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Mechanical Stress-induced DNA damage and rac-p38MAPK Signal Pathways Mediate p53-dependent Apoptosis in Vascular Smooth Muscle Cells

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ABSTRACT

Recently, we demonstrated that biomechanical stress induces apoptosis of vascular smooth muscle cells (SMCs) (Mayr et al., *FASEB J*. 2000; 15:261–270). In this article we investigated the molecular mechanisms of mechanical stress-induced apoptosis. When SMCs were subjected to cyclic strain, tumor-suppressor p53 was activated as evidenced by gel mobility shift assays and Western blot analyses. p53 activation was largely attenuated if SMCs were pretreated with SB202190, a specific p38MAPK inhibitor, or were stably transfected with dominant negative rac, an upstream signal transducer of p38MAPK pathways. Kinase assays provided direct evidence that p38MAPKs phosphorylated p53 within 30 min of cyclic strain. Additionally, mechanical stress resulted in oxidative DNA damage as detected by the presence of 8-oxoguanine. Treatment with the antioxidant U-74389G abrogated p53 activation. p53 activation was followed by expression and mitochondrial translocation of the proapoptotic protein Bax. Likewise, mechanical stress resulted in up-regulation of anti-apoptotic Bcl-2 proteins, including Bcl-2 and Bcl-xL. However, a marked loss of mitochondrial membrane potential occurred in wild-type, but not in p53–/–, SMCs. The latter lost their ability to express Bax and showed no apoptosis in response to cyclic strain. Taken together, our data provide the first evidence that SMC apoptosis induced by mechanical stress is p53-dependent.

Key words: signaling • cyclic strain stress • cell death • oxidative stress • arteriosclerosis

The vessel wall is an integrated functional component of the circulatory system that is subjected to shear stress and cyclic strain or stretch stress. Although shear stress is sensed primarily by endothelial cells, the pulsatile nature of blood flow exposes all vascular cell types to stretch stress (1). A common feature of vascular diseases is altered or elevated biomechanical stress. For instance, spontaneous atherosclerotic lesions are prone to form in the branch where cyclic strain is elevated (2). Vein vessels do not develop atherosclerosis in their normal low-pressure environment, but accelerated atherosclerosis is observed when veins are grafted to arteries, where the vessels bear increased biomechanical forces. Obviously, biomechanical stress is a crucial factor in atherogenesis (3).

Smooth muscle cells (SMCs), as a main cell component of the vessel wall, are believed to play an important role in the development of vascular diseases. Although previous studies concerning the role of SMCs emphasize cell proliferation, the pathogenetic relevance of SMC apoptosis is increasingly recognized in arteriosclerosis (4, 5). Recently, we demonstrated that SMC apoptosis is an early cellular event of vein bypass graft arteriosclerosis, in which mechanical stress plays a key role (6). The tumor suppressor protein p53, a crucial regulator of cell proliferation and death, is involved in atherosclerosis (7) and SMC apoptosis (8). Numerous genes have been identified as transcriptional targets regulated by p53, including the cyclin-dependent kinase inhibitor $p21^{WAF-1}$ (9) and the proapoptotic gene Bax (10). In response to apoptotic stimuli, Bax translocates from the cytosol to mitochondria where it releases apoptogenic factors, such as cytochrome *c*, from the inner mitochondrial space by opening the permeability transition pores. Anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, are located at the outer mitochondrial membrane and mitigate the proapoptotic effect of Bax (11). Because p53 regulates expression of apoptosis-related genes (12), p53 could be a determinant for cell fate; that is, proliferation or apoptosis, in the development of cardiovascular diseases.

Mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, encompass the extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal protein kinases (JNKs), or stress-activated protein kinases (SAPKs), and p38 MAPKs (13). MAPKs are strongly activated in SMCs stimulated with biomechanical stress *in vivo* and *in vitro* (14, 15). Recently, we provided evidence that mechanical stress induces SMC apoptosis, for which p38MAPK activation is, at least in part, responsible (6). To further scrutinize the molecular mechanisms of mechanical stress-induced SMC apoptosis, the present study was designed to investigate signal pathways that regulate gene expression leading to SMC death. We demonstrate that SMC apoptosis stimulated by cyclic strain is dependent on p53, in which p38 MAPK activation and oxidative DNA damage are crucial for signal initiation. We found that p53 regulates Bax expression, which leads to mitochondrial dysfunction in stressed SMCs.

MATERIALS AND METHODS

Cell culture

SMCs were isolated by enzymatic digestion of aortas from rats, mice, and humans, as described previously (16, 17), and cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Linz, Austria) supplemented with 20% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and were passaged by treatment with 0.05% trypsin/0.02% ethylene diamine tetraacetate (EDTA) solution. Experiments were conducted on SMCs achieving subconfluence at passages 7 to 15.

Cyclic strain

SMCs were seeded on silicone elastomer-bottomed culture plates (Flexcell, McKeesport, PA) at 1.5×10^5 cells per well, grown for 48 h in medium with 20% FCS, and subjected to cyclic strain. The Cyclic Stress Unit, a modification of the unit initially described by Banes et al (18), consisted of a computer-controlled vacuum unit and a base plate to hold the culture plates

(FX3000 AFC-CTL, Flexcell). A vacuum (15 to 20 kPa) was applied repetitively to the elastomer-bottomed plates via the base plate. Cyclic deformation (60 cycles⁄min) with elongations ranging from 5 to 25% in different experiments was applied for 10 min up to 6 h in a humidified incubator with 5% CO_2 at 37˚C. Inhibitors, that is, the p38 MAPK inhibitor SB202190 or the 21-Aminosteroid U-74389G (Calbiochem, Darmstadt, Germany), were added 1 h before the onset of cyclic strain.

Electrophoretic gel mobility shift analysis (EMSA)

Nuclear protein extracts were prepared as described by Verhaegh et al (19). For gel shift analysis, 10 µg of nuclear protein extracts were incubated with 0.5 ng of oligonucleotide encompassing the double-stranded p53 consensus binding sequence $p53^{con}$ (5´- GGA CAT GCC CGG GCAT GTC C -3´). Binding assays containing nuclear extract (10 µg), sonicated salmon testes DNA (1 µg; Sigma, St. Louis, MO), bovine serum albumin (8 µg), DTT (6.5 mM) were adjusted to a final volume of 15 µl. All reactions were performed in the presence of the monoclonal antibody to p53 (Ab-1) (100 ng; Oncogene, San Diego, CA) and incubated on ice for 30 min. This antibody recognizes the C-terminal epitope AA 371-380 of human p53. It is required to stabilize the p53-protein complex and to detect stable binding of p53 in cellular extracts. Finally, 1.5 ng of double-stranded oligonucleotides end-labeled with γ-P^{32} ATP (Amersham, Uppsala, Sweden) were added. Samples were electrophoresed through a 4% polyacrylamide gel, dried, and analyzed by autoradiography in a phosphoimager (BAS 2500, Fuji, Japan). The specificity of the binding was controlled extensively by competition by using cold and mutant oligonucleotides. The latter contained a substitution in the p53 binding motif (Santa Cruz Biotech, Santa Cruz, CA).

Stable transfection

Plasmids expressing dominant negative rac1 were provided by G. Baier (Institute for Medical Biology and Human Genetics, University of Innsbruck, Austria). Rat SMCs were transfected stably with N17 rac (pEF-rac1 N17) plasmids by using a SuperFect Kit (Qiagen). We cultured transfected cells overnight, divided them one to four and placed them in culture medium supplemented with 150 µg/ml G418 (Sigma) to guarantee selection of cells carrying a neomycin-resistant plasmid. Rac N17-transfected SMCs were identified by Northern blotting and/or Western blotting, as described previously (20). Transfected cells were used for experiments on passages 20 to 30.

Protein extraction

After strain stress, SMCs were washed twice with cold PBS. Cellular extracts were harvested according to an established protocol (20). We performed subcelullar fractionation as described by Cook et al (21). Nuclear proteins were extracted from the pellet of cell nuclei and unbroken cells similar to the procedure in gel-mobility shift assay. Protein concentration was measured by the Bio Rad assay (Bio Rad Laboratories, Hercules, CA).

Kinase assay

For kinase assays, we incubated 0.5 ml of the supernatant containing 0.5 mg protein with 10 μl of antibody against mammalian pan- or phospho-p38 MAPK (Santa Cruz Biotech) for 2 h at 4˚C with rotation. Subsequently, 40 μl of protein G-agarose suspension (Santa Cruz Biotech) was added, and rotation was continued for 1 h at 4˚C. Immunocomplexes were precipitated by centrifugation and washed twice with buffers A (20 mM/L *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES, pH 7.4), 2 mM/L EDTA, 50 mM/L β-glycerophosphate, 1 mM/L DTT, 1 mM/L Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 100 μM/L phenylmethylsulfonyl fluoride), B (500 mM/L LiCl, 100 mM/L Tris, 1 mM/L DTT, and 0.1% Triton X-100, pH 7.6), and C (20 mM/L MOPS, 2 mM/L EGTA, 10 mM/L $MgCl_2$, 1 mM/L DTT, and 0.1% Triton X-100, pH 7.2), respectively. p38 MAPK activity in the immunocomplexes was measured by using glutathione S-transferase-p53 as substrate (GST-p53, the plasmid was provided by J.Y.-J. Shyy, Dept. of Bioengineering, University of California, CA), produced in competent cells and isolated with gluthatione-sepharose 4B Redi Pack Columns (Amersham Co., Little Chalfont, UK) according to the manufacturer's protocol. The p38 MAPK assay was performed as described previously (22). In brief, immunocomplexes were incubated with GST-p53 (6 μg) and [γ-P^{32}] ATP (5 μCi) for 20 min at 30˚C. To stop the reaction, 15 μl of 4× Laemmli buffer was added, and the mixture was boiled for 5 min. Proteins in the kinase reaction were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel) and quantified with a phosphoimager (Fuji).

Measurement of oxidative DNA damage

A specific fluorescent probe for 8-oxoguanine (DNA damage assay, Biotrin, Dublin, Ireland) was used for direct detection of oxidized DNA. We performed tests according to manufacturer's instruction. Briefly, cells were fixed with 4% paraformaldehyde and de- and rehydrated with graded methanol on ice. Samples were blocked for 1 h at 37°C and were incubated with the fluorescein isothiocyanate (FITC)-conjugated probe for 8-oxoguanine overnight at 4°C. After washing, samples were analyzed by confocal microscopy (Microradiance, Bio Rad). The fluorescent probe dihydrorhodamine 123 (DHR 123, Molecular Probes, Eugene, OR) was used to detect reactive oxygen species in SMCs (10 μM/L).

Western blot analysis

The procedure used for Western blot analysis was similar to that described elsewhere (20). Briefly, 50–150 μg of proteins were separated by electrophoresis through a 10–12% SDS-polyacrylamide gel and were transferred onto nitrocellulose membranes. Blots were probed with antibodies against mouse p53 (NCL-p53-CM5p; Novocastra, Newcastle, UK); cytochrome *c* (7H8.2C12, Pharmingen, San Diego, CA); Bax, Bcl-2, Bcl-xL, ERK 2 (Santa Cruz Biotech); myc-tagged rac1 (a gift from G. Baier, Innsbruck); or heat shock protein 60 (Hsp60, II-13, 23). Reactions were visualized by ECL Western Blot Detection Kit (Amersham) after incubation with peroxidase conjugates.

Annexin V and JC-1 staining

Annexin V staining was performed according to the manufacturer's instructions (Pharmingen). In brief, adherent SMCs were trypsinized 18 h after mechanical stress, washed twice with cold PBS (4˚C), resuspended in binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM

NaCl, 2.5 mM $CaCl_2$, and incubated with 5 µl FITC-labeled annexin V and 10 µl propidiumiodide (stock solution 50 µg/ml) for 15 min in the dark at room temperature. SMCs were analyzed by flow cytometry. After appropriate markings for negative and positive populations were set, the percentage of annexin V^+/PI^- cells was determined and compared with unstressed controls (6). Similarly, SMCs were labeled with JC-1 (10 µg/ml; Molecular Probes) at 37˚C for 15 min and were analyzed immediately by flow cytometry or confocal microscopy.

Statistical analysis

Statistical analyses were performed on a Macintosh computer with StatView SE+Graphics software. We performed ANOVA for multiple comparisons. We used an unpaired Student's *t*-test to assess differences between two groups. Results are given in means ± SD. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Mechanical stress induced p53-activation.

To elucidate whether mechanical stress induces p53 activation in SMCs, we analyzed DNA binding activity of p53 by electrophoretic gel mobility shift assay (EMSA). The DNA-damaging reagent methyl methanesulfonate (MMS) served as a positive control. As shown in Figure 1A, the slower migrating band designated with a black arrow represented the specific p53 DNA binding complex (19). It completely disappeared by using a mutated p53 oligonucleotide that has been shown to disrupt binding of the transcription factor.

Aortic SMCs of wild-type and p53–/– mice were subjected to cyclic strain as described previously (6). Tensile stimuli with 15% elongation of original size resulted in p53 activation in p53+/+ cells within 30 to 60 min, but maximum DNA binding activity was not achieved until 3 to 6 h after mechanical stress (Fig. 1B). We obtained similar results for human and rat SMCs. As expected, no p53 binding complex was seen in p53–/– SMCs (data not shown).

Figure 1C demonstrates the specificity of mechanical stress-induced p53 activation: Mutant oligonucleotides and cold oligonucleotides corresponding to the p53 binding site but not to unrelated binding sites, such as SP-1, abrogated the specific p53 DNA binding activity.

In accordance with the EMSA experiments, Western Blot analyses revealed that levels of p53 increased in the cytoplasm and cell nuclei in response to mechanical stress (Fig. 1D).

p38 MAPK contributes to mechanical stress-induced p53 activation

Our previous findings indicated that p38 MAPK may be involved in transducing signals leading to cell death after mechanical stress (6). Pretreatment with SB202190 (Fig. 2A), a specific inhibitor of p38 MAPKs, significantly reduced mechanical stress-induced p53 activation. Additionally, stable transfection with dominant negative rac 1 (rac1 N17), a member of the ras superfamily of small GTP-binding proteins and a key upstream signal transducer in p38 MAPK signaling (22), inhibited p53 activation after mechanical stress compared with vector-transfected cells (Fig. 2B). Quantification of optical densities (OD) from three EMSA experiments revealed

that inhibition of p38 MAPK by SB202190 (Fig. 2C) or dominant negative rac-1 N17 (Fig. 2D) reduced DNA binding activity of p53 in mechanical stressed SMCs.

To obtain direct evidence of p53 phosphorylation by p38 MAPK, p38 MAPK from mechanically stressed SMCs was isolated and kinase assay was performed by using GST-p53 as a substrate. Phosphorylation of GST-p53 paralleled p38 MAPK activation (Fig. 2E) and peaked after 10 to 30 min of mechanical stress (Fig. 2F). Pretreatment with the p38 MAPK inhibitor SB202190 markedly inhibited GST-p53 phosphorylation (Fig. 2F).

p53 activation by oxidative DNA damage

It has been demonstrated that grafting veins to arteries (24) or exposing SMCs to pulsatile stretch (25) increases oxidative stress. Therefore, we used a FITC-labeled fluorescent probe to detect 8-oxoguanine, a well-established surrogate for oxidative DNA damage (26). No staining was observed in unstressed SMCs, but oxidative nucleotide modifications were present 3 to 6 h after mechanical stress (Fig. 3A). The appearance of 8-oxoguanine paralleled the production of reactive oxygen species in stressed SMCs as demonstrated by DHR 123 staining (Fig. 3B). As expected, exposure to ultraviolet light (UV; 10J/m^2) caused oxidative DNA damage and served as a positive control (data not shown). When SMCs were preincubated with the aminosteroid U-74389G, an antioxidant inhibiting lipid peroxidation, mechanical stress-induced p53 activation was abrogated in EMSA experiments (Fig. 3C). However, U-74389G did not influence p38 MAPK activation (Fig. 3D).

Increased expression of Bcl-2 family proteins in response to mechanical stress

The expression of Bcl-2 protein family members was assessed in subcellular extracts of stressed and unstressed SMCs. Increased protein levels of Bax were detected in both the cytosolic and mitochondrial fractions after mechanical stress (Fig. 4A), indicating *de novo* transcription and mitochondrial translocation, respectively. Levels of Bcl-xL and Bcl-2 showed a similar increase in the mitochondria, but not in the cytosol. Bcl-2 was found in the nuclear fraction, but its levels did not increase by mechanical stress.

Time-course analysis (1 Hz, 15% elongation) revealed an up-regulation of Bax and Bcl-xL at 3 to 6 h (Fig. 4B). Furthermore, Bax and Bcl-xL increased in a strength-dependent manner (1 Hz, 6 h, 5%, 15%, and 25% elongation; Fig. 4B). In comparison, Bcl-2 was activated earlier and at lower levels of elongation.

Mitochondrial translocation of Bax was observed in p53+/+, but not in p53–/– SMCs (Fig. 4C). Western blot analysis of total protein extracts revealed that p53–/– SMCs lost their ability to express Bax in response to mechanical stress (Fig. 4D.

SMC apoptosis is p53-dependent and associated with mitochondrial dysfunction

Because Bcl-2 family members are known to control the opening of membrane permeability transition pores, we measured the mitochondrial potential ($\Delta\psi$) after mechanical stress. JC-1, a fluorescent dye that concentrates in mitochondria, exists as green monomers at a low mitochondrial potential and as red fluorescent J-aggregates at a high mitochondrial potential.

Mechanical stress resulted in a fluorescence emission shift from red to green in JC-1 labeled SMCs (Fig. 5A), indicating mitochondrial depolarization. Representative examples of flow cytometry analysis are shown in Figure 5B. Cells that lost their mitochondrial potential appeared in the lower right quadrant compared with viable cells with high potential in the upper-left quadrant. H_2O_2 was used as a positive control. Notably, mitochondrial depolarization was observed in p53+/+, but not in p53–/– SMCs. The FL2/FL1 ratio decreased in a time-dependent manner and reached significant differences from unstressed cells 18 h after mechanical stress (Fig. 5C; $P<0.01$).

In wild-type cells, mitochondrial depolarization coincided with a leakage of cytochrome *c* into the cytosol (Fig. 5D). Treatment with UV ($10 J/m^2$) and pan ERK2 were used as a positive or loading control, respectively. Hsp60 served as mitochondrial marker. In contrast to cytochrome *c*, no major change in Hsp60 distribution occurred in stressed SMCs.

Cells undergoing apoptosis can be identified by annexin V labeling due to their changes in plasma membrane asymmetry, such as phosphatidylserine exposure. Early stages of apoptosis can be distinguished from necrosis and late stages of apoptosis by doublestaining with propidium iodide (PI) that labels cells with permeable plasma membranes. Data shown in Figure 5B indicate induction of apoptosis in response to mechanical stress reaching a twofold increase of annexin V-FITC$^+$/PI$^-$ cells 18 h after the onset of mechanical stress. A population of cells progressing to late stages of apoptosis became double-positive (Fig. 5F). Consistent with our previous results, SMCs of p53–/– mice were resistant to mechanical stress-induced apoptosis.

DISCUSSION

SMC apoptosis is a striking feature of arteriosclerotic lesions (27, 28). p53, as a functional link between cell growth and apoptosis, is hyperexpressed in atherosclerotic lesions and colocalized with increased Bax (8) and p21 expression (7). SMCs derived from atherosclerotic lesions are more sensitive to p53-mediated apoptosis (29). In vein graft arteriosclerosis, we demonstrated previously that biomechanical stress is primarily responsible for cell death and that MAPKs partially mediate the process of mechanical stress-induced signaling (6). In the present study, we provide the first evidence that mechanical stress is a novel factor for p53 activation, which is initiated by oxidative DNA damage and rac-p38MAPK activation. We demonstrate that apoptosis occurs as a result of mitochondrial dysfunction due to changes in the ratio of pro- and anti-apoptotic Bcl-2 family members. Because of the importance of p53-dependent apoptosis and of mechanical stress in the pathogenesis of vascular diseases, our data provide a link between mechanical force applied to vascular SMCs and cell death.

Mechanical stress-induced p53 activation may be of general importance in the development of vascular disease via controlling cell density. Antisense strategy against p53 resulted in abnormal SMC growth and increased lesion formation (30). Cytomegalovirus aggravates restenosis after angioplasty by its immediate early gene product, IE2-84, that binds and inactivates p53, thereby protecting SMCs from undergoing apoptosis and predisposing them to accumulation (31, 32). Based on recent observations in our mouse model of vein graft arteriosclerosis, loss of p53 accelerated neointima formation, which was attributed primarily to SMC accumulation (33). Less apoptotic cells were detected in p53-deficient vein grafts, suggesting that p53 is required for SMC apoptosis *in vivo*.

Vascular SMCs contain potent oxidant-generating systems, including NADPH oxidase (34). Reactive oxygen species such as superoxide, peroxide, hydroxyl radicals as well as peroxynitrite are generated in atherosclerosis and recent studies provide evidence for oxidative DNA damage in experimental atherosclerosis (35). DNA sequences with high-guanosine content are susceptible particularly to oxidative modifications (26), which appreciably alter the backbone structure of DNA strands and compromise the fidelity of transcription and replication (36). In the present study we demonstrate that mechanical stress induces free radical generation to result in oxidative DNA damage as evidenced by the presence of 8-oxoguanine. DNA damage recognizing proteins; for example, p53, not only repair the damage but also turn on apoptosis to remove potentially mutagenized cells (37). Thus, mechanical stress-induced oxidative DNA damage could be an important signal for p53 activation.

p53 activity is regulated by several events, including transcription, phosphorylation, and de-phosphorylation (38–40). MAPKs are highly activated by mechanical stress and have been implicated in both cell proliferation and apoptosis (6, 15). A novel finding in the present study is that mechanical stress-induced signaling pathway rac–p38 MAPKs enhance DNA binding activity of p53. We demonstrated that p38 MAPKs from mechanically stressed SMCs directly phosphorylate p53. p53 activation was attenuated by pretreatment with p38 MAPK inhibitor or rac-N17 transfection, but not completely blocked. p38 MAPK can phosphorylate the N-terminal transactivation domain in serine 33 (39) and the C-terminal regulation domain in serine 392 of human p53, the homologue serine 389 of the murine p53 protein (38). The latter is particularly important because the regulatory domain serves as a constitutive negative regulator of the DNA binding domain. Phosphorylation of the C-terminus induces conformational changes that expose the sequence-specific DNA binding domain of p53. N-terminal phosphorylation events have been implicated in the release of p53 from its inhibitor Mdm2. Therefore, p38 MAPK can transfer p53 into a preactivated state where it can sense DNA damage. However, because of the variety in p53 species, it is likely that other signals may concomitantly contribute to p53 activation (41).

As mentioned above, p53 regulates expression of many genes related to cell apoptosis and growth arrest (9), including members of the Bcl-2 protein family (12). The ratio of Bcl-2 and Bcl-xL to Bax is a dynamic interaction and provides a preset rheostat for determining the cellular response to death signals (42). Our data clearly show that p53-dependent Bax expression is responsible for mitochondrial dysfunction induced by mechanical stress, further supporting the importance of p53 in mechanical stress-induced SMC apoptosis. Notably, p53–/– cells are still susceptible to other death stimuli apart from mechanical stress (33). The increase of anti-apoptotic proteins is likely sufficient to afford some protection against apoptosis, particularly at lower levels of mechanical stress. At extreme levels of mechanical stress, such as balloon injury, down-regulation of anti-apoptotic proteins is associated with a more rapid onset of apoptosis (43). Similar changes might also occur in vein grafts.

Obviously, mechanical force can induce p53 activation that leads to SMC apoptosis. What are possible mechanosensors for p53 activation? One pathway identified in the present study involves rac-p38 MAPKs. However, upstream activators of this pathway are not clarified yet. Our previous studies indicated that suramin could inhibit platelet-derived growth factor (PDGF) receptor phosphorylation induced by stretch stress and neointima hyperplasia of mouse vein

grafts was reduced by locally applied suramin *in vivo* (17). We found that suramin could inhibit ERK, but not JNK activation, and enhance the p38 MAPK phosphorylation, indicating that growth factor receptor-independent pathways lead to increased p38 MAPK phosphorylation (22). Similarly, pre-incubation with U-74389 G did not abrogate p38 MAPK activation, indicating that oxidative stress might not be primarily responsible for p38 MAPK activation. On the other hand, integrins that bind to collagen or flexible membrane might be activated in response to mechanical stress (44). In endothelial cells, integrins mediate JNK/SAPK activation in response to shear stress (45). Similarly, we found that activation of rac-p38 MAPK by mechanical stress might involve an integrin-dependent pathway in SMCs (Wernig et al, unpublished observations). Thus, the transformation of mechanical force into a biological response might involve receptor-dependent and—independent pathways; that is, integrins and oxidative stress, respectively.

We hypothesize that mechanical stress stimulates mechanosensors, possibly integrins, leading to rac-p38MAPK activation. Phosphorylation of p53 by p38 MAPK (38, 39) could be an early event in p53 activation, and enhanced later by oxidative DNA damage. Both signals converge at the level of p53 activation leading to apoptosis. Additionally, p38 MAPKs might indirectly activate or regulate oxidase activation and p53 itself might contribute to DNA damage by increasing free radical generation (46, 47) providing a positive feedback loop for p53 activation. Therefore, our results suggest that mechanical stress exerts its role in cell apoptosis via p53 activation in the development of vascular diseases. These studies may bring new insights into molecular mechanisms of biomechanical stress-induced apoptosis and open up new therapeutic targets for vascular diseases.

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Fig. 1

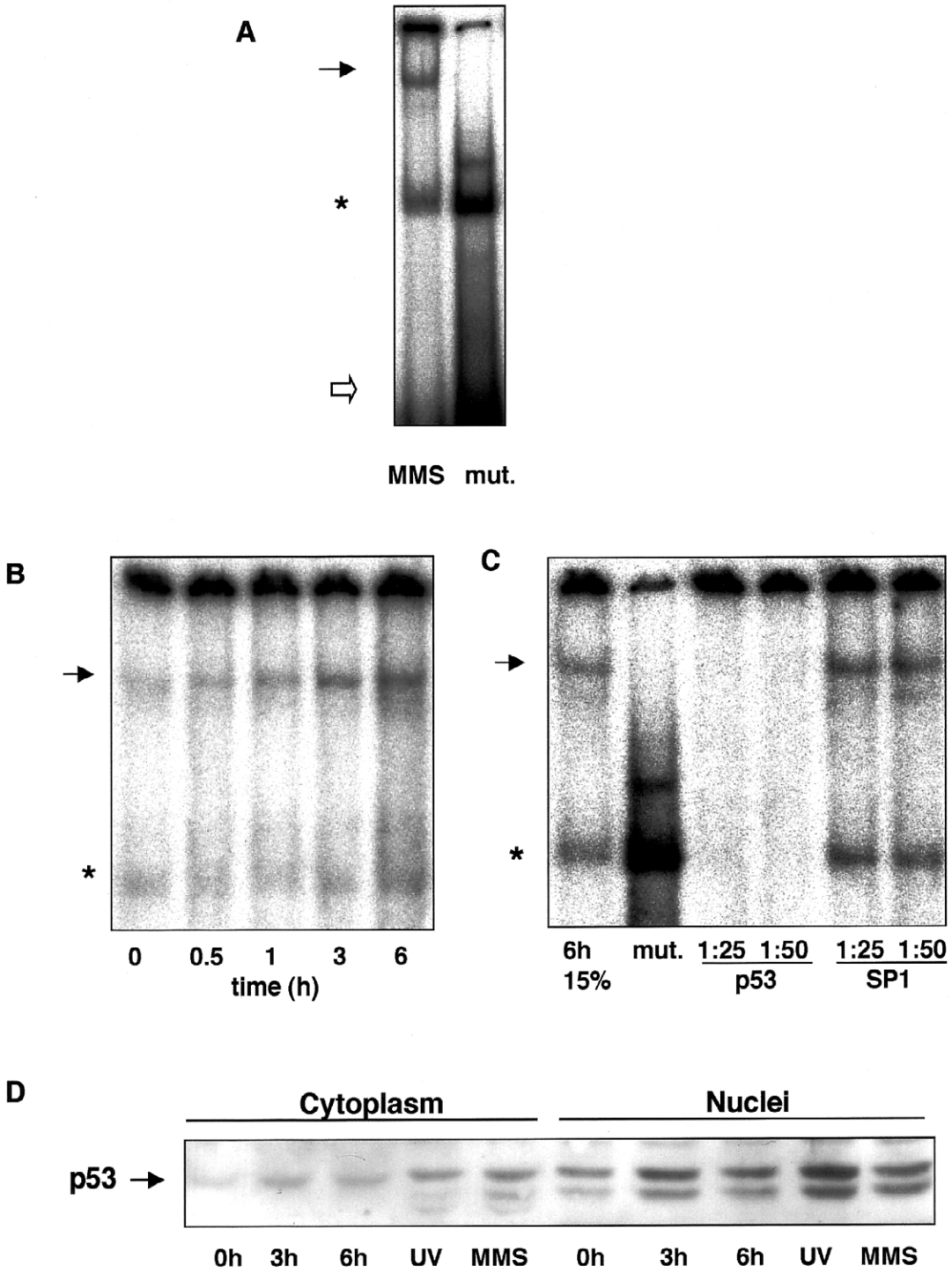

Figure 1. Analysis of p53-activation in protein extracts of SMCs. Aortic SMCs were cultivated on flexible membranes and were subject to mechanical stress (15% elongation, 1 Hz). Nuclear proteins were prepared as described in Materials and Methods. Gel mobility shift assay was performed in 4% gel by using a monoclonal antibody to p53 (Ab-1, Oncogen) to supershift the specific p53-DNA complex. **A)** p53-DNA binding in SMCs treated with MMS (1 mM) for 6 h. The positions of the p53-DNA-Ab1 complexes (filled arrow), of complexes corresponding to an unknown protein that binds to DNA in a non-sequence specific manner (*), and of the free probe (white arrow) are indicated; *mut* indicates competition using mutant oligonucleotides. **B)** Time course of p53-DNA binding activation in SMCs subjected to mechanical stress. **C)** Nuclear extracts of stressed SMCs were incubated with ^{32}P-labeled p53 consensus or mutant oligonucleotides, the former in the presence or absence of unlabeled p53 or SP1 oligonucleotides. **D)** Western blot analysis of p53 protein levels in cytoplasmic and nuclear extracts of stressed SMCs.

Fig. 2

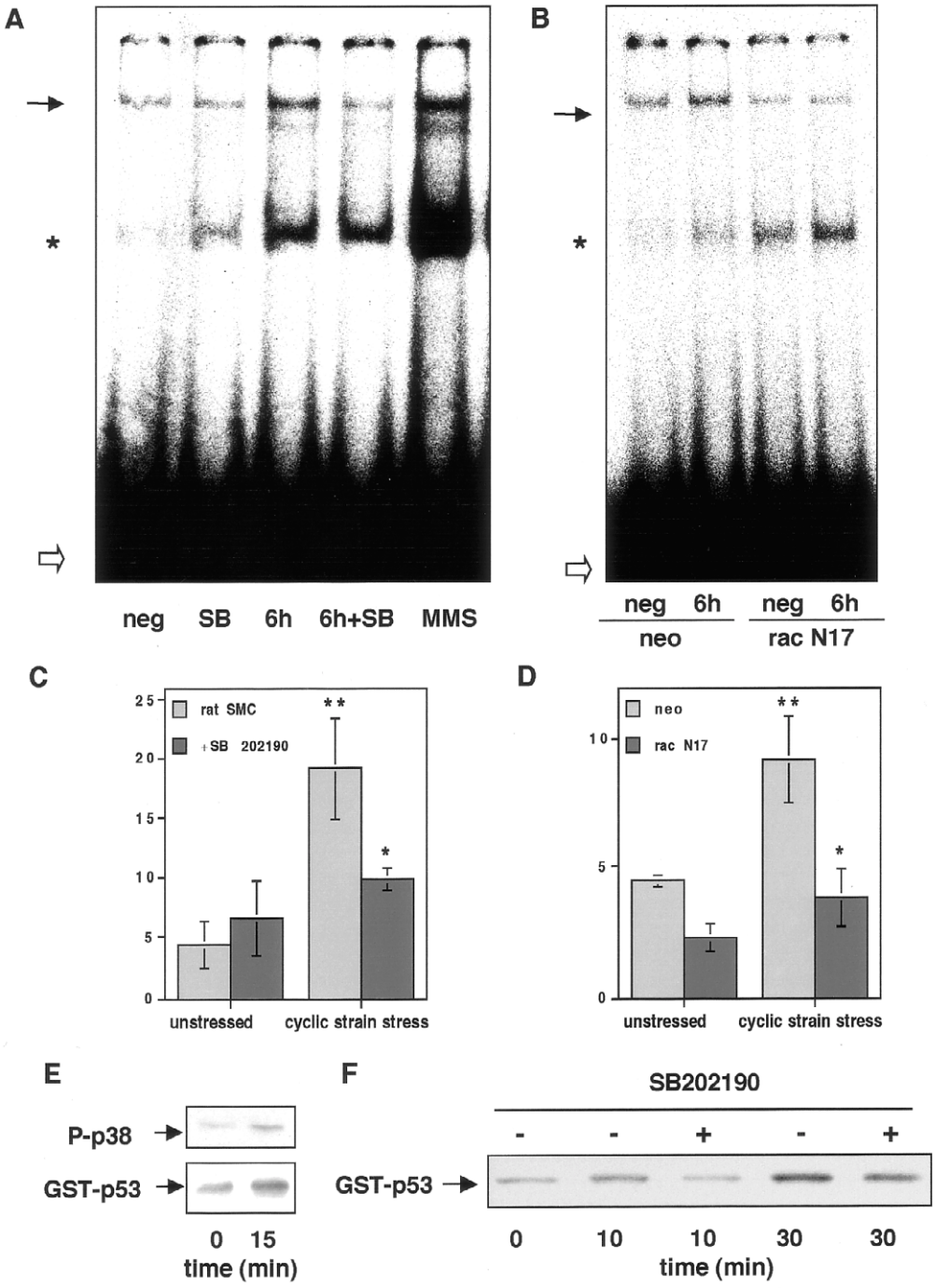

Figure 2. p38 MAPK and p53 activation. A) SMCs were pretreated with SB202190 (SB, 5 μM/L) for 1 h, subject to mechanical stress (15% elongation, 1 Hz) for 6 h, and harvested for nuclear protein isolation. EMSA experiments for p53 activation were performed as described in Materials and Methods and in **Figure 1**; *neg* stands for negative control. **B)** Rat SMCs were stably transfected with constructs expressing dominant negative rac (pEF-rac1 N17) or vector (pEF-neo) as published previously (20). p53 activation was analyzed by use of gel mobility shift assays as mentioned above. **C, D**) Statistical data on p53 activation from SMCs treated with SB 202190 or dominant negative rac. (**)Significant difference compared with unstressed controls. (*)Significant difference compared with positive controls. $P<0.05$. **E)** SMCs were serum-starved for 3 days and stressed for 15 min at 15% elongation. Protein extracts were prepared and analyzed for p38 MAPK phosphorylation by Western blot (upper panel). In parallel, p38 MAPKs were immunoprecipitated from the protein extracts, and kinase activity was measured based on phosphorylation of GST-p53 substrate (lower panel). **F)** Time course of GST-p53 phosphorylation in kinase assays by p38 MAPK in SMCs pretreated with or without SB202190 (SB, 5 μM/L) for 1 h.

Fig. 3

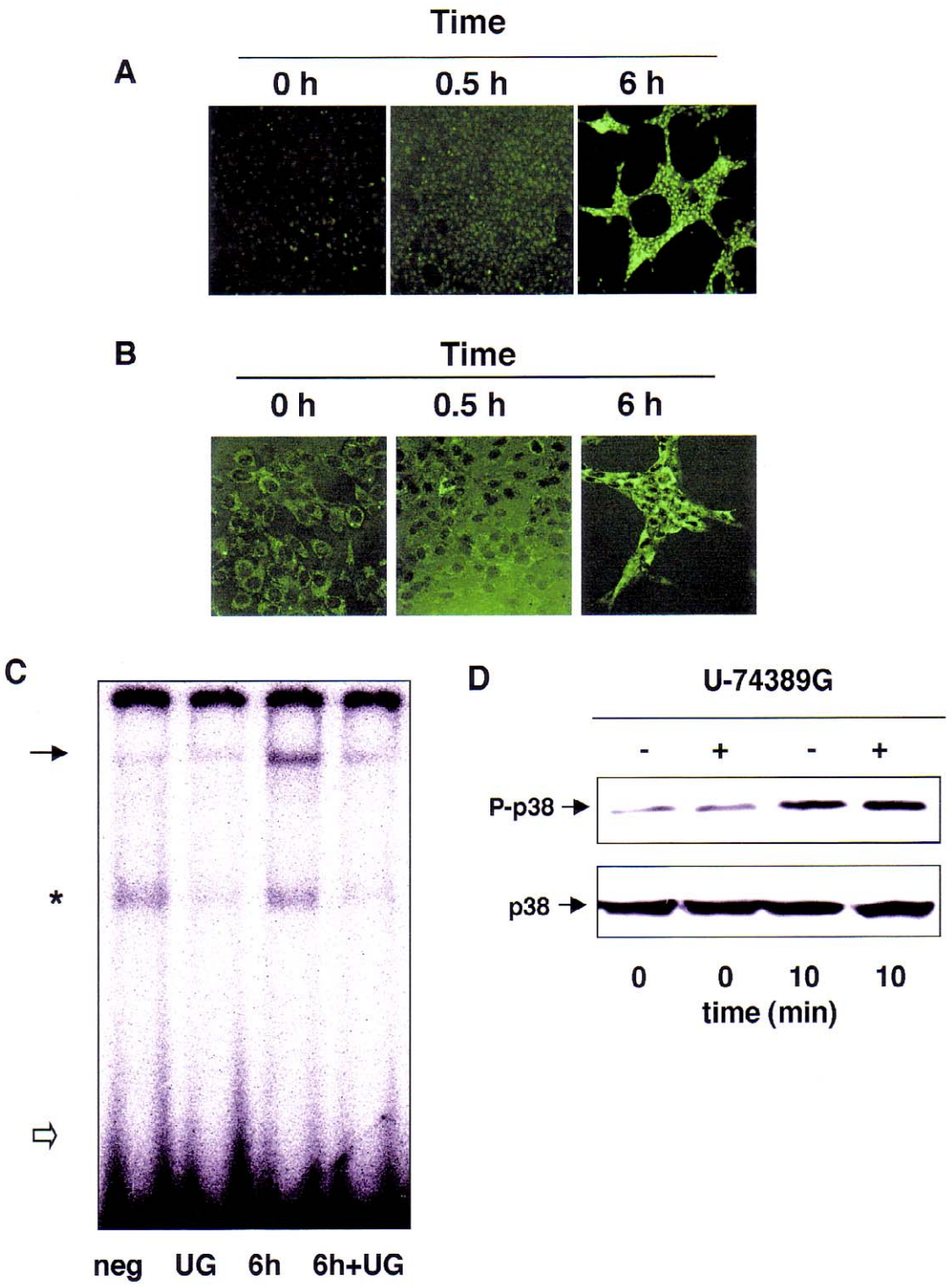

Figure 3. Oxidative DNA damage and p53 activation. A) Rat SMCs were subject to cyclic mechanical strain for 0.5 h and 6 h (15% elongation, 1 Hz), fixed with paraformaldehyde and incubated with a FITC-labeled probe for 8-oxoguanine (Biotrin) at 4˚C overnight. Oxidative DNA damage was visualized by confocal microscopy in stressed SMCs. **B)** Oxidative stress was measured simultaneously by the presence of the oxidant-sensing fluorescent probe DHR123. **C)** SMCs were preincubated with 100 μM of the antioxidants U-74389G (UG) for 1 h and stressed, and nuclear extracts were harvested for EMSA experiments. Filled arrows indicate specific binding; (*) indicates nonspecific binding; open arrows indicate free probe. **D)** SMCs were serum-starved for 3 days and stressed for 10 min at 15% elongation in the presence or absence of U-74389G. Protein extracts were prepared and analyzed for p38 MAPK phosphorylation by Western blot.

Fig. 4

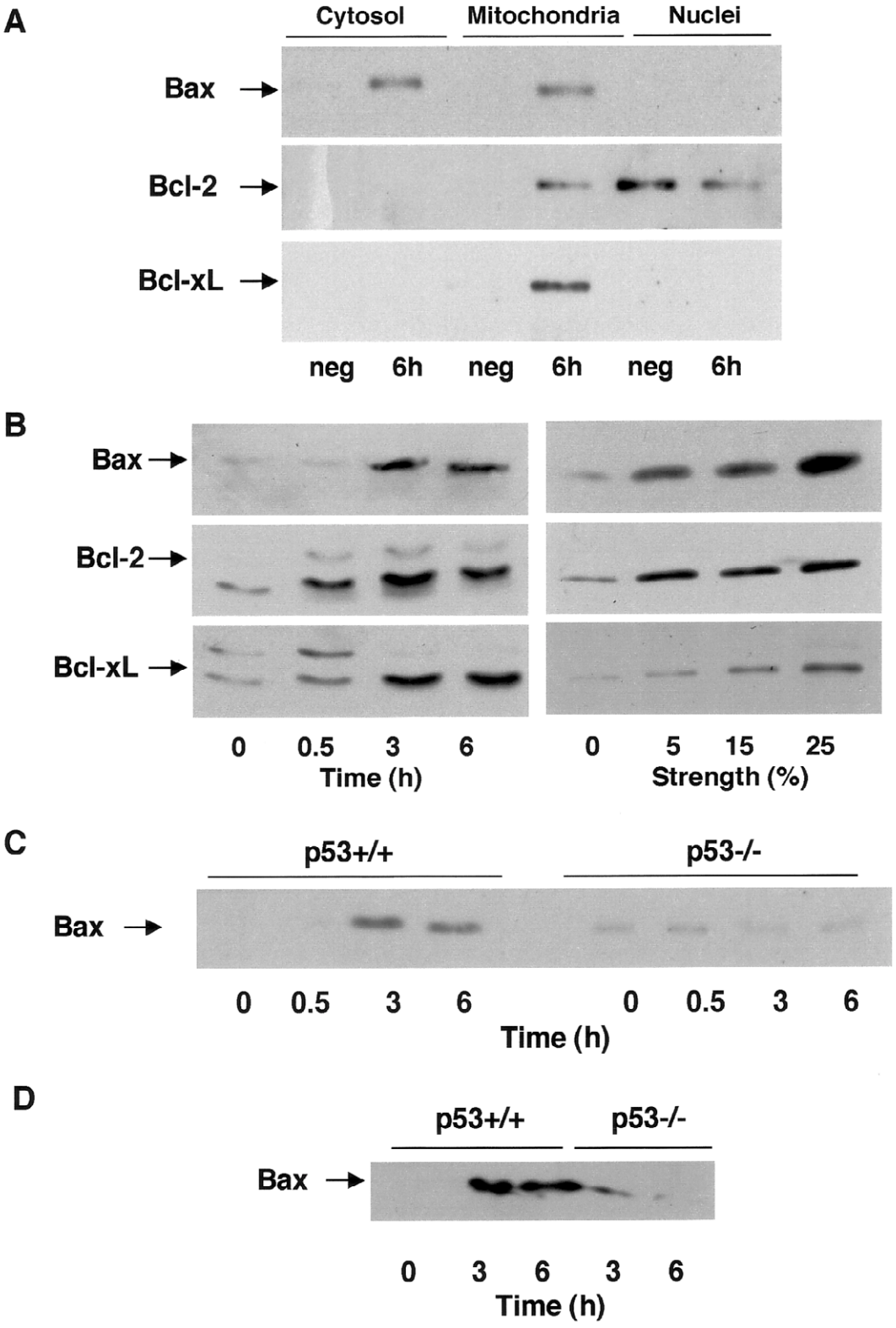

Figure 4. Bcl-2 family protein expression after mechanical stress. Subcellular protein extracts were prepared by differential centrifugation 24 h after the onset of mechanical stress and were separated on 12% SDS-polyacrylamide gel, transferred to membranes, and probed by using antibodies to Bax, Bcl-2, and Bcl-xL. **A)** Bcl-2 family protein expression in cytosol, mitochondria, and nucleus of stressed SMCs. **B)** Time- and strength-dependent expression of Bcl-2 family protein members in mitochondrial extracts of stressed SMCs. **C, D)** Western blot analysis of Bax levels in mitochondrial and total protein extracts of p53 +/+ and –/– SMCs, respectively.

Fig. 5

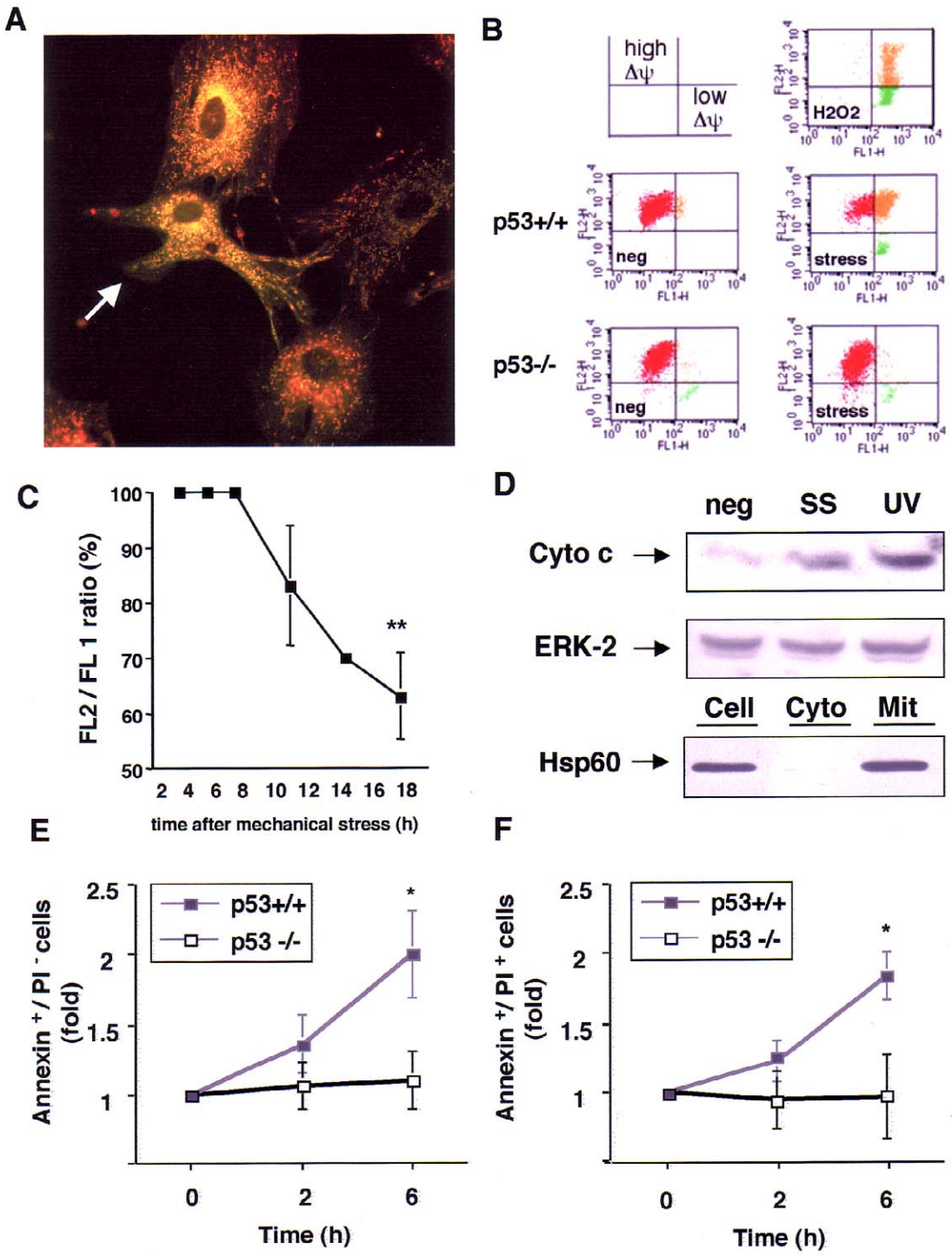

Figure 5. p53 activation and apoptosis. Stressed SMCs (15% elongation, 1 Hz) were stained with JC-1 (10 μM) for 15 min at 37˚C and analyzed by confocal microscopy. **A)** Arrow indicates a cell with low ΔΨ) or flow cytometry (**B**). The fluorescence shift from red to green was recorded as FL2/FL1 ratio in FACS analysis (**C**); (**) significant decrease compared with unstressed controls. $P<0.01$. **D)** Cytosolic extracts of stressed and unstressed cells were separated by SDS-PAGE on 15% gels and processed with antibodies to cytochrome *c* (upper panel) and ERK-2 (middle panel). Whole protein and cytosolic and mitochondrial extracts were probed with a monoclonal antibody to the mitochondrial marker Hsp60 (II-13) in Western blot analysis (bottom panel). Stressed SMCs derived from p53 +/+ and –/– mice were incubated for 12 h before they were harvested and analyzed in the flow cytometer by annexin V and PI staining. The increase in annexin V^+/PI^- (**E**) and annexin V^+/PI^+ (**F**) cells was compared with unstressed controls, respectively. *Significant difference compared with unstressed controls, $P<0.05$.