Oncogenic Ras mediates apoptosis in response to Protein Kinase C inhibition through the generation of reactive oxygen species

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Running Title: Ras induces apoptosis by ROS
Ras is a well-established modulator of apoptosis. Suppression of PKC activity can selectivity induce apoptosis in cells expressing a constitutively activated Ras protein. We wished to determine whether reactive oxygen species serve as an effector of Ras-mediated apoptosis. Ras-transformed NIH/3T3 cells contained higher basal levels of intracellular H$_2$O$_2$ compared to normal NIH/3T3 cells, and PKC inhibition upregulated ROS to five-fold greater levels in Ras-transformed cells than in normal cells. Treatment with N-acetyl-L-cysteine reduced both the basal and inducible levels of intracellular H$_2$O$_2$ in NIH/3T3-Ras cells and antagonized the induction of apoptosis by PKC inhibition. Culturing NIH/3T3-Ras cells in low-oxygen conditions, which prevents ROS generation, also inhibited the apoptotic response to PKC inhibition. These results suggest that reactive oxygen species are necessary as downstream effectors of the Ras-mediated apoptotic response to PKC inhibition. However, the generation of ROS alone is not sufficient to induce apoptosis in Ras-transformed cells because inhibition of cell cycle progression prevented the induction of apoptosis in NIH/3T3-Ras cells without inhibiting the generation of intracellular H$_2$O$_2$ observed after PKC inhibition. These findings suggest that continued cell cycle progression of Ras-transformed cells during PKC inhibition is also necessary for the induction of apoptosis.
INTRODUCTION

The Ras proto-oncogene serves as a molecular switch controlling a variety of cellular processes including proliferation (1), differentiation (2), and senescence (3). Point mutations that cause single amino acid substitutions in the normal cellular Ras protein lead to its constitutive activation (4). This dominant mutant form of Ras plays a major role in the multi-step progression of tumorigenesis in many human tumors, with oncogenic ras mutations occurring in approximately 30% of all human tumors (5). Strikingly, over 90% of pancreatic tumors and 50% of colorectal tumors analyzed contain ras mutations.

Our laboratory and others have established Ras as a modulator of apoptosis (6-8). Paradoxically, Ras can either inhibit or promote apoptosis, with the outcome probably dependent upon cell type and the presence of other pro-apoptotic or anti-apoptotic signals (6). Ras can inhibit apoptosis induced by a variety of stimuli and protection against these apoptotic stimuli by Ras is thought to contribute to tumorigenesis. Studies have shown that phosphoinositide-3-OH kinase (PI3K), a direct downstream effector of Ras, plays a crucial role in mediating Ras protection against apoptosis by activating PKB/AKT (9,10). PKB/AKT activation may prevent apoptosis by phosphorylating the pro-apoptotic protein BAD (11), allowing it to be sequestered by 14-3-3 proteins (12). Alternatively, PKB/AKT may also activate NF-κB as a protective mechanism against apoptosis (13), as NF-κB activity is known to be protective against various forms of apoptosis (14), including Ras-mediated apoptosis. Inhibition of NF-κB activity results in apoptosis of Ras-transformed cells (15). Similarly, expression of the transcriptional repressor protein Par-4, which can inhibit NF-κB transcriptional activity, also induces apoptosis in Ras-transformed cells (16). In addition to activation of PI3K by Ras, activation of the Raf-MAP
kinase pathway may also be necessary for Ras-mediated protection against apoptosis, downstream of growth factor signaling (17). Curiously, Ras activation of the Raf-MAP kinase pathway may actually enhance apoptosis induced by c-Myc (9).

Ras can sensitize cells to apoptosis induced by a variety of stimuli (6), and high level expression of oncogenic Ras alone can induce apoptosis as well (18). Our laboratory has demonstrated that the inhibition of PKC activity can also induce apoptosis in cells expressing activated Ras (7,8). Like NF-κB, PKC can also protect against many forms of apoptosis (19) and basal levels of PKC activity may protect against Ras-induced apoptosis. Although the mechanisms by which Ras provides protection against apoptosis are being elucidated, less is understood about how Ras can induce apoptosis or sensitize cells to different apoptotic stimuli. Some studies have reported that the activation of ERK and/or JNK is important for Ras-mediated apoptosis (18,20), while others show no role for ERK or JNK activation (21,22). Elucidation of the role of ERK activation in Ras-mediated apoptosis is complicated by the fact that activation of the Raf-MAP kinase pathway can also inhibit apoptosis (17,23,24).

One downstream effector of Ras that could potentially mediate or initiate an apoptotic process is reactive oxygen species (ROS)

ROS can influence numerous intracellular pathways, including those leading to programmed cell death (25,26). As intracellular second messengers, ROS also control a variety of Ras-mediated cellular effects (27). We wished to determine whether ROS mediated apoptosis of Ras-transformed cells in which PKC was inhibited. We report here that the generation of ROS is necessary for Ras to initiate apoptosis when PKC is inhibited. However, upregulation of ROS alone is not sufficient for the induction of apoptosis by PKC. Our studies indicate that cell cycle progression is also necessary for Ras-mediated apoptosis to occur, independent of ROS generation.
EXPERIMENTAL PROCEDURES

**Cell culture.** The NIH/3T3 mouse embryo fibroblast line and Balb/3T3-clone A31 mouse embryo fibroblast line were obtained from ATCC (American Type Culture Collection, Rockville, MD). NIH/3T3-Ras cells were produced by stable transfection of NIH/3T3 cells with v-Ha-ras, and selected and maintained in 0.5 μg Geneticin/ml (Gibco BRL). KBalb cells were produced by stable transfection of Balb cells with v-Ki-ras and have been described previously (28). KBalb-Bcl2 cells were produced by stable transfection of KBalb cells with a retroviral Bcl-2 expression vector (29). Cells were maintained in Dulbecco Modified Eagle medium (DME) supplemented with 10% DCS, 2 mM L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (Gibco BRL).

**Cell viability assay.** Cells were cultured in 24-well plates at 2 x 10^4 cells/well and treated in triplicate with phorbol 12-myristate 13-acetate (PMA) (Sigma) or 1-O-hexadecyl-2-O-methyl-rac-glycerol (HMG) (Calbiochem) for the times indicated. Background control wells were treated with 0.19% saponin for 10 minutes prior to assay. Wells were washed once with phosphate buffered saline (PBS) and 500 μL of 1 μM calcein-AM (Molecular Probes) in PBS was added to each well. Plates were incubated at room temperature for 30 minutes. Fluorescence was quantified on a Cytofluor 2300 fluorescent plate reader (PerSeptive Biosystems) at excitation/emission wavelengths of 485/530 nm.

**DNA fragmentation and cell cycle analysis.** Cells were plated at 1 x 10^5/plate in 60 mm dishes and treated for the indicated times. Cells were harvested with trypsin/EDTA, and fixed in 35% ethanol/65% DME. Cells were washed once in ice-cold PBS, and resuspended in 25 μg propidium iodide /ml and 50 μg RNase A /ml in PBS. Samples were incubated at 37°C for
2 hours and DNA profiles were analyzed by FACS using a FACScan flow cytometer (Becton Dickenson).

**Measurement of ROS.** Cells were plated at 1 x 10^5/plate in 60 mm dishes and treated for the indicated times. Cells were harvested with trypsin/EDTA, washed once in PBS, and resuspended in 5 μg 2’-7’ dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes) /ml in Hank’s balanced salt solution (HBSS). Samples were incubated for 10 minutes at room temperature and analyzed immediately by FACS. For manipulation of ROS levels, cells were either treated with 20 mM NAC (Sigma) or placed in sealed chambers (Billups-Rothenberg), which had been flushed for 30 minutes with 95% N_2/5% CO_2 as previously described (30) for 24 hours before treatment with HMG.

**In vitro PKC assay.** Cellular PKC activity was measured using a commercial assay, following the manufacturer’s protocol (Upstate Biotechnology) and described previously (7). Briefly, cells were treated with 150 μM HMG for 24 hours or left untreated in normal culture conditions before harvesting with trypsin/EDTA. Cells were lysed in 25 mM Tris-HCl (pH 7.5), 1% Triton X-100, 20 mM MgCl_2, and 150 mM NaCl and extracts were normalized for protein concentration. Subsequently, 100 μg of extract was incubated with a PKC-specific peptide substrate, [^{32}P]ATP, and inhibitors of PKA and calmodulin kinase for 10 minutes at 30°C. ^{32}P incorporated into the substrate was separated from residual ^{32}P using p81 phosphocellulose paper and quantified by scintillation counting.

**Measurement of Ras activity.** NIH/3T3-Ras cells were treated as indicated for 24 hours, either in low-oxygen culture conditions, or with sodium butyrate (5 mM) or TsA (100ng/ml). For control, NIH/3T3 cells were serum-starved in medium containing 0.5% DCS for 24 hours and endogenous Ras was activated by PDGF (30ng/ml) stimulation for 15 minutes. Ras activity was
measured using the technique developed by de Rooij and Bos (31). Briefly, cells were lysed in a
buffer containing 25mM HEPES, pH 7.5, 150mM NaCl, 1% Igepal CA-630, 0.25% sodium
deoxycholate, 10% glycerol, and 25 mM NaF. Protein was normalized to 1ug/ul and activated
Ras was affinity precipitated by mixing 1mg of cell lysate with 10ug of Raf-1 RBD agarose bead
conjugate (Upstate Biotechnology), end over end for 30 minutes at 4°C. Agarose bead conjugates
were washed 3 times in lysate buffer, resuspended in laemmlie sample buffer, and boiled for 5
minutes. Beads were pelleted and supernatant was loaded onto a 10% SDS-PAGE gel and
electrophoresed. Proteins were transferred onto PVDF membrane and immunoblotted with a pan-
Ras antibody (Oncogene Research Products). Ras protein was detected using ECL-Plus
chemoluminescent reagent (Amersham).
RESULTS

Our laboratory has previously shown that downregulation of PKC by chronic, high-dose PMA treatment (32) could selectively induce apoptosis in Jurkat human T lymphoblastoid cells stably-expressing v-Ha-ras (PH1 cells) when compared to normal Jurkat cells (7,8). Downregulation of PKC by high-dose PMA treatment also selectively induced cell death in both v-Ki-ras transformed Balb fibroblasts (KBalb) and v-Ha-ras transformed NIH/3T3 fibroblasts (NIH/3T3-Ras) when compared to normal Balb and NIH/3T3 cells (Fig. 1a). Treatment with 100 nM PMA, a concentration that activates PKC but is insufficient to cause downregulation of PKC, consistently caused less than 10% loss in cell viability of the Ras-transformed fibroblasts. In contrast, chronic high-dose treatment of Ras-transformed fibroblasts with 500 nM PMA, a concentration known to cause downregulation of PKC after prolonged exposure (32), caused between 85%-95% cell death after 72 hours. No loss in cell viability was seen in normal Balb or NIH/3T3 fibroblasts with either 100 nM or 500 nM PMA treatment. These findings demonstrate that the suppression of PKC activity, but not activation of PKC, can trigger cell death in the presence of oncogenic Ras activity.

To determine whether a pharmacological inhibitor of PKC could also selectively induce cell death in Ras-transformed cells, the DAG antagonist HMG was used to treat both normal and Ras-transformed fibroblasts. Treatment of KBalb and NIH/3T3-Ras fibroblasts with 150 μM HMG caused Ras-specific cell death, similar to that achieved using chronic, high-dose PMA downregulation of PKC. Ras-transformed cells treated with 150 μM HMG underwent 80%-90% loss in cell viability after 48 hours, while less than 10% loss of viability was observed after treatment of normal Balb and NIH/3T3 cells (Fig. 1b). Other selective PKC inhibitors, such as Ro-31-8220 and GF109203X, were also able to differentially induce cell death in Ras-
transformed cells. Overexpression of Bcl-2 in KBalb cells by transfection with a retroviral expression vector inhibited the apoptosis induced by HMG treatment, consistent with our previous findings that Bcl-2 overexpression effectively inhibited death induced by chronic, high-dose PMA treatment in cells expressing activated Ras (8). To verify that the dose of HMG that selectively induced cell death was also inhibiting PKC activity, an in vitro PKC activity assay was performed. Treatment with 150 µM HMG suppressed the cellular PKC activity of cells growing logarithmically in 10% serum-containing medium by greater than four-fold (Fig. 1c).

Photomicroscopy of NIH/3T3-Ras cells treated with HMG showed changes in morphology consistent with apoptosis (Fig. 1d). Quantification of apoptosis by propidium iodide staining and FACS analysis showed that NIH/3T3-Ras cells underwent increasing amounts of DNA fragmentation over a 72 hour period, with over 70% of the population containing a hypodiploid DNA content at 72 hours (Fig. 2a). In contrast, no increase in DNA fragmentation was observed in parental (normal) NIH/3T3 after 72 hours of HMG treatment when compared to basal levels (Fig. 2b). Concurrent analysis of cell cycle indicated that over 98% of parental NIH/3T3 cells were arrested in the G0/G1 phase after PKC inhibition (Fig. 2c). This cell cycle arrest was reversible, and NIH/3T3 cells resumed cell cycle progression once HMG was washed out of culture.

To determine if reactive oxygen species served as effectors of Ras-mediated apoptosis, intracellular ROS levels were measured by FACS using the peroxide-sensitive fluorescent indicator, DCF. NIH/3T3-Ras cells showed higher basal levels of ROS compared to parental NIH/3T3 cells (Fig. 3a). KBalb cells also showed higher basal ROS levels compared to parental Balb cells. HMG treatment for 24 hours caused an upregulation of ROS levels in both NIH/3T3 and NIH/3T3-Ras (Fig. 3b). However, while ROS levels were upregulated by only two-fold in
NIH/3T3 cells in response to HMG, ROS levels increased by more than seven-fold in NIH/3T3-Ras cells. Treatment with the structurally-unrelated PKC inhibitors Ro-31-8220 and GF109203X also resulted in similar upregulation of ROS levels, suggesting that ROS generation by HMG correlated with PKC inhibition.

To establish whether the upregulation of ROS levels is necessary for Ras-mediated apoptosis, the antioxidant N-acetyl-cysteine (NAC) was used to pre-treat Ras-transformed cells. Treatment of NIH/3T3-Ras cells with 20 mM NAC for 24 hours reduced the basal ROS to levels similar to those in parental NIH/3T3 (Fig. 4a). Moreover, pre-treatment of NIH/3T3-Ras cells with NAC completely inhibited the upregulation of ROS levels induced by HMG, reducing ROS to levels comparable to those found in untreated NIH/3T3-Ras cells (Fig. 4b). Pre-treatment of NIH/3T3-Ras cells with NAC also effectively inhibited the DNA fragmentation induced by HMG. DNA fragmentation was reduced from a thirteen-fold increase in cells unprotected by NAC treatment, to two-fold in cells pre-treated with NAC (Fig. 4c). Culturing cells under a low oxygen environment is another method for inhibiting intracellular ROS generation (33). NIH/3T3-Ras cells were cultured in either normoxic or hypoxic conditions for 24 hours before treatment with HMG. After PKC inhibition, cells were maintained in normoxic or hypoxic conditions for another 48 hours before analysis of DNA fragmentation by FACS. While NIH/3T3-Ras cells maintained in normoxic conditions underwent a ten-fold increase in DNA fragmentation upon HMG treatment, DNA fragmentation was reduced to only two-fold in NIH/3T3-Ras cells cultured in hypoxia (Fig. 5a). In contrast, serum-deprivation, which can induce apoptosis independently of ROS generation (34), was able to induce DNA fragmentation in NIH/3T3 cells, regardless of normoxic or hypoxic culture conditions (Fig. 5b).
To determine a mechanism by which PKC inhibition generates ROS, studies were performed using diphenylene iodonium (DPI), an inhibitor of NAD(P)H oxidase (35). Pretreatment of NIH/3T3-Ras cells with DPI did not inhibit the upregulation of ROS by HMG, suggesting the lack of a role for NAD(P)H oxidase in generating ROS downstream of PKC inhibition (data not shown). Since mitochondria play an important role in many forms of apoptosis and are also a major source for ROS production (36), we investigated the involvement of mitochondria as the source of ROS downstream of PKC inhibition. To explore the possibility that the inhibition of PKC generates ROS as a consequence of the opening of the mitochondrial permeability transition pore (PTP), studies were performed using cyclosporine A (CsA), an inhibitor of the mitochondrial PTP (37). Pretreatment of NIH/3T3-Ras cells with CsA did not affect the basal level of ROS in the cells, but effectively blocked the upregulation of ROS by HMG treatment (Fig. 6a). Moreover, the inhibition of HMG-induced ROS generation by CsA correlated with the inhibition of apoptosis. NIH/3T3-Ras cells pretreated with CsA exhibited nearly a ten-fold reduction in DNA fragmentation induced by HMG (Fig. 6b).

Previous results from our laboratory suggested that cell cycle progression may also be necessary for Ras-mediated apoptosis (7). Attempts to use the cell cycle-arresting agents hydroxyurea or aphidicolin to inhibit the cell cycle progression of Ras-transformed cells resulted in high levels of cytotoxicity. However, the cell cycle-arresting agents sodium butyrate and trichostatin A (TsA) were relatively nontoxic to NIH/3T3-Ras cells and were therefore tested for their ability to inhibit Ras-mediated apoptosis induced by PKC inhibition. Pre-treatment of NIH/3T3-Ras cells with either sodium butyrate (5 mM) (Fig. 7a) or TsA (100ng/ml) (Fig. 7b) reduced the induction of DNA fragmentation in cells by HMG treatment from over thirteen-fold to less than two-fold. Concurrent cell cycle analysis showed inhibition of cell cycle progression
and accumulation of cells in $G_1$ induced by both sodium butyrate and TsA treatments (Fig. 8a). Treatment with either sodium butyrate or TsA reduced the percentage of cells in S phase by more than five-fold. In contrast, treatment with isobutyramide, an analog of sodium butyrate (38), had no effect on the cell cycle distribution of NIH/3T3-Ras cells (Fig. 8a) and also did not prevent induction of apoptosis by HMG$^2$. Thus, inhibition of apoptosis by these compounds correlated with inhibition of cell cycle progression. Our laboratory has shown that continued Ras activity is necessary to mediate apoptosis induced by PKC inhibition, as inhibition of Ras activity with a farnesyltransferase inhibitor can inhibit apoptosis$^3$. To rule out the possibility that the inhibition of apoptosis by hypoxic culture conditions, sodium butyrate treatment, or trichostatin A treatment were due to effects on Ras activity, Ras activity was analyzed by affinity-binding to a Raf Ras-binding-domain (RBD) peptide which only binds the activated (GTP-bound) form of Ras. Stimulation of serum-starved NIH/3T3 cells with PDGF (30 ng/ml) resulted in an increase in Ras activity as measured by the Raf-RBD-GST pulldown assay (Fig. 8b1). NIH/3T3-Ras cells constitutively expressed a relatively high level of activated Ras and culturing NIH/3T3-Ras cells under hypoxic conditions, or treatment with either sodium butyrate or TsA, had no inhibitory effect on the levels of Ras activity (Fig. 8b2).

Interestingly, despite the strong protection against apoptosis, pre-treatment of NIH/3T3-Ras cells with sodium butyrate also had no significant effect on either basal ROS levels, or the upregulation of ROS levels by HMG (Fig. 8c). These results, together with the protective effects of antioxidants agents like NAC, suggest that the observed increase in ROS levels after PKC inhibition are necessary for Ras-mediated apoptosis, but that increased ROS levels alone are not sufficient, and that enforced cell cycle progression may be required as well. To determine whether the transition between specific phases of cell cycle might be necessary for Ras-mediated
apoptosis, the mitotic inhibitor nocodazole was used to arrest NIH/3T3-Ras cells in M phase. Pre-treatment of NIH/3T3-Ras cells with nocodazole (400 ng/ml) did not prevent the DNA fragmentation induced by PKC inhibition (Fig. 9a), suggesting that transition through M phase is not necessary for Ras-mediated apoptosis. Previous reports have shown that nocodazole treatment can induce endoreduplication, the process in which cells bypass mitosis but continue to pass through the other cell cycle phases and replicate DNA (39), and we also observed a large fraction of NIH/3T3-Ras cells treated with nocodazole undergoing endoreduplication. FACS analysis showed that after 24 hours of nocodazole treatment, more than 80% of NIH/3T3-Ras cells were arrested in the M phase of cell cycle and contained a 4n quantity of DNA (Fig. 9b). By 72 hours though, only 20% of the population remained at 4n, while 52% of the cells contained an 8n quantity of DNA, and thus had passed at least twice through S phase. We conclude from these experiments that mitosis is not necessary for Ras-mediated apoptosis induced by PKC inhibition, but the results using sodium butyrate or TsA suggest that transition through other phases of cell cycle may be important.
DISCUSSION

The concept of using oncogenically-mutated Ras as a molecular target for cancer therapy is an appealing one, but it has not been clear how Ras is able to induce apoptosis or sensitize cells to apoptosis in response to various apoptotic stimuli. We have shown that downregulation of PKC by chronic, high-dose PMA treatment selectively induces apoptosis in cells expressing oncogenic Ras. Since suppression of PKC activity by chronic high-dose PMA exposure is not an approach that can be translated into in vivo studies, we wished to establish a model for studying Ras-mediated apoptosis using a pharmacological inhibitor of PKC that could potentially be developed as a clinical therapeutic against human tumors containing oncogenically-mutated Ras. The PKC inhibitor, 1-O-hexadecyl-2-O-methyl-rac-glycerol (HMG), was first discovered as a metabolite of its phosphocholine analog, 1-O-hexadecyl-2-O-methylglycero-3-phosphocholine (HMG-PC), and shown to inhibit PKC activity both in vitro and in vivo (40). HMG has also been reported to inhibit the PKC-dependent respiratory burst in human neutrophils (41), the PKC-dependent activation of phospholipase D in human fibroblasts by bradykinin (42), and the PKC-dependent suppression of apoptosis in T lymphocytes by IL-2 (43).

Our studies show that HMG can be used to inhibit cellular PKC activity, and to selectively induce apoptosis in Ras-transformed cells while sparing normal cells. Interestingly, HMG-PC was first described to have anti-neoplastic activity against the promyelocytic leukemia line HL-60, but not against the erythroblastic leukemia line K562 (44). HMG-PC has also been reported to be non-cytotoxic to either normal human neutrophils or skin fibroblasts (45). The mechanism for the differential cell line-specific cytotoxicity of HMG-PC observed in these previous studies is unclear. No correlation was found between cell-specific sensitivity to the effects of HMG-PC and either alkyl cleavage enzyme activity (as a mechanism for drug
activation) (46), or incorporation of drug into the plasma membrane and changes in membrane fluidity (47). However, HL-60 cells contain an activating mutation of N-ras while K562 cells do not contain any ras mutations (48). This raises the possibility that when HMG-PC accumulates in the plasma membrane and is metabolized into HMG (40), the resulting inhibition of PKC activity triggers cell death in HL-60 cells by a Ras-mediated mechanism. Furthermore, the ether phospholipid ET-18-OCH3 also inhibits PKC (49), and has been reported to both increase ROS levels (50) and induce apoptosis in HL-60 cells (51), consistent with our studies showing that Ras-mediated apoptosis in response to PKC inhibition by HMG is accompanied by ROS generation.

The source of the increased basal and inducible H$_2$O$_2$ levels we observed in Ras-transformed cells has not yet been defined. Our findings that the intracellular peroxide levels in Ras-transformed mouse fibroblasts are higher than in normal fibroblasts are consistent with the report by Irani, et al. that Ras-transformed NIH/3T3 cells produced larger amounts of superoxide than normal NIH/3T3 under basal conditions (52). This superoxide production is thought to be mediated by a Rac-dependent activation of NADPH oxidase localized to the cellular membrane in non-phagocytic cells (52-54). Though Irani, et al. did not measure the intracellular peroxide levels in Ras-transformed NIH/3T3 cells, they suggested that increased intracellular H$_2$O$_2$ levels were likely to coexist, presumably from the rapid dismutation of superoxide. Indeed, infection of primary human diploid fibroblasts with an oncogenic Ras-expressing retrovirus induced increased intracellular levels of H$_2$O$_2$ (55). In that particular case, however, the increase in intracellular H$_2$O$_2$ was determined to be mitochondria-derived and not via a Rac-dependent pathway.
Functionally, ROS have been shown to serve as intracellular second messengers of Ras, as well as messengers for growth factors that activate Ras. PDGF stimulation increases intracellular H\textsubscript{2}O\textsubscript{2} in vascular smooth muscle cells and this increase in H\textsubscript{2}O\textsubscript{2} is necessary for PDGF-mediated changes, such as MAP kinase activation and DNA synthesis, because inhibition of H\textsubscript{2}O\textsubscript{2} generation by NAC blocks these cellular responses to PDGF (56). EGF stimulation of PC12 cells also results in an increase in ROS that can be inhibited by expression of a dominant-negative N\textsuperscript{17} Ras (57). Elevated ROS levels may contribute to a transformed phenotype by increasing proliferation in a Raf-MAPK independent manner (52) and inducing the transformed morphology by Rac-dependent changes in the actin cytoskeleton (58,59). Moreover, ROS may contribute to the oncogenic process by damaging DNA, increasing genetic instability, and causing the loss of tumor suppressors (60,61). Indeed, our laboratory has shown that Ras-transformed fibroblasts demonstrate increased genetic instability and decreased p53 expression (62). Finally, the induction of senescence by Ras was shown to be dependent on the generation of H\textsubscript{2}O\textsubscript{2}, as treatment with NAC or culturing under low oxygen rescues these cells from Ras-induced senescence (55).

We have shown that the inhibition of PKC can cause an increase in intracellular peroxide levels in both normal and Ras-transformed cells, with much greater ROS induction in Ras-transformed cells. The level of ROS induced by PKC inhibition in normal cells was not sufficient to induce apoptosis, while the higher level of ROS production induced by PKC inhibition in Ras-transformed cells was necessary for apoptosis, as inhibition of intracellular H\textsubscript{2}O\textsubscript{2} generation by NAC treatment or low-oxygen culture conditions inhibited apoptosis. This finding is significant, as ROS generation is not necessary for many forms of apoptosis (63). The mechanism by which PKC inhibition leads to increased ROS generation is unclear. We have previously reported that
Bcl-2 can inhibit Ras-mediated apoptosis (8), and that the ability of Bcl-2 to inhibit Ras-mediated apoptosis depends on its phosphorylation (64). Others have reported that phosphorylation of Bcl-2 by mitochondrial PKCα may be necessary for suppression of apoptosis (65). Many mechanisms for how Bcl-2 inhibits apoptosis have been reported, one mechanism being the prevention of ROS generation (66), and another being the prevention of the opening of the mitochondrial permeability transition pore (67). Our results with CsA suggest that the opening of the mitochondrial permeability transition pore is responsible for ROS generation downstream of PKC inhibition, potentially due to reduced Bcl-2 phosphorylation and function at the mitochondria.

While ROS generation is necessary for Ras-mediated apoptosis in response to PKC inhibition, it is not sufficient. Sodium butyrate treatment inhibited apoptosis in Ras-transformed cells without affecting the generation of ROS caused by PKC inhibition. Sodium butyrate treatment coincidentally inhibited cell cycle progression, and the structurally-unrelated compound TsA was also able to inhibit both cell cycle progression and apoptosis. These findings corroborate previous results from our laboratory showing that anti-sense cyclin E treatment can inhibit apoptosis induced by high-dose PMA downregulation of PKC in Jurkat cells stably-expressing activated Ras (7), and suggest that cell cycle progression is a necessary component of the Ras-mediated apoptotic response to PKC inhibition. The literature also supports the hypothesis that enforced cell cycle progression by activated Ras is required for Ras-induced apoptosis. Re-entry into cell cycle is necessary for the induction of apoptosis in small intestinal villus enterocytes expressing oncogenic Ras (68). Furthermore, the inhibition of cell cycle progression by dominant-negative Ras prevents the apoptosis of serum-deprived neuronal PC12 cells (69).
The cell cycle is thought to have an interdependent connection with the cellular apoptotic program as a mechanism to restrict uncontrolled cell growth and oncogenesis (70,71). Though the connection between cell cycle and apoptosis is not well understood, the cell death program appears to be induced when growth regulatory signals are inappropriate in amplitude or temporal sequence, or otherwise conflicting. We have shown here and in previous studies that suppression of PKC activity induces a reversible G1 arrest in normal cells. Therefore, we believe apoptosis is triggered when a strong growth-stimulating signal, such as oncogenic Ras activity, induces cell cycle progression while the cells are simultaneously receiving a growth-inhibitory signal such as the suppression of PKC activity. Agents that were effective in inducing cell cycle arrest in Ras-transformed cells, such as sodium butyrate and TsA, protected those Ras-transformed cells from apoptosis upon PKC inhibition. In contrast, nocodazole, which blocked mitosis but still allowed cells to re-enter S phase, was ineffective at preventing Ras-induced apoptosis, suggesting that progression through S phase may be the critical cell cycle event in initiating apoptosis. The mechanism for the induction of apoptosis by Ras appears to be p53-independent (15,62,68), a finding of potential clinical importance as p53 is frequently lost during tumorigenesis (72). Future elucidation of the factors connecting cell cycle progression and apoptosis in Ras-transformed cells may provide new insights into the normal cellular mechanisms that prevent tumorigenic progression, and may also yield new therapeutic strategies against tumor cells.
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FIGURE LEGENDS

Figure 1. Suppression of PKC activity induces cell death in Ras-transformed fibroblasts. Loss of cell viability was assessed using a calcein fluorescent viability assay for Ras-transformed and normal mouse fibroblasts treated with a) 100 nM or 500 nM PMA for 72 hours or b) 150 µM HMG for 48 hours. *, p < 0.05 compared to normal parental cells c) cellular PKC activity was measured using an in vitro PKC assay for NIH/3T3 cells growing under normal culture conditions, in the presence or absence of 150 µM HMG for 24 hours. *, p < 0.05 compared to control treated cells. d) Photomicroscopy at 100x magnification of NIH/3T3 and NIH/3T3-Ras cells treated with 150 µM HMG for 48 hours. Results shown are representative of experiments performed at least three times.

Figure 2. PKC inhibition causes apoptosis in Ras-transformed cells and G0/G1 arrest in normal cells. a) NIH/3T3-Ras cells were treated with 150 µM HMG for the times indicated before propidium iodide staining and determination of DNA fragmentation by FACS analysis of hypodiploid DNA content. The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. b) NIH/3T3 cells were treated with HMG for 48 hours, stained with propidium iodide, and analyzed by FACS for DNA fragmentation (log fluorescence scale). The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. c) NIH/3T3 cells were treated with HMG for 48 hours, stained with propidium iodide, and analyzed by FACS for cell cycle arrest (linear fluorescence scale). The percentage of the population of cells in S phase and G2/M phase for each condition are indicated. Results shown are representative of experiments performed at least three times.
Figure 3. **Effects of Ras and PKC inhibition on intracellular ROS levels.** a) NIH/3T3 and NIH/3T3-Ras cells were loaded with the peroxide indicator, DCF, and analyzed by FACS. As a positive control for peroxide-specific increase in fluorescence, NIH/3T3-Ras cells were also treated with 1 mM H$_2$O$_2$ for 10 min. prior to analysis. b) NIH/3T3 and NIH/3T3-Ras cells were treated with HMG for 24 hours prior to loading with DCF and analysis by FACS. Results shown are representative of experiments performed at least three times.

Figure 4. **NAC inhibits both ROS generation and DNA fragmentation induced by PKC inhibition.** a) NIH/3T3 and NIH/3T3-Ras cells were treated with 20 mM NAC for 24 hours prior to analysis of ROS. b) NIH/3T3-Ras cells were treated with 20 mM NAC for 24 hours or left untreated before inhibition of PKC by HMG for 24 hours. Cells were then analyzed for ROS levels. c) NIH/3T3-Ras cells were treated with 20 mM NAC for 24 hours or left untreated before inhibition of PKC by HMG for 48 hours. Cells were then stained with propidium iodide and analyzed by FACS for DNA fragmentation. The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. Results shown are representative of experiments performed at least three times.

Figure 5. **Low oxygen inhibits DNA fragmentation caused by PKC inhibition.** Cells were cultured in a low-oxygen environment in sealed chambers for 24 hours, or left in normoxic conditions. Subsequently, a) NIH/3T3-Ras cells were treated with 150 µM HMG, or b) NIH/3T3 cells were serum-starved as control. Cells cultured in low-oxygen were returned to sealed chambers for another 48 hours before analysis of DNA fragmentation. The percentage of the
population of cells containing less than 2n of DNA in each condition are indicated. Results shown are representative of experiments performed two times.

Figure 6. Cyclosporine A inhibits both ROS generation and DNA fragmentation caused by PKC inhibition. a) NIH/3T3-Ras cells were treated with 10 µM CsA for 24 hours or left untreated before inhibition of PKC by HMG for 24 hours. Cells were then analyzed for ROS levels. b) NIH/3T3-Ras cells were treated with 10 µM CsA for 24 hours or left untreated before inhibition of PKC by HMG for 48 hours. Cell were then stained with propidium iodide and analyzed by FACS for DNA fragmentation. The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. Results shown are representative of experiments performed at least three times.

Figure 7. Sodium butyrate and trichostatin A inhibit DNA fragmentation caused by PKC inhibition. NIH/3T3-Ras cells were treated with a) 5 mM sodium butyrate or b) TsA (100 ng/ml) for 24 hours or left untreated before inhibition of PKC with 150 µM HMG for 48 hours. DNA fragmentation was analyzed by FACS. The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. Results shown are representative of experiments performed at least three times.

Figure 8. Sodium butyrate and trichostatin A inhibit cell cycle progression, but do not affect Ras activity or intracellular ROS levels. a) NIH/3T3-Ras cells were treated with 5 mM isobutyramide, 5 mM sodium butyrate, or TsA (100 ng/ml) for 24 hours before staining with propidium iodide and cell cycle analysis by FACS. The percentage of the population of cells in S
phase for each condition are indicated. **b1)** NIH/3T3 cells were serum-starved in media containing 0.5% DCS for 24 hours and left untreated (lane 1) or stimulated with PDGF (30 ng/ml) for 15 minutes (lane 2). Cells were subsequently analyzed for Ras activity by GST-Raf-RBD (Ras Binding Domain) pull-down of activated Ras and immunoblot analysis. **b2)** NIH/3T3-Ras cells were grown in normal culture conditions (lane 1), cultured in low-oxygen (lane 2), treated with 5 mM sodium butyrate (lane 3), or treated with TsA (100 ng/ml) (lane 4). After 24 hours, cells were analyzed for Ras activity by GST-Raf-RBD pull-down of activated Ras and immunoblot analysis. **c)** NIH/3T3-Ras cells were treated with 5 mM sodium butyrate for 24 hours or left untreated before inhibition of PKC by HMG for 24 hours and analysis of ROS levels by FACS. Results shown are representative of experiments performed two times.

Figure 9. **Transition through M phase is not necessary for apoptosis induced by PKC inhibition.** **a)** NIH/3T3-Ras cells were treated with nocodazole (400ng/ml) for 24 hours or left untreated before inhibition of PKC by HMG for 48 hours and analysis of DNA fragmentation by FACS. The percentage of the population of cells containing less than 2n of DNA in each condition are indicated. **b)** NIH/3T3-Ras cells were treated with nocodazole (400ng/ml) for 24 hours or 72 hours before cell cycle analysis by propidium iodide staining and FACS. Results shown are representative of experiments performed two times.

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1 Abbreviations used in this paper: PKC, Protein Kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; HMG, 1-O-Hexadecyl-2-O-methyl-rac-glycerol; ROS, reactive oxygen species; DCF, dichlorodihydrofluorescein diacetate; FACS, fluorescent-activated cell sorter; DPI, diphenylene iodonium; TsA, trichostatin A.

2 Unpublished observation

3 Manuscript in preparation
FIGURE 2

a)

NIH/3T3-Ras

< 2N = 8%

+150μM HMG: 24 Hrs

< 2N = 34%

+150μM HMG: 48 Hrs

< 2N = 67%

+150μM HMG: 72 Hrs

< 2N = 72%

FL2-H

b)

NIH/3T3

< 2N = 8%

+150μM HMG

< 2N = 8%

FL2-H
c)

NIH/3T3

S+G2/M = 20%

+150μM HMG

S+G2/M = 2%

FL2-A
FIGURE 3

LEGEND

a) 1. NIH/3T3
2. NIH/3T3-Ras
3. NIH/3T3-Ras + 1mM H2O2

b) 1. NIH/3T3
2. NIH/3T3-Ras
3. NIH/3T3 + 150uM HMG
4. NIH/3T3-Ras + 150uM HMG
FIGURE 4

LEGEND
1. NIH/3T3
2. NIH/3T3-Ras
3. NIH/3T3 +20mM NAC
4. NIH/3T3-Ras +20mM NAC

LEGEND
5. NIH/3T3
6. NIH/3T3-Ras
7. NIH/3T3-Ras +150uM HMG
8. NIH/3T3-Ras +20mM NAC +150uM HMG

FL1-H

Events

FL2-H

< 2N = 4%

< 2N = 52%

< 2N = 4%

< 2N = 8%
FIGURE 6

LEGEND
1. NIH/3T3
2. NIH/3T3-Ras
3. NIH/3T3-Ras + 10mM CsA
4. NIH/3T3-Ras + 150mM HMG
5. NIH/3T3-Ras + 10mM CsA + 150mM HMG

a)

b)

NIH/3T3-Ras
+10mM CsA

<2N=4%

<2N=4%

NIH/3T3-Ras
+150mM HMG

<2N=77%

NIH/3T3-Ras
(10mM CsA pre-Rx)
+150mM HMG

<2N=8%
FIGURE 9

a) NIH/3T3-Ras
   +400ng/ml Nocodazole
   < 2N = 10%

   +150uM HMG
   < 2N = 77%

   +400ng/ml Nocodazole/150uM HMG
   < 2N = 82%

 FL2-H

b) NIH/3T3-Ras
   4n=25%
   +Nocodazole: 24 Hrs
   4n=80%
   +Nocodazole: 72 Hrs
   4n=20%
   8n=52%

 FL2-A
Oncogenic Ras mediates apoptosis in response to Protein Kinase C inhibition through the generation of reactive oxygen species
James S. Liou, Chang-Yan Chen, James S. Chen and Douglas V. Faller

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