AGE-DEPENDENT DECLINE IN EGF-INDUCED SIGNALING IS INDEPENDENT OF INTRACELLULAR THIOLS

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ABSTRACT

Loss of proliferative capacity is common in many tissue types during aging. We have shown that mitogenic signaling through the epidermal growth factor (EGF) receptor declines in hepatocytes from old rats. Specifically, we showed that in old hepatocytes there is a decrease in autophosphorylation of EGF receptor at Tyr-1173. This results in loss of recruitment of the adapter protein Shc to the membrane and decreased ERK MAP kinase pathway activation. Because EGF receptor signaling also requires intracellular generation of H₂O₂, we next questioned whether altering the intracellular GSH/thiol concentration may also affect the age-dependent decline in EGF receptor signaling. Surprisingly, decreased intracellular GSH had no effect on EGF receptor signaling in hepatocytes from either young or old animals. However, increasing the thiol concentration dramatically attenuated EGF receptor signaling in hepatocytes from both young and old animals. Unexpectedly, loss of EGF receptor signaling was due to a specific decrease in EGF receptor number, but not in other components of the EGF receptor signaling pathway such as ERK MAP kinase. These results suggest that age-dependent mechanisms of alteration in EGF receptor signaling are independent of thiol regulation of EGF receptor signaling.

INTRODUCTION

The proliferative potential of several tissues, including the liver, declines with age (1,2). Decreased hepatocyte proliferation may be consequential to the elderly in their ability to recover from the hepatotoxic side effects of medications and/or exposure to environmental toxins. Proliferation of hepatocytes is induced by a number of

growth factors and cytokines. The age-dependent decline in response to growth factors and cytokines has been shown by Bucher et al., (1), and Beyer et al., (2), who reported diminished DNA synthesis in epidermal growth factor (EGF)-treated hepatocytes from old rats.

Cellular proliferation is activated by a cascade of signals initiated at the cell membrane in response to growth factors binding specific transmembrane receptors. Upon ligand binding, the EGF receptor kinase is activated and the receptor is phosphorylated, leading to membrane recruitment of adapter and exchange proteins for activation of Ras and subsequent Raf kinase activation (3-5). Downstream of Raf kinase, activation of a series of kinases proceeds to activate primarily ERK MAP-kinase in response to EGF (6,7). Proteins of the MAP kinase family are activated by dual-phosphorylation of thr-X-tyr residues (reviewed in 8,9). Activation of ERK-MAP kinase results in phosphorylation of a number of target proteins including the ternary complex factor, Elk-1. Elk-1 mediates transcriptional activation of many genes, including c-fos and, consequently, AP-1 target genes (10-14).

We have recently identified an early step in the EGF-stimulated pathway as at least a component part of the mechanism for the age-related difference in mitogenic responsiveness (15). The ability of the adapter protein, Shc, to bind the activated EGF receptor is diminished in hepatocytes from old animals as compared with young. This reduced association is due, in part, to the decreased phosphorylation of tyrosine 1173 on the EGF receptor. Decreased Shc association in EGF-stimulated hepatocytes from old animals leads to a decline in activation of both Raf kinase and ERK-MAP kinase.

Early in the pathway, the reactive oxygen species, H₂O₂, is generated and required for EGF-induced signal transduction (16). However, the mechanism by which H₂O₂ functions in signal transduction is unknown. The cellular redox potential is highly regulated to maintain a reduced environment inside the cell. Glutathione, the major intracellular redox metabolite, is present at mM levels. One theory of aging, the free radical theory, hypothesizes that aging is the result of exposure to oxidants over time and/or an age-related decline in the ability to protect against oxidant exposures (17). Decreased exposure to ROS and/or increased protection

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by dietary/supplemental antioxidants has been proposed to retard aging. Liver glutathione levels do not change with age. However, the ability to recover from loss of glutathione in response to oxidative stress is diminished in old animals compared with young (18). Given our previous identification of at least part of the molecular defect in age-dependent decline in the EGF receptor signaling pathway and the fact that ROS are required in the pathway, we questioned whether intracellular glutathione might have an age-related effect on EGF-stimulated signal transduction. Therefore, we tested the effect of decreasing or increasing the intracellular thiol concentration in hepatocytes from young and old rats on the regulation of EGF-induced signaling.

MATERIALS AND METHODS

Hepatocyte isolation: Hepatocytes were isolated from young (4-6 mo) or old (>30 mo) male F1 F344XBN rats by perfusion of the liver with Type 2 collagenase (Worthington Biochemical Corporation, Freehold, NJ.) according to the procedure of Seglen (19), with the modification of using a HEPES (10 mM) based buffer (20). Cell viability (>85%) was determined by trypan blue exclusion. The cells (5.5×10^6 per 100 mm dish) were plated in DMEM supplemented with 10% FBS, penicillin (100 μ g/ml) and streptomycin (100 μ g/ml) on tissue culture dishes precoated with Matrigel (1.2 mg/100 mm dish, Collaborative Research, Lexington, MA). Matrigel supports the differentiated state of hepatocytes similar to that in the native liver (20). Two hours after plating, the medium was removed, cells were washed gently 2X with DMEM alone and treatment medium was added (DMEM supplemented with 0.5% FBS plus glucose, 5 mM; insulin, 0.14 μ M; hydrocortisone, 5 μ M; sodium selenite, 0.2 μ g/ml; and transferrin, 1 μ g/ml).

Pretreatment of cells with buthionine sulfoximine (BSO) or n-acetyl cysteine (NAC): BSO was added to the medium 4 hours after plating (final concentration of 200 μ M). A stock solution of NAC (200 mM) in DMEM was adjusted to pH 7.4, sterile filtered, and added to the medium 4 hours after plating (final concentration of 20 mM). Preliminary data showed that addition of NAC for 2 hours did not elevate cellular glutathione levels. Therefore, preincubation with NAC for 16-18 hours was done in an attempt to elevate the glutathione levels. The hepatocytes were resistant to elevation of glutathione levels.

EGF treatment and cell harvest: Following plating of the cells (18-20 h), hEGF (50 ng/ml; Life Technologies, Gaithersburg, MD) was added to the medium. Cells were harvested by washing 2X with ice cold PBS, scraped into PBS, pelleted by centrifugation, and protein was prepared by extraction into a whole cell extract buffer (25 mM HEPES, pH 7.5; 0.3 M NaCl; 1.5 mM MgCl_2 ; 0.2 mM EDTA; 0.05% Triton-X 100; 20 mM β -glycerophosphate; 0.1 mM orthovanadate; 0.5 mM DTT; 0.4 mM phenylmethylsulfonyl fluoride (PMSF); leupeptin (1 mg/ml); pepstatin (1 mg/ml)) for 30 min. at

4° C. Extracted cells were centrifuged at $12,000 \times g$ for 15 min. Supernatants were frozen in aliquots at -70° C.

Cell toxicity measured by lactate dehydrogenase (LDH) release into the medium: Medium was collected and cell lysates were prepared for LDH analysis as described (TOX-7 kit, Sigma Chemical Co.). BSO pretreatment had no effect on cell toxicity as measured by % LDH released into the medium. The % LDH in the medium for control and EGF-treated cells from young or old animals ranged from 13-18%. Likewise, the % LDH in the medium of NAC pretreated-cells from young animals (16%) was similar to the control value (14%). Cells from old animals pretreated with NAC had slightly higher LDH release into the medium (controls – 15.5%; NAC 26%).

Intracellular glutathione (GSH) determination: To monitor age-related differences in changes in glutathione levels in response to BSO or NAC pretreatment, glutathione levels were assayed using the method of Sedlak (21). The GSH content was normalized to 10^6 cells.

Immune complex kinase assay: ERK-MAP kinase and Raf kinase activities were measured using an immune complex kinase assay (23). Briefly, whole cell extract (50 μ g of protein) was immunoprecipitated using specific antibodies (Santa Cruz Biotechnology, Inc.) bound to protein A-sepharose beads (Repligen Corp., Needham, MA). Myelin basic protein (Sigma, St. Louis, MO) was used as the substrate for the p44 ERK kinase. Purified MEK protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as substrate for Raf kinase. Activity was visualized by autoradiography of the dried 10% SDS-PAGE gel and quantitation was assessed by phosphorimage analysis using Molecular Dynamics phosphorimaging equipment.

Immunoprecipitation and Western blot analysis: Specific antibody was bound to Protein-A Sepharose beads (Repligen Corp., Needham, MA). Human Shc antibody and phosphotyrosine antibody, PY20, were obtained from Transduction Laboratories, Lexington, KY. All other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Whole cell extracts (100-400 μ g protein) were immunoprecipitated with the bead/antibody complex. The beads were washed with Triton lysis buffer (0.1% Triton X-100; 20 mM Tris, pH 7.4; 137 mM NaCl; 2 mM EDTA; 25 mM β -glycerophosphate; 1 mM sodium ortho vanadate; 2 mM sodium pyrophosphate; 10 % glycerol; 0.4 mM PMSF; leupeptin (1 mg/ml); pepstatin (1 mg/ml)), and the protein was eluted into 4X Laemmli's buffer, boiled and electrophoresed by SDS-PAGE. Western blot analysis was done by standard procedures after protein transfer to nitrocellulose membranes. Proteins were detected by a chemiluminescent procedure (NEN Life Science Products, Boston, MA).

RESULTS

Glutathione is the major intracellular redox metabolite. In order to determine if alteration of the intracellular

redox affects the EGF-induced response of hepatocytes from old animals compared with young, isolated hepatocytes were treated to alter the glutathione levels. Buthionine sulfoximine blocks γ -glutamyl synthetase, the rate-limiting enzyme of glutathione synthesis, resulting in depletion of glutathione. BSO pretreatment of hepatocytes from young or old rats resulted in similar depletion of glutathione to 10% of non-treated levels (Figure 1). NAC provides the precursor, cysteine, for the synthesis of glutathione. Administration of NAC can increase cellular glutathione in some cells. Using similar conditions of preincubation, the glutathione content of A431 cells was elevated 50 to 100% by preincubation with NAC for 2 or 24 hours (data not shown). NAC also functions to prevent the loss of glutathione in the presence of an oxidative challenge (22). The level of glutathione in hepatocytes from young and old animals, is already high and was resistant to any basal increase, even with an 18 hour pretreatment of NAC (Figure 1). Even without changes in glutathione, NAC can increase protection against reactive oxygen species by the increase in cysteine, which protects oxidation of critical cellular sulfhydryl groups (23).

Figure 1: Total glutathione (GSH) content of hepatocytes from young and old rats. Hepatocytes were pretreated with BSO or NAC as described in **Materials and Methods**. Results represent an average of 2 animals.

To determine if changes in intracellular redox would affect age-dependent EGF-induced signal transduction, we first analyzed the activity of ERK-MAP kinase in cells with altered thiol levels. As shown in Figure 2, ERK-MAP kinase is activated eleven-fold in response to EGF treatment in cells from young animals. These results are similar to those reported for other cell types (7, 24). In hepatocytes from old rats, this activation is diminished to 5-fold over basal levels (15, 25). BSO pretreatment to deplete cellular glutathione did not affect the activation of ERK-MAP kinase in hepatocytes from either young or old rats (Figure 2). However, pretreatment of these cells with NAC diminished the activation of ERK-MAP kinase by EGF by 50% at 15 minutes and to basal levels by 40

minutes (Figure 2). These results indicate that decreased levels of glutathione have no effect on EGF signal transduction. Rather, an increased thiol concentration can strongly alter EGF-dependent signaling to activate ERK-MAP kinase.

Figure 2: ERK-MAP kinase activation in response to EGF in hepatocytes from young and old rats following pretreatment with BSO or NAC. A. Representative activity of ERK-MAP kinase after 15 or 60 min of EGF treatment (50 ng/ml). The activity was determined by an immune complex kinase assay using myelin basic protein as a substrate. B. Graphic results of ERK-MAP kinase activity in response to treatments of hepatocytes from two pairs of animals. Results are normalized to the young control. C. Effect of EGF +/- BSO or NAC on ERK-MAP kinase protein levels in primary hepatocytes as detected by Western blot analysis using an ERK-1 antibody.

In order to understand the mechanism of the NAC effect on ERK-MAP kinase activation, we examined events up-stream of ERK-MAP kinase activation in response to EGF. Pretreatment with NAC diminished the EGF-induced increase in Raf kinase activity in hepatocytes from young rats (Figure 3). Similarly, NAC pretreatment reduced the Raf kinase activation in hepatocytes from old animals. However, the age-related decline in Raf kinase activation to near basal levels lessened the ability to detect an effect of NAC pretreatment on Raf activity in hepatocytes from old rats. These results confirm the observation that increased intracellular thiol strongly regulates the EGF-stimulated ERK-MAP kinase activation pathway.

Figure 3: Raf kinase activity in response to EGF treatment in hepatocytes from young and old rats following pretreatment with NAC. A. Representative activity of Raf kinase after 15 min of EGF treatment (50 ng/ml) +/- pretreatment with NAC (20 mM). The activity was determined by an immune complex kinase assay using MEK-1 as a substrate. B. Graphic results of Raf kinase activity in response to EGF treatment of hepatocytes from four pairs of animals. Results are expressed as the mean +/- s.e. and normalized to the young control. C. Effect of EGF on Raf kinase protein levels in primary hepatocytes as detected by Western blot analysis using a Raf-1 antibody.

Upstream of Raf kinase the adapter protein, shc, mediates recruitment of the Ras exchange factor, SOS, and subsequent Ras activation in response to EGF. The complex formed between shc, the EGF receptor and the adapter protein, grb2, to recruit SOS depends on the SH2 domain of shc and its affinity for phosphotyrosine moieties of the activated receptor as well as the affinity of the SH2 domain of grb2 for the EGF-induced shc phosphotyrosine residues. We have shown an age-dependent decline in the ability of shc to associate with the EGF receptor due, at least in part, to decreased phosphorylation of the EGF receptor at pY-1173. To assess the effect of NAC pretreatment, we examined the association of shc with the EGF receptor. Whole cell extracts were immunoprecipitated with antibody for shc and immunoblotted with phosphotyrosine antibody. Pretreatment of the cells with NAC abolished the association of Shc with the EGF receptor in both young and old

and decreased tyrosine phosphorylation of shc (Figure 4). Since association with the EGF receptor and shc tyrosine phosphorylation are dependent on phosphorylation of the EGF receptor, we hypothesized that NAC was affecting the phosphorylation of the receptor itself.

Figure 4. Tyrosine phosphorylation of Shc and proteins associated with Shc in response to EGF treatments of hepatocytes pretreated with NAC. A. Whole cell extracts were immunoprecipitated with the Shc antibody and immunoblotted with an antibody for phosphotyrosine. The results shown are representative of three pairs of animals. B. Shc protein levels were determined by Western blot analysis of whole cell extracts. The results are representative of 3 experiments, each with one old/one young pair of animals.

To determine the effect of NAC pretreatment on phosphorylation of the EGF receptor, cell extracts were immunoprecipitated with EGF receptor antibody and immunoblotted with phosphotyrosine antibody. NAC pretreatment reduced the tyrosine phosphorylation of the EGF receptor in hepatocytes from either young or old animals (Figure 5A). Surprisingly, this reduced phosphorylation appears to be due to decreased receptor number, as total EGF receptor protein is also diminished by NAC pretreatment (Figure 5B). These results clearly indicate that increased thiol concentrations alters EGF-induced signaling directly at the level of the receptor.

DISCUSSION

ROS function to mediate the signal transduction of several growth factors and cytokines (16, 26-28). The mechanism of their generation as well as their down-

A **B**

Figure 5. Tyrosine phosphorylation and total amount of the EGF receptor in response to EGF in hepatocytes pretreated with NAC (N). A. Total tyrosine phosphorylation of the EGF receptor was determined by immunoprecipitating the whole cell extracts with the EGF receptor antibody followed by immunoblotting with an antibody for phosphotyrosine. The results shown here are representative of analysis done on 3 pairs of animals. B. The amount of EGF receptor was analyzed by Western blot using the human EGF receptor antibody. The results shown here are representative of analysis done on 3 pairs of animals.

stream signaling targets are not well understood. The small G-protein, rac, and activation of membrane-associated NADPH oxidase activity were identified to be in the pathway of EGF-, PDGF-, $\text{TNF}\alpha$ -, and IL-1 β -induced mitogenesis (29). Clearly, the involvement of ROS in mitogenic signal transduction suggests that intracellular redox status may affect the ability of a cell to respond to stimuli.

It has not been definitively shown whether mitogen-induced ROS production is decreased in hepatocytes from old animals; however, it appears that it may not be involved. Bae et al., (16) have shown that, in the presence of EGF, cells containing the EGF receptor kinase-inactive mutant do not generate H_2O_2 . We (15) and others (30) have shown no difference in total phosphorylation of the EGF receptor with age, suggesting that the intrinsic kinase activity of the receptor does not differ with age. We therefore tested whether alteration of intracellular redox would have an age-dependent effect on EGF-induced signaling. Depletion of glutathione by BSO might be expected to prolong the EGF-induced signaling due to decreased capacity to inactivate the H_2O_2 generated upon EGF stimulation. However, the data in Figure 2 show no effect on ERK-MAP kinase activation after BSO pretreatment in hepatocytes from either young or old rats. Elevation of the intracellular reducing capacity by NAC pretreatment could be expected to diminish signaling. Indeed, NAC pretreatment resulted in decreased Shc phosphorylation, as well as

Raf and ERK-MAP kinase activation in hepatocytes from both young and old animals (Figure 2-4). NAC pretreatment has been previously reported to decrease arsenite- and IL-1 β -induced MAP-kinase activation (31,32). These data suggest that the regulation of EGF-induced ROS generation can be regulated by elevation of intracellular thiols. However, there is no age-dependent difference on the effect of altered thiol status on EGF-induced signaling (Figure 2-5).

Reduction of ROS, an upstream mediator of the signal, may be part of the mechanism to diminish activation of Raf and ERK-MAP kinases upon preincubation with NAC (Figure 2-3). However, preincubation with NAC for 18 hours resulted in decreased amounts of EGF receptor protein in hepatocytes from both young and old rats (Figure 5). Therefore, it is likely that a decrease in receptor number is the major mechanism for diminished EGF-induced signaling. Previously, decreased tyrosine phosphorylation of the EGF receptor upon NAC pretreatment has been shown in UVB-treated human epidermal cultures (33) and UVC irradiated HC11 mouse mammary cells (34). EGF receptor protein levels were not reported. Our data show the effect on protein level was specific to the EGF receptor protein, as no changes in the amount of Shc, Raf or ERK-MAP kinase proteins were observed with NAC pretreatment (Figure 2-4).

While the NAC-induced decrease in EGF receptor protein levels may account for the decrease in EGF receptor signaling, it is possible that other mechanisms may also contribute to the effect of NAC on EGF signal transduction. We previously showed an age-dependent difference in EGF phosphorylation at specific site(s), such as tyrosine 1173. Therefore, it seems likely that an age-dependent difference in a phosphatase may be responsible for the decreased proliferation of hepatocytes from old animals. Interestingly, Knebel et al., (35) have proposed an intriguing hypothesis about the regulation of UV-induced ROS-induced tyrosine phosphorylation by phosphatases. They suggested that reversible SH-group oxidation at the active site of one or several protein tyrosine phosphatases by UV-induced ROS or thiol agents will inhibit the dephosphorylation of the EGF receptor. Such an hypothesis emphasizes the importance of phosphatases in the regulation of signal transduction. Our data suggest increased activity of a specific tyrosine phosphatase in hepatocytes from old rats. The similar effects of BSO and NAC on EGF-induced signaling in cells from young and old rats is consistent with the idea that a thiol-sensitive phosphatase may be key in regulating the age-related differences in signal transduction. NAC may function to maintain or enhance the activity of such a phosphatase, therefore reducing the EGF-induced signaling in both young and old animals.

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