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Interferon-γ Induces Reactive Oxygen Species and Endoplasmic Reticulum Stress at the Hepatic Apoptosis

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Abstract Interferon-γ (IFN-γ) induces cell-cycle arrest and p53-independent apoptosis in primary cultured hepatocytes. However, the detailed mechanism, including regulating molecules, is still unclear. In this study, we found that IFN-γ induced generation of reactive oxygen species (ROS) in primary hepatocytes and that pyrrolidinedithiocarbamate (PDTC), an anti-oxidant reagent, completely suppressed IFN-γ-induced hepatic apoptosis. PDTC blocked apoptosis downstream from IRF-1 and upstream from caspase activation, suggesting that the generation of ROS occurred between these stages. However, IFN-γ also induced the generation of ROS in IRF-1-deficient hepatocytes, cells insensitive to IFN-γ-induced apoptosis. Moreover, a general cyclooxygenase (COX) inhibitor, indomethacin (but not the cyclooxygenase 2-specific inhibitor, NS-398) also inhibited the apoptosis without blocking the generation of ROS. Both PDTC and indomethacin also blocked IFN-γ-induced release of cytochrome c from mitochondria. These results suggest that ROS are not the only or sufficient mediators of IFN-γ-induced hepatic apoptosis. In contrast, we also found that IFN-γ induced endoplasmic reticulum (ER) stress proteins, CHOP/GADD153 and caspase 12, in wild-type primary hepatocytes, but induced only caspase 12 and not CHOP/GADD153 protein in IRF-1-deficient hepatocytes. These results suggest that IFN-γ induces ER stress in primary hepatocytes. Both the ROS and ER stress induced by IFN-γ may be complementary mediators that induce apoptosis in primary hepatocytes. J. Cell. Biochem. 89: 244–253, 2003.

Key words: IFN-γ; reactive oxygen species; ER stress; apoptosis; hepatocytes

Hepatitis is an inflammatory liver disease with various causes, such as viral infection, bacterial infection, and alcohol or drug injury. Although, there are many unsolved problems concerning the mechanisms involved, it is clear that hepatocytes are the major target cells damaged in hepatitis. However, it is not clear what kinds of molecules and what kinds of regulation are fundamentally involved in hepatic damage. Tumor necrosis factor-α (TNF-α), interleukins-1 and -6 (IL-1, IL-6), and interferon-γ (IFN-γ) are important mediators in the pathogenesis of immune-mediated hepatitis [McClain and Cohen, 1989; Ehlers et al., 1992; Morita et al., 1995; Mizuhara et al., 1996]. We have previously reported that of these cytokines, only IFN-γ directly induces p53 expression, cell-cycle arrest, and p53-independent cell death in primary cultured hepatocytes [Morita et al., 1995; Kano et al., 1997] and that the signaling is mediated by IFN-γ-induced interferon regulatory factor (IRF-1) [Kano et al., 1999]. The critical mediators between IRF-1 and apoptosis remain to be elucidated.

Reactive oxygen species (ROS) are usually generated during the reactions of mitochondrial respiration and are involved in a number of physiological cellular phenomena, including DNA and membrane injury and cellular aging [Nose, 2000]. However, researchers are now focusing on cytokines and growth factors that induce the generation of ROS via activation of oxygenases in the plasma membrane [Bae et al., 1997]. Some recent studies suggested the possibility that cytokine-induced ROS mediate the signaling for apoptosis [Herrera et al., 2001].

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It has been reported that TGF-β, another hepatotoxic cytokine, induces apoptosis in fetal hepatocytes via ROS generation [Gores et al., 1989; Herrera et al., 2001]. In this study, we have examined the possible involvement of ROS in IFN-γ-induced hepatic apoptosis. Although, IFN-γ induced the generation of ROS in primary hepatocytes, it is likely that the generated ROS were an essential but not sufficient mediator for the induction of apoptosis. We also found that IFN-γ induced both caspase 12 and CHOP/GADD153 expression in hepatocytes. The expression of these molecules is induced by ER stress [Zinszner et al., 1998; Nakagawa et al., 2000]. CHOP/GADD153 was induced by IFN-γ in wild-type hepatocytes but not in IRF-1-deficient hepatocytes, whereas caspase 12 was induced in both cell types, suggesting that IFN-γ induced ER stress and that CHOP/GADD153 is another potential mediator for IFN-γ-induced hepatic apoptosis.

MATERIALS AND METHODS

Reagents and Animals

Recombinant mouse IFN-γ was purchased from Genzyme, Inc. (Cambridge, MA), and recombinant human insulin from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The female C57BL/6 mice used in the experiments in this study were purchased from Charles River Japan, Inc. (Kanagawa, Japan). IRF-1-deficient mice were obtained from Jackson Laboratory, ME. All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

Cell Preparation

Parenchymal hepatocytes were prepared as previously described [Morita et al., 1994]. Briefly, a liver was perfused with a 0.0125% collagenase solution. After the liver had been excised, parenchymal hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50*g* for 90 s. The dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia). The viable parenchymal hepatocytes were suspended in Williams' E medium containing antibiotics and then plated at a density of 1×10^4 cells/well in flat-bottomed 96-well plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) pre-coated with collagen. The hepatocytes were incubated at 37°C for 3 h to allow them to adhere to the collagen-coated plates. Before the experiments, the medium was changed to one containing 5% fetal calf serum (FCS), 10 ng/ml epidermal growth factor (EGF), and 10^{-7} M insulin.

Analysis of Hepatocyte Cell Death

For the estimation of cell death, the activity of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis and one of the commonly used hallmarks of cellular cytotoxicity [Decker and Lohmann-Matthes, 1988], was measured in the supernatants of the samples using a CytoTox 96™ Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) [Korzeniewski and Callewaert, 1983], following the manufacturer's instructions. LDH release was expressed as a percentage of maximum release.

Measurement of Caspase Activity

Caspase activity was measured as described previously [Kano et al., 1999]. Briefly, cell lysates were prepared by repeated freezing and thawing of cells in 100 μl extraction buffer containing 50 mM PIPES–NaOH (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Cell lysates were then diluted with a standard buffer (100 mM HEPES–KOH [pH 7.5], 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml ovalbumin) to adjust the protein concentration. The diluted samples were mixed with 1 μM fluorescent substrate (DEVD-AMC) at 37°C for 12 h in a 96-well microplate. The fluorescence of cleaved substrate was determined with a spectrofluorometer (MTP-32, Corona Electronic Co., Ibaragi, Japan) set at an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

RT-PCR Analysis

Cytoplasmic RNA was extracted from stimulated or non-stimulated cells with TRIzol (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD). The primers used for the PCR were: actin, P1-GGCTGCAGCTCGTCGTCGACAACGGC, P2-CAGGTCCAGACGCAGGATGGCATG; IRF-1, P1-AGCTGTGTGCAGATGTTAGCC, P2-CGTGAAGACATGTTGTATGCC; caspase-12, P1-A-

TAACAAAGGCCCATGTGGA, P2-CTTGACTGGGAACTGCATGA; CHOP, P1-CCTAGCTTGGCTGACAGAGG, P2-CTGCTCCTTCTCCTTCATGC. The PCR was carried out over 25 thermal cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

Preparation of Cellular Components

The cytosolic fraction (S-100 fraction), the ER, and nuclear fraction were isolated as described [Herrera et al., 2001]. Briefly, cultured hepatocytes were washed with cold phosphate-buffered saline (PBS) after incubation for the indicated times, and then lysed with cold lysate buffer A (20 mM HEPES [pH 7.5], 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 250 mM suc rose, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 10 μg/ml leupeptin). The ER (plasma membrane), nuclear fraction, and S-100 fraction were isolated by stepwise centrifugation.

Western Blotting

After SDS–PAGE on a 7.5% gel, proteins were electroblotted onto PVDF membranes (Amersham, IL) in cold transfer buffer for 3 h at 60 V. The filters were incubated with anti-MMP-9 antibodies for 1 h at room temperature after blocking with 3% skimmed milk. They were washed with Tris-buffered saline (TBS), and then incubated with the peroxidase-labeled secondary antibody for 1 h. Bands were detected with an enhanced chemiluminescence (ECL) kit (Amersham).

ROS Detection

ROS generated in hepatocytes were detected using 2′,7′-dichloro-dihydrofluorescein diacetate (H_2-DCFDA; Molecular Probes, Inc.) [Herrera et al., 2001]. Cells were washed with Krebs–Ringer solution (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 22 mM $NaHCO_3$) for 20 min at 37°C, then incubated with the reagent (10 μM). These cells were washed again with Krebs–Ringer solution and observed with a confocal microscope (excitation 494 nm/emission 520 nm).

The data are representatives of three or four experiments. Experiments were performed in triplicate, and the error bars in figures mean standard deviation (SD) of a single experiment.

RESULTS

IFN-γ Induces ROS in Primary Cultured Hepatocytes and Anti-Oxidant Reagent Inhibits IFN-γ-Induced Hepatic Apoptosis

We first examined the possibility that IFN-γ induces apoptosis in primary hepatocytes via the generation of ROS. Hepatocytes generate ROS immediately after isolation, probably as the result of stimulation. Therefore, we stimulated cells 12 h after isolation. The early ROS generation was quenched within 12 h (data not shown). IFN-γ strongly induced the generation of ROS in primary hepatocytes and prolonged this generation for up to 48 h. ROS generation was quenched completely by the anti-oxidant reagent pyrrolidinedithiocarbamate (PDTC) (Fig. 1A). Furthermore, PDTC suppressed IFN-γ-induced hepatic apoptosis. PDTC-treated hepatocytes treated with IFN-γ were morphologically intact (Fig. 1B) and the cytotoxicity of IFN-γ was suppressed dose-dependently by PDTC (Fig. 1C).

Next, we investigated the points at which PDTC inhibits the IFN-γ-induced hepatic apoptosis pathways. PDTC did not affect the induction of IRF-1 mRNA, which is rapidly induced by IFN-γ signaling via signal transducers and activators of transcription (STATs) (Fig. 2A). Therefore, PDTC does not block the upstream signaling of IRF-1 induction by IFN-γ. In contrast, PDTC dose-dependently and effectively inhibited the activation of caspase 3 in IFN-γ-stimulated hepatocytes (Fig. 2B). To examine whether PDTC functions as a direct inhibitor of caspase 3, caspase 3-like activity in the cell lysates from IFN-γ-treated hepatocytes was measured in the presence of PDTC. As a result, PDTC did not have any direct effect on caspase 3 activity (Fig. 2C). Therefore, we concluded that the reagent blocked pathways between IRF-1 and caspase 3 activation.

ROS Is Not Sufficient Mediator to Induce Apoptosis in IFN-γ-Stimulated Hepatocytes

The results above suggest that the ROS induced by IFN-γ play a critical role in the apoptosis in hepatocytes. To confirm this possibility, we used IRF-1-deficient hepatocytes, which are resistant to IFN-γ-induced apoptosis (Fig. 3A). Intriguingly, IFN-γ induced ROS generation in IRF-1-deficient hepatocytes (Fig. 3B). This result suggests that ROS are not an essential or sufficient mediator to induce apoptosis in

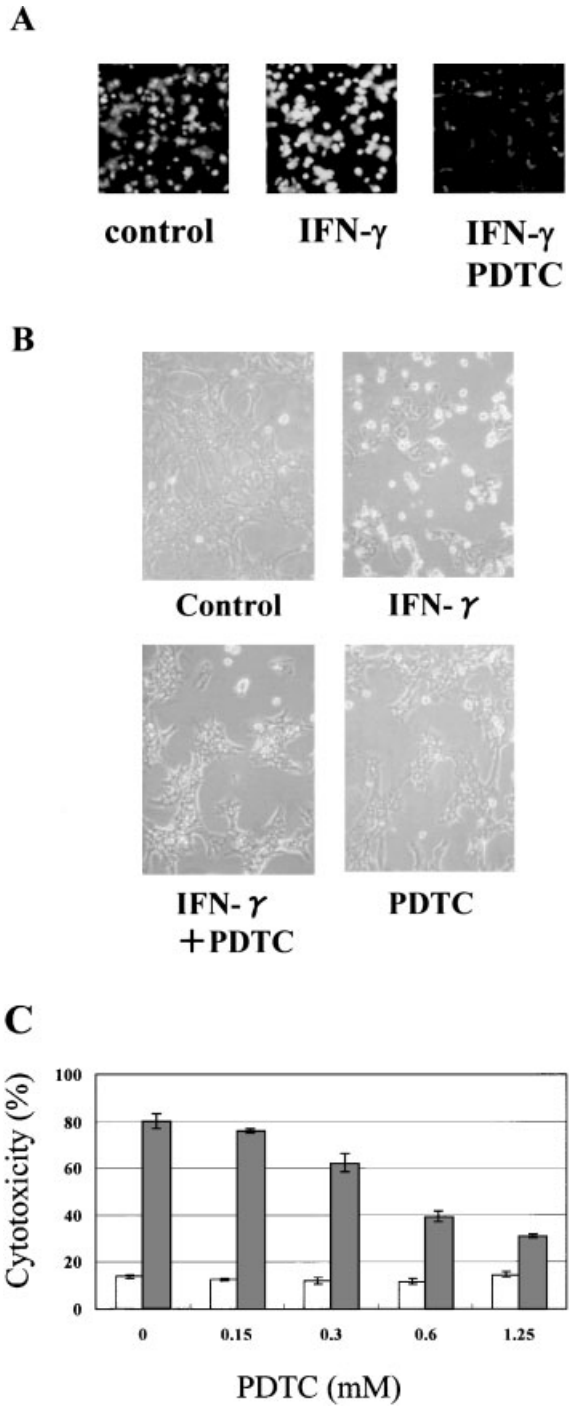

Fig. 1. PDTC inhibited both IFN-γ-induced ROS generation and apoptosis in primary hepatocytes. **A**: Freshly isolated hepatocytes were cultured for 12 h and stimulated with IFN-γ (100 U/ml) for 24 h. ROS generation was observed using H_2-DCFDA under a confocal microscope. Hepatocytes were cultured with IFN-γ (100 U/ml) in the presence or absence of PDTC (1.25 mM in (**B**) and at the indicated concentrations in (**C**)) for 48 h, and the cells were observed under a microscope (B). The LDH in the supernatants was measured as described (C). Gray bars, IFN-γ-treated; blank bars, control.

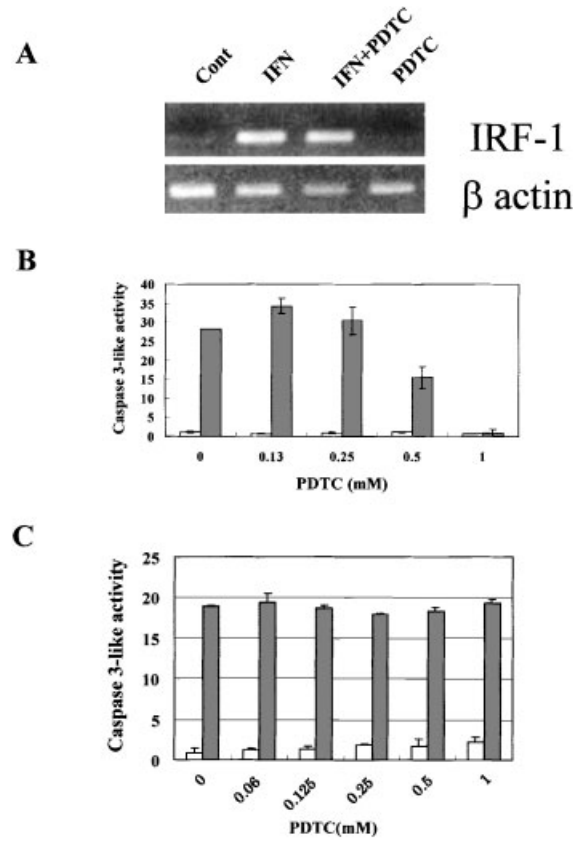

Fig. 2. PDTC suppressed caspase 3-like activation but not IFN-γ signaling. **A**: Hepatocytes were stimulated with IFN-γ (100 U/ml) in the presence or absence of PDTC (1 mM) for 24 h. PCR was performed for IRF-1 and β-actin using the cDNA of these cells. PDTC did not suppress the induction of IRF-1 mRNA by IFN-γ. **B**: Cell lysates were extracted from IFN-γ-treated hepatocytes together with various concentrations of PDTC, and the caspase 3-like activity in the samples was measured with DEVD-AMC substrate. **C**: Cell lysates were prepared from IFN-γ alone-treated hepatocytes, and then caspase 3-like activity in the lysates was measured in the presence of PDTC at various concentrations. PDTC did not directly affect caspase 3-like activity. Gray bars, IFN-γ-treated; blank bars, control.

hepatocytes. However, it became clear that ROS generation did not occur downstream from IRF-1 signaling in the pathways of IFN-γ-stimulated hepatocytes. Furthermore, we found that a cyclooxygenase (COX) inhibitor, indomethacin, also inhibited IFN-γ-induced hepatic apoptosis in a dose-dependent manner (Fig. 4A). COXs are known to induce ROS generation via synthesis of prostaglandin J_2 (PGJ_2) [Gores et al., 1989]. However, the inhibitory effect of indomethacin on IFN-γ-induced hepatic apoptosis is not attributed to the inhibition of COXs for the following reasons (1) indomethacin is known to mainly

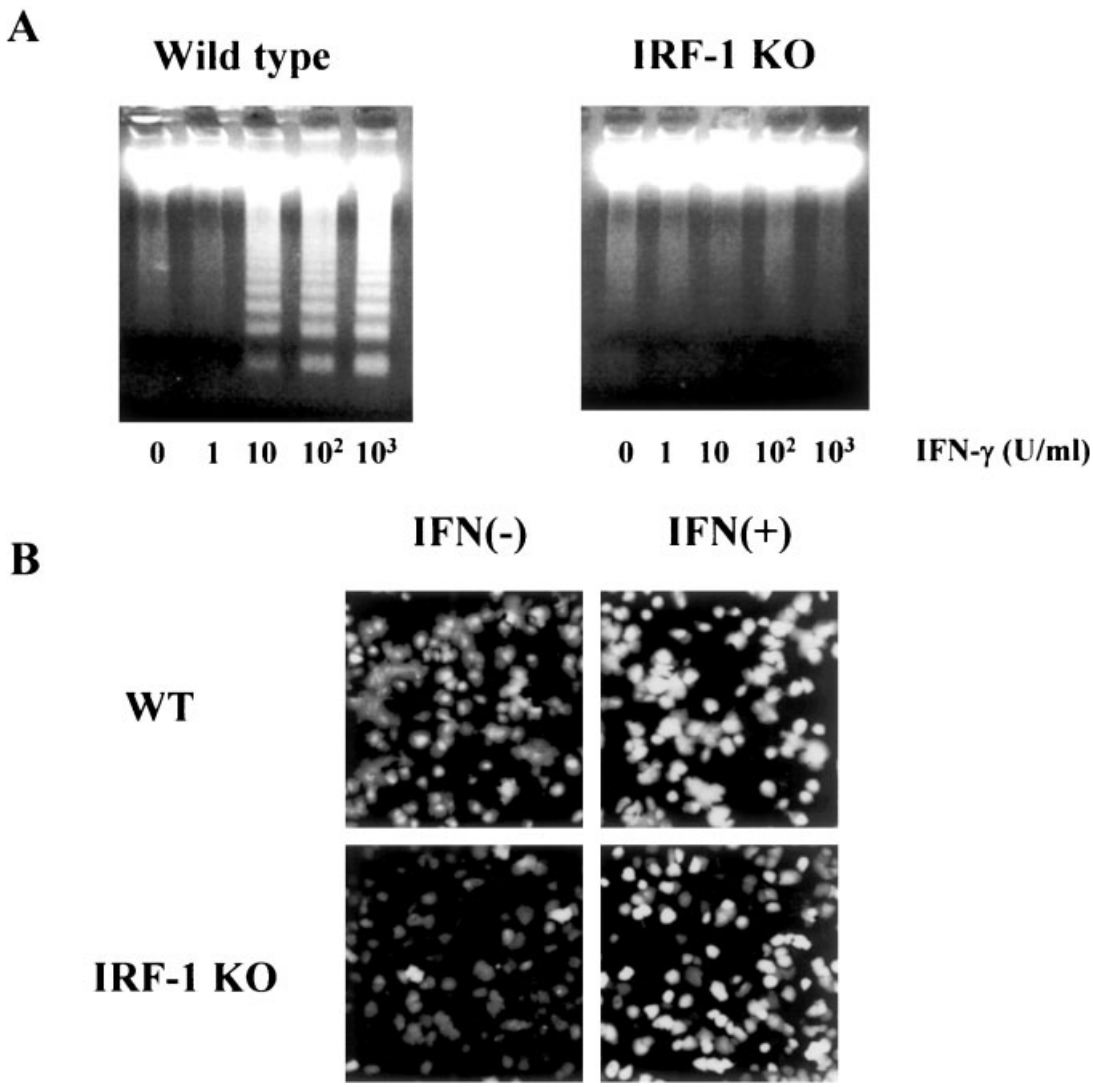

Fig. 3. IFN-γ generated ROS in IFN-γ-resistant IRF-1-deficient hepatocytes. **A**: Primary hepatocytes from wild type or IRF-1-deficient mice were treated with IFN-γ at various concentrations for 48 h. The genomic DNA from these cells was extracted and subjected to electrophoresis on agarose gel. **B**: Hepatocytes from wild type or IRF-1-deficient mice were treated with IFN-γ (100 U/ml) for 24 h, and were subjected to ROS analysis under a confocal microscope.

inhibit the inducible cyclooxygenase, COX2. However, the COX2-specific inhibitor (NS-398) did not inhibit IFN-γ-induced hepatic apoptosis (Fig. 4B); (2) although COX2 induction is regulated by IRF-1 in macrophages [Blanco et al., 2000], IFN-γ did not induce COX2 in primary hepatocytes (Fig. 4C); and (3) finally, indomethacin did not inhibit ROS generation in IFN-γ-stimulated hepatocytes (Fig. 4D). These results strongly support the conclusion that ROS are not the only mediator inducing apoptosis in hepatocytes.

PDTC and Indomethacin Block Apoptosis Signaling by IFN-γ Upstream From Mitochondrial Disruption

Because cytochrome c release into the cytosol from mitochondria is the critical stage in the apoptosis of hepatocytes, we examined the effect of PDTC and indomethacin in the pathway. As shown in Figure 5, both these inhibitors blocked cytochrome c release from mitochondria to the cytosol, indicating that these reagents suppress the signaling upstream of

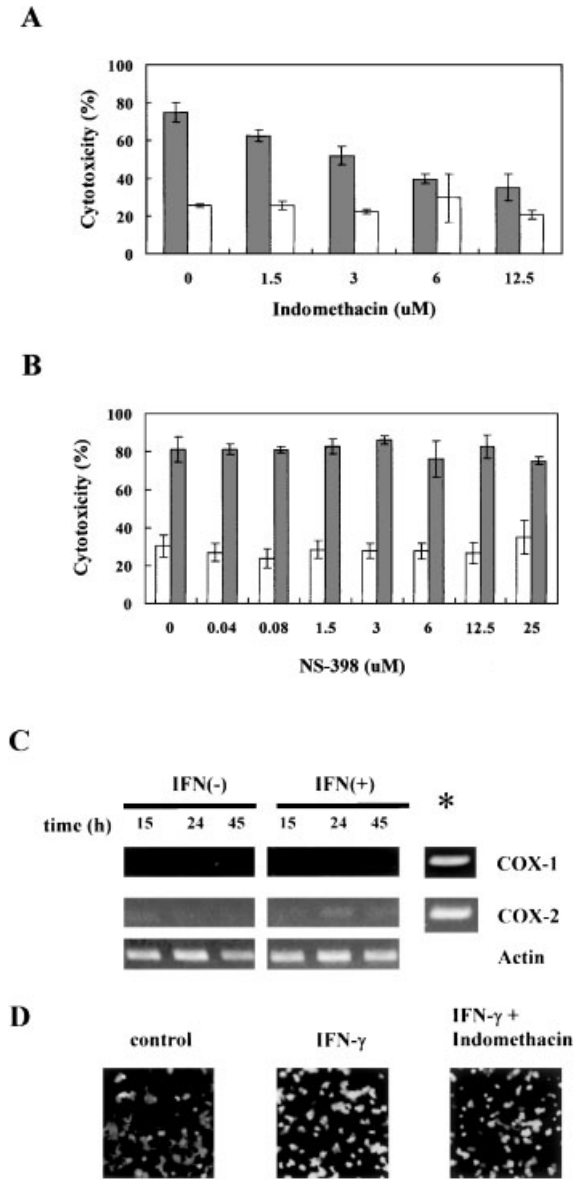

Fig. 4. Cyclooxygenase 2 (COX2) is not involved in IFN-γ-induced ROS generation and apoptosis. Primary hepatocytes were treated with IFN-γ (100 U/m) for 48 h in the presence of indomethacin (**A**) or a selective COX2 inhibitor, NS-398 (**B**), at various concentrations. LDH released into the supernatants was then measured as described. Gray bars, IFN-γ-treated; blank bars, control. **C**: PCR for *COX1* and *COX2* was performed with cDNA from IFN-γ-treated hepatocytes. *Positive control from LPS-treated macrophage cell line, RAW. **D**: Hepatocytes treated with IFN-γ (100 U/ml) for 24 h in the presence or absence of indomethacin (50 μM) were subjected to ROS analysis.

mitochondrial disruption. Although some Bcl-2 family molecules that induce mitochondrial disruption have been reported, IFN-γ did not affect the expression of Bid, Bad, Bax, or Bak at either the mRNA or protein levels (data not shown). Therefore, it is unlikely that these reagents affected these molecules. These results indicate that IFN-γ stimulates the pathway downstream from IRF-1 and upstream from mitochondrial disruption.

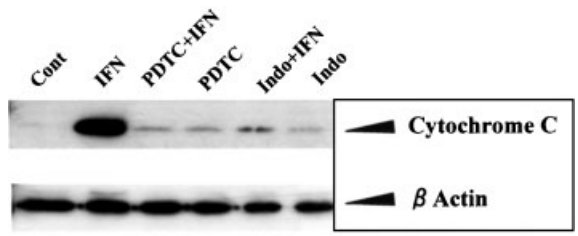

Fig. 5. PDTC/indomethacin blocked apoptotic signaling of IFN-γ upstream of mitochondria. The cytosolic fraction (S-100) from IFN-γ-treated hepatocytes with PDTC (1 mM) or indomethacin (50 μM) was extracted and subjected to Western blotting for cytochrome c and β-actin.

IFN-γ Induces ER Stress in Primary Hepatocytes

During a number of trials to elucidate the pathway, we previously found that IFN-γ induces caspase-12 in primary hepatocytes [Shinzawa et al., 1997]. Recently, this caspase has been reported to be a manifestation of ER stress [Nakagawa et al., 2000]. Therefore, we checked whether IFN-γ induces ER stress in hepatocytes. CHOP/GADD153, as well as caspase-12, has recently been reported to be an ER stress protein [Wang et al., 1996]. We focused on this protein because CHOP/GADD153 induces cell-cycle arrest and apoptosis in cells [Sylvester et al., 1994; Matsumoto et al., 1996; Kaufman, 1999]. As expected, IFN-γ induced caspase-12 mRNA in both wild type and IRF-1-deficient hepatocytes (Fig. 6). In contrast, IFN-γ enhanced CHOP/GADD153 mRNA in wild type but not in IRF-1-deficient hepatocytes (Fig. 6A). This is the first apoptosis-related molecule identified as selectively induced in wild type but not in IRF-1-deficient hepatocytes by IFN-γ. This selectivity was confirmed at the protein level. Figure 6B shows that IFN-γ induced CHOP/GADD153 protein in the nuclear fraction only in IFN-γ-stimulated wild-type hepatocytes. The expression kinetics show two peaks at 15 and 45 h, which correspond to the onset of IFN-γ-induced cell-cycle arrest and apoptosis in hepatocytes [Kano et al., 1997].

DISCUSSION

IFN-γ induces apoptosis in a few types of normal cells. The apoptosis induced in primary

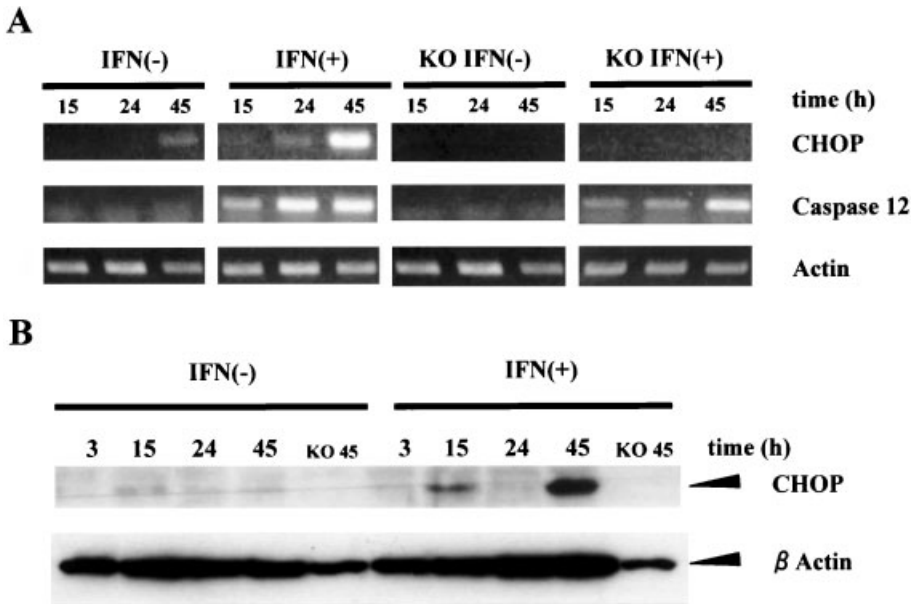

Fig. 6. IFN-γ selectively induced CHOP/GADD153 in wild type but not in IRF-1-deficient hepatocytes. **A**: PCR for CHOP/GADD153, caspase 12, and β-actin was performed with cDNA from IFN-γ-treated (0–45 h) wild type or IRF-1-deficient hepatocytes. **B**: Nuclear fractions were prepared from IFN-γ-treated (0–45 h) wild type or IRF-1-deficient hepatocytes. These samples were subjected to Western blotting for CHOP/GADD153. KO45, IRF-1-deficient hepatocytes incubated for 45 h.

cultured hepatocytes by IFN-γ is thought to be physiologically important because this cytokine is involved in the pathogenesis of hepatitis. However, the mechanism of IFN-γ-induced hepatic apoptosis is still unclear. In this study, we have shown that ROS and ER stress are involved in this apoptosis.

We have shown that IFN-γ induced the generation of ROS in primary hepatocytes. According to the results using IRF-1-deficient hepatocytes, ROS generation and caspase-12 expression are IRF-1-independent. Because PDTC blocked IFN-γ-induced hepatic apoptosis in parallel with the complete suppression of ROS, we suggest that ROS are essential but not sufficient for the apoptosis we observed. ROS may act coordinately with IRF-1-dependent molecules such as CHOP/GADD153 to induce mitochondrial permeabilization. In fact, it has been reported that ROS are involved in TGF-β-induced apoptosis of fetal hepatocytes [Sanchez et al., 1996]. However, because PDTC has a variety of functions [Verhaegh et al., 1997; Iseki et al., 2000; Herrera et al., 2001], we cannot rule out the possibility that PDTC affects other critical pathways in IFN-γ-induced apoptosis. ER also generates ROS as a second messenger, in response to stress. Several oxidases localized to the ER membrane (cyclooxygenases, lipoxygenases, cytochrome P450, etc.) produce ROS as a by-product of their peroxidase activities [Kaufman, 1999]. Therefore, ER-derived ROS may play a critical role in apoptosis and PDTC may suppress the generation of ROS downstream from the ER.

PDTC is known to be an NF-κB inhibitor [Lauzurica et al., 1999; Iseki et al., 2000], whereas indomethacin does not have this inhibitory function [Tegeder et al., 2001]. Moreover, because suppression of NF-κB activation promotes apoptosis in many cell types [Schwenger et al., 1997; Shao et al., 1997; Xu et al., 1998; Millet et al., 2000; Chaisson et al., 2002], it is unlikely that NF-κB mediates the inhibitory effects of these reagents on IFN-γ-induced hepatic apoptosis. In fact, transduction of mutant IκB into primary hepatocytes by an adenoviral vector instead increased their sensitivity to IFN-γ-induced apoptosis (unpublished data).

We first thought that ROS generation by IFN-γ in hepatocytes is mediated by COX2 because (1) COX2 expression is controlled by IRF-1 in macrophages [Blanco et al., 2000]; (2) COX2 induces ROS generation via the synthesis of PGs [Kondo et al., 2001]; and (3) COX-induced

ROS are involved in cellular apoptosis [Kim et al., 1993]. However, we have demonstrated that COX2 is not involved in the IFN-γ-induced generation of ROS. Our results are consistent with a report that COX2 expression is induced in fetal hepatocytes but not in mature hepatocytes [Martin-Sanz et al., 1998]. Moreover, IFN-γ-induced COX2 protects cells from NO-mediated apoptosis in a macrophage cell line [Knethen and Brüne, 1997]. Taking into consideration the results described above and the fact that IRF-1 controls COX2 expression [Blanco et al., 2000], we suggest that mature hepatocytes have cell-type-specific mechanisms to suppress the expression of COX2.

To our knowledge, this is the first report showing that indomethacin, a non-steroidal anti-inflammatory drug (NSAID), suppresses apoptosis in normal cells. Indomethacin inhibited cytochrome c release from mitochondria, whereas it did not suppress CHOP/GADD153 induction (data not shown). Therefore, this reagent blocked the apoptotic pathway between CHOP/GADD153 and mitochondrial permeabilization. Another representative NSAID, aspirin, has a similar effect (data not shown). Some NSAIDs, including salicylate, are known to block NF-κB activation and promote apoptosis in various tumor cell lines [Kopp and Ghosh, 1994; Schwenger et al., 1997, 1998; Wahl et al., 1998; Xu et al., 1998; Jones et al., 1999; Tegeder et al., 2001]. It is important to elucidate the mechanisms for these contradictory effects to explain the physiological variety in NSAID functions.

As a protein-folding compartment, the ER is exquisitely sensitive to alterations in homeostasis. These stresses include calcium depletion from the ER lumen, inhibition of glycosylation, and reduction of disulfide bonds. When protein misfolding occurs and unfolded proteins accumulate and aggregate in the ER, there is a signal that selectively activates the transcription of various genes. This signal is appropriately termed the "unfolded protein response" (UPR). These stress-induced proteins trigger apoptosis at the end stage. During this process, translocation of activated caspase-7 from the cytosol to the ER occurs and translocated caspase-7 induces the expression of caspase-12 [Rao et al., 2001]. Because non-ER stress stimulation, such as Fas or serum depletion, does not induce caspase-12 [Rao et al., 2001], caspase-12 is considered a marker of ER stress. It has been reported that ER stress has specific pathways by which to activate caspase-9 via caspase-12 in a myoblast cell line [Morishima et al., 2002]. However, since IFN-γ induces cytochrome c release from mitochondria, which is blocked by the apoptosis inhibitors PDTC and indomethacin, it is likely that the mitochondrial pathway is critically involved in IFN-γ-induced hepatic apoptosis.

CHOP/GADD153 was originally identified as a transcription factor induced by DNA damage and growth arrest [Fornace et al., 1988]. Subsequent studies have demonstrated a strong correlation between UPR and CHOP/GADD153 expression [Kaufman, 1999]. Thus, CHO/GADD153 is now recognized as an ER stress protein. IFN-γ induced two ER stress proteins involved in apoptosis, indicating that IFN-γ induces ER stress in primary hepatocytes. Most previous evidence supports the idea that CHOP/GADD153 induces cell-cycle arrest and apoptosis [Barone et al., 1994; Zhan et al., 1994; Matsumoto et al., 1996; Zinszner et al., 1998]. CHOP/GADD153 is the first apoptosis-related molecule reported that is not induced in IRF-1-deficient hepatocytes by IFN-γ. Levels of caspase-12 or the Bcl-2 family (Bid, Bad, Bax, and Bak) were not significantly different in wild type and IRF-1-deficient hepatocytes. Recently, Wilkinson and Dickson reported that only stimulation by monolayer cultures induces CHOP/GADD153 expression in rat hepatocytes [Wilkinson and Dickson, 2001]. In fact, IFN-γ enhanced expression at the mRNA level (Fig. 6A) and induced the protein in the nuclear fraction. There may be a variety of factors, such as molecular distribution, species differences, and medium and culture conditions, that regulate the expression of this molecule [Wilkinson and Dickson, 2001]. Furthermore, to establish whether CHOP/GADD153 is essential for IFN-γ-induced apoptosis in hepatocytes requires further experiments using CHOP/GADD153-deficient hepatocytes. It is noteworthy, and first reported here, that CHOP/GADD153 expression is dependent on IRF-1 because the promoter of CHOP/GADD153 shows little homology with the ER stress-response element (ERSE), and its expression is thought to be controlled by Ire [Kaufman, 1999].

In conclusion, IFN-γ induces ER stress and the generation of ROS, followed by apoptosis, in primary hepatocytes. However, there are still missing pathways connecting these stages

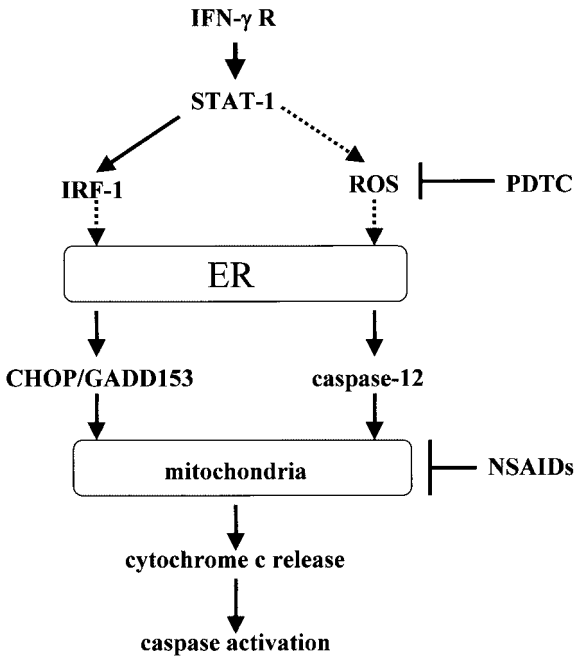

Fig. 7. Putative pathways of IFN-γ-induced hepatic apoptosis. IFN-γ receptor signaling induces two different pathways: IRF-1-dependent and IRF-1-independent pathways. CHOP/GADD153 expression is IRF-1-dependent, whereas ROS generation and caspase-12 induction are IRF-1-independent. PDTC suppresses the generation of ROS, whereas NSAIDs function upstream from the mitochondria. For details, see text. Dotted arrow lines indicate unknown mechanisms.

(see Fig. 7). This study demonstrates that IFN-γ triggers multiple and mutually independent pathways to induce apoptosis in hepatocytes.

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