Expression of the Pro-apoptotic Gene *gadd153/chop* Is Elevated in Liver with Aging and Sensitizes Cells to Oxidant Injury*

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Aging is generally accompanied by reduced tolerance to oxidative stress and altered responsiveness to proliferative signals. We have shown that hepatocytes derived from aged rats (24–26 months) exhibit greater sensitivity to H$_2$O$_2$ treatment and reduced proliferation following epidermal growth factor (EGF) treatment than cells of young adult rats (5–6 months). Here we examined the effects of aging and calorie restriction (CR) on expression of the oxidative stress-inducible and pro-apoptotic gene *gadd153* (chop) in these hepatocytes, and we investigated its influence on sensitivity to oxidants. We show that aging was associated with elevated expression of *gadd153*, both basally and in response to H$_2$O$_2$ treatment. CR, which attenuates age-associated declines in stress tolerance, prevented the age-related increase in *gadd153* expression. EGF treatment also resulted in *gadd153* induction in old cells. This effect was absent in young cells and in old cells of CR rats. *gadd153* induction by EGF was reactive oxygen species-dependent and correlated with heightened sensitivity to subsequent H$_2$O$_2$ treatment, suggesting that elevated *Gadd153* contributes to the greater sensitivity of EGF-pretreated old cells to oxidative stress. Additional support for this hypothesis was provided by experiments with Rat1 fibroblasts in which conditional expression of *Gadd153* conferred increased sensitivity to H$_2$O$_2$. We propose a model whereby the diminished ability of old hepatocytes to overcome an EGF-triggered reactive oxygen species load leads to induction of proapoptotic genes (*gadd153*), which, in turn, sensitizes the cells to oxidant injury. Our findings point to *gadd153* expression levels as an important factor in liver aging.

Reactive oxygen species (ROS)$^*$ constitute a double-edged sword for mammalian cells. Many proteins involved in growth control are subject to redox regulation, and transient generation of ROS by growth factor treatments is essential for proliferative signals. We have shown that hepatocytes derived from aged rats (24–26 months) exhibit greater sensitivity to H$_2$O$_2$ treatment and reduced proliferation following epidermal growth factor (EGF) treatment than cells of young adult rats (5–6 months). Here we examined the effects of aging and calorie restriction (CR) on expression of the oxidative stress-inducible and pro-apoptotic gene *gadd153* (chop) in these hepatocytes, and we investigated its influence on sensitivity to oxidants. We show that aging was associated with elevated expression of *gadd153*, both basally and in response to H$_2$O$_2$ treatment. CR, which attenuates age-associated declines in stress tolerance, prevented the age-related increase in *gadd153* expression. EGF treatment also resulted in *gadd153* induction in old cells. This effect was absent in young cells and in old cells of CR rats. *gadd153* induction by EGF was reactive oxygen species-dependent and correlated with heightened sensitivity to subsequent H$_2$O$_2$ treatment, suggesting that elevated *Gadd153* contributes to the greater sensitivity of EGF-pretreated old cells to oxidative stress. Additional support for this hypothesis was provided by experiments with Rat1 fibroblasts in which conditional expression of *Gadd153* conferred increased sensitivity to H$_2$O$_2$. We propose a model whereby the diminished ability of old hepatocytes to overcome an EGF-triggered reactive oxygen species load leads to induction of proapoptotic genes (*gadd153*), which, in turn, sensitizes the cells to oxidant injury. Our findings point to *gadd153* expression levels as an important factor in liver aging.

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The abbreviations used are: ROS, reactive oxygen species; AL, ad libitum; CR, calorie-restricted; EGF, epidermal growth factor; gadd, growth arrest and DNA-damage-inducible; RT, reverse transcriptase; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; ER, endoplasmic reticulum.

†‡‡ The expression of *gadd153* appeared higher in old hepatocytes relative to young cells. Gadd153 belongs to the CCAAT/enhancer-binding protein fam-

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MATERIALS AND METHODS

Animals—Male Fischer 344 rats 4–6 months old (NIA Contract Colonies at Harlan Sprague-Dawley) were maintained on a 12-h light/dark cycle in a controlled environment with water supplied at all times. AL fed rats were offered unlimited quantities of regular NIH-31 pellets diet, whereas calorie-restricted (CR) rats received a vendor-provided pellet-enriched NIH-31 diet equal to 60% of the average daily caloric intake of the AL fed rats (GRC ACUC protocol NJH-058-Ra/Mi).

Cell Culture and Treatment—Hepatocytes were isolated from young (4–6-month-old) and old (24–26-month-old) rats by the method of Se-glen (43). Rat1 cells were obtained from Dr. C. C. Ling (44). Gadd153i cells were derived from Rat1 fibroblasts and express ectopic gadd153 under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, as described previously (41). Rat1 and Rat1-My-cGadd153 (AB4) cells lines were grown in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum and antibiotics. AB4 and Gadd153i cells were maintained as described above in medium containing 300 μg of hygromycin B (Sigma) per ml. H2O2 was from Sigma; EGF was from Inovitrogen; and AG1478, compound 56, ebelen, and N-acetylcysteine were from Calbiochem. Cell viability was determined by trypan blue dye.

Northern and Western Blot Analyses and Real Time RT-PCR—Northern blot analysis was carried out using total RNA as described previously (35). Hybridizations were carried out using radiolabeled cDNA probes specific for rat gadd153, gadd45, and grp78. A radio-labeled oligonucleotide complementary to 18 S rRNA was used to verify RNA integrity and loading differences (35).

Real-time PCR determinations were performed using the iCycler IQ™ Real Time Detection System (Bio-Rad) using a SYBR® Green PCR Master Mix (PE Biosystems, Foster City, CA). Assays were carried out in triplicate, and PCR products were examined by agarose gel electrophoresis. Primers for amplification of histone H1 sequence were AGA-TCCGCAAGTCAGTTGTGC and GTTGAAGTTCTCGAGGCTG; primers for amplification of the GADD153 sequence were CCTGAAGCAGAACCACCCTGTC and CCTCCACCCACGCTGCACC. Reactions were performed as follows: 1 cycle at 95 °C for 7 min and 40 cycles at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. Standard curve and slope values were derived from 10-fold cDNA dilutions (1:10, 1:100, 1:1000, and 1:10000). Input amounts of RNA (IA) were calculated using Equation 1 provided by ABI Prism 7700,

\[ \text{IA} = 10^{\text{Ct} - b} \]

where Ct = value from RT-PCR data file; b = y intercept of standard curve line; m = slope of standard curve line (from equation \( y = mx + b \) for standard curve line). Histone H1 was used as reference gene, because its expression was not changed with age.

Whole-cell lysates (50–150 μg, prepared as reported previously (35)) were size-fractionated by electrophoresis through Tris-glycine gels (In-vitrogen) and transferred to polyvinylidene difluoride membranes. For protein detection, a polyclonal anti-Gadd153 antibody was used (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After secondary antibody incubation, signals were visualized by enhanced chemiluminescence (Amersham Biosciences).

Gadd153 Promoter-Luciferase Reporter Assays—The gadd153-LUC construct containing the −778 to +21 promoter region of the hamster gadd153 gene was obtained from Dr. S. Howell (45). Cells were transiently transfected for 12 h using 100 μl of LipofectAMINE 2000 (In-vitrogen) with 1.6 μg of DNA per dish. After transfection, cells were incubated in serum-free medium for 6 h before treatment with 100 ng/ml of EGF for 12 h. Cells were then incubated in fresh serum-free medium for 6 h, washed twice with phosphate-buffered saline, and lysed for assessment of luciferase activity using the Luciferase Assay System kit (Promega, Madison, WI). Relative light units were normalized to protein concentrations that were determined in parallel.

Statistical Analysis—Statistical analysis was performed using a one-way analysis of variance. Differences between individual age or treatment groups were evaluated using the unpaired two-tailed Student’s t test.

RESULTS

Basal and H2O2- and EGF-induced Gadd153 Expression in Young and Old Hepatocytes—Preliminary experiments using Northern analysis suggested that gadd153 mRNA expression might be elevated with aging. However, basal expression of gadd153 is very low and often undetectable by Northern analysis, precluding accurate quantitative measurement with this method. Therefore, real time RT-PCR was used to examine expression of the gene in young versus old cells obtained from rats either AL fed or maintained on a CR diet. As shown in Fig. IA, gadd153 mRNA expression was higher in old hepatocytes from AL rats relative to young cells from AL rats. However, old CR hepatocytes showed significantly lower gadd153 expression relative to their AL counterparts. Elevations in Gadd153 protein expression with aging were also observed in whole liver tissue as shown in Fig. 1B, but as shown for mRNA expression in hepatocytes, CR reduced them.
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As oxidative damage increases gadd153 expression in many cell types, we next examined gadd153 mRNA levels in young and old cells following their exposure to 300 or 600 μM H2O2. We have shown previously (28) that H2O2 induces apoptosis in a dose-dependent manner in both young and old hepatocytes, but with old cells exhibiting greater sensitivity. Consistent with the old cells being more sensitive to H2O2, these cells showed higher induction of gadd153 mRNA relative to young cells (Fig. 2).

We next investigated gadd153 expression following treatment with EGF. As shown in Fig. 3A, the absence of gadd153 mRNA induction by EGF in young cells is consistent with the general view that gadd153, as implied by its name, is regulated by conditions of growth arrest and is not responsive to proliferative signals. Much to our surprise, however, we observed significant induction of gadd153 mRNA by EGF in old cells (Fig. 3A). The abundance of mRNAs encoding gadd45 and grp78, two other stress-related genes whose expression often coincides with that of gadd153 during conditions of stress, was not affected by EGF treatment (Fig. 3A). Increased gadd153 mRNA expression was associated with increased Gadd153 protein (Fig. 3B). Importantly, the EGF-induced Gadd153 expression was greatly attenuated in cells of old CR rats relative to cells of old AL rats. To determine whether EGF induced gadd153 expression through increases in transcription, cells were transiently transfected with a construct carrying the gadd153 promoter linked to a luciferase reporter gene. As shown in Fig. 3C, no significant activation of the promoter occurred in young cells. By contrast, a marked increase in gadd153 promoter activity was seen following EGF treatment of old hepatocytes (Fig. 3C).

ROS Contribute to Increasing Gadd153 Levels by EGF Treatment—EGF stimulation leads to the transient generation of ROS downstream of EGF receptor activation (1). To investigate the importance of ROS in mediating Gadd153 induction by EGF, cells were treated with EGF in the presence of either N-acetylcysteine, a glutathione precursor, or eboserin, a glutathione peroxidase mimetic, both of which reduce the ROS load (46, 47). Both antioxidants prevented Gadd153 induction in old cells in response to EGF (Fig. 4A), and they inhibited Gadd153 induction in old and young cells in response to H2O2 treatment (Fig. 4C). Gadd153 induction in response to both H2O2 and
EGF was likewise dependent on EGF receptor activation as co-treatment of cells with either AG1478 or compound 56, inhibitors of EGF receptor phosphorylation, markedly reduced Gadd153 induction (Fig. 4, A and C).

Influence of Gadd153 on Survival—Induction of Gadd153 is frequently correlated with stresses that result in cell death. Evidence that Gadd153 expression in fact correlated with relative survival in H$_2$O$_2$-treated cells is shown in Fig. 4D. This observation suggested that EGF treatment may indeed constitute a stress for these cells. However, examination of EGF-treated old cells revealed no evidence of apoptosis (Fig. 4B). In previous studies using several different cell lines, we found

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![Figure 4](image-url)

**Figure 4.** Effect of antioxidants and inhibitors of EGF receptor on Gadd153 induction and cell death after treatment with either EGF or H$_2$O$_2$. Hepatocytes derived from young and old AL-fed rats were pretreated with either N-acetylcysteine (NAC) (500 µM), ebselen (10 µM), AG1478 (AG) (5 µM), or compound 56 (1 µM) for 30 min as indicated and then treated with 100 ng/ml of EGF (A) or 600 µM of H$_2$O$_2$ (C), and Gadd153 levels were examined 8 h later by Western blot analysis. Cont., control. B and D, cells were treated same as described above, and 24 h later cell viability assay was assessed (see “Materials and Methods”). Shown are the means ± S.E. of three independent experiments. *, p < 0.05 comparing values for young and old AL cells.

![Figure 5](image-url)

**Figure 5.** Effect of combined treatment with EGF and H$_2$O$_2$ on gadd153 induction and cell death. A, hepatocytes derived from young AL and old AL rats were treated with either 100 ng/ml EGF, 600 µM H$_2$O$_2$, or both. Top, Gadd153 levels were examined 8 h later by Western blot analysis as described under “Materials and Methods.” B, cells were treated as described in A, and cell viability was assessed 24 h later. Cont., control. Shown are the means ± S.E. of three independent experiments. *, p < 0.05 comparing values for young and old AL cells; **, p < 0.05 comparing values for young and old AL cells; ***, p < 0.05 comparing values for young and old AL cells.

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that elevated Gadd153 expression alone did not lead to cell death but did enhance cell sensitivity to ER stress (41). Therefore, we sought to determine whether up-regulation of Gadd153 in old hepatocytes in response to EGF treatment would increase their sensitivity to an oxidative insult. To this end, cells were treated with either EGF, H2O2, or both, and Gadd153 expression and cell survival were assessed 8 or 24 h later, respectively. As shown in Fig. 5A, EGF and H2O2 treatments resulted in similar levels of Gadd153 induction in old cells, although only H2O2 resulted in apoptosis. Importantly, however, simultaneous exposure of old cells to EGF and H2O2 potentiates Gadd153 expression, and this was associated with greater apoptosis than that seen with H2O2 alone. No such potentiation of Gadd153 expression was seen with combined treatment of young cells, nor was the sensitivity to H2O2 altered by combined exposure with EGF (Fig. 5A).

The above studies suggest that EGF-stimulated increases in Gadd153 expression lead to heightened sensitivity of old hepatocytes to oxidant exposure. If so, then ectopic expression of high levels of Gadd153 should likewise increase cell sensitivity to oxidants. Unfortunately, such experiments cannot be performed in primary hepatocytes, as we are unable to achieve a high enough efficiency of transfection. However, support for this notion was obtained with Myc-transformed Rat1 fibroblasts (Rat1-Myc) in which Gadd153 expression is constitutively elevated (A94 cells). Parental Rat1-Myc and A94 cells were treated with various concentrations of H2O2 and 24 h later evaluated for cell viability. Constitutive expression of Gadd153 led to a marked increase in cell death compared with that seen in Rat1-Myc cells (Fig. 5B). Similar findings were obtained using another model in which ectopic (FLAG-tagged) Gadd153 expression was driven via an IPTG-inducible promoter in untransformed Rat1 cells (Fig. 5C). In the absence of IPTG, Gadd153 protein is undetectable in these cells. IPTG treatment (resulting in the accumulation of Gadd153) alone did not significantly induce cell death. However, it markedly enhanced the sensitivity of cells to H2O2. These findings support the view that elevated Gadd153 expression in aged hepatocytes contributes to their enhanced sensitivity to oxidative stress.

DISCUSSION

We have reported previously (28) that aging in rats is associated with reduced tolerance of hepatocytes to oxidative injury, an effect that can be attenuated by maintaining the animals on CR. Having preliminarily identified the stress-inducible gene gadd153 as up-regulated in the aged hepatocytes, we sought here to investigate further the relationship between gadd153 expression and oxidative stress tolerance in this model. The major findings presented are as follows: 1) gadd153 expression is elevated both basally and in response to oxidative injury as a function of aging, but CR reduces these elevated levels; 2) aberrant induction of gadd153 occurs in response to EGF stimulation selectively in old hepatocytes; and 3) elevated Gadd153 sensitizes cells to oxidative insults. Coupled with our previous observations, these new findings argue strongly that elevated Gadd153 is an important factor in liver aging and specifically that it contributes to the enhanced susceptibility of aged cells to oxidative insults.

A previous study (48), employing microarray screening to identify genes altered by aging in mouse brain, reported that gadd153 expression was increased 1.8-fold with aging in the neocortex but not the cerebellum. In that study, CR did not alter the effect of aging on gadd153 expression. By using a similar approach to examine age-related changes in gene expression in mouse heart, it was recently reported that gadd153 expression is not affected by aging but is reduced >3-fold by CR (49). Such disparate findings point to important organ/cell type-specific differences in the aging process of mammals. However, it is worth noting that in neither of the two previous studies were the findings for gadd153 obtained with the arrays verified by other methods. Here we have demonstrated the up-regulation of gadd153 with aging in rat hepatocytes and its attenuation by CR by using several different approaches including real time RT-PCR and Northern and Western blot analyses. The fact that CR up-regulates EGF-activated Gadd153 expression in old cells, but not young cells, supports the notion that, in liver, CR does not directly influence gadd153 expression but rather acts to prevent age-related alterations leading to elevated expression of the gene.

The mechanisms contributing to age-related alterations in gadd153 expression remain to be determined. As an oxidative stress-inducible gene, basal increases in gadd153 expression are consistent with the idea that aging is associated with elevated levels of ROS and chronic oxidative stress (4, 50). However, it is also possible that changes in gadd153 contribute to elevations in ROS, as we have shown that ectopic expression of Gadd153 in rat fibroblasts leads to increased levels of ROS (41). Elevations in basal ROS levels with aging could be an important factor contributing to EGF-induced gadd153 expression as well. Whereas transient generation of ROS is an important and necessary consequence of growth factor stimulation for proliferation, if imposed on cells already containing elevations in ROS, it could shift the cell into a pro-oxidant state, resulting in oxidative stress. The ability of CR (which is known to reduce the levels of oxidative damage with aging) to prevent EGF-induced gadd153 expression is consistent with this hypothesis.

 Whereas a role for gadd153 in mediating apoptosis in response to ER stress is well established (40, 42), its contribution to cell death in other stress paradigms is less clear. Such an effect cannot be taken for granted, as we found that elevated gadd153 expression did not affect the cellular response to ionizing radiation in Myc-transformed rat fibroblasts (41). Our findings here using two different model systems of gadd153 overexpression, as well as experiments in rat hepatocytes, support its pro-apoptotic function during conditions of oxidative stress and point to elevated gadd153 expression as an important factor in the increased susceptibility of aged hepatocytes to oxidative injury. Strategies aimed at reducing gadd153 expression in aged cells and tissues could have anti-aging benefits.

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