Protection of Human Endothelial Cells From Oxidative Stress
Role of Ras-ERK1/2 Signaling

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Background—Reactive oxygen species play a critical role in inducing apoptosis. The small GTPase p21 Ras and the ERK1/2 MAPK have been proposed as key regulators of the signaling cascade triggered by oxidative stress (H₂O₂). Harvey-Ras (Ha-Ras) and Kirsten-Ras (Ki-Ras) isoforms are so far functionally indistinguishable, because they activate the same downstream effectors, including ERK1/2. Moreover, ERK1/2 signaling has been involved in both protection and induction of apoptosis.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were subjected to H₂O₂, and apoptosis was detected by fluorescence-activated cell sorting analysis, fluorescence microscopy, and caspase-3 activation. Transfection of Ha-Ras and Ki-Ras genes in HUVECs was performed to evaluate the response to H₂O₂. We have found that, whereas Ha-Ras decreases tolerance to oxidative stress, Ki-Ras has a potent antiapoptotic activity. Both effects are mediated by ERK1/2. Tolerance to H₂O₂ is encoded by a unique stretch of lysines at the COOH terminus of the Ki-Ras, lacking in Ha-Ras, and it is relatively independent of the farnesylated anchor. Inhibition of p21 Ras signaling by farnesylation inhibitors increased the resistance to apoptosis in Ha-Ras–expressing cells.

Conclusions—These findings explain the opposite effects of ERK1/2 stimulation on apoptosis found in different cell types and suggest that local activation of ERK1/2 signaling may account for the opposing response to oxidative stress by Ha-Ras or Ki-Ras–expressing cells. Modulation of cell reactivity to oxidative stress by p21 Ras points to the specific and predictive effects of Ras inhibitors in vivo as potential therapeutic drugs in disorders produced by increase of reactive oxygen species inside the cells. (Circulation. 2002;105:968-974.)

Key Words: endothelium ■ apoptosis ■ stress ■ free radicals ■ hypoxia

High reactive oxygen species (ROS) levels have been detected in several human disorders such as neurodegenerative diseases, cancer, ischemia, atherosclerosis, and aging.1–5 A growing body of evidence suggests that the small GTPase p21 Ras is an essential mediator of the signal transduction pathway leading to cell apoptosis after oxidative injury.6–8 Oxidative stress and p21 Ras activate a group of kinases, the mitogen-activated protein kinase (MAPK) family,9 including the extracellular signal–regulated kinases (ERK1/2), c-Jun NH₂-terminal kinase (JNK or SAPK), and p38 MAPK.10

ERK1/2 signaling has been involved both in protection11,12 and induction13,14 of apoptosis. To date this paradoxical effect(s) has not been fully understood. To address this question, we have explored the biological activity of different isoforms of p21 Ras. The 3 major isoforms of this small GTPase protein are N-Ras, Kirsten Ras (Ki-Ras), and Harvey Ras (Ha-Ras). Specifically, Ki-Ras and Ha-Ras share a high degree of structural and functional homology. Both Ras proteins are farnesylated and localized in the inner face of the plasma membrane.15 The sequences of Ki-Ras and Ha-Ras proteins are almost identical. They diverge only in the 20–amino acid hypervariable domain at the COOH termini. Although their functions remain indistinguishable,16 very recently, evidence for differential activities of the Ras isoforms has started to accumulate.17 We have found recently that the effects of Ha-Ras and Ki-Ras on ROS levels are...
complementary; Ha-Ras activates, and Ki-Ras reduces, intracellular ROS.18,19

In the present study, we show that Ha-Ras protein increases the number of cells undergoing apoptosis after oxidative stress; conversely, the Ki-Ras isoform has a protective effect, encoded by a polybasic stretch of lysine residues at the COOH terminus of the molecule, not present in the Ha-Ras sequence.

Methods

Cells

HUVECs from American Type Culture Collection were maintained in RPMI 1640 (Life Technologies) with 10% FBS (Life Technologies). HUVECs were grown to 70% to 80% confluence and treated for 2 hours with 2 mmol/L H2O2. When needed, cells were preincubated overnight with PD98059 (40 μmol/L) (Calbiochem) or FTI-277 (20 μmol/L) (Calbiochem). Six hours after the removal of the stimulus, apoptosis was detected either by fluorescence microscopy or FACS analysis (FACSCalibur) using propidium iodide (PI) or annexin V (Clontech). To measure caspase-3 activity, cells were cultured under the same conditions. After oxidative injury, cells were collected and lysed in appropriate buffer. Total cell extract (30 to 50 μg) was electrophoresed onto a 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The blot was incubated with a polyclonal anti–caspase-3 antibody (BD-PharMingen), and the presence of total and active caspase-3 was revealed by chemiluminescence (Amersham-Pharmacia Biotech).

The concentration and the exposure time to H2O2 were selected by determining dose-response and time curves. Under these conditions, the assay was linearly dependent on H2O2 concentration and incubation time. Longer incubation periods or higher concentrations of H2O2 resulted in massive apoptosis, and the difference in the cytoprotection between Ha-Ras– and Ki-Ras–expressing cells was abolished.

Biochemical Assays

For Western blotting with the antibodies against the phosphorylated form of ERK1/2 and JNK, HUVECs were grown to confluence, lysed in radioimmunoprecipitation assay buffer, separated by a 12.5% SDS-PAGE, and immunoblotted with a monoclonal anti-phospho-ERK1/2 or anti-phospho-JNK (New England BioLabs). After being “stripped,” the membranes were reprobed with polyclonal antibodies against the total (phosphorylated and unphosphorylated) ERK1/2 and JNK (Santa Cruz Biotechnology).

For immunodetection of Ha-Ras and Ki-Ras, cells were lysed in Triton buffer (1% Triton X-100 and (in mmol/L) NaCl 150, Tris-HCl [pH 7.5] 20, and EDTA 2) supplemented with protease inhibitors; lysates were cleared by centrifugation and incubated overnight at 4°C with a polyclonal anti–pan-Ras antibody (Santa Cruz Biotechnology), followed by a 1-hour incubation at 4°C with protein G beads. The immunoprecipitates were washed 3 times with washing buffer (50 mmol/L Tris-HCl, pH 7.5), centrifuged, and resuspended in 5× SDS sample buffer. Blots were developed with specific monoclonal antibodies against Ha-Ras and Ki-Ras (Santa Cruz Biotechnology).

ROS levels were determined by 2',7'-dichlorofluorescin fluorescence using the oxidation-sensitive probe, 5,6-carboxy-2',7'-dichlorofluorescin diacetate.19

Statistical Analysis

All data are shown as mean±SEM. Statistical analysis between groups was performed according to the χ2 test. A P value <0.05 was considered significant.

Results

Oxidative Stress Induces Apoptosis in Endothelial Cells

HUVECs were stimulated for 2 hours with 2 mmol/L H2O2. After the removal of the oxidative stress, the cells were incubated in the normal medium and apoptosis was determined as described in Methods. Figure 1a shows that 6 hours after the removal of oxidative stimulus, the fraction of
apoptotic cells significantly increased. Later (24 hours), the nuclei of apoptotic cells stained with PI. Apoptosis was monitored also by determining the activation of caspase-3 (Figure 1b). The ratio between the 33-kDa procaspase-3 and the 17-kDa active caspase-3 is a measure of apoptosis induced by H2O2. The histogram in Figure 1b shows a significant accumulation of active caspase-3 in H2O2-treated cells.

The molecular signaling of oxidative stress was investigated by determining the activation of stress-related kinases. HUVECs were stimulated with 2 mmol/L H2O2 for 10, 20, 30, and 60 minutes, and the lysates were probed with specific anti-phospho-ERK1/2 and anti-phospho-JNK antibodies. Figure 1c shows the activation pattern of the 2 kinases. Although ERK1/2 activation appears to be stronger than that of JNK, both kinases reach the maximal peak at the 20-minute time point, suggesting a common mechanism of activation. Note that these are early events in the apoptotic process (compare Figure 1a and 1b). The activation of stress kinases and the onset of apoptosis were inhibited by 30 mmol/L of the reducing agent N-acetyl cysteine (data not shown).

Figure 1. Oxidative stress induces apoptosis in endothelial cells. HUVECs were stimulated with H2O2 and apoptosis was detected by fluorescence microscopy as described in Methods. a, Time course of annexin V-FITC (closed symbols) and PI (open symbols) fluorescence in the presence (• and □) or absence (○ and ◆) of oxidative injury. b, Immunoblot developed with a polyclonal antibody against caspase-3 in the presence or absence of H2O2 stimulation. Diagram shows densitometric analysis of active caspase-3. AU indicates arbitrary units. c, Time course of stress-kinase activation after oxidative injury. Immunoblot developed with antibodies against the active, phosphorylated, and total forms of ERK1/2 and JNK. Diagram below figure shows relative quantification by densitometric analysis. All data are mean±SEM of 3 independent experiments.

Ha-Ras Amplifies, and Ki-Ras Inhibits, H2O2-Induced Apoptosis

Because stress-activated kinases are regulated by p21 Ras, we have tested whether expression of Ras influences the response to oxidative stress. HUVECs grown on coverslip slides were transiently transfected with either the Ki-Ras- or Ha-Ras-expressing vectors. Transfection efficiency was evaluated by determining the number of the cells expressing the GFP encoded by the same polycistronic mRNA (see Methods). After stimulation with H2O2, the oxidative stress was removed and the apoptosis was detected 6 hours later by fluorescence microscopy (Figure 2a). Cells overexpressing Ha-Ras gene (green) showed a higher degree of PI staining (yellow) compared with mock-transfected cells, suggesting a proapoptotic effect of the p21 Ha-Ras protein. Conversely, a significant decrease in PI staining was detected in Ki-Ras-transfected endothelial cells, indicating a protective role of the Ki-Ras gene product on oxidative stress-induced apoptosis.

To obtain quantitative data of these Ras effects on H2O2-induced apoptosis, we carried out FACS analysis of Ras-transfected cells by using annexin V-cy3 (red). Figure 2b shows that FACS analysis confirms microscopy data.

Induction (Ha-Ras) and Inhibition (Ki-Ras) of H2O2-Induced Apoptosis Are Mediated by ERK1/2 Signaling

To demonstrate the specificity of Ras-elicited effects, the dominant negative Leu61-Ser186 Ras reversed the pro- and antiangiogenic effects of the 2 Ras genes. Histograms represent the percentage of annexin V-positive cells, determined in 3 independent experiments. All data are mean±SEM. *P<0.05 vs control; **P<0.05 vs Ha-Ras or Ki-Ras controls.

Figure 2. Ha-Ras amplifies and Ki-Ras inhibits H2O2-induced apoptosis. a, HUVECs transfected with Ha-Ras and Ki-Ras pIRES plasmids were subjected to oxidative injury. Apoptotic cells are shown by PI staining (red). Yellow cells derive from the overlay of green (GFP) and red (PI) fluorescence. Red cells are untransfected HUVECs undergoing apoptosis. b, FACS analysis of Ha-Ras– and Ki-Ras–expressing cells (described in panel a) stained with annexin V–cy3 (red) in the presence or absence of the MEK inhibitor PD98059 (40 μmol/L, 18 hours). Cotransfection of Ha-Ras– or Ki-Ras–expressing cells with the dominant negative Leu61-Ser186 Ras reversed the pro- and antiangiogenic effects of the 2 Ras genes;
To rule out the effects caused by overexpression of Ras exogenous gene(s), we sought to determine whether the endogenous Ras was also able to modulate H$_2$O$_2$-induced apoptosis. We first analyzed the expression pattern of Ras genes in HUVECs and then measured the sensitivity of the cells to H$_2$O$_2$. Figure 3 shows that (1) Ha-Ras was the predominant isoform expressed by these cells (Figure 3a); (2) H$_2$O$_2$-induced activation of stress kinases was affected by FTI-277, a farnesyl transferase inhibitor;20 (Figure 3b); and (3) oxidative stress–induced apoptosis was reversed by PD98059 or by FTI-277 (Figure 3c and 3d). The 2 compounds showed a comparable degree of inhibition of caspase-3 activation and apoptosis, although FTI-277 inhibited both ERK1/2 and JNK, whereas PD98059 prevented only ERK1/2 activation (data not shown). Taken together, these data indicate that Ha-Ras and ERK1/2, but not JNK signaling, are necessary for oxidative stress–induced apoptosis in endothelial cells (Figures 2b, 3c, and 3d).

Molecular Determinants Underlying Ki-Ras Tolerance to Oxidative Stress

To identify the molecular determinants that mediate the inhibition of H$_2$O$_2$-induced apoptosis by Ki-Ras, we mutated the lysine residues of the polybasic region of Ki-Ras to glutamic acid (Lys$^\gamma$), the polybasic stretch (Lys$^\beta$), or both (Lys$^\gamma$/Lys$^\beta$). A, Percentage of cells undergoing apoptosis (annexin V–cy3) after H$_2$O$_2$ stimulation. Data are mean±SEM. *P<0.05 vs control; **P<0.05 vs wild-type. Inset shows immunoblot with anti–Ki-Ras 4B–specific antibodies (Santa Cruz Biotechnology, C-19). Longer exposures of the film showed the band of the endogenous gene (first lane). B, Time course of ROS production by Fe$^{3+}$ ascorbate (0.1 mmol/L) oxidative stress in Ha-Ras and Ki-Ras wild-type and derivative mutants. HUVECs were transfected as indicated in panel a. Data are mean±SEM. *P<0.05 vs wild-type Ki-Ras and Ki-Ras vs control; **P<0.05 vs wild-type Ki-Ras. C, Confocal microscopy (Leica, Model TCS SP2) of HUVECs transfected with wild-type and mutant HA-tagged Ki-Ras. Exogenous protein was detected by immunofluorescence against the HA tag. Generation of Ki-Ras mutants is described in Methods. Different planes of the cell are shown, above (Cys$^\gamma$) or inside (Cys$^\gamma$ and Lys$^\beta$) the nucleus.
Ki-Ras partially reduced tolerance to oxidative injury compared with Ki-Ras wild type–expressing cells, whereas a significant increase in apoptosis was observed in Lys/Cys–mutant transfected cells. The double Lys/Cys mutation abolished completely the Ki-Ras protective effect. Note that the effect of cysteine mutation, albeit small compared with that of the Lys–mutant, was significant and reproducible (Figures 4a and 5a). These findings indicate that the protection from apoptosis by the Ki-Ras isoform was mainly determined by the polybasic region at the COOH terminus. ERK1/2-dependent transcription was not affected in Lys–mutants. Determination of ROS in Ki-Ras–transfected cells indicates that low ROS levels parallel resistance to H₂O₂-induced apoptosis. Moreover, mutation of lysines at the COOH terminus of Ki-Ras significantly increased ROS levels, although Ki-Ras Cys–also showed a partial effect (Figure 4b). These data suggest that susceptibility to H₂O₂-induced apoptosis is linked to the ability of Ki-Ras to lower ROS levels.

Immunoblot with anti–Ki-Ras antibody shows that transfected cells expressed comparable levels of Ras proteins (Figure 4a, inset). Staining of transfected cells with an antibody against the HA tag of the exogenous proteins shows that the Cys–mutation resulted in a significant loss of the canonical plasma membrane signal without significant changes in intracellular dotlike fluorescence (Figure 4c). Conversely, in Lys– Ki-Ras–expressing cells, the dotlike fluorescence disappeared. The double-mutant protein was completely diffuse throughout the cell (Figure 4c). Although the immunofluorescence data shown do not identify the specific intracellular compartment(s) where Ki-Ras mutants are localized, they suggest a major modification in the localization pattern relative to the wild-type gene.

**How the Ki-Ras Lipid Anchor Contributes to the Protection of Endothelial Cells From H₂O₂-Induced Apoptosis**

The data shown above indicate that the main lipid anchor signal, the cysteine within the CAAX box, does not have a dominant role in the tolerance to H₂O₂-induced apoptosis in Ki-Ras–expressing cells. To assess independently the role of Ki-Ras CAAX box farnesylation signal in the development of tolerance to oxidative injuries in HUVECs, we used the farnesyl transferase inhibitor, FTI-277, which prevents farnesylation of Ras proteins. Control cells and Ha-Ras and Ki-Ras wild-type and mutant transfected cells were incubated, before the application of the oxidative stress, with FTI-277 (Figure 5a). This compound significantly inhibited apoptosis in control and Ha-Ras–expressing cells. Conversely, the protective effect of Ki-Ras wild type was only partially affected. Treatment with FTI-277 resulted in a partial stimulation of apoptosis, in cells expressing either Lys– or Cys–Ki-Ras. Figure 5 indicates that FTI-277 treatment does not eliminate the protective effect of Ki-Ras, as does the mutation in the lysine stretch at the COOH terminus. A reproducible effect of FTI-277 treatment can only be detected in Lys– Ki-Ras–expressing cells. FTI-277 has a limited effect on wild-type or Cys–Ki-Ras–expressing cells. Taken together, these data indicate that farnesylation has a minor role in cytoprotection by Ki-Ras.

To test the effect of FTI-277 on the intracellular localization of Ha-Ras and Ki-Ras proteins, we transiently expressed fusion proteins containing the wild-type or mutant COOH termini of Ki-Ras and GFP at the NH₂ terminus. GFP in these fusion proteins contains the natural Ki-Ras localization signals (see Methods). The fluorescence staining of FTI-277–pretreated cells indicates that the compound efficiently displaced the GFP-fusion protein carrying the COOH terminus of Ha-Ras. On the other hand, a redistribution of membrane localization, without any significant changes in the intracellular fluorescent dots, was observed in cells expressing the GFP-fusion protein carrying the COOH terminus of Ki-Ras (Figure 5b).

At present we do not know whether the dots shown by the Ki-Ras fluorescent signal indicate a specific membrane subcompartment or a different organelle where Ki-Ras is local-
ized. In both cases, this location appears to be important for the tolerance of oxidative stress by Ki-Ras.

Discussion

The production of intracellular ROS has been implicated in the pathogenesis of several human disorders (Alzheimer’s and Parkinson’s diseases, cancer, cerebral and myocardial ischemia, atherosclerosis, etc), as well as aging. Recent insights into the p21 Ras ERK1/2 signaling cascade suggest that Ras is directly involved in the regulation of the intracellular redox state.

HUVeCs, exposed to 2 mmol/L H2O2 for 2 hours, undergo apoptosis 6 to 24 hours later. This is accompanied by activation of stress-related kinases that culminates in the apoptotic death of stressed cells. This process is initiated by H2O2, which increases intracellular ROS and triggers Ras activation. Ras (specifically Ha-Ras) stimulates intracellular ROS levels by activating NADPH oxidase, further amplifying the cascade initiated by H2O2. Ki-Ras and Ha-Ras regulate H2O2-induced apoptosis in a complementary fashion; Ki-Ras increases the tolerance, whereas Ha-Ras decreases it and promotes apoptosis induced by H2O2. Both Ki-Ras and Ha-Ras effects are dependent on the Ras signaling cascade because the transdominant negative Ras variant Leu61-Ser186 completely suppresses both the Ki- and the Ha-effects (Figure 2). Ras activation of MEK and ERK1/2 signaling is required for stimulation of H2O2-dependent apoptosis (Figure 2b) or protection (Figure 3c and 3d). Because these cells express predominantly Ha-Ras, it is likely that inhibition of H2O2-dependent apoptosis by MEK inhibitor PD98059 or farnesyl transferase inhibitor FTI-277 is dependent on ERK1/2 signaling triggered by Ha-Ras (Figure 3c and 3d). On the other hand, cells transfected with Ki-Ras are resistant to H2O2-dependent apoptosis, and they became sensitive when treated with the MEK inhibitor PD98059 (Figure 2b). These data are puzzling, because they indicate that the same enzymatic cascade induces opposing phenotypes in the same cell type (Figures 2b and 3). In fact, there are reports showing that ERK1/2 signaling inhibits apoptosis or that ERK1/2 signaling inhibits apoptosis. The analysis of the available data may shed some light on this apparent paradox. Ha-Ras is an important activator of the NADPH oxidase complex, because it participates in the assembly under the plasma membrane of the oxidase complex. The activation of the NADPH oxidase complex results in a significant increase of cellular ROS. ROS can directly activate GTP binding proteins, such as Ras or GTP binding proteins or increase cellular levels of ROS. Ha-Ras stimulates the activity of the NADPH oxidase complex, which in turn increases ROS levels. Ki-Ras, on the other hand, stimulates powerfully ERK1/2 signaling and activates both transcription and mitochondrial MnSOD, which reduces superoxide anion levels in combination with catalase. This might be accomplished either by a different membrane compartmentalization or by a discrete subcellular localization.

Figure 6. Regulation of cellular ROS by Ha-Ras and Ki-Ras. Schematic diagram showing effects of H2O2 treatment on Ras signaling. Oxidative stress initiated by H2O2 may activate directly Ras or GTP binding proteins or increase cellular levels of ROS. Ha-Ras stimulates the activity of the NADPH oxidase complex, which in turn increases ROS levels. Ki-Ras, on the other hand, stimulates powerfully ERK1/2 signaling and activates both transcription and mitochondrial MnSOD, which reduces superoxide anion levels in combination with catalase. This might be accomplished either by a different membrane compartmentalization or by a discrete subcellular localization.

massive apoptosis if not restrained by a complementary activity. Ki-Ras performs this function by reducing cellular ROS and antagonizing Ha-Ras. Ki-Ras effects on ROS are dependent on a polybasic lysine stretch at the COOH terminus and not on the classical farnesylation box that is known to be essential for Ras activity (for review, see Reference). We have recently found that Ki-Ras but not Ha-Ras stimulates the mitochondrial enzyme manganese superoxide dismutase (MnSOD) via ERK1/2 signaling and that the polybasic region of Ki-Ras is essential for this induction. Taken together, the data suggest that Ki-Ras reduces H2O2-induced apoptosis by buffering the production of endogenous ROS via MnSOD-ERK1/2 signaling (Figure 6). The difference between ERK1/2 activated by Ha-Ras or Ki-Ras is probably accounted for by a different membrane compartmentalization or by a discrete organelle localization encoded by the polybasic lysine stretch.

The protective effect of Ki-Ras on H2O2-induced apoptosis is abolished by Ras dominant negative variants and MEK inhibitor, but not by FTI-277 (Figures 2, 3, and 5). This is not surprising, given that Ki-Ras 4B is relatively insensitive to FTIs. On the other hand, total ERK1/2 activity in HUVeCs is almost totally dependent on Ha-Ras, which is the predominant isoform expressed by these cells (Figure 3a). Thus, PD98095 induces cytoprotection both in Ha-Ras–expressing cells and in control HUVeCs. Conversely, cells expressing Ki-Ras are more sensitive to apoptosis when treated with MEK inhibitor. It is likely that overexpression of Ki-Ras masks the activation of ERK driven by the endogenous Ha-Ras gene. Similarly, the antiapoptotic effect of FTIs in HUVeCs stressed with H2O2 can be explained by selective inhibition of Ha-Ras.

The data we have presented contain some general implications. In cells or tissues with high Ki-Ras/Ha-Ras ratio, the reduction of ROS by extracellular stimuli is dominant over...
the production of ROS, and it may result in profound changes in oxidative metabolism. Conversely, endothelial cells, expressing predominantly Ha-Ras (Figure 2), are exquisitely sensitive to oxidative stress. This notion is supported also by recent data showing that activated Ha-Ras, but not Ki-Ras, can induce apoptosis.31

In conclusion, our data highlight a new, modulatory role of the 2 Ras genes in endothelial cell response to oxidative injuries and point to the potential utilization of p21 Ras as an alternative molecular target in disorders associated with high levels of intracellular ROS.

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