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Various Fatty Acids Induce Cell Damages Differently in CYP2E1-transduced HepG2 Cells, E47 Cells, Compared to C34 Cells

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The differential effects of various fatty acids such as n-3 and n-6 types or degrees of unsaturation on the CYP2E1 induction and the production of lipid peroxidation (LPO) were investigated. The CYP2E1-transduced human hepatoma HepG2 cells (E47) were cultured in RPMI 1640 media containing different concentrations of various fatty acids up to 48 h incubation compared to C34 cells and CYP2E1-null cells. Treated fatty acids were linoleic acid (LA:n-6, C18:2), arachidonic acid (AA:n-6, C20:4) and docosahexaenoic acid (DHA:n-3, C22:6). The cell survival rate was decreased corresponding to the degree of unsaturation (LA>AA≅DHA) and to LPO production in E47 and C34 cells. The four or five unsaturation degree of fatty acids, AA and DHA, caused time- and dose-dependent cell death in E47 cells but not as much as in C34 (without CYP2E1), suggesting an important role of CYP2E1 in the DHA mediated damage. In the levels of lipid peroxides (LPO), AA also elevated LPO by 3- and 5- fold compared to DHA or LA treated E47 cells. However, AA did not increase LPO until 48 h incubation in C34 cells. In conclusion, the polyunsaturated fatty acids induced CYP2E1 induction might be changed by the elevated levels of lipid peroxide (LPO) and oxidative stress through the connection of CYP2E1 and degrees of unsaturated fatty acids.

Key words: Polyunsaturated fatty acids (PUPA), n-3 · n-6 · n-9 type of fatty acids, Lipid peroxides (LPO), CYP2E1

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INTRODUCTION

The microsomal P450 enzyme of the liver is the heme protein which is dealing with oxidization and deoxidation in the body, and is a complex type of monooxygenase.[1,2] Out of more than 150 isozymes of P450, CYP4A1 and CYP2E1 are increased due to toxic substances and nutritional components. Ethanol-inducible P450 2E1 (CYP2E1), present in the liver and other extra-hepatic tissues, is known to metabolize various small molecules of potentially toxic substrates: ethanol, acetaldehyde, dimethyl nitrosamine, acetaminophen, CCl_4, benzene, unsaturated fatty acids, etc.[3] CYP2E1, a loosely bound P450 enzyme, mediated metabolism of these substrates usually leads to the production of more toxic or carcinogenic metabolites such as reactive or free radical metabolites and lipid peroxides.[1,4] Cederbaum and colleagues established a stable human HepG2 hepatoma cell line with transduced CYP2E1 (E47 HepG2 cells) to study the role of CYP2E1 in cell damage caused by fatty acids.[1,2,5,6] Arachidonic acid, n-6 fatty acid, as a CYP2E1 substrate, was shown to induce much more damage to E47 cells than C34 control hepatoma cells, demonstrating the role of CYP2E1 in the metabolic activation and subsequent cell dama.[5] It can be assumed that the production of oxygen free radicals is further facilitated by unsaturated fatty acids.[7] When n-3-fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) enter the cell membrane, they enter in competition with n-6 fatty acids such as arachidonic acids (AA) and linolenic acids (LA), affecting the n-6/n-3 ratio. Furthermore, DHA is known to lower the incidence of cancer when it is used alone or combined with other dietary fish oils such as EPA. DHA and EPA have been demonstrated to decrease tumor cell proliferation both *in vitro* and *in vivo* models .[8-10] On the other hand, the P450 reduces carcinogenesis but it is different from the original roles as a monooxygenase mechanism control of healthy cells from foreign substances like free radicals. The oxygen free radicals participate in the process acting as a mediator that can be shared with DNA of the cell that

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has been given carcinogens. This shows their close connection with CYP2E1 control.

The purpose of this research was to validate the control of CYP2E1 and LPO production as one of markers for the changes in cell membrane structure due to dietary fatty acids. Also, this research was performed to validate the offsetting role of peroxide formation by selecting unsaturated fatty acids as positive CYP2E1 regulating factor. In addition, the importance of the levels of LPO in the PUFA-mediated cell damages were compared with those in E47 cells and C34 cells.

MATERIALS AND METHODS

1. Materials

Minimal essential media (MEM) and other materials for cell culture including gentamicin, antibiotics, L-glutamine and fetal bovine serum (FBS) were purchased from InVitrogen (San Diego, CA, USA). C34 and E47 HepG2 cells were kindly provided from Dr. A. Cederbaum (Mount Sinai Medical School, New York, NY). MTT cell proliferation kit and LDH cytotoxicity kit were purchased from Roche Diagnotics GmbH (Indianapolis, USA). Lipid peroxidation (LPO) kit and SP100625 were purchased from Calbiochem Co. (San Diego, USA). CYP2E1 monoclonal antibody was purchased from PharMingen (San Diego, CA, USA). Other materials not specified were the same as described.[11,12]

2. Cell Culture and Fatty Acid Supplements

The human HepG2 hepatoma cell line with transduced CYP2E1 (E47 HepG2 cells) was established by Cederbaum & colleagues. The stability of E47 cells was confirmed after ethanol treated cells to study the role of CYP2E1 in cell damage caused by its substrates, including fatty acids (Fig. 1). Both C34 and E47 cells were maintained in MEM with glutamine, 10% FBS and antibiotics (gentamycin sulfate) at 37 ℃ in a humidified incubator containing 5% CO_2. For cell viability and Hoechast staining, cells were plated on 96-well or 24-well plates at a density $2.5\times10^4/cm^2$. To study the effects of DHA on cell growth, E47 and C34 hepatoma cells were exposed to different concentrations of fatty acids for 12, 24, 36, or 48 h. Fatty acids completely dissolved in ethanol were dried under argon gas and immediately exposed to 100% FBS, as described0.[12] Final concentrations of fatty acids 25 to 100 uM and FBS in media were 1%.

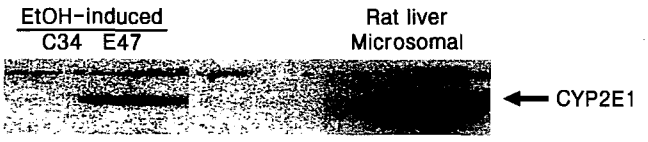

Fig. 1 A human HepG2 hepatoma cell line with transduced CYP2E1 (E47 HepG2 cells) was established by Cederbaum & colleagues. The stability of E47 cells was confirmed after ethanol treated cells to study the role of CYP2E1 in cell damage caused by its substrates including fatty acids.

3. Cell Proliferation

Viability of E47 and C34 cells after treatment with fatty acids was determined by following the protocol supplied in the CellTiter 96 NonRadioactive Cell Viability Assay Kit (Promega, Madison, WI) by using MTT as a substrate. Lactate dehydrogenase (LDH) activity was also measured in the supernatant fraction after treatment with fatty acids for indicated times using the Cytotoxicity Detection kit (Roche Molecular Biochemicals, Summerville, NJ). E47 cells were placed in 24-well plates and treated with 50 uM DHA for 24 and 48 h as described above.

4. Measurement of Lipid Peroxides (LPO)

Both 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) end products derived from peroxidation of PUFA were measured as convenient indices for lipid peroxidation after culturing the cells under the same conditions. Whole cell lysates from 3×10^6 cells were prepared by three repeated cycles of freeze/thawing in distilled water. Diluted samples (200 ul volume) were added into 650 ul of 10.3 mM N-methyl 2-phenylindole. Methane sulfonic acid (15.4 M; final volume 150 ul) was added to mix the sample, which was then incubated at 45 ℃ for 40 min. After cooling on ice, 4-HNE and MDA products were measured at 596 nm in comparison to the standard curves.

5. CYP2E1 Induction

Following the hepatoma cell culture, the cells were homogenized using 0.15 M KCl and 10,000×g centrifugally separated for 30min. For microsome separation, the upper layer fluid was again centrifugally separated for 60 min and only a pellet was stored at -80 ℃. After the CYP2E1 was purified using DEAE-sepharose column, it was measured using the Western blot analysis which utilizes polyclonal antibody.[13] The Western blot analysis was executed with SDS-PAGE, utilizing the anti-CYP2E1 IgG antibody, and horseradish peroxidase was used for color development. The relative value of CYP2E1 was measured using both densitometer (BMS) and Hewlett Packard dual-wavelength thin-layer scanner.

6. Protein Quantification

Protein quantification was measured according to the Bradford method.[14] Bovine serum albumin was used as the standard.

7. Statistical Analysis

Statistical analyses were performed using Student's *t*-test for the comparisons between two groups and one-way ANOVA test for the comparisons among the three or more groups. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

1. Concentration - and time - Dependent Cell Death in E47 and C34

To study the effect of fatty acids on hepatoma cell growth rates in E47 or C34, we determined the dose - and time - dependent changes in cell morphology, cell viability (MTT). As shown in Fig. 2 & 3, dose dependent DHA and AA fatty acids drastically decreased cell survival according to incubation times up to 48 h in E47. As the degree of unsaturation increased, the cell survival rate was decreased. AA or DHA groups had similar effect on cell death but AA had stronger effect than that in DHA at 48 h. Generally, more cells died after longer exposure and higher concentrations of DHA and AA than shorter exposure and lower DHA and AA concentrations. For instance, less than 5% of E47 cells died after exposure to 25 uM DHA for 12 h, 25% died after exposure to 100 uM DHA for 12 h and more than 65% cells died when cells were exposed to 100 uM or 200 uM DHA for 48 h. However, AA and LA did not show same results for cell survival in C34 cells except DHA. DHA treated C34 cells died more in 48 compared to 12 h incubation but cell death was not decreased according to higher concentration at 12 or 48 h. Similar tendencies were observed in the pilot study, when the levels of treatment were increased according to increasing times. As conclusion, although HepG2 cell's survival rate was decreased as the fatty acid's degree of unsaturation increased, DHA showed similar results to AA even though DHA has higher degree of unsaturation than AA. This indicates that the type of unsaturation (n-6 or n-3) plays more important than the degree of unsaturation.

Previous studies indicated that dietary PUFA killed various cancer cells although the cell death depends on the cell type, concentration and duration of treatment. Chen *et al.* reported that AA efficiently caused cell death of HepG2 cells which continuously express CYP2E1, possibly through the production of lipid peroxides, which were raised by about 2-fold (up to about 7 uM) over the control cells after exposure to 30 uM AA.[5] Oleic acid (OA, C18:1) did not cause cell death on both of E9 and MV5 cells which does not express CYP2E1. These results suggest that higher degrees of unsaturated fatty acids are more toxic to CYP2E1-containing cells through elevated levels of LPO and oxidative stress.[5,15,16]

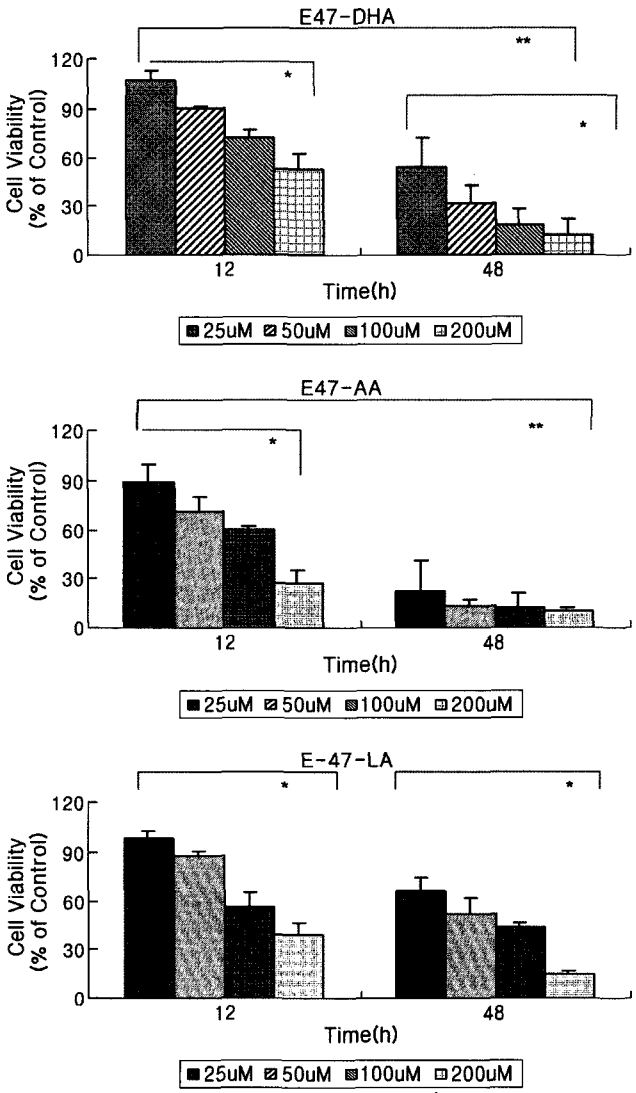

Fig. 2 Time - and dose - dependent effects of fatty acids on the cell viability in E47 cells compared to control.

More cells died after longer exposure and higher concentration of fatty acids. (* P<0.05, ** P<0.01) LA was not stronger on cell death in E47 cells compared to DHA and LA fatty acids treated.

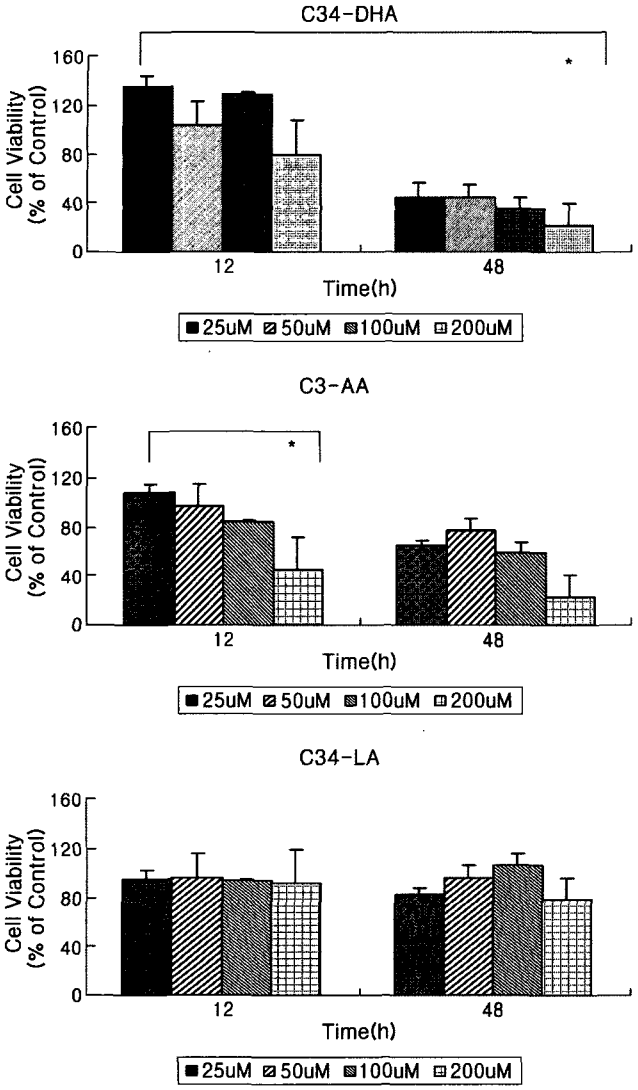

Fig. 3 Time - and dose - dependent effects of fatty acids on the cell viability in C34 cells compared to control.

DHA and AA treated C34 cells died in longer incubation times, otherwise, LA was not different between 12 and 48 h times.

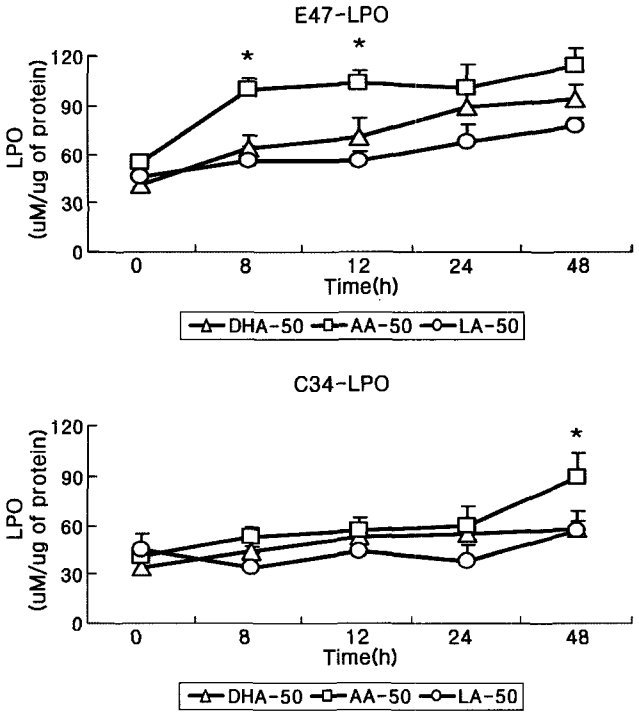

Fig. 4 Effect of different fatty acids on LPO production in E47 and C34 cells.

Both cells were exposed to 50 μmol/L DHA, AA and LA up to 48 h incubation times. AA increased LPO compared to other fatty acids in E47 (significant differences with $P<0.01$ at 8 and 12 hs) AA treated C34 cells significantly increased LPO at 48 h.

2. LPO Production in Different Fatty Acids Treated Cells

For all fatty acid groups, 50 uM was treated because the concentration of 100 and 200 uM FA induced cell toxicity (cell necrosis) in E47 cells. Theoretically, DHA, the highest degree of unsaturation, was expected as the highest production of peroxide production, followed by AA>LA. However, AA treated E47 cells showed the highest LPO production and LPO levels were similar between LA and DHA. However, we could not find different effects of fatty acids on LPO production in C34 cell except in the case of AA 48 hr incubation (Fig. 4). Compared to E47 and C34, AA had higher LPO production in E47 rather than that in C34. Both the pilot study and this research showed that for all types of fatty acids, as the administered amount increased, or as the degree of unsaturation increased, the amount of MDA was also increased. In the DHA group, which has the highest degree of unsaturation, the increase of conjugated diene repressed the proliferation of cancerous cells. Peroxide, which indicates changes in healthy cells, also repressed the proliferation of cancerous cells. There was also a report that said peroxide levels, which indicate cytotoxicity of cancerous cells, did not affect healthy cells such as skin fibroblast or human glioblastoma cell line.[16] It appears to be that cancerous cells are especially sensitive to peroxides and the production of peroxide lowers the cell multiplication rate of n-3-type fatty acids, which have high peroxidizability.[17-19] Many cancer therapies such as radiotherapy, chemotherapy and hyperthermia, kill cancer cells in their process involving free radical generation leading to LPO. Accordingly, the increase of PUFA has a higher sensitivity to reactive oxygen species in tumor cells because of cytoxicity of LPO and its products.[20] On the other hand, the killing mechanism of LPO was various in different cell type systems. The two possible mechanisms for LPO induced

cell damage through CYP2E1-mediated metabolism of fatty acids would be suggested. CYP2E1 directly oxidized PUFA (omega-1 hydroxylation) to reactive radicals that produced the toxicity or CYP2E1 generated reactive radicals such as superoxide or H_2O_2 that reacted with PUFA and produced lipid peroxides.[20,21]

CONCLUSION

We hypothesized that CYP2E1 was involved in fatty acids-mediated cell damage, since our current results suggest that DHA and AA caused more damage to E47 cells than C34 cells. LPO are believed to play an important role in E47 cell damage because CYP2E1 is known to increase the levels of reactive oxygen species (ROS) that usually lead to increased LPO production.[12,15] Our results supported a role of CYP2E1 in increasing LPO (the sum of MDA and 4-HNE) production after DHA or AA exposure and suggested LPO might be involved in higher unsaturation of FA such as DHA or AA -treated cell death.

Acknowledgement

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