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Protective Effect of Linoleic Acid on IFN γ-Induced Cellular Injury in Primary Culture Hepatocytes[1]

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We have previously demonstrated that treatment of hepatocytes with IFN γ results a series of cellular injury processes, including DNA synthesis arrest, membrane breakage and apoptosis. In the present work, we show that IFN γ suppresses cellular respiration and protein synthesis in hepatocytes, and that cellular respiration suppression is an early event in the IFN γ-induced cellular injuries. Polyunsaturated fatty acids (PUFAs) increased cellular respiration of hepatocytes, but only linoleic acid showed some protective effect against IFN γ-induced cellular respiration suppression. Linoleic acid also reduced other IFN γ-mediated cellular injuries, including membrane breakage and protein synthesis inhibition. Like linoleic acid, fetal bovine serum also inhibited IFN γ-induced cellular damage. Increased NAD levels were found in both IFN γ-treated and non-treated hepatocytes following the addition of PUFAs, but clofibrate, a peroxisome proliferator, bromophenacyl bromide (BPB), an inhibitor of phospholipase, nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase, and arachidonic acid, a metabolite of linoleic acid, did not inhibit IFN γ-induced cellular injury. In addition, the combination of linoleic acid and IFN γ induced nitric oxide (NO) synthesis in hepatocytes. These results suggest that fatty acid may play an important role in liver homeostasis during chronic inflammatory states and sepsis.

Key words: cellular injury, hepatocyte, IFN γ, linoleic acid, nitric oxide.

Although hepatocytes are the major target cells in hepatitis, the critical factors and mechanism involved in the cellular injury remain unclear. Various cytokines such as TNF α, IL-1β, IL-6, and IFN γ are involved in this inflammatory liver disease (*1*). *In vitro*, incubation of hepatocytes with IFN γ induced serious injury to hepatocytes, including DNA synthesis arrest and protein synthesis inhibition (*2*). In animals, IFN γ was shown to be a critical factor in the induction of hepatitis in mice (*3, 4*). Increased IFN γ is often seen in patients with hepatitis (*5*) or in bacterially infected liver (*6*). IFN γ receptors are expressed on hepatocytes in diseased liver, but not in normal liver (*7*). Our previous work showed that among IFN γ, TNF α, IL-1β, and IL-6, only IFN γ induced membrane breakage and apoptosis in primary-cultured hepatocytes (*8*). Therefore, it seems that IFN γ is involved in the liver damage and is a key factor in the onset of hepatitis (*9*).

IFN γ is a multifunctional cytokine secreted by activated T lymphocytes and NK cells. It plays a crucial role in host defense due to its antiviral (*10*), antiproliferative (*11*), pro-inflammatory, and immunoregulatory (*12, 13*) activities. IFN γ induces cell damage and cell death (*14*). Its effect in epithelial cells is biphasic. The first phase consists of cell proliferation arrest, which is followed by the second phase of cell death, which has the characteristics of programmed cell death (*15*). However, little information, if any, is available on the relationships among the various IFN γ-induced cellular injuries. Here, we show that IFN γ-induced cellular respiration suppression is an early event of IFN γ-mediated cellular injury. Linoleic acid improved the cellular respiration and showed some protective effects against IFN γ-induced cellular injuries.

MATERIALS AND METHODS

Materials—Flavianic acid (NYS), BSA (fatty acid-free), clofibrate, nordihydroguaiaretic acid (NDGA), and bromophenacyl bromide (BPB) were purchased from Sigma. Murine recombinant IFN γ and TNF α were gifts from Genentech Inc. Human recombinant HGF was a gift from

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Abbreviations: ALA, α-linolenic acid; BPB, bromophenacyl bromide; BSA, bovine serum albumin; CF, clofibrate; EPA, eicosapentaenoic acid; FBS, fetal calf serum; HGF, hepatocyte growth factor; IFN γ, interferon γ; IL-1β, interleukin 1β; LDH, lactose dehydrogenase; Lin, linoleic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaiaretic acid; NO, nitric oxide; Ole, oleic acid; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; TNF α, tumor necrosis factor α.

Snow Brand Milk Products Co. Female ICR mice were from Charles River Japan.

Hepatocyte Isolation and Culture—Parenchymal hepatocytes were isolated from an adult mouse by the modified *in situ* perfusion method (*16*). The liver was first perfused *in situ* through the thoracic inferior vena cava with Ca^{2+}-free Hank's solution supplemented with 5 mM EDTA and 5 mM glucose at 37°C until the blood in the liver was completely removed. Then the perfusion solution was changed to 0.0125% collagenase solution. After a few minutes of perfusion, the liver was excised and dispersed in cold Hank's solution. The resulting cell suspension was filtered through 300-gauge mesh. Parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at $50\times g$ for 90 s. The dead parenchymal hepatocytes were removed by density gradient centrifugation in Percoll. The liver parenchymal hepatocytes were seeded at a density of 3×10^4 cells/cm^2 in 96-well plates with RPMI 1640 containing 2×10^{-9} M insulin, 2×10^{-9} M dexamethasone, 1.0% BSA (fatty acid free), and antibiotics.

Measurement of Cellular Respiration—Mitochondrial respiration was measured by the mitochondrial-dependent reduction of MTT to formazan (*17*) with some modification. Briefly, after removal of the culture medium at the indicated time, cells were incubated with fresh medium containing MTT (0.2 g/ml) at 37°C for 1.5 h. Thereafter, the cells were washed with NKT solution (6.0 g NaCl, 0.2 g KCl, and 3.0 g Tris in 1,000 ml dH_2O, pH 7.4) three times, and the formazan formed in the cells was dissolved with 100 μl of lysing buffer (45% dimethylformamide, 10% SDS, pH 4.7). The absorbance of each well at 570 nm was measured by a micro plate reader, MT-120 (Corona Electronic).

Cellular Protein Determination—Quantitative binding and extraction of the dye flavianic acid (NYS) were used for determining cellular protein (*18*). After removal of the culture medium, cells were washed with NKT solution three times, then fixed with 10% trichloroacetic acid for 30 min. Fixed cells were stained with 0.2% flavianic acid (100 μl/well) at room temperature for another 30 min. Unbound dye was removed by washing the cells with 1% acetic acid 4 times. The plates were air-dried and the bound dye was extracted with 10 mM unbuffered Tris base. The absorbance of each sample was read at 415 nm.

Measurement of Cellular NAD^+ Levels—Cells were exposed to IFN γ for 48 h in the presence or absence of linoleic acids (300 μM). They were extracted in 0.25 ml of 0.5 N $HClO_4$, scraped, neutralized with 3 M KOH, and centrifuged for 2 min at $10,000\times g$. The supernatant was assayed for NAD by using a modification of the colorimetric method (*19*). The rate of increase in the absorbance at 560 nm was read immediately after addition of the NAD samples and after 10 and 30 min incubation at 37°C against a blank.

Lactate Dehydrogenase (LDH) Release Assay—LDH activities in the culture medium were measured using a CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega, Madison, USA). A 50 μl aliquot of culture medium of hepatocytes was mixed with 50 μl of substrate solution. The mixture was kept at room temperature for 20 min, and the reaction was stopped by adding 50 μl of stop solution to the mixture. The absorbance of each well at 415 nm was measured using a micro plate reader, and the cell membrane damage was expressed as percentage lysis.

Determination of Nitric Oxide (NO) Production—NO in the growth medium was detected by using Griess' reagent. A 50 μl aliquot of culture medium was mixed with 50 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 2.5% H_3PO_4 solution), and kept at room temperature for 20 min. The optical absorbance values of the samples were read at 570 nm using a micro plate reader MTP-120 (Corona Electronic).

Analysis of Chromosomal DNA—Cells were incubated with the lysis buffer (10 μg/ml proteinase K, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% SDS) for 15 h at 37°C. Chromosomal DNA was obtained by phenol/chloroform extraction and ethanol-precipitation. The sample in TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 1 μg/ml RNase was incubated for 1 h at 37°C. The same amount of DNA from each sample was subjected to electrophoresis through 1.0% agarose gel containing 0.1 μg/ml ethidium bromide.

RESULTS

Cellular Respiration Suppression Is an Early Event of IFN γ-Mediated Cellular Injury—Our previous work showed that among various cytokines expected to be in-

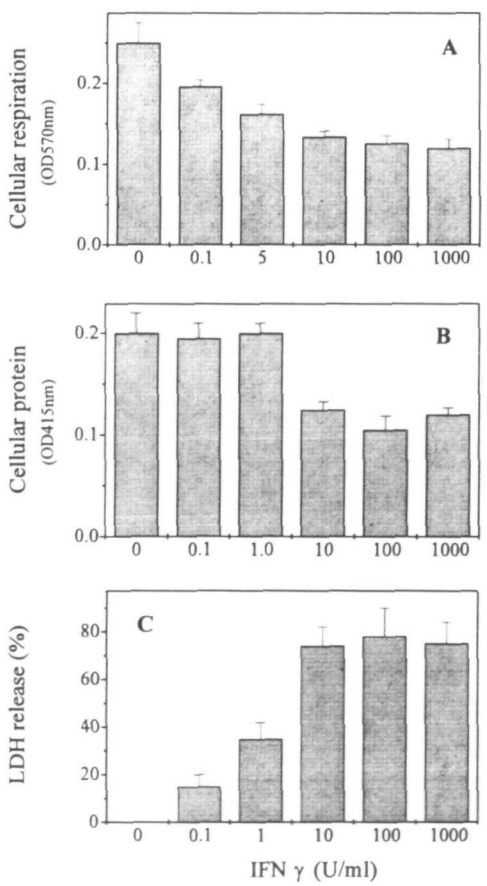

Fig. 1. IFN γ-induced cellular damage in primary hepatocyte cultures. Hepatocytes were exposed to IFN γ stimulation at 20 h after seeding. Cellular respiration (A), cellular protein (B), and LDH released into the culture medium (C) was detected according to the procedures outlined in "MATERIALS AND METHODS" after a further 38 h incubation of hepatocytes with IFN γ.

volved in cellular injury in hepatitis, only IFN γ induces membrane breakage and apoptosis in hepatocytes (*8*). In this experiment, we found that IFN γ also depresses cellular respiration and inhibits protein synthesis in hepatocytes (Fig. 1). As in the case of cell membrane damage, DNA arrest, and apoptosis (*8*), IFN γ at the concentration of 10 U/ml was enough for the induction of cellular injury, and 100 U/ml was the concentration inducing maximal cellular damage in hepatocytes.

To examine whether there are relationships among the various IFN γ-induced various cellular injuries, we compared the induction times of the IFN γ-mediated injuries. As shown in Table I, different IFN γ-induced cellular injuries occurred at different stages of incubation. Cellular respiration suppression occurred immediately after IFN γ addition (Table I), implying that the suppression of mitochondrial respiration is an early event of IFN γ-mediated cellular injury.

Linoleic Acid Increases Cellular Respiration and Inhibits IFN γ-Induced Cellular Damage—It is well known that the β-oxidation of long-chain fatty acids provides the liver with its main supply of energy for synthetic and transport processes (*20*). Therefore, we next examine whether polyunsaturated fatty acids (PUFAs) have any effect on IFN γ-induced cellular injury. As shown in Fig. 2A, all four tested PUFAs increased the cellular respiration of hepatocytes. Elevated respiration of hepatocytes began at 2 h and was maximal at 12-24 h after fatty acid addition (Fig. 2A). Thereafter, cellular respiration decreased progressively. However, among these four PUFAs, only linoleic acid showed a significant protective effect on IFN γ-induced cellular respiration depression (Fig. 2B); the other three PUFAs (Ole, ALA, and EPA) had little effect.

Although IFN γ suppressed cellular respiration in a dose-dependent fashion, it hardly affected cellular respiration of hepatocytes in the presence of 200 μM linoleic acid

TABLE I. **Comparison of various IFN γ-mediated cellular injuries.** Hepatocytes were exposed to 100 U/ml IFN γ at 8.0 h after seeding, then the incubation was started. Cellular respiration, cellular protein, DNA fragmentation (apoptosis), and cell membrane damage (LDH release) were estimated by the methods described under "MATERIALS AND METHODS."

	Respiration suppression	Protein synthesis inhibition	Apoptosis	Membrane breakage
Induction time (h)	2	10	16	24

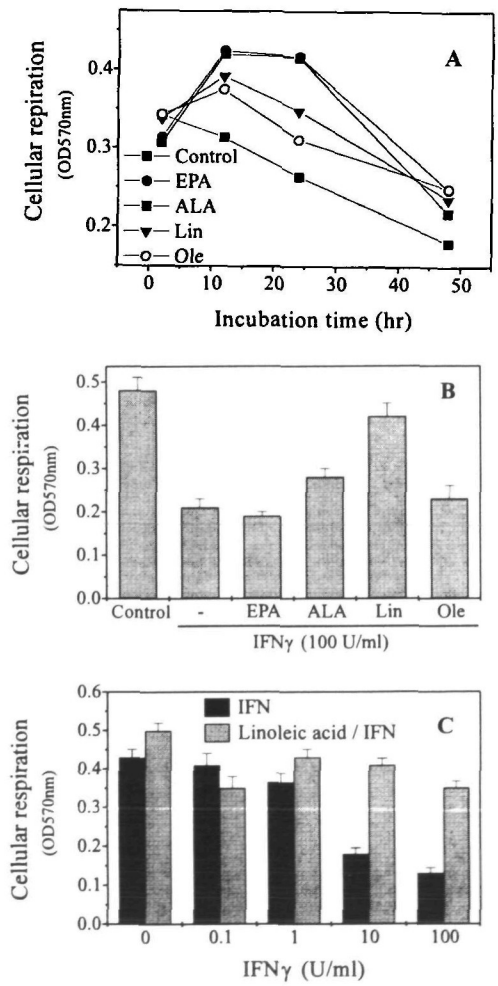

Fig. 2. **Effects of PUFAs on cellular respiration.** A, hepatocytes were exposed to PUFAs (100 μM) at 8 h after seeding, then the incubation was started, and cellular respiration was assayed at the time indicated on the abscissa; B, hepatocytes were exposed to PUFAs (200 μM) and IFN γ (100 U/ml) at 24 h after seeding. Cellular respiration was measured after another 38 h incubation of hepatocytes with PUFAs and IFN γ; C, hepatocytes were exposed to linoleic acid (200 μM) and IFN γ at 20 h after seeding. Cellular respiration was measured after another 38 h incubation of hepatocytes with linoleic acid and IFN γ.

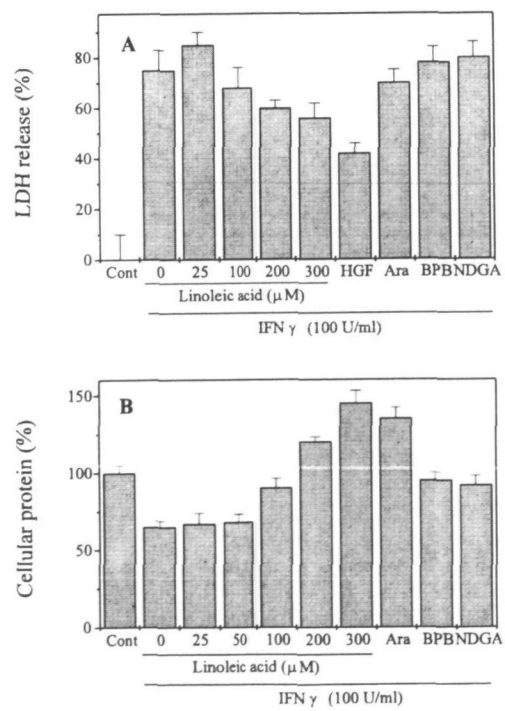

Fig. 3. **Protective effects of linoleic acid on IFN γ-induced cellular injury.** Hepatocytes were exposed to linoleic acid, HGF (100 ng/ml), arachidonic acid (Ara, 200 μM), BPB (4 mM), NDGA (4 mM), and IFN γ (100 U/ml) at 20 h after seeding. LDH released into the culture medium (A) and the cellular protein of hepatocytes (B) was measured after another 38 h incubation of hepatocytes with various factors.

(Fig. 2C). In addition, linoleic acid treatment also reduced IFN γ-induced LDH release (Fig. 3A) and protein synthesis inhibition (Fig. 3B) in hepatocytes. Compared with hepatocyte growth factor (HGF), linoleic acid showed weaker protective effects on IFN γ-induced LDH release (Fig. 3A). In fact, linoleic acid at low concentration showed some synergistic effect on IFN γ-induced LDH release (Fig. 3A). Arachidonic acid, a metabolite of linoleic acid, bromophenacyl bromide (BPB), an inhibitor of phospholipase, and nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase, did not affect the protective action of linoleic acid against IFN γ-induced cellular injury (Fig. 3), implying that arachidonic acid-mediated signal transduction was not involved in linoleic acid inhibition of IFN γ-induced cellular injury in hepatocytes.

Roles of Fetal Bovine Serum and NAD Levels in IFN γ-Induced Cellular Damage—Considerable amounts of fatty acids (~300 μM) exist in the serum. In our previous work, we found that fetal bovine serum (FBS) enhances the inhibitory effects of HGF on IFN γ-induced cellular injury, including apoptosis and cell membrane damage, but FBS itself does not show any protective effect against IFN γ-induced cellular injury (*8*). However, in that experiment, hepatocytes had been cultured in medium containing 10% FBS for 10 h before exposure to IFN γ (*8*). To avoid any effects due to the pre-treatment of hepatocytes with FBS, hepatocytes were starved in serum-free medium for 20 h after seeding, and then incubated with 100 U/ml IFN γ for another 38 h. As shown in Fig. 4, compared with FBS-treated hepatocytes, serum-starved hepatocytes became more sensitive to IFN γ stimulation. FBS itself showed some inhibitory effect on IFN γ-induced cellular injury (Fig. 4).

IFN γ induces apoptosis in hepatocytes (*8*). Increased DNA breaks may activate the poly (adenosine 5′-diphosphoribose) synthetase (PARS), and this rapidly leads to consumption of NAD and energy depletion. This causes cell

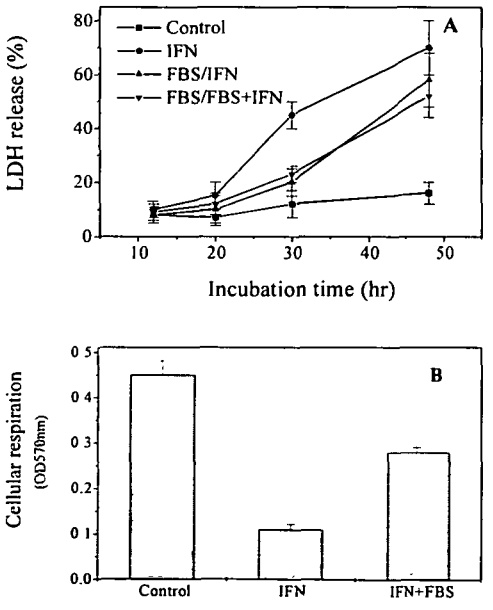

Fig. 4. **Protective effect of FBS on IFN γ-induced cellular injury.** A, hepatocytes were cultured in serum-free medium (●, ■) or medium supplemented with 5% FBS (▲, ▼) for 20 h after seeding. Thereafter, cells were exposed to IFN γ (100 U/ml) in the same medium supplemented with (▼) or without (●, ▲) 5% FBS. Activities of LDH released into the culture medium were determined with the indicated substrate as described under "MATERIALS AND METHODS"; B, cells were cultured in the medium containing 5% FBS for 20 h, and then IFN γ (100 U/ml) was introduced into the culture medium. After another 38 h incubation, cellular respiration of hepatocytes was measured by MTT reduction assay as described under "MATERIALS AND METHODS."

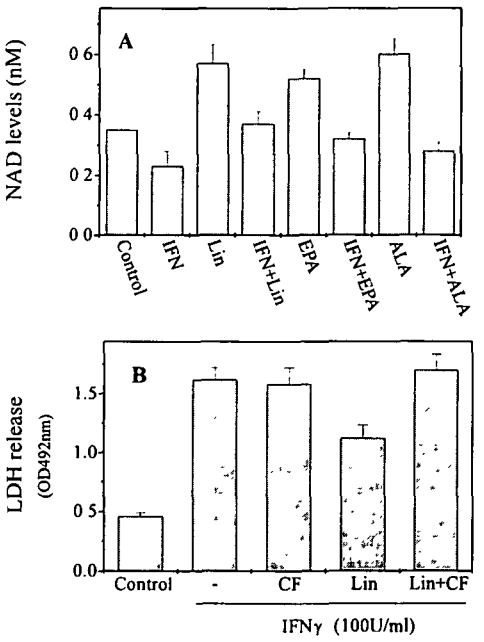

Fig. 5. **Role of peroxisomal oxidation in linoleic acid inhibition of IFN γ-induced cellular injury of hepatocytes.** A: Hepatocytes were maintained with or without PUFAs (300 μM) for 24 h after seeding. After removal of PUFAs, cells were incubated with the medium supplemented with or without 100 U/ml IFN γ for another 24 h. NAD levels was estimated by the method described in "MATERIALS AND METHODS." B: Hepatocytes were maintained with or without linoleic acid (300 μM) for 24 h after seeding. After removal of linoleic acid, cells were incubated with 100 U/ml IFN γ in the presence or absence of 0.3 mM clofibrate for another 38 h. LDH released into the culture medium was measured by the procedures described in "MATERIALS AND METHODS."

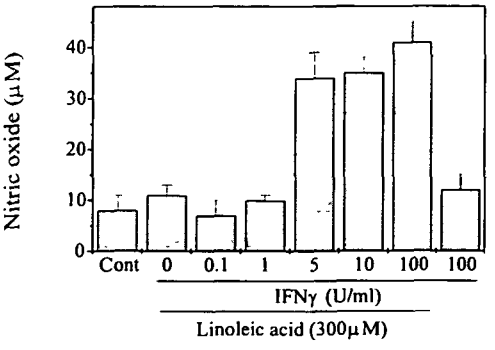

Fig. 6. **Induction of nitric oxide synthesis by linoleic acid and IFN γ.** Hepatocytes were exposed to linoleic acid (300 μM) and IFN γ at 24 h after seeding. Nitric oxide released into the culture medium was estimated by using Griess' reagent as described in "MATERIALS AND METHODS" after another 38 h incubation of hepatocytes with IFN γ and linoleic acid.

death (*21*). On the other hand, maintenance of intracellular NAD level by addition of nicotinamide preserves the growth and functioning of cultured hepatocyte (*22*). Therefore, we further examine the effects of linoleic acid on the NAD levels in hepatocytes. As shown in Fig. 5A, although IFN γ reduced the NAD levels in hepatocytes, linoleic acid increased the NAD levels in both IFN γ-treated and untreated hepatocytes (Fig. 5A). Other PUFAs (EPA and ALA) also showed protective effects on NAD levels of hepatocytes (Fig. 5A), but clofibrate (CF), a peroxisome proliferator, did not inhibit IFN γ-induced cellular injury in hepatocytes (Fig. 5B).

Induction of Hepatocyte Nitric Oxide Synthesis by a Combination of Linoleic Acid and IFN γ—It is known that IFN γ can induce nitric oxide synthase expression in hepatocytes. The maximal induction of nitric oxide synthesis in hepatocytes is afforded by a combination of IFN γ and another nitric oxide synthesis stimulator, such as lipopolysaccharide, TNF α, or IL-1β. Although linoleic acid or IFN γ alone had little effect on hepatocyte nitric oxide synthesis, the combination of linoleic acid and IFN γ strongly induced nitric oxide synthesis in hepatocytes (Fig. 6)

DISCUSSION

Our observations may be summarized as follows. (1) Mitochondrial respiration suppression is an early event in IFN γ-mediated cellular injuries in hepatocytes. Linoleic acid increased mitochondrial respiration and inhibited cellular injury in IFN γ-treated hepatocytes. (2) The combination of linoleic acid and IFN γ induced nitric oxide synthesis in hepatocytes.

Long-chain polyunsaturated fatty acids (PUFAs) are essential components of membrane phospholipids. In recent years, interest has been aroused in the possible use of *n*-3 PUFAs-rich oils for protection against and treatment of various diseases. Polyunsaturated fatty acids modulate membrane properties (*23, 24*) and modify signal transduction across the cell membrane, as indicated by changes in ligand-induced receptor activation and second messenger formation after enrichment with *n*-3 and *n*-6 PUFAs (*25, 26*). The biological effectiveness of PUFA may be partly due to their role as precursors of the eicosanoids. Recently arachidonic acid and its metabolites have been identified as a novel class of intracellular second messengers that modulate expression of growth-related genes and cell growth after mitogenic stimulation in various cell lines (*27, 28*). However, because neither arachidonic acid nor phospholipase and lipoxygenase inhibitors influence the inhibitory effects of linoleic acid (Fig. 3), it is unlikely that arachidonic acid metabolism is involved in the inhibitory actions of linoleic acid on IFN γ-induced cellular injury. It is interesting that FBS itself shows some inhibitory effect on IFN γ-induced cellular injury (Fig. 4). Because there is no difference of cellular damage between FBS/FBS+IFN and FBS/IFN-treated hepatocytes (Fig. 4A), it is likely that FBS inhibition of IFN γ-induced cellular injury may involve changed metabolic or redox states of hepatocytes.

Linoleic acid shows reciprocal actions on cell membrane breakage of hepatocytes: high concentrations of linoleic acid decrease, but low concentrations increase the cell membrane damage of IFN γ-treated hepatocytes (Fig. 3A). Stimulated mitochondrial fatty acid β-oxidation generates a large amount of reactive oxygen species (ROS), which increases cellular injury. However, in addition to mitochondrial β-oxidation, there are other two fatty acid oxidation pathways located in peroxisomes and endoplasmic reticulum of hepatocytes (*29*). Under conditions of impairment or overload of mitochondrial β-oxidation, increased fatty acid oxidation *via* the microsomal and peroxisomal pathways occurs (*30, 31*). Peroxisomes are important for detoxification because catalase is present at a high concentration in peroxisomes and scavenges the reactive and potentially harmful H_2O_2, producing O_2, and H_2O (*32*). On the other hand, endotoxin reduces peroxisomal β-oxidation and catalase activities both *in vivo* and *in vitro* (*33*). However, since a peroxisome proliferator (CF) could not block IFN γ-mediated cellular injury (Fig. 5B), stimulation of peroxisomal β-oxidation by overload of mitochondrial β-oxidation is not the major reason for the inhibitory action of linoleic acid on IFN γ-mediated cellular injury. In addition, it was reported that a stimulator of fatty acid peroxisome β-oxidation can increase NAD synthesis from tryptophan (*34*). Because clofibrate does not protect hepatocytes from IFN γ-mediated cellular damage (Fig. 5B) and other PUFAs show the same protective effect as linoleic acid on the NAD levels of hepatocytes (Fig. 5A), the possibility can be excluded that increased NAD levels due to linoleic acid are responsible for the linoleic acid inhibition of IFN γ-induced cellular damage.

The mechanism by which linoleic acid enhances IFN γ-mediated nitric oxide synthesis in hepatocytes (Fig. 6) is not clear. Duval *et al.* (*35*) and we (*36*) showed that cytokine-mediated hepatocyte nitric oxide synthesis is related to the redox and metabolic states of hepatocytes, and both ROS and reduced glutathione are required. Since a low concentration of linoleic acid does not affect IFN γ-mediated nitric oxide synthesis (data not shown), it is likely that nitric oxide induction by IFN γ and linoleic acid is at least partially due to linoleic acid peroxidation. As in other organs, the biological role of nitric oxide in liver is ambiguous (*37*). However, it is noteworthy that NO may behave as a pro-oxidant or antioxidant in iron-mediated oxidative stress in hepatocytes and play a critical role in protecting the liver from oxidation stress (*38*). Therefore, it is possible that NO acts as antioxidant in linoleic acid and IFN γ-induced oxidative stress and inhibits IFN γ-mediated cellular injury in hepatocytes. It is clear that further research is needed to define the mechanism of linoleic acid and IFN γ-mediated hepatocyte NO synthesis and the role of nitric oxide in IFN γ-induced cellular damage.

Although polyunsaturated fatty acid can perturb a number of cellular functions, such as platelet aggregation, lymphocyte mitogenesis, surface receptor capping, cell-to-substrate adhesion, and secretion, little is known of the pathophysiologic relevance of PUFAs and the cytokines in inflammatory liver diseases. Our present study suggests that PUFAs may play potent roles in liver homeostasis during chronic inflammatory states and sepsis. Further understanding of the interaction between PUFAs and cytokines should be of great important for evaluation and preservation of hepatic function during states of severe illness and sepsis.

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