Scavenging of Extracellular \( \text{H}_2\text{O}_2 \) by Catalase Inhibits the Proliferation of \( \text{HER}-2/\text{Neu} \)-transformed Rat-1 Fibroblasts through the Induction of a Stress Response*

Received for publication, May 29, 2000, and in revised form, December 21, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.M004617200

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High levels of reactive oxygen species (ROS) are associated with cytotoxicity. Alternatively, nontoxic levels of ROS like hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) can mediate the transmission of many intracellular signals, including those involved in growth and transformation. To identify pathways downstream of endogenous cellular \( \text{H}_2\text{O}_2 \) production, the response of Rat-1 fibroblasts exhibiting differential HER-2/Neu receptor tyrosine kinase activity to removal of physiological \( \text{H}_2\text{O}_2 \) concentrations was investigated. The proliferation of all cells was abolished by addition of the \( \text{H}_2\text{O}_2 \) scavenger catalase to the culture medium. HER-2/Neu activity was not significantly affected by catalase treatment, suggesting that the target(s) of the \( \text{H}_2\text{O}_2 \) signal lie downstream of the receptor in our model. ERK1/2 phosphorylation was blocked by catalase in fibroblasts expressing wild type Neu, however such a response did not occur in cells possessing activated mutant Neu. This indicates that the ERK1/2 response contributes little to the growth inhibition observed. By contrast, JNKK1 activity increased following the addition of catalase or \( \text{H}_2\text{O}_2 \), regardless of Neu activity or level of cell transformation. Phosphorylation of p38 MAPK was induced by \( \text{H}_2\text{O}_2 \) but not by catalase. These observations suggest that scavenging of \( \text{H}_2\text{O}_2 \) from the cellular environment blocks Rat-1 proliferation primarily through the activation of stress pathways.

Oxidative stress is known to induce the cellular apoptotic program by the activation of defined ROS-responsive signals: the stress-activated protein kinase (SAPK) and nuclear factor kappa B (NF-\( \kappa \)B) pathways ((1), for review see Ref. 2). Activation of c-Jun/AP-1 transcription factors by the c-Jun N-terminal kinase (JNK) members of the SAPKs has been shown to induce apoptosis in several cell types (3). This signal is in contrast to the other characterized roles of c-Jun/AP-1 activity in survival and growth. For example, Jun proteins associate with Nrf 1 and 2 transcription factors to up-regulate the antioxidant/electrophile response element-mediated gene expression as a defense against oxidative stress (4). Similarly, NF-\( \kappa \)B was originally identified as a mediator of tumor necrosis factor \( \alpha \)- and ROS-induced apoptosis but more recently has also been characterized as part of a survival response by stimulating antioxidant response element-regulated and anti-apoptotic protein expression (5, 6). Indeed, ROS, like \( \text{H}_2\text{O}_2 \), are associated with the regulation of multiple cellular processes via interaction with proteins and lipid species (7). These observations have led to the idea that ROS are required “cofactors” in the regulation of many intracellular signal transduction cascades. Addition of micromolar concentrations of \( \text{H}_2\text{O}_2 \) to cells in vitro can activate plasma membrane receptor tyrosine kinases, including the epidermal growth factor receptor/ErbB-1 (8), platelet-derived growth factor receptor (9), and insulin receptor (10). The p21 Ras-MAPK (mitogen-activated protein kinase) pathway, a key growth signal linking membrane receptors to the nucleus, is also stimulated by oxidative stimuli such as \( \text{H}_2\text{O}_2 \) (11, 12). Rapid induction of cellular \( \text{H}_2\text{O}_2 \) generation has been observed to follow the addition of a variety of peptide growth factors to cells, suggesting an autocrine and/or paracrine role for ROS in growth signaling (13–15). The specific molecular targets that are critical for \( \text{H}_2\text{O}_2 \)-mediated mitogenesis have yet to be determined; the importance of a given pathway is also dependent on factors such as cell type, environment, and level of differentiation.

It follows that the deregulation of ROS levels may be a key factor in neoplasia. Elevations in \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) are seen in some human tumor cell lines (16), and their production from mitochondrial and plasma membrane sources appears to be a requirement of tumor viability and growth (17, 18). Mitochondria-localized manganese-containing superoxide dismutase is proposed to act as both an inhibitor (19) and activator (20) of growth and transformation. A higher oxidative capacity of cancer cells may also accompany an increase in resistance to ROS stress, which could in turn affect drug resistance. For instance, resistance to \( \text{H}_2\text{O}_2 \) stress by the acquired up-regulation of catalase activity and glutathione levels was shown to be crucial for maintenance of cell viability as well as resistance to cisplatin treatment (21). In theory, the oxidative stress response may be shifted from growth arrest and death to proliferation and transformation for cells exhibiting defects in growth pathway regulation and the apoptotic program (22). If the \( \text{H}_2\text{O}_2 \) signal is involved in these processes, they should be inhibited by its removal from the cellular environment.

In the present study the requirement of low levels of \( \text{H}_2\text{O}_2 \) for
Catalase-mediated Cell Growth Inhibition

Rat-1 fibroblast proliferation was investigated. The effects on defined growth- and stress-associated signaling pathways, produced by the scavenging of physiological H₂O₂ levels from the extracellular environment, were assessed. Rat-1 clones exhibiting differential growth and transformation properties resulting from specific alterations of the HER-2/Neu receptor were utilized as a model for the responses to removal of the H₂O₂ stimulus (23, 24). Multiple signals downstream of Neu are implicated in oncogenesis, and Neu overexpression and/or activating mutation is observed in 25–30% of human breast and ovarian cancers (for review see Ref. 25). The activities of the Neu receptor, extracellular signal-regulated kinase 1 and 2 (ERK1/2) MAPK and JNK1 effector pathways were studied after the addition of the H₂O₂ scavenger catalase. All Rat-1 clones displayed comparable sensitivities to growth inhibition by this treatment regardless of the level of Neu activity. It was found that scavenging of H₂O₂ caused stable inhibition of the ERK1/2 signal, transient induction of the JNK1 pathway, and no effect on p38 MAPK. Constitutive Neu activation could rescue the catalase-mediated block of ERK1/2 activity while only slightly increasing resistance to growth inhibition. Therefore, these results indicate that removal of extracellular H₂O₂ can both down-regulate growth signals and activate stress-associated signals, however, the stress response appears to play a larger role in the anti-proliferative effects observed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—All Rat-1 fibroblast clonal cell lines were kindly provided by Dr. W. J. Muller and have been previously characterized (23, 24). Rat-1NeuN (17) clone expresses Neu having a 12-amino acid (aa 641–652) deletion in the extracellular region of the receptor proximal to the transmembrane domain, resulting in its constitutive dimerization. Rat-1NeuNT (3) is another highly transformed clone expressing Neu with an activating point mutation (V654E) in the transmembrane domain (26). Rat-1NeuNT-NYPD (6) is a transformation-impaired clone expressing the NeuNT receptor with C-terminal autophosphorylation sites (Tyr-1028, Tyr-1144, Tyr-1201, Tyr-1226/7, Tyr-1253) replaced with phenylalanine residues. Cells were maintained at early passage number in 100-mm diameter tissue culture plates supplemented with 10% heat-inactivated fetal calf serum (Life Technologies). Rat-1NeuNT-NYPD clones displayed comparable sensitivities to growth inhibition by this treatment regardless of the level of Neu activity. It was found that scavenging of H₂O₂ caused stable inhibition of the ERK1/2 signal, transient induction of the JNK1 pathway, and no effect on p38 MAPK. Constitutive Neu activation could rescue the catalase-mediated block of ERK1/2 activity while only slightly increasing resistance to growth inhibition. Therefore, these results indicate that removal of extracellular H₂O₂ can both down-regulate growth signals and activate stress-associated signals, however, the stress response appears to play a larger role in the anti-proliferative effects observed.

**Quantitation of Cellular DNA Synthesis**—The cell growth rate was assessed by measurement of the total cell number after 4 days of culture. 1000 cells/well were seeded onto a 96-well plate (Falcon, Becton Dickinson) at 37 °C in a humidified atmosphere of 5% CO₂, 95% air. Rat-1 lines were grown in Dulbecco’s modified Eagle’s medium with 1-glutamine (Life Technologies, Inc.), and all media were supplemented with 1% heat-inactivated fetal calf serum (Life Technologies, Inc.), except in serum-starved controls. Cells were passaged by trypsinization (1× trypsin-EDTA; Life Technologies, Inc.).

Cultures were treated with various agents to manipulate extracellular H₂O₂ levels and detect cellular functions scavenging bovine liver or fungal (Aspergillus niger) catalase preparations (0–2000 units/ml Sigma), exogenous H₂O₂ (BDH inc.), and the MEK1 inhibitor PD98059 (20–30 μM, New England BioLabs).

**RESULTS**

**Low Level Generation of H₂O₂ by Rat-1 Fibroblasts Is Required for Efficient Growth in Culture**—Addition of catalase preparations to the culture media has been shown by our laboratory to block the proliferation of a variety of immortalized cell types in a dose-dependent and reversible manner. Extracellular catalase treatments have also been utilized to inhibit the effects of exogenous H₂O₂ in cell culture (27) and forced overexpression of catalase can inhibit DNA synthesis and cell growth (28). To determine whether exogenous catalase activity could produce a similar response in our Rat-1 clones, cells were treated with doses of catalase or heat-inactivated preparation, and the total cell number after 4 days culture was observed (Fig. 1A). The growth of all Rat-1 lines was blocked after catalase addition; removal of this treatment during incubation or heat inactivation of the enzyme reversed this effect. Catalase treatment caused growth arrest without significant induction of the apoptotic program, as observed by cell mor-

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2 T. J. Preston, W. J. Muller, and G. Singh, unpublished observations.
phology and trypan blue exclusion (not shown). The cell cycle profile of fibroblasts at increasing times of treatment (6–48 h) was assessed by propidium iodide staining of total DNA and flow cytometry (not shown). A discreet block of the cell cycle at the G1/S or G2/M checkpoints was not observed, however, an accumulation of cell populations in S and G2 phases suggests a prolonged S phase and inhibition of entry into mitosis. Coincubation of cells with an H2O2-generating glucose oxidase preparation in addition to catalase also rescued the proliferation block (Fig. 1B), providing evidence that catalase-mediated effects are indeed a result of changes in H2O2 levels. Treatment with glucose oxidase alone at activities greater than 4 \( \times 10^2 \) units/ml was toxic to cells, suggesting that a defined window of extracellular H2O2 concentration is required for cell survival and growth.

Consistent with the observations of lower cell number and a prolonged S phase and mitosis, active DNA synthesis was slowed; 2- to 4-fold by 2000 units/ml catalase as observed by BrdUrd (Fig. 2A) or [methyl-\(^3\)H]thymidine (Fig. 2B) incorporation into cellular DNA. Also, the addition of 200 \( \mu \)M H2O2 increased DNA synthesis in all lines tested, in accordance with its well-documented growth-promoting effects. Various mammalian cell types have been shown to secrete H2O2 into the surrounding culture medium (15, 16). This generation is thought to reflect cell growth potential, because manipulation of cellular H2O2 production results in changes to both the amount of extracellular H2O2 and to levels of proliferation and transformation (19, 20). The endogenous generation of H2O2 by Rat-1 cells was examined (not shown), with the amounts of H2O2 produced by each clone (5–15 pmol/h/50,000 cells) corresponding to the rate of growth in culture. Addition of A. niger catalase preparation to the media efficiently lowered extracellular H2O2 levels (not shown). Rat-1Neu8142 cells consistently demonstrated a 2-fold or higher increase in H2O2 production compared with the Rat-1NeuN line. Because of the differential in HER-2/Neu activity, extracellular H2O2 generation, and level of transformation exhibited by these two clones, they were compared directly in subsequent experiments focusing on molecular responses to catalase treatment.

P44/42 MAPK Phosphorylation Is Blocked by Catalase Treatment, and This Effect Is Rescued by Constitutively-activated Neu—The Ras-MAPK signaling cascade has also been shown to be activated by H2O2 (11, 29). We wished to determine whether the removal of H2O2 would cause ERK1/2 MAPK down-regulation, as further evidence for the redox sensitivity of this pathway. Thr-202/Tyr-204-phosphorylated ErK1 and 2 were detected using phospho-MAPK-specific primary antibodies. Levels of phosphorylated ERKs were compared with total ERK1/2 protein levels. Rat-1NeuN cells treated with H2O2 displayed an increase in p44/42 MAPK phosphorylation, whereas the addition of catalase for 24 h (\( \pm 10\)-min exposure to H2O2) abolished MAPK activity (Fig. 3A). Heat-inactivated catalase did not produce such an effect. In contrast, this decrease in ERK1/2 phosphorylation was not observed after the identical treatment of Rat-1Neu8142 cells (Fig. 3B). The MEK1 inhibitor PD98059 (30) was added to cells for 1 h as a control for
ERK1/2 down-regulation. These observations demonstrate that the alteration of Neu receptor activity can modulate the response of the MAPK pathway to a decrease in H$_2$O$_2$ levels. However, because the growth response of Rat-1Neu8142 cells to catalase treatments differed little from that of Rat-1NeuN cells, this proliferation block appears largely independent of the p44/42 MAPK signal.

The response of Rat-1 clones to a range (0–500 units/ml) of catalase activities was compared (not shown). Rat-1Neu8142 cells displayed a slightly higher resistance to the effects of catalase than Rat-1NeuN: This may result from differences in MAPK pathway activity or in the amount of endogenous H$_2$O$_2$ production observed between the two clones. To test for the later possibility, SK-OV-3 cells (human ovarian adenocarcinoma) were also examined for resistance to catalase (not shown). These cells generate extracellular H$_2$O$_2$ at a level ~6-fold higher than the Rat-1Neu8142 line. This increased H$_2$O$_2$ did not correlate to any increase in resistance; indeed the growth of SK-OV-3 cells was extremely sensitive to the scavenging treatment. Sensitivity of cells to removal of H$_2$O$_2$ in the presence of the MEK1 inhibitor PD98059 was also investigated (not shown). This concurrent treatment blocked the growth of Rat-1NeuN cells to a greater extent than catalase treatment alone, whereas this effect was less marked in the Rat-1Neu8142 line, which exhibited increased p44/42 MAPK phosphorylation. Such increased MAPK activity did not rescue cell growth to a significant degree, however. Thus it appears that, although the H$_2$O$_2$ signal can regulate ERK1/2 activities, there are also other intracellular signals that are responsible for blocking proliferation.

**Extracellular Catalase Activates the JNK1 Pathway**—Because the effects of H$_2$O$_2$ scavenging could not be fully attributed to the down-regulation of mitogenic MAPK signals, we examined the response of a SAPK signal; the JNK1 pathway. The N-terminal phosphorylation of c-Jun by JNK1 (p46 JNK) is a well-characterized cell stress response (1, 31). JNK1 activity was examined by an in vitro kinase assay (Fig. 4). All treatments induced a transient increase in JNK1 kinase activity as quantitated by level of substrate (c-Jun peptide) phosphorylation. In Rat-1NeuN cells (Fig. 4A) a toxic level of H$_2$O$_2$ (2 mM) activated JNK1 up to 10-fold over basal levels, with peak
activity occurring 1 h after treatment. Nontoxic levels of H\textsubscript{2}O\textsubscript{2} could not induce JNK1 activity. 500 units/ml catalase (IC\textsubscript{75}) induced the JNK1 signal 5-fold with peak activity after 30 min. Heat-inactivated catalase (500 units/ml) could also activate JNK1 to a lesser extent (2-fold over basal levels). Such stimulation did not result in any change in cell viability and growth as discussed. The response of Rat-1NeuN cells (Fig. 4B) was similar, except that 2 mM H\textsubscript{2}O\textsubscript{2} increased phosphorylation of the c-Jun substrate in an identical manner as catalase treatment (5-fold increase, peak at 30 min). These observations demonstrate that opposing directions of H\textsubscript{2}O\textsubscript{2} imbalance, namely, the addition of high concentrations of H\textsubscript{2}O\textsubscript{2} or removal of endogenous H\textsubscript{2}O\textsubscript{2} levels by catalase, can stimulate the JNK1 pathway in a similar fashion. Also, the activation of this SAPK by catalase is unaffected by differential Neu receptor activity, suggesting that it may be a critical component of the growth inhibition observed with all Rat-1 fibroblasts studied. These data indicate that the up-regulation of JNK1 is a common response to removal of extracellular H\textsubscript{2}O\textsubscript{2} in our Rat-1 fibroblast model.

**P38 MAPK Phosphorylation Is Induced by H\textsubscript{2}O\textsubscript{2} but Not by Catalase Treatment**—In a similar manner to that observed with JNK1 signal stimulation, the stress-induced p38 MAPK was phosphorylated upon addition of exogenous H\textsubscript{2}O\textsubscript{2} in all cells tested (Fig. 5). However, this response to oxidative stress was sustained over time, and no p38 activation was observed after treatment of cells with catalase or heat-inactivated catalase preparations. Levels of induction of p38 phosphorylation in both Rat-1NeuN and Rat-1Neu8142 lines by 2 mM H\textsubscript{2}O\textsubscript{2} was comparable to that observed under hyperosmotic conditions (0.2 M NaCl for 30 min). The phosphorylation of one p38 MAPK substrate, the transcription factor ATF2, was also investigated under the same conditions using an in vitro kinase assay (Fig. 5). No phosphorylation of this substrate was observed. C-6 extract, untreated C-6 glioma cell protein extract (negative control; NEB); C-6 + Anisomycin, anisomycin-treated C-6 extract (positive control; NEB); AT2, no ATP2 substrate included in reaction; Untr., no treatment.
DISCUSSION

This is the first report describing the effects of the removal of endogenously produced H₂O₂ upon specific mitogenic and stress-responsive signaling pathways. To remove H₂O₂ from the cellular environment, a catalase preparation was directly added to the culture media. This strategy was adopted so that the molecular signaling responses observed could be attributed to changes in H₂O₂ status at the plasma membrane. One potential problem with this approach is enzyme purity, raising concerns that the effects observed could result from contaminants. To address this issue, we compared the effects of catalase to heat-inactivated catalase in all experiments. Incubation with the heat-inactivated solution did not affect cell viability and growth, nor did it affect Neu receptor or ERK activities. JNK1 activity did increase upon the addition of heat-inactivated catalase, but at a 2- to 3-fold lower level than that observed after treatment with active catalase. A comparable A. niger catalase preparation markedly reduced extracellular H₂O₂ levels, whereas heat inactivation of this treatment blocked the effect. In addition, catalase-induced growth inhibition was rescued upon cotreatment of cells with H₂O₂-generating glucose oxidase. The levels of glucose oxidase activity required for this rescue were cytotoxic if added alone, indicating that the two enzymes are inversely affecting H₂O₂ levels. These results provide evidence that the responses to the catalase preparation used specifically reflect its H₂O₂ scavenging activity. It is likely that changes in the concentration of extracellular H₂O₂ will in turn affect intracellular redox status, because H₂O₂ can freely diffuse across cell membranes. Therefore, the effects of the catalase treatment used here are best described as a response to the alteration of the “pericellular” redox environment, which is both outside and inside of the plasma membrane. Certainly the fact, that Neu receptor activity was not greatly changed by the addition of catalase to cell cultures whereas various MAPK signaling cascades were, indicates that direct manipulation of intracellular signals by this type of treatment occurs.

One such affected signal was the ERK1/2 pathway. The inhibition of ERK1/2 phosphorylation observed with catalase addition (up-regulation observed with H₂O₂) to Rat-1NeuN cells agrees with the previous findings that demonstrate the responsiveness of the MAPK cascade to H₂O₂ (11, 12). Constitutive activation of the Neu receptor in Rat-1 Neu8142 cells induced basal ERK1/2 activity and protected it from catalase-induced inhibition. Neu interacts with several signaling cascades, including the Ras/Raf/MEK pathway, important in ERK1/2 regulation (25). The rescue of ERK phosphorylation was accomplished by only a slight increase in resistance of fibroblasts to growth inhibition by catalase. This result indicates that, although contributing to the cellular responses observed, ERK1/2 signal down-regulation is not critical for them.

Unlike the ERK response, the stress-activated JNK1 signal was rapidly and transiently induced by treatments that both increased and decreased H₂O₂ in Rat-1NeuN and Rat-1Neu8142 lines. JNK1 is known to be responsive to oxidative stress (2, 32), but our findings suggest an additional role for this signal in response to an antioxidant. Rac1 and Cdc42, members of the Rho family of small GTPases involved in actin cytoskeleton regulation and cell transformation, have been shown to activate the JNKs through interaction with p21-activated kinase (33, 34). In addition, Rac1 is a cytoplasmic component of the plasma membrane-localized NAD(P)H oxidase complex that directs the extracellular generation of O₂⁻ and its dismutase product H₂O₂ (35). Interestingly, the function of this complex appears to be essential not only for ROS generation but also for cell survival and growth (15, 36). It follows that Rac may stimulate the JNK pathway through p21-activated kinase as well as H₂O₂ production.

Because our data demonstrate MAPK inhibition and SAPK activation after catalase treatment, it could be that loss of the H₂O₂ stimulus blocks the Ras pathway while inducing other JNK-dependent signals like phosphoinositide 3-OH kinase (PI3K) and Rac. Recent evidence suggests that PI3K can play dual roles: the survival-promoting PI3K target Akt (protein kinase B) was shown to phosphorylate Rac1 at serine 71 and abolish its GTPase activity (37). Treatment of our Rat-1 fibroblast lines with a 5 μM concentration of the PI3K inhibitor LY294002 (38) inhibited cell proliferation to a comparable extent regardless of HER-2/Neu activity levels (not shown). Thus, changes to the PI3K/Akt signal might contribute to the catalase-induced effects upon JNK1 observed; however, this remains to be determined.

The role of JNK in cell cycle control is not known. c-Jun contributes to a number of contrasting biological processes as part of the AP-1 transcriptional complex (3). The degree of SAPK activity balanced with other AP-1-inducing signals such as the ERKs could decide which process is followed. In our system, ERK inhibition and JNK activation are observed with cell growth arrest. Studies by Wisdom et al. (31) demonstrate that JNK-mediated phosphorylation of c-Jun is specific for stress responses only, whereas c-Jun involvement in cell cycle progression is triggered by other factors. Another report indicates that the JNK signal can prolong the cell cycle at S and G₂/M phases in a p57-independent manner (39), however, the mechanisms underlying this effect have yet to be elucidated. Indeed, we observed an increase of the proportion of cells in S and G₂ phases after incubation with catalase (results not shown). Because cells remain trapped in the late stages of the cell cycle, apoptosis may then be triggered by a “mitotic crisis.”

In conclusion, this work demonstrates that H₂O₂ regulates components of various MAPK cascades to allow for the efficient growth of cultured Rat-1 fibroblasts. ERK1/2 activities are induced upon the addition of H₂O₂ and inhibited by its removal. Expression of constitutively activated Neu receptor reversed this inhibition but had little effect on the catalase-induced block of proliferation. However, the stress-activated JNK1 pathway was induced by both the addition and removal of the H₂O₂ signal, regardless of Neu status. Another stress signal, the p38 MAPK pathway, was activated by toxic levels of H₂O₂ but not by its removal, indicating that the JNK1 response might be specific. A discreet range of JNK activation may act as a cell sensor of oxidative and reducing stresses, leading to a response of growth arrest and death.

Acknowledgments—We thank Drs. A. Dorward, P. Siegel, N. Singh, J. Lee, M. Rozakis-Adcock, R. Austin, and W. Duivenvoorden for their manuscript preparation.

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