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Redox regulation of apurinic/apyrimidinic endonuclease I activity in Long-Evans Cinnamon rats during spontaneous hepatitis

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Abstract

The Long-Evans Cinnamon (LEC) rat is an animal model for Wilson's disease (WD). This animal is genetically predisposed to copper accumulation in the liver, increased oxidative stress, accumulation of DNA damage, and the spontaneous development of hepatocellular carcinoma (HCC). Thus, this animal model is useful for studying the relationship of endogenous DNA damage to spontaneous carcinogenesis. In this study, we have investigated the apurinic/apyrimidinic endonuclease 1 (APE1)-mediated excision repair of endogenous DNA damage, apurinic/apyrimidinic (AP)-sites, which is highly mutagenic and implicated in human cancer. We found that the activity was reduced in the liver extracts from the acute hepatitis period of LEC rats as compared with extracts from the age-matched Long Evans Agouti (LEA) rats. The acute hepatitis period had also a heightened oxidative stress condition as assessed by an increase in oxidized glutathione level and loss of enzyme activity of glyceraldehyde 3- phosphate dehydrogenase, a key redox-sensitive protein in cells. Interestingly, the activity reduction was not due to changes in protein expression but apparently by reversible protein oxidation as the addition of reducing agents to extracts of the liver from acute hepatitis period reactivated APE1 activity and thus, confirmed the oxidation-mediated loss of APE1 activity under increased oxidative stress. These findings show for the first time in an animal model that the repair mechanism of AP-sites is impaired by increased oxidative stress in acute hepatitis via redox regulation which contributed to the increased accumulation of mutagenic AP-sites in liver DNA.

Keywords

APE1; DNA damage; DNA repair; HCC

Introduction

Considerable evidence has linked chronic inflammation and oxidative damage with increased risk of cancer [1, 2]. Reactive oxygen species (ROS) are generated in different

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subcellular locations as a function of oxygen-mediated biochemical reactions [3]. The ROS levels are also elevated by transition metals, such as iron or copper, and by exogenous agents, such as ionizing radiation or ozone [4].

Certain diseases that are characterized by oxyradical overload, such as Wilson's disease (WD), have been associated with chronic inflammation and a higher risk of hepatocellular carcinoma (HCC) [5]. The Long-Evans Cinnamon (LEC) rat is a model for Wilson's Disease (WD) and is characterized by a mutation in the *Atp7b* gene. As in WD this mutation leads to defective copper excretion and copper accumulation in the liver, thereby leading to increased ROS production. This may result in oxidative damage in susceptible regions such as the liver, brain and kidney. The LEC rat has been shown to be very useful for studying the mechanisms of inflammation-mediated DNA damage and spontaneous carcinogenesis. Recent investigations revealed considerable increases in DNA single-strand breaks and 8-oxoguanine levels in the brain, liver, and kidney of affected animals [6–10]. In addition, other findings have suggested the involvement of lipid peroxidation in Cu-mediated toxicity in the LEC liver [11]. To eliminate the deleterious effects of oxidized bases, organisms have developed efficient repair mechanisms. Oxidized base lesions are removed by enzymes of the base excision repair (BER) pathway. Endonuclease III (NTH1) and 8-oxoguanine DNA glycosylase (OGG1) play significant roles in the removal of oxidized bases such as oxidized pyrimidines and 8-oxoguanine [12–15]. Previously, we found that expression and activity of both NTH1 and OGG1 were significantly reduced during the course of liver tumorigenesis in LEC rats [10].

Mammalian AP-endonuclease (APE1), a major enzyme in BER pathway, initiates the repair of abasic sites (AP-sites), which are directly formed by free radicals during cellular metabolism, inflammatory diseases, carcinogenesis, aging, anti-tumoricidal agents and by excision of damaged bases by DNA glycosylases via the BER pathway. APE1 catalyzes the hydrolytic cleavage of the phosphodiester bond immediately 5′ to the AP-site [16]. Being a multifunctional protein, APE1 has recently been found to be involved in RNA metabolism [17] and acts as a redox factor to activate various transcription factors and is involved in gene regulation [18, 19]. Moreover, APE1 or acetylated APE1 interacts with various transcription factors to control transcription of several genes in various biological pathways (19). APE1 itself is a redox sensitive protein and its activity is redox regulated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [20–24]. The nuclear redox function of GAPDH is involved in transcriptional regulatory function of various transcription factors as well [25].

To understand the biology of APE1 and its role in carcinogenesis, transgenic mice with conditional APE1 gene knock-out have been developed [26–28]. It has not been possible yet to show the direct relationship of APE1 deficiency and AP-site accumulation with tumorigenesis as the APE1 null mouse is embryonically lethal and in conditional knock-out mice tumorigenesis studies are complicated by high levels of apoptosis [26]. This complication was demonstrated in both APE1-null mouse and APE1 knockdown in cultured human cells, which underwent cell death due to AP-site accumulation and deficiency in transcriptional regulation in part [26, 29]. In this study, we have investigated the excision repair capacity of AP-sites, a potentially protective cellular mechanism. We found that

APE1 activity was reduced in the liver extracts from the acute hepatitis period of LEC rats as compared with extracts from age-matched Long Evans Agouti (LEA) rats carrying wild type *Atp7b* gene. The activity reduction was not due to expressional changes, but by reversible protein oxidation. These findings suggest that the repair of endogenous AP-sites is marred by heightened oxidative stress in acute hepatitis through redox regulation which contributed to the increased accumulation of mutagenic AP-sites in liver DNA.

Materials and Methods

Animals and liver tissues

LEC and LEA animals were maintained under standard conditions as described earlier following IACUC approved protocol [10]. Liver tissues were collected from LEC and LEA rats of various age groups (8–40 weeks), as described previously [10].

Cell lines and Cell Culture Conditions

HepG2 cells were obtained from the Tissue Culture Shared Resource (TCSR) of Lombardi Cancer Center, Georgetown University, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and 1% Penicillin-Streptomycin (Mediatech Inc., Manassas VA). Huh- 7 ($p53^{-/-}$) cells were kindly provided by Brent E. Korba (Department of Microbiology and Immunology, Georgetown University Medical Centre, Washington DC), cells were cultured in DMEM, (4.5g/liter glucose, L-glutamine, and sodium pyruvate) 10% FBS, 1% Penicillin-Streptomycin. The cells were treated by Glucose Oxidase (Sigma Chemical, St Luis, MO) at 70% confluence at 50, 100 and 150 ng/ml for 1h. Cell viability was not affected by the treatment as determined by the growth rate. The cells were maintained at 37°C incubator in 5% CO_2 and subcultured according to protocol, using trypsin.

DNA isolation

Genomic DNA from the liver tissues was extracted by a guanidine/detergent based DNA isolation method using DNeasy Blood and Tissue kit (Qiagen). The DNA was then measured by Nano-Drop ND1000 spectrophotometer (Thermo scientific, DE) and stored in aliquots at −80°C for AP-site measurements.

Protein isolation

Liver nuclear extracts were prepared under usual reducing conditions from 20 mg of liver tissues of LEC and LEA rats using NE-PER nuclear extraction kit (Thermo scientific, DE) following manufacturer's protocol. However, the nuclear extracts in the non-reducing condition were prepared based on a published procedure, except the reducing agent, dithiothreitol (DTT), was omitted in the buffers [30].

Western blot analysis

The liver nuclear extracts (20 μg) from LEC and LEA rats of various ages were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane. Western blotting was carried out with the affinity-purified anti-

APE1 (1:1000; Novus Biologicals, CO), anti-GAPDH (1:1000), and β-actin (1:1000; Sigma-Aldrich, MO) monoclonal antibodies and a horseradish peroxidase-linked anti-mouse secondary antibody (1:1000; Amersham Pharmacia Biotech, NJ) using the enhanced chemiluminescence technique (Santa Cruz Biotechnology, CA) according to the manufacturer's protocol. Image capture and quantification of the protein bands were carried out using the Chemi Genius 2 Bio-Imaging System. Western blot from the HePG2 and Huh7 cell extract (5 g) were performed by running 4–12% gradient gel using Invitrogen gel running and transfer system (Invitrogen).

AP-site detection

AP sites were measured in DNA from liver tissues of LEC and LEA rats of different ages using Aldehyde Reactive Probe (ARP) assay-based DNA damage quantification kit (Dojindo Molecular Technologies, Inc., MD) following manufacturers protocol and as described previously [31].

Substrate preparation

A 50-mer double stranded oligonucleotide containing a tetrahydrofuran (THF) at the 27^{th} position was [^{32}P] labeled at the 5`-end as described previously [32] and was used as substrate for APE1 activity assay.

AP endonuclease 1 activity assay

APE1 activity assay was performed following a published method [32]. Briefly, liver nuclear extracts (10 ng) from various ages of LEC and LEA rats were incubated with 5` ^{3}2P-labeled THF-containing duplex oligonucleotide substrate (1.5 nM) in the presence or absence of 1.0 mM DTT in an assay buffer containing 50 mM Tris HCl (pH 7.5), 0.1mg/ml bovine serum albumin (BSA), 2 mM $MgCl_2$, 50 mM KCl for 7 min at 37°C in 20 μl of reaction volume. The reactions were then terminated followed by separation of the substrate and the product in the reaction mixture as described previously [32].

Effect of DTT and α-Lipoic acid on AP endonuclease 1 activity assay

Prior to performing the assay, liver tissue extracts from 16 weeks of LEC and LEA rats were incubated with varying concentrations of DTT (0–2 mM) and α-lipoic acid (0–1 μM) for 1 hour on ice and the extracts (10 ng) were used for APE1 activity assay as described previously [32].

GAPDH activity assay

GAPDH activity from liver tissue nuclear extract was measured by using a 96-well plate format colorimetric assay kit (KD alert GAPDH assay kit, Ambion Inc., Tx). Briefly, liver nuclear extracts (5 μg) from various ages of LEC and LEA rats or the diluted GAPDH pure enzyme were used with the GAPDH substrate, the appropriate reaction buffer (provided in the kit) and followed manufacturer's instructions for measurement.

Assay of Glutathione

Total [reduced (GSH) and oxidized glutathione (GSSG)] and GSSG from the liver tissue extracts of different ages of LEC and LEA rat were measured using a 96 well-plate glutathione assay kit (Cayman Chemical Co, Ann Arbor, MI). Briefly, 50 μl of liver tissue extracts and the standards (provided in the kit) were used for the measurement of total glutathione and GSSG levels following manufacturer's instructions.

Statistical analysis

Statistical analyses were performed on data collected from three to six independent experiments using three animals per group. Student's *t* test (one-tailed) was used to evaluate the statistical difference between LEC and LEA rats. The differences were considered statistically significant when $P<0.05$.

Results

Increased accumulation of AP-sites in LEC rat liver DNA during acute hepatitis

Comparison of AP-site accumulation in genomic liver DNA from age-matched mutant LEC and wild type LEA rats showed a statistically significant increase (up to 30–40%) in AP-site levels, particularly during the period of acute hepatitis (16–18 weeks) and early chronic hepatitis (24 weeks; Fig. 1 B). However, both the pre-hepatitis (Fig. 1 A, 8 weeks) and the chronic hepatitis periods (Fig. 1 A, 40 weeks) displayed no significant changes in AP-site levels in LEC compared to LEA rat livers (Fig. 1 B). To our knowledge this is the first time that an increase in AP-sites, which can be generated by oxidative stress [26], has been observed during hepatitis and inflammation prior to preneoplastic foci formation and HCC.

APE1 activity is unchanged in LEC rat liver during hepatitis

Increase in AP-site generation can be due to reduced repair activity of APE1-initiated BER pathway; so, we tested this idea during different disease stages of LEC rats. It is well known that APE1 is an essential enzyme to initiate the repair of AP-sites by the BER pathway [16]. We examined APE 1 activity in liver tissue extracts from different ages of wild type LEA and copper overloaded mutant LEC rats. The activity was measured by an APE1-mediated incision of a 50- mer oligonucleotide containing a single THF, a stable analog of AP-site. Figure 2A and B shows that there are no changes in the activity of APE1 from livers of LEC rats of ages 8–40 weeks when compared to LEA rats of the same age. Earlier we found that activity and expression of other BER enzymes, NTH1 and OGG1, that initiate the repair of oxidized pyrimidines and 8-oxo-dG, respectively, were correlated at different stages of disease in LEC rats [10]. Thus, we tested whether APE1 activity is also correlated with APE1 expression level, which we determined by Western blotting. Like activity, we did not observe any significant difference in protein expression (Fig. 2C) in the livers of LEC compared to wild type LEA rats, even in the period of acute (16–18 weeks) or early chronic (24 weeks) hepatitis.

APE1 activity is affected by its redox state in LEC rat liver during hepatitis

It was previously reported that oxidation or reduction of APE1 can markedly alter its activity *in vitro* [20], affecting BER-mediated repair of AP-sites in cultured cells [21]. In the prior APE1 activity experiment (Fig. 2 A and B), we used the tissue nuclear extract prepared under reducing conditions, i.e., in presence of DTT. We also performed the APE1 activity assay in presence of DTT and found no change in activity of APE1 at any time point. We hypothesized that APE1 protein was oxidized in the hepatitis period, which decreased activity and contributed to an increase in AP-site accumulation. Addition of DTT during extract preparation, however, reduced the oxidized APE1 protein and artificially reactivated its enzymatic activity. To test this hypothesis, we prepared liver tissue extracts from different ages of LEC and LEA rats in the absence of DTT and then examined APE1 activity in an assay buffer also lacking DTT. Figure 3A and B shows that these extracts from both LEC and LEA rat livers had typical APE1 activity in absence of DTT. Interestingly, we found that under such reaction conditions, APE1 activity was decreased by 20–30% during the acute hepatitis period (16–18 weeks) in the mutant LEC compared to LEA rats (Fig. 3 A and B). Although we did not observe any significant difference in protein expression in the livers of LEC compared to wild type LEA rats (Fig. 3C). These results are highly reproducible and statistically significant and indicate that the increase in AP-site accumulation during the acute hepatitis period (Fig. 1) is due to reduced activity of APE1. However, the sustained increase in accumulation of AP-sites at 24 weeks (early chronic hepatitis phase) may be in part due to carryover of AP-sites from earlier time points.

The possibility that the difference in APE1 activity among LEA and LEC rat liver tissues of different ages could be attributable to differential APE1 protein enrichment was tested by actin immune-staining of protein gels containing the same protein extracts used for APE1 activity assay. The results showed equal loading of the same extracts and nullified that possibility (Fig. 3 A).

Effect of reducing agents on APE1 activity during acute hepatitis

Comparison of Figures 2 A, B and 3 A, B suggests that addition of DTT in the extracts could have reactivated the APE1 activity possibly via reduction of oxidized APE1 [20]. We tested different concentrations of two reducing agents, DTT, which is commonly used in the laboratory, and biologically relevant α-lipoic acid [36] for their restoration capability of APE1 activity in LEC rat liver extracts from an acute hepatitis time point (16 weeks), as the extracts from this period showed the maximum decrease in APE1 activity (Fig. 3A and B). We found that, compared to LEA rat liver extracts from the same time period, the APE1 activity was restored and stimulated in LEC rat extracts with addition of increasing concentrations of α-lipoic acid and also DTT (Fig. 4 A-C). The results indicate that APE1 is oxidized in acute hepatitis period and can be reactivated via reduction of APE1 protein.

Increased level of oxidized glutathione and reduced activity of GAPDH, a known redox-sensitive protein in LEC rat liver during acute hepatitis

In order to determine whether oxidative stress is actually increased in the acute hepatitis period to cause APE1 oxidation, we measured the glutathione level and activity of GAPDH, a known redox-sensitive enzyme at various time points in LEC and LEA rats. Comparison

of the oxidized glutathione (GSSG) and total glutathione showed a statistically significant increase (up to 60– 90%) in GSSG level during the period of acute hepatitis (16–18 weeks) and chronic hepatitis (40 weeks; Fig. 5 A). Next, we measured GAPDH activity in LEC and LEA rat liver tissue extracts from pre-hepatitis to chronic hepatitis stages. We found that GAPDH activity was significantly reduced by 25–50% in the period of acute hepatitis (16–18 weeks) in the mutant LEC compared to wild type LEA rats (Fig. 5 B). However, we did not observe any change in the expression of GAPDH protein as detected by Western blot analysis of liver tissue extracts from the same time points (Fig. 5 C). Thus, the reduced activity (Fig. 5 B), not the expression *per se*, of GAPDH (Fig. 5 C) and the increased level of oxidized glutathione (Fig. 5 A) appear to correlate with decreased activity of APE1 (Fig. 3 A and B) in the acute hepatitis period.

These results indicate high oxidative stress during inflammatory periods, which could be the cause of APE1 oxidation and loss of its repair activity.

Discussion

In this study we showed for the first time that AP-sites, which are highly mutagenic and carcinogenic, are significantly elevated during hepatitis in the Wilson's disease-mimicking LEC animal model. Recently, in a methionine adenosyltransferase 1a knockout (*Mat1a* KO) mouse model, AP-sites were also shown to be increased due to increased oxidative stress along with decreased expression of APE1 at both mRNA and protein levels and development of HCC [37]. However, we did not observe any change in APE1 expression (Fig. 2C) in our model. Rather, a unique redox modification of APE1 protein and consequent loss of its activity (Figures 3 and 4) seem to play a crucial role in increased AP-site accumulation and eventually liver carcinogenesis. Of note, it has not been possible yet to show in an animal model the direct relationship of APE1 deficiency and AP-site accumulation with tumorigenesis as the APE1 knockout is embryonically lethal [26]. However, the AP-site accumulation in APE1-deficient cells has been shown to be the primary cause of cell death [29], and this fact might have impeded the tumorigenesis experiments in biological model systems. APE1 polymorphisms as modulated by smoking habits seem to play a role in human bladder cancer [38]. It is now well established that smoking increases oxidative stress in addition to other carcinogenic effects in humans [39, 40].

APE1 has multifactorial roles. Other than its repair function in the BER pathway, APE1 is also known as REF1 as it is involved in redox regulation of several transcription factors that regulate various genes in many different pathways (19). Recently, it has been shown that DNA repair endonuclease activity of APE1 can be redox modulated by known redox sensitive enzymes, such as GAPDH in in vitro reactions [21]. In fact, the functional domains of REF1 and APE1 are distinct, and even the redox active site cysteines are distinct for two different functions [41, 42, 20] In this study we showed for the first time in an animal disease model of HCC that elevated endogenous oxidative stress as shown by increased oxidized glutathione level (Fig. 5 A) and reduced activity of a redox-sensitive protein GAPDH (Fig. 5 B) may have significant effect on the decreased AP-site cleavage activity of APE1 (Fig. 3 A and B) in the acute hepatitis period of LEC rats. The overall cellular redox

state shifts dramatically toward an oxidized state during acute hepatitis and returns to the pre-hepatitis redox state during the chronic phase of hepatitis. The activity of APE1 correlates with these redox spectrum swings within the cell. Indeed, the loss of activity of APE1 during acute hepatitis of LEC rats (Fig. 3 A and B) was due to protein oxidation as addition of reducing agents, α-lipoic acid and DTT, to the extracts could rescue the activity of APE1 to the normal level observed in wild type LEA rats (Figs. 2 A and B and 4 AC). Therefore, the acute hepatitis period is at once characterized by both high levels of oxidative stress and low levels of BER activity, a particularly precarious position for the hepatocytes.

We have shown previously that repair of other oxidative DNA damages, such as oxidized pyrimidines and 8-oxoguanine, which are also toxic and mutagenic, are also inhibited in this LEC rat liver cancer model [10]. Notably, these oxidized bases are also repaired by the BER pathway and are initiated by specific DNA glycosylases, including NTH1, NEIL1, and OGG1. Other than polynucleotide kinase, APE1 is the only endonuclease known so far to process the reaction products of DNA glycosylases prior to completion of the BER pathway by DNA polymerase and ligase [16]. Therefore, APE1 plays a crucial role in processing AP-sites whether those are formed directly by oxyradicals or as repair intermediates.

The current results of this APE1 study, along with our previous results regarding BER glycosylases, indicate that the decreased BER of endogenous DNA adducts induced by increased oxyradicals in inflammation may play a role in carcinogenesis. It was reported in LEC rat liver that preneoplastic foci were first evident at 24-weeks and peaked at 48-weeks of age [43]. Therefore, it is intriguing that the reduction of repair of AP-sites (Fig. 3 A and B) and other oxidative DNA damage [10] during 16–18 weeks of age leads to the appearance of preneoplastic foci and eventually tumorigenesis of LEC rat liver [43]. Moreover, the LEC rat liver displays higher cell proliferation and relatively lower apoptosis [43]. Thus, both the reduction of APE1 activity due to protein oxidation and the elevation of AP-sites due to DNA oxidation coupled with continuous cell proliferation during hepatitis may predispose individuals to increased mutation load and cancer risk [5]. The restored APE1 activity during the chronic hepatitis period suggests that BER activities may help the hepatocytes to survive in the presence of additional cytotoxic DNA adducts, such as AP-sites, which are toxic as they block DNA replication [16] and mutagenic when replication occurs [16]. Thus, APE1 possibly protects normal cells against oxidative DNA damage and subsequent tumorigenesis, but once the tumors are formed, APE1 may protect the tumor cells against additional cytotoxic DNA damage. The latter is supported by ample evidence from studies of APE1 overexpression in human cancers [44]. Overall the present study highlights a remarkable imbalance between the accumulation of mutagenic lesions and the crucial repair of those lesions in the highly oxidative environment of the inflamed liver, which itself is responsible for crippling the necessary repair enzymes and creating the imbalance.

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Abbreviations

LEC	Long Evans Cinnamon
LEA	Long Evans Agouti
APE1	AP-endonuclease 1
ROS	reactive oxygen species
WD	Wilson's disease
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

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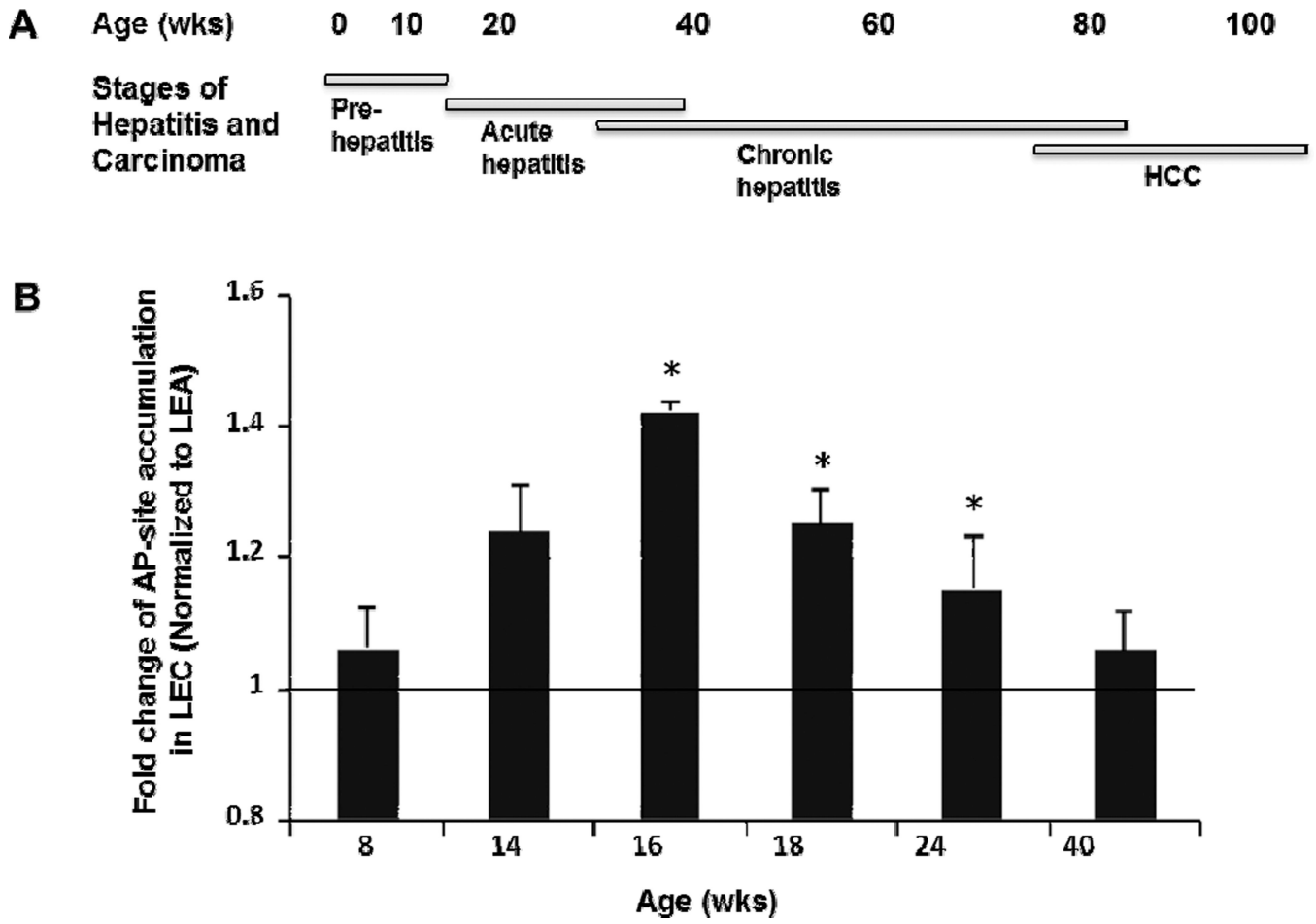

Figure 1. AP site accumulation in mutant LEC vs. wild type LEA rats

(A) Different stages during hepatitis and hepatocellular carcinoma. (B) DNA extracted from the liver tissues of various ages of LEC and LEA rats was used to measure the number of AP sites in DNA using Aldehyde Reactive Probe (ARP) assay as described in "Materials and Methods" section. The data presented are the values (mean ± SD) derived from three independent experiments from three animals per age group of each strain. (* denotes p-value < 0.05.)

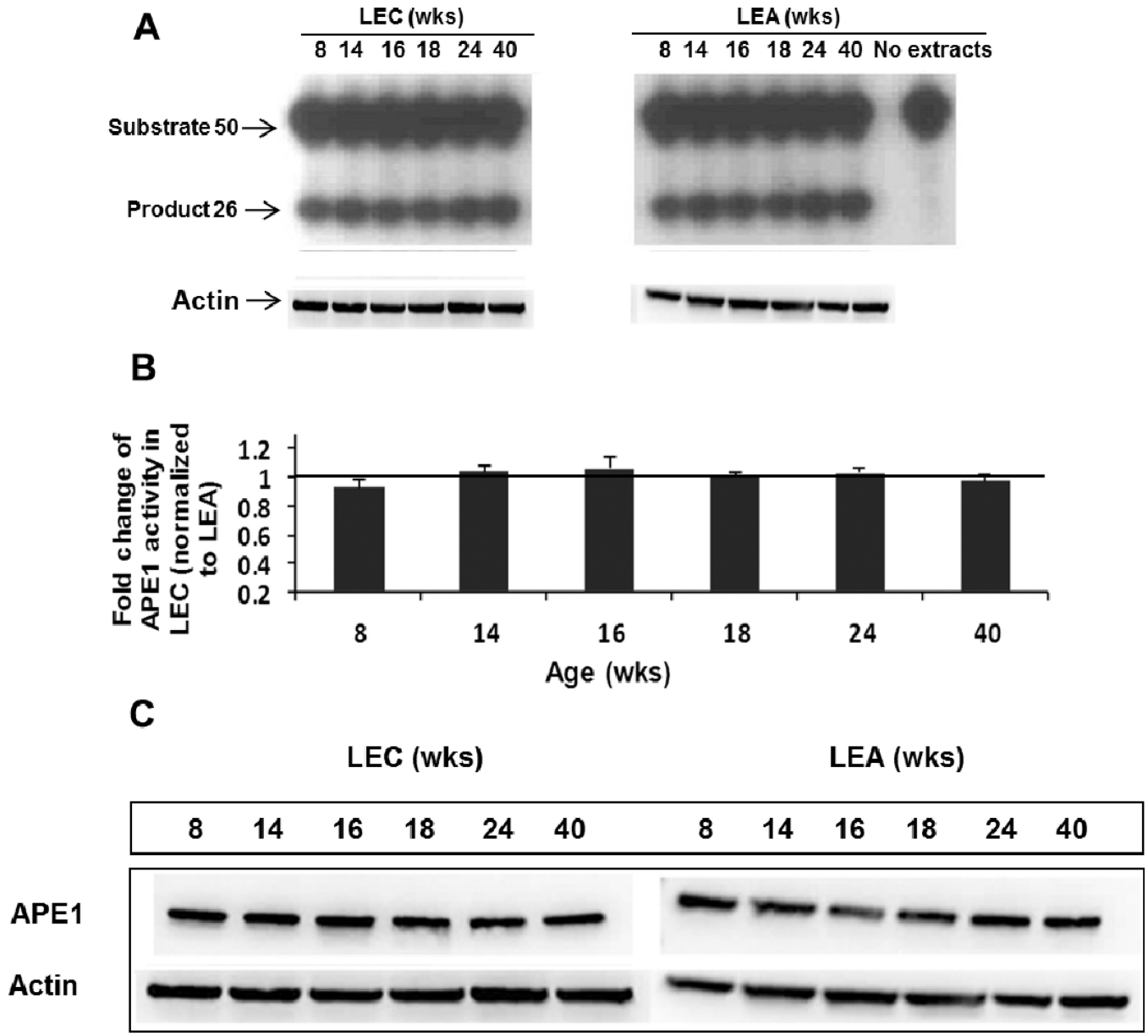

Figure 2. APE1 activity in liver extracts from LEC and LEA rat

(A) Typical autoradiograms of denaturing gels showing APE1 activity in liver extracts. The details of the assay procedure are described under “Materials and Methods”. Substrate 50, 50-mer oligonucleotide containing a single THF; Product 26, incised 26-mer oligonucleotide. Loading control is shown as the actin staining of the same protein extracts used for APE1 activity. (B) Quantification of APE1-mediated incision activity. (C) Western blotting of protein expression of APE1 in LEC and LEA rat liver extracts. Image of APE1 Western blotting of liver extracts of two rat strains from different ages. β-actin was used as a loading control and for normalization of APE1 expression. The details of the assay procedure are described under “Materials and Methods.” The data presented are the values (mean ± SD) derived from six independent experiments from three animals per age group of each strain.

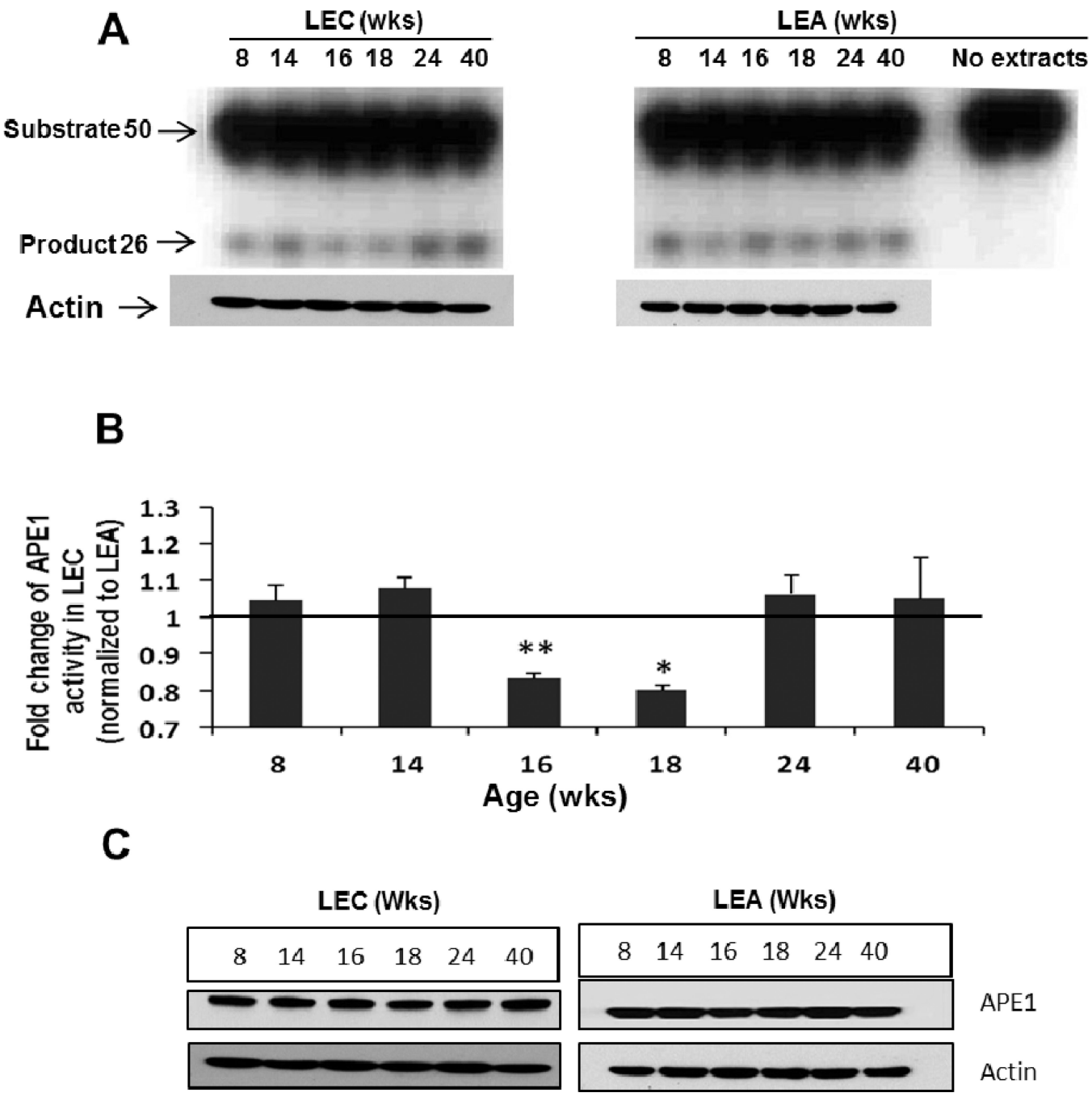

Figure 3. APE1 activity in liver extracts from LEC and LEA rats under nonreducing conditions

(A) Typical autoradiograms of denaturing gels showing APE1 activity in liver extracts lacking DTT. The details of the assay procedure are described under "Materials and Methods." Designations of substrate and product and description of loading control are as described in Figure 2A. (B) Quantification of APE1-mediated incision activity. (C) APE1 protein expression in LEC and LEA rat liver extracts by western blotting, β-actin was used as a loading control and for normalization of APE1 expression. The values (mean ± SD)

from the data presented are from six independent experiments from three animals per age group of each strain. (* and ** denote p-value <0.05 and <0.01, respectively).

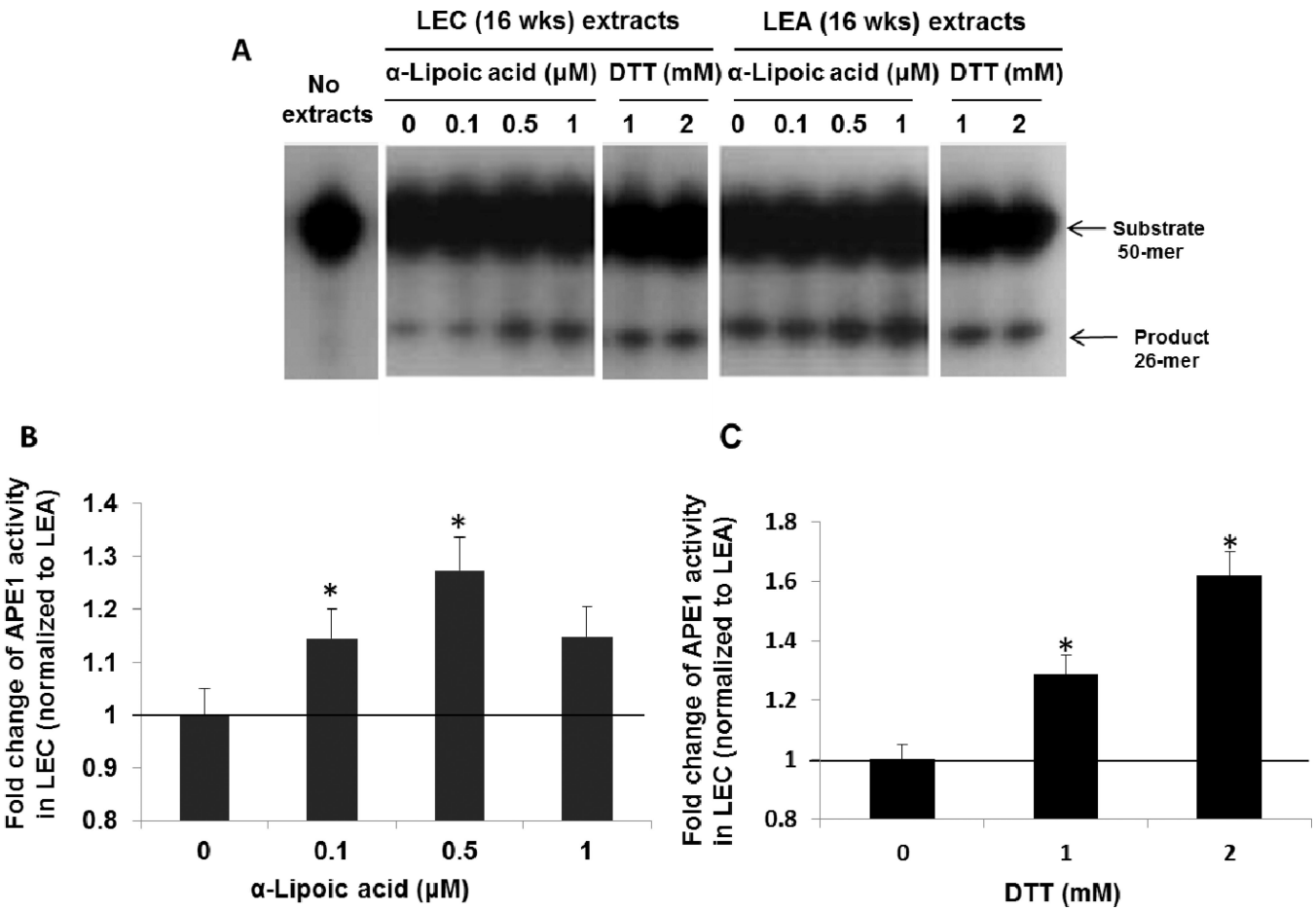

Figure 4. Effect of α-lipoic acid and DTT on APE1 activity in rat liver tissue extract

Liver tissue extracts from LEC and LEA rats (age 16 weeks) were incubated with various concentrations of α-lipoic acid and DTT. (A) Typical autoradiograms of denaturing gels showing APE1 activity in liver extracts after pre-treatment with various concentrations of α-lipoic acid and DTT. (B) Quantification of APE1-mediated incision activity after pre-treatment of extracts with α-lipoic acid. (C) Quantification of APE1-mediated incision activity after pretreatment of extracts with DTT. The details of the assay procedure are described under "Materials and Methods." The data presented in panels (B) and (C) are the values (mean ± SD) derived from three independent experiments from three animals of each strain. (* denotes p-value <0.05). The "No extract" control and all LEC and LEA liver extracts samples were resolved on the same gel but were separated from each other by several lanes, as depicted by the division in the gel figure.

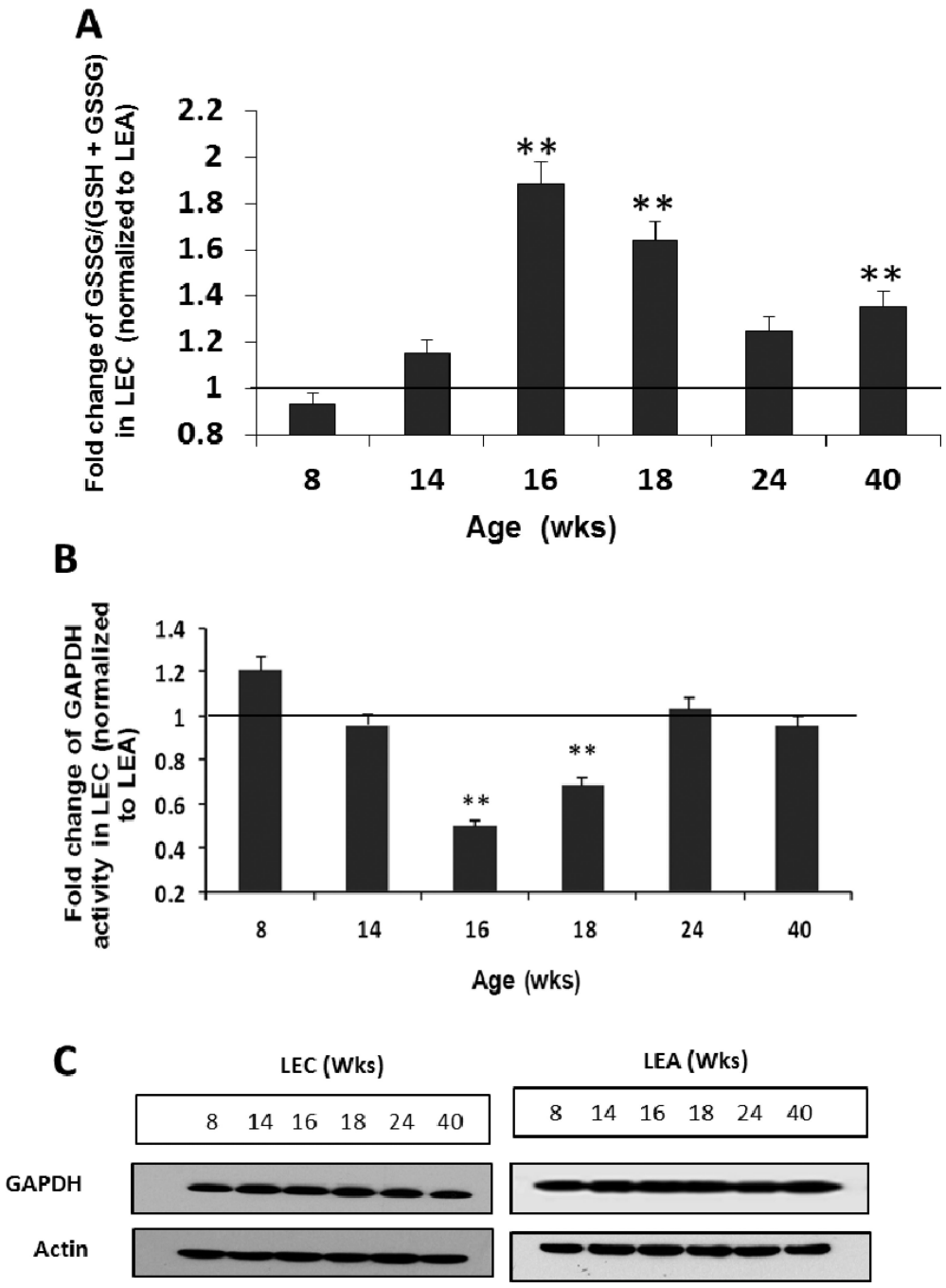

Figure 5. Oxidative stress during hepatitis in LEC rat

(A) Levels of oxidized glutathione in liver extracts from LEC and LEA rats. Deproteinated extracts from liver tissues of various ages of LEC and LEA rats were used to measure levels of oxidized and total glutathione utilizing the glutathione assay (B) GAPDH activity in liver extracts from LEC and LEA rats under nonreducing condition. (C) Western blotting of GAPDH protein expression in LEC and LEA rat liver extracts. Western blotting was carried out for the GAPDH protein expression along with the housekeeping protein β-actin as loading control. The details of glutathione assay and the western blotting procedure are

described under “Materials and Methods.” The data presented are the values (mean ± SD) derived from three independent experiments from three animals per age group of each strain. (** denotes p-value < 0.01.)