylatable form of human HSP27 is associated with membrane blebbing in Chinese hamster fibroblasts exposed to H₂O₂ when ERK is blocked. CCL39 fibroblastic cell line expressing only the neo selection gene (a, d, and g) or overexpressing wild-type HSP27 (b, e, and h) or a nonphosphorylatable form of human HSP27 (c, f, and i) were pretreated for 60 min with vehicle (DMSO 0.25%, a–f) or PD098059 (25 μM, g–i) and then treated or not (a–c) with H₂O₂ for 60 minutes (800 μM, d–i). Cells were stained for actin and examined by confocal microscopy. Representative fields are shown. Bar, 25 μm.
One possibility is that in endothelial cells, cleavage of PARP involves a SAPK2-dependent protease unrelated to DEVDases. Indeed, it is known that PARP can be cleaved by proteases different from caspases (de Murcia and Ménilsier-de Murcia, 1994). Another possibility is that SAPK2 collaborates with caspase-3 to the cleavage of PARP. For example, PARP and/or caspase-3 might require phosphorylation downstream of SAPK2 to interact to each other. Whereas there is no evidence that caspases are phosphorylated, phosphorylation of PARP has been observed both in vitro and in vivo (Bauer et al., 1992; Ruscetti et al., 1998). The protective mechanism of SB203580 on PARP cleavage is under investigation.

The intense membrane blebbing activity that resulted from inhibition of ERK in cells exposed to H2O2 was associated with an accumulation of F-actin at the periphery of the cells and at the perimeter of the blebs. In fact, membrane blebbing was strictly dependent on actin polymerization activity, being blocked by cytochalasin D at a concentration that has no effect of the organization of the actin filaments in control cells. The concentration of cytochalasin D (10 nM) was 100 times lower than that generally used to disrupt the actin filaments and is thought to only compensate for new actin polymerization activity. This finding suggests that the inhibition of membrane blebbing by blocking SAPK2 activity with SB203580 is also likely due to the inhibition of the actin polymerization activity generated by SAPK2 activation. Modulation of actin dynamics by SAPK2 requires phosphorylation of HSP27 by MAPKAP kinase-2/3, a physiological substrate of SAPK2 (Rouse et al., 1994; McLaughlin et al., 1996; Guay et al., 1997; Huot et al., 1997). Activation of SAPK2 and phosphorylation of HSP27 are involved in an important pathway regulating F-actin dynamics, membrane ruffling, and pinocytosis in both endothelial cells and other cells stimulated by stress and growth factors (Lavoie et al., 1993; Huot et al., 1996, 1997; Pietrowicz and Levin, 1998). HSP27 behaves in vitro as an F-actin cap binding protein.

Figure 8. ERK inhibition in presence of H2O2 does not induce DEVDase activity on DNA fragmentation in Chinese hamster fibroblasts. (a) DEVDase activity in CCL39 clonal cell lines and HUVECs (control clone 3, black bars; clone V expressing the nonphosphorylatable form of human HSP27, dark gray bars; clone B12 expressing the wild-type form of HSP27, white bars; HUVECs, light gray bars) pretreated for 60 min with vehicle (DMSO 0.12%) or PD098059 (25 μM for CCL39 clonal cell lines and 50 μM for HUVECs) and then treated or not with 800 μM H2O2 for 60 min (500 μM for 30 min for HUVECs). At 3, 6, and 10 h after treatment (6 h for HUVECs), adherent cells were pooled with floating cells and processed for DEVDase activity assay as described in Materials and Methods. Values are means of two separate experiments. (b) CCL39 clonal cell lines and HUVECs were pretreated for 60 min with PD098059 (25 μM) and then treated with 800 μM H2O2 for 60 min (500 μM for 30 min for HUVECs) or left untreated (−) for 0 (CCL39) or 6 h (HUVECs). At 6, 12, and 24 h after treatment (6 h for HUVECs), adherent cells were pooled with floating cells and processed for DNA extraction as described in Materials and Methods. Then, DNA was run into 1% agarose gel. DNA marker is shown (M).

Figure 9. Overexpression of wild-type form of human HSP27 is associated with SB203580-sensitive membrane blebbing in Chinese hamster fibroblasts exposed to cis-platinum. CCL39 fibroblastic cell line expressing only the neo selection gene (a and b) or overexpressing wild-type HSP27 (c–f) were pretreated for 60 min with vehicle (DMSO 0.25%, a−d) or SB203580 (25 μM, e and f) and then treated or not (a, c, and e) with cis-platinum for 60 min (100 μM, b, d, and f). Cells were stained for actin and examined by confocal microscopy. Representative fields are shown. Bar, 25 μm.
and has a phosphorylation-modulated inhibitory function on F-actin polymerization (Benndorf et al., 1994). A pool of HSP27 has been found associated with the basolateral membrane of endothelial cells (Pietrowicz and Levin, 1998). Its rapid phosphorylation upon activation of SAPK2 would release its binding to actin, allowing polymerization.

We found that blebs contained enhanced amounts of HSP27, suggesting that this local concentration of HSP27 may be involved in modulating the SAPK2-actin-dependent formation of membrane blebbing. The role of HSP27 phosphorylation-mediated changes of F-actin dynamics in membrane blebbing formation was more firmly demonstrated by showing that fibroblasts, which expressed HSP27 in amounts comparable to HUVECs, responded similarly to endothelial cells when they were treated with H$_2$O$_2$. As in HUVECs, F-actin reorganized in these cells into transcytoplasmic cables after exposures to H$_2$O$_2$. Furthermore, they formed blebs when they were exposed to H$_2$O$_2$ and PD098059. This contrasts with the effect observed in control fibroblasts, where H$_2$O$_2$ treatment resulted in the fragmentation of preexisting stress fibers but not in bleb induction. These data strongly suggest that the SAPK2 activation by H$_2$O$_2$ modulates membrane blebbing formation through HSP27 phosphorylation. In corollary, these results indicate that the strong actin polymerization–generating activity of SAPK2/HSP27, which in normal conditions may be useful for mediating endothelial remodeling, can be toxic during H$_2$O$_2$ treatment unless appropriate ERK activity is concomitantly induced. Interestingly, in most cases, the ERK pathway is not activated strongly by stress signals, and most apoptotic stimuli induce preferentially the stress-activated kinase pathways involving SAPK1 and SAPK2 (Verheij et al., 1996; Juo et al., 1997; Sanchez-Perez et al., 1998). In fact, cis-platinum also induced a SB203580-sensitive blebbing activity at a concentration that did not affect or weakly increased ERK activity (data not shown and Sanchez-Perez et al., 1998) expanding the concept that activation of the SAPK2/HSP27 pathway can, depending on the cell context, be responsible for induction of membrane blebbing activities in response to stress. Inhibition of the ERK pathway was not, however, sufficient by itself to induce membrane blebbing in HUVECs. FGF, which strongly activated ERK without affecting SAPK2 (Huot et al., 1997), did not induce bleb formation when ERK was blocked with PD098059. Furthermore, inhibiting ERK activation during cell stimulation with VEGF, which like H$_2$O$_2$ activates both ERK and SAPK2 and produced a SAPK2-mediated actin reorganization (Rousseau et al., 1997), did not produce cell blebbing. Thus, a strong imbalance between activation of SAPK2 and ERK signaling pathways was also not sufficient to induce membrane blebbing. Membrane blebbing would therefore require, in addition to high SAPK2 activity and low ERK activity, some stress-induced alterations that prevent proper organization of F-actin into transcytoplasmic stress fibers.

Although several reports have illustrated the role of F-actin in the membrane blebbing process, very little is known on the exact mechanisms involved. Membrane blebbing induction may share mechanistic features with the formation of membrane protrusions, like lamellipodia and filopodia involved in cell motility (Mitchison and Cramer, 1996). The formation of motile protrusions involves actin polymerization, which coupled with the action of unconventional myosin pushes the membrane forward. It also involves conventional myosin that drives the retraction of the cytoplasm (Mitchison and Cramer, 1996). Recently, strong evidence has been presented implying a role of conventional and unconventional myosins in the formation of blebs in serum-deprived cells (Mills et al., 1998). It has been proposed that the centripetal force generated by myosin II would cause extrusion of the cytoplasm in regions where there exist weak anchor points between the membrane and actin. Similarly, unconventional myosin, which in normal conditions pushes F-actin outwards in the protrusions, would be responsible for bleb retraction in regions where contact of F-actin with the substratum are not appropriate. Bundling of actin into transcytoplasmic stress fiber formation as well as formation of motile cell protrusion are tightly associated with formation of focal adhesions to which actin filaments are anchored (Nobes and Hall, 1995). Focal adhesions are sites of tight adhesion between the membrane and the extracellular matrix on one hand, and the membrane and the cytoskeleton on the other. They are assembled after the recruitment of signaling molecules such as FAK and paxillin, and of structural and membrane actin-anchoring proteins such as talin, vinculin, tensin, and α-actinin, which link the microfilament network to the adhesive molecule integrins at their sites of clustering (Burridge et al., 1997). These proteins provide a structural link allowing the anchorage of stress fibers to the membrane and to integrins. We found here that H$_2$O$_2$ and VEGF, which both activate ERK and SAPK2, induce a SAPK2-dependent formation of transcytoplasmic stress fibers associated with recruitment of vinculin to ventral adhesion plaques. In the presence of PD098059, H$_2$O$_2$ but not VEGF, induces a major defect in the assembly of focal adhesions, which was characterized by the accumulation of vinculin at the periphery of the cells instead of forming the typical elongated pattern at the ventral face. This quick redistribution of focal adhesions induced by H$_2$O$_2$ suggests that it may be causal to membrane blebbing. Interestingly, oxidants have been shown to impair formation of focal adhesion by stimulating the activity of calpain leading to the degradation of talin and α-actinin. In hepatocytes, this effect has been proposed to underlie membrane blebbing (Miyoshi et al., 1995). Moreover, H$_2$O$_2$ treatments induce a rapid translocation from the membrane to the cytoplasm of filamin—a protein involved in anchoring actin to membrane proteins (Hastie et al., 1997)—a redistribution of integrins from focal adhesion plaques to the apical and lateral surface of the cells (Bradley et al., 1995), and activation of MLCK and phosphorylation of MLC (Zhao and Davis, 1998). We thus propose that a weakening of the membrane–actin and actin–substratum linkage at the focal adhesions combined with a stronger myosin contractile force and with increased actin polymerization activity generated by SAPK2 may all contribute to formation of blebs during exposure to H$_2$O$_2$. Finding how inhibition or low activity of the ERK pathway contributes to the misassembly of focal adhesions and to the increase in bleb formation by H$_2$O$_2$ in endothelial cells may provide a clue for the general protec-
tive function described for the ERK pathways during exposure of cells to H\textsubscript{2}O\textsubscript{2} and other apoptotic stimuli.

In several cell systems, activation of SAPK2 has been correlated with cellular toxicity and with the onset of apoptosis (Kummer et al., 1997; Schwenger et al., 1997). The major contribution of our study is to describe a mechanism by which SAPK2 activation can lead to membrane blebbing. The blebbing activity depends first on the induction of a stress-sensitive misassembly of focal adhesions, which is amplified in the absence of ERK activation, and second on a strong F-actin reorganization activity generated by phosphorylation of the F-actin cap binding protein HSP27. Endothelial cells, smooth muscle cells, and many tumor cells express high levels of HSP27, suggesting that the described mechanisms of SAPK2-induced cell blebbing may be of wide occurrence. In other cellular contexts, activation by stress of other actin signaling pathways may similarly activate blebbing activity. For example, RhoA also leads to membrane blebbing through a stimulation of actin polymerization activity and through an increase in myosin-dependent contractile forces (Watanabe et al., 1997; Mills et al., 1998). In summary, our results suggest a key role played by actin organization and dynamics in modulating membrane blebbing generated by stresses, and they illustrate how the intermingling balance between biological transduction pathways can modulate the fine tuning regulation of survival and toxic responses.

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