

Age-related Decline in Mitogen-activated Protein Kinase Activity in Epidermal Growth Factor-stimulated Rat Hepatocytes*

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A number of studies have demonstrated that the proliferative capacity of cells declines with aging. In particular, epidermal growth factor (EGF)-stimulated DNA synthesis is reduced in hepatocytes from aged rats relative to young rats. Growth factor stimulation activates a genetic program in large part regulated by a family of mitogen-activated protein kinases (MAPK) that phosphorylate and thereby activate transcription factors involved in controlling the expression of proliferation-associated genes. In the present study, we compared the activation of the extracellular signal-regulated kinase 2 (ERK2) and c-Jun N-terminal kinase 1 (JNK1) MAPK in EGF-stimulated hepatocytes derived from young (6-month) and aged (24-month) rats. JNK activity was not appreciably altered by EGF treatment of cells from either age group. In contrast, ERK2 was highly activated by EGF treatment, but the magnitude of activation was significantly lower in hepatocytes of aged animals compared to those of young animals (7-fold versus 20-fold, respectively). The reduced ERK2 activity in response to EGF was associated with decreased *c-fos* and *c-jun* mRNA expression and lower levels of AP-1 transcription factor DNA binding activity in the aged hepatocytes. Finally, the basal expression of MAPK phosphatase 1, a MAPK-regulated gene involved in regulating MAPK activity, was higher in aged hepatocytes. Taken together, these findings suggest that an alteration in the balance between MAP kinase-phosphatase activities could contribute to the age-related decline in proliferative capacity.

Mitogen-activated protein kinases (MAPKs),¹ which include the extracellular signal-regulated kinases (ERK) and the c-Jun N-terminal kinases (JNK), are important regulatory proteins through which a wide variety of extracellular signals are transduced into intracellular events (1–6). Both ERKs and JNKs are themselves activated through complex phosphorylation cascades (1, 5, 6), and their activation, in turn, leads to the phos-

phorylation and activation of a variety of proteins including a number of transcription factors involved in regulating the expression of genes controlling cellular proliferation (7–10).

The cellular events leading to activation of ERK in response to growth factors have been studied extensively (1, 5, 6). Briefly, growth factor binding results in receptor autophosphorylation, leading to the sequential activation of Ras and Raf. Raf then phosphorylates MAPK/ERK kinase (MEK), which in turn activates ERK through phosphorylation of threonine and tyrosine residues. JNK is also activated by growth factor stimulation (3, 4). However, recent studies indicate that its activation is achieved through an alternative signaling cascade involving small GTPases, MEK kinase (MEKK) and the MEK homolog SEK1/MKK4/JNKK (11–17).

The activities of MAPKs are also subject to negative regulatory influences including inactivation by phosphatases (18), and several threonine/tyrosine dual specificity phosphatases exhibiting high selectivity for MAPKs have been identified (19–23). MAP kinase phosphatase 1 (MKP-1), in particular, inactivates ERK and JNK both *in vitro* and *in vivo* and has been implicated in the regulation of mitogenic processes (20, 24, 25).

Evidence from several different model systems has indicated that, during aging, cells lose their capacity to proliferate (26, 27). In *in vitro* human diploid fibroblasts, the decline in proliferative capacity is correlated with a decrease in *c-fos* expression (27), as well as with reduced DNA binding activity of serum response factor and AP-1 transcription factors (28, 29). Liver regeneration following partial hepatectomy has been used for studying the proliferative response *in vivo*. Following partial hepatectomy, DNA synthesis is delayed and reduced in magnitude in aged rats compared to young adults (30). Epidermal growth factor (EGF)-stimulated DNA synthesis in primary hepatocytes from aged rats is also significantly lower than that seen in EGF-treated hepatocytes from young animals (31).

In order to gain insight into the mechanisms responsible for the loss of responsiveness to EGF with aging, we compared the activation of ERK2 and JNK1 MAPK in EGF-stimulated primary hepatocytes from young and aged rats. We report here that hepatocytes of aged rats show significantly lower levels of ERK2 activation in response to EGF stimulation compared to hepatocytes from young rats. The activation of JNK in response to EGF, on the other hand, did not differ between young and old hepatocytes. Consistent with the reduced ERK2 activity, induction of *c-fos* and *c-jun* expression and AP-1 DNA binding activity following EGF stimulation were also lower in aged hepatocytes. Interestingly, aged hepatocytes showed significantly higher basal levels of MAP kinase phosphatase 1 (MKP-1) expression compared to that of young hepatocytes, although induction of MKP-1 following EGF treatment was lower in the

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¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ATF-2, activating transcription factor-2; AP-1, activator protein 1; MKP-1, MAP kinase phosphatase 1; MEK, MAPK/ERK kinase; MEKK, MEK kinase; GST, glutathione S-transferase; MBP, myelin basic protein.

old cells. Taken together, these results suggest that an alteration in the balance of kinase-phosphatase activities could contribute to the age-related decline in proliferative capacity.

MATERIALS AND METHODS

Isolation, Culture, and Treatment of Rat Hepatocytes—Hepatocytes were isolated from male Wistar rats by the collagenase perfusion method of Seglen as described (32). The isolated cells were plated in Williams E medium containing 5% fetal bovine serum and cultured for 2 h at 37 °C under 5% CO₂ to allow attachment. The medium was then replaced with Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum, and the cells were cultured for an additional 16 h prior to treatment. This procedure results in less than 2–5% contamination with non-hepatocytes. EGF was added to the medium to a final concentration of 100 ng/ml.

Western and Northern Blot Analyses—For Western blot analysis, total cell lysates (30 µg of protein) were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane using standard techniques (25). Following immunoblotting of membranes with a monoclonal antibody against ERK2 (Transduction Laboratory, Lexington, KY), the immune complexes were visualized using an enhanced chemiluminescence detection kit (Amersham).

For RNA analysis, cells were harvested at various time points following treatment, and total RNA was extracted using Stat 60 (Teltest "B," Friendswood, TX). Northern blot analysis was performed using rat *c-jun*, *c-fos*, MKP-1 cDNA probes, and 18 S oligonucleotide as described previously (25).

Immunoprecipitation and Protein Kinase Assays—Hepatocytes (at approximately 70% confluence) were treated with EGF and harvested at various times thereafter for assay as described previously (25). Briefly, endogenous ERK2 or JNK1 was immunoprecipitated from the cell extracts using rabbit polyclonal antibodies against p42^{ERK2} or p46^{JNK1} (Santa Cruz Biotechnology Inc., Santa Cruz, CA), respectively. ERK2 and JNK1 activities were assayed as described previously using bovine brain myelin basic protein (MBP) and recombinant GST-c-Jun-1–135 (4) substrates, respectively (25). After completion of the reaction, the proteins were resolved by SDS-polyacrylamide gel electrophoresis. Incorporation of ³²P into the substrates was quantitated using a PhosphorImager (Molecular Dynamics).

Gel Retardation Analysis—Protein extracts (20 µg of protein) were mixed with 0.5 ng of ³²P-labeled double strand oligonucleotide containing a consensus AP-1-binding sequence (5'-CGCTTGATGACTCAGC-CGGAA-3') in a total volume of 50 µl containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 1 µg of poly(dI-dC). The binding reactions were incubated for 30 min at room temperature and loaded onto a 4% native polyacrylamide gel. After electrophoresis, the gels were dried and subjected to autoradiography.

Statistical Analysis—Unpaired Student's *t* test was used to assess statistical significance of differences. A *p* value of <0.05 was considered significant.

RESULTS

EGF-stimulated ERK2 Activation—ERK2 activation was assessed by both Western blot analysis and immune complex kinase assays. Fig. 1A shows a representative Western blot comparing ERK2 protein levels in young (6-month) and old (24-month) hepatocytes following EGF treatment. ERK2 abundance did not change in response to EGF treatment and did not differ between young and old cells. In young hepatocytes, ERK2 was rapidly phosphorylated in response to EGF treatment, as evidenced by a shift in the mobility of the protein; the slower migrating (phosphorylated) form appeared within 15 min and persisted up to 45 min post-treatment. However, EGF-stimulation of old hepatocytes resulted in substantially less phosphorylation of the ERK2 protein.

ERK2 activity was assessed over an extended time course in these EGF-treated hepatocytes (Fig. 1B). Consistent with the phosphorylation of ERK2 seen above, the activity of ERK2 in young hepatocytes increased greatly in response to EGF stimulation. Maximum kinase activity (>20-fold elevation) was achieved within 15 min. The activity declined thereafter, returning to baseline levels by 2 h post-treatment. By compari-

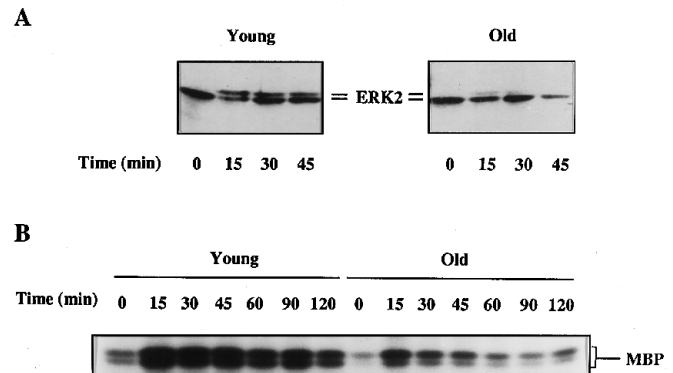


FIG. 1. EGF-triggered ERK activation in hepatocytes from young and old rats. A, effect of EGF stimulation (100 ng/ml) on ERK2 protein levels in young and old hepatocytes. ERK2 was detected by Western blot analysis using ERK2-specific monoclonal antibody. B, kinetics of ERK2 activation in young and old hepatocytes. ERK2 activity was determined with an immune complex kinase assay using MBP as a substrate.

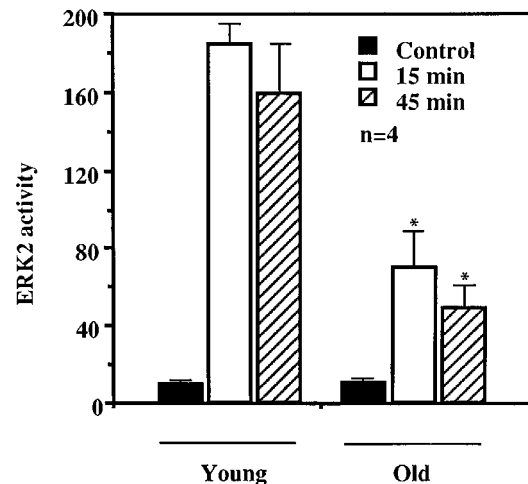


FIG. 2. Statistical analysis of ERK2 activation in hepatocytes from multiple young and old rats. ERK2 activity was assayed in hepatocytes from 4 young and 4 old animals after 15- and 45-min treatments with 100 ng/ml EGF. Kinase activity is expressed as the mean value of ³²P incorporation into MBP quantitated by a PhosphorImager. Data are mean ± S.D. of 4 independent experiments. *, significantly different from young animals (*p* < 0.05).

son, EGF stimulation of old hepatocytes resulted in significantly less activation of ERK2 (<7-fold elevation) than seen in young cells. A quantitation of the results of EGF-treated hepatocytes from 4 young and 4 old animals is summarized in Fig. 2. Aged hepatocytes showed a significant decrease in ERK2 activity (>60% decline) relative to young hepatocytes.

JNK1 Activity in Young and Old Hepatocytes—Since JNK also constitutes an important growth factor-activated MAPK signaling pathway, we examined JNK1 activity in EGF-treated hepatocytes. Results of a representative experiment with young hepatocytes is shown in Fig. 3A. Primary hepatocytes contain high basal JNK1 activity, but EGF treatment had little effect on JNK1 activity (<2-fold activation). Additional experiments revealed no age-related differences in JNK1 activation (Fig. 3B) or JNK1 protein levels (data not shown).

***c-fos* and *c-jun* Expression in EGF-stimulated Hepatocytes**—*c-fos* and *c-jun* are immediate-early genes whose induction by growth factors occurs through ERK- and JNK-mediated pathways (24, 25, 33–35). Since the aged hepatocytes showed reduced ERK activation in response to EGF treatment, we compared *c-fos* and *c-jun* mRNA expression following EGF

FIG. 3. Effect of EGF treatment on JNK1 activity in young and old hepatocytes. A, time course of JNK1 activation in EGF-treated young hepatocytes. Young hepatocytes were stimulated with 100 ng/ml EGF for the indicated times. JNK1 activity was assayed by an immune complex kinase assay using bacterial recombinant GST-c-Jun as a substrate. B, JNK1 activity in EGF-treated hepatocytes from 2 young and 2 old animals.

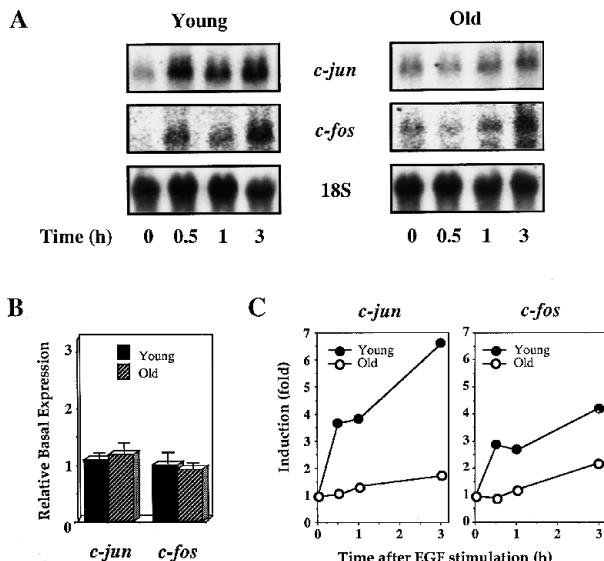
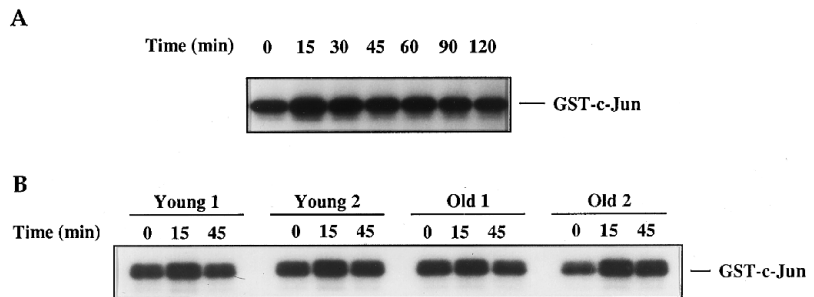


FIG. 4. *c-jun* and *c-fos* expression in EGF-stimulated hepatocytes. A, representative Northern blot showing the time course for *c-jun* and *c-fos* induction in EGF-stimulated hepatocytes from young and old rats. The 18 S signals were used to normalize for differences in loading and transfer among samples. B, basal levels of *c-jun* and *c-fos* expression in hepatocytes from 4 young and 4 old animals. Data are mean \pm S.D. of 4 independent experiments. C, *c-jun* and *c-fos* induction by EGF in young and aged hepatocytes. Results presented are the average of 2 independent experiments. All signals were normalized to those for 18 S.

stimulation in young and old hepatocytes (Fig. 4). Although basal levels of *c-fos* and *c-jun* mRNA did not differ between young and old hepatocytes (Fig. 4B), expression following EGF treatment was markedly different (Fig. 4C). While both *c-fos* and *c-jun* mRNAs were significantly elevated in young hepatocytes treated with the growth factor, the response to EGF was greatly attenuated in the aged cells.

Members of the c-Fos and c-Jun protein family dimerize to form AP-1 transcription factor complexes that regulate the expression of other genes (34, 35). AP-1 transcription factor activity is regulated at two different levels in response to growth factor stimulation: phosphorylation of pre-existing and newly synthesized c-Fos and c-Jun proteins and induction of *c-fos* and *c-jun* gene expression. Since both mechanisms are dependent on MAPK activation (34, 35), we examined the AP-1 DNA binding activity in cell extracts of old and young hepatocytes stimulated with EGF (Fig. 5). AP-1 DNA binding activity increased over time with EGF treatment in lysates of young hepatocytes. In contrast, little AP-1 DNA binding activity was evident in either control or EGF-treated aged hepatocytes.

Basal and EGF-induced MKP-1 Expression—ERK activity is regulated via reversible phosphorylation (18), and MKP-1 is believed to contribute to the dephosphorylation and inactivation of ERK. Since MKP-1 is itself induced in response to proliferative stimuli (24, 36), we examined the expression of

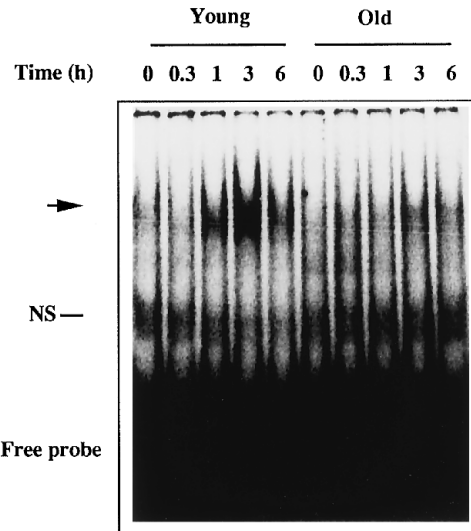


FIG. 5. AP-1 activation in EGF-stimulated young and old hepatocytes. AP-1 DNA binding activity was assayed using a gel retardation assay. Protein extracts were prepared at various times following EGF stimulation and incubated with a 32 P-labeled oligonucleotide containing an AP-1 binding site. The reaction mixtures were separated by electrophoresis on a 4% polyacrylamide gel. Arrow indicates specific AP-1 binding. NS, nonspecific binding.

MKP-1 in EGF-stimulated hepatocytes from young and aged rats (Fig. 6). Interestingly, old hepatocytes contained significantly higher basal levels of MKP-1 mRNA expression than young hepatocytes (Fig. 6B). However, EGF treatment did not affect MKP-1 mRNA expression in the aged cells, while MKP-1 mRNA was elevated 4- to 5-fold in young hepatocytes treated with EGF.

DISCUSSION

Although a number of studies using a variety of models have provided evidence that aging is associated with a decline in proliferative capacity, the molecular basis of this age-related loss in function is poorly understood (26–32). In previous studies, Ishigami *et al.* (31) had demonstrated that EGF-induced DNA synthesis was reduced in aged hepatocytes. Since EGF is known to exert its mitogenic effects through activation of MAPK, we compared the relative level of ERK and JNK activation in old *versus* young hepatocytes. Although no age-related differences in JNK activity were found, a marked reduction in the magnitude of ERK2 activation in response to EGF treatment was found in aged hepatocytes. Reduced activation was shown to be associated with decreased expression of the MAPK-regulated genes *c-fos* and *c-jun* and diminished AP-1 DNA binding activity. While the cause for the age-related loss in ERK activation by EGF remains to be determined, it is clearly not the result of a change in ERK protein levels. It also appears not to be due to alterations in either the number or affinity of EGF receptors on hepatocytes, as previous studies by Ishigami *et al.* (31) showed no age-related differences in these

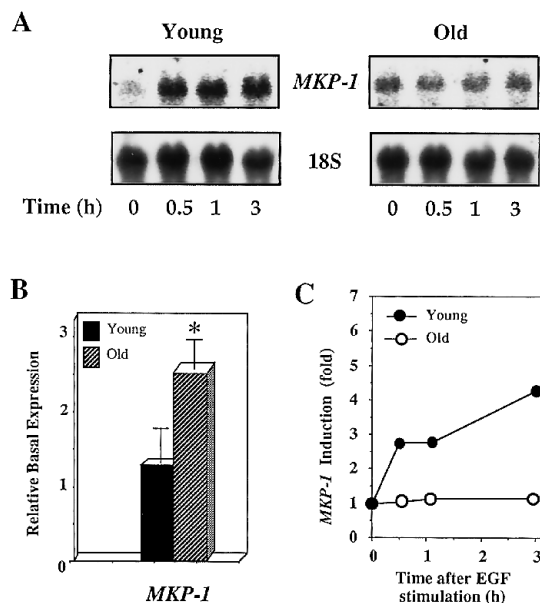


FIG. 6. MKP-1 expression in young and old hepatocytes. *A*, representative Northern blot analysis showing the time course for MKP-1 expression in EGF-stimulated hepatocytes from young and old rats. The 18 S signals were used to normalize for differences in loading and transfer among samples. *B*, basal levels of MKP-1 expression in hepatocytes from 4 young and 4 old animals. Data are mean \pm S.D. of 4 independent experiments. *, significantly different from young animals ($p < 0.05$). *C*, MKP-1 induction by EGF in young and aged hepatocytes. Results presented are the average of 2 independent experiments. All signals were normalized to those for 18 S.

properties. However, we cannot rule out the possibility that the EGF receptor is functionally altered with age.

The cascade initiated through EGF stimulation of the EGF receptor that ultimately leads to ERK activation involves several steps and numerous regulatory molecules including Ras, Raf, and MEK (1, 6). Altered activity of any one of these regulatory molecules could contribute to reduced ERK activity. However, our observation that MKP-1 expression is elevated in aged hepatocytes suggests an alternative explanation for the reduced ERK activity. The ability of MKP-1 to dephosphorylate and thereby inactivate ERK is well established (20). Even in the absence of alterations in the initiation/activation of the response to EGF, an elevation in basal MKP-1 levels would be expected to perturb the balance between the positive (kinase) and negative (phosphatase) influences on ERK activity and thus result in attenuation of ERK activity. This would further lead to reduced expression of MAPK-regulated genes (as seen for *c-fos* and *c-jun*) involved in the mitogenic response and diminished proliferative capacity.

MKP-1 expression is highly induced by mitogenic stimulation (24, 36), presumably through an ERK-mediated pathway.

However, MKP-1 can also be induced by treatments in the absence of significant ERK activation (19, 25), suggesting that alternative pathways contribute to the regulation of MKP-1. Further elucidation of the mechanisms regulating MKP-1 expression could provide additional insight into the cause and consequence of its elevation in aged hepatocytes.

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