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Induction of indoleamine 2,3-dioxygenase by interferon-gamma in human lens epithelial cells: Apoptosis through the formation of 3-hydroxykynurenine

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Abstract

Interferon-gamma (IFN-γ) is known to cause apoptosis of lens epithelial cells and cataract formation, but the molecular mechanisms underlying these effects are unknown. IFN-γ induces the expression of indoleamine 2,3-dioxygenase (IDO) and thereby enhances the production of kynurenines from L-tryptophan. The present study was designed to investigate the role of IDO and kynurenines in the IFN-γ-mediated apoptosis of lens epithelial cells and to determine the signaling pathways involved. IFN-γ stimulated the synthesis of IDO and activated the JAK-STAT1 signaling pathway in human lens epithelial cells (HLE-B3) in a dose-dependent manner. Meanwhile, fludarabine, an inhibitor of STAT1 activation, blocked IFN-γ-mediated IDO expression. N-formylkynurenine, kynurenine (Kyn) and 3-hydroxykynurenine (3OHKyn) were detected in cells, with 3OHKyn concentrations being higher than those of the other kynurenines. The intracellular production of kynurenines was completely blocked by 1-methyl-DL-tryptophan (MT), an inhibitor of IDO. Kyn- and 3OHKyn-modified proteins were detected in IFN-γ-treated cells. The induction of IDO by IFN-γ in HLE-B3 cells caused increases in intracellular ROS, cytosolic cytochrome c and caspase-3 activity, along with a decrease in protein-free thiol content. These changes were accompanied by apoptosis. At equimolar concentrations, 3OHKyn caused higher levels of apoptosis than the other kynurenines in HLE-B3 cells. MT and a kynurenine 3-hydroxylase inhibitor (Ro61-8048) effectively inhibited IFN-γ-mediated apoptosis in HLE-B3 cells. Our results show that the induction of IDO by IFN-γ is JAK-STAT1 pathway–dependent and that this induction causes 3OHKyn-mediated apoptosis in HLE-B3 cells. These data suggest that IDO-mediated kynurenine formation could play a role in cataract formation related to chronic inflammation.

Keywords

Interferon-γ; Indoleamine 2,3-dioxygenase; Kynurenine; Apoptosis: Lens epithelial cells

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1. Introduction

Indoleamine 2,3-dioxygenase (IDO) catalyzes the first reaction in the kynurenine pathway that converts L-tryptophan to N-formyl kynurenine (Nfk). This step involves the reduction of the heme iron with either superoxide or cytochrome b5 (King and Thomas, 2007, Maghzal et al., 2008). Nfk thus produced is deformylated to kynurenine (Kyn), which is hydroxylated by kynurenine 3-hydroxylase to 3-hydroxykynurenine (3OHKyn). This molecule is further metabolized by a series of steps to eventually produce nicotinamide adenine dinucleotide (NAD) (King and Thomas, 2007).

In the human lens and cornea, kynurenines are thought to act as UV light filters (they absorb UV light at 300-400 nm) that protect the retina from photodamage (Serbecic and Beutelspacher, 2006, Wood and Truscott, 1993). 3OHKyn is glycosylated to form 3OHKyn glucoside (3OHKynG), which along with a glutathione (GSH) derivative of 3OHKyn, is a major component of the kynurenines in the human lens (Snytnikova et al., 2008). Although they serve a protective role by shielding the retina from UV damage, they can also cause damage to lens proteins if GSH levels are inadequate to prevent the reaction of kynurenines with proteins (Taylor et al., 2002). Kynurenines undergo spontaneous deamination to form α, β-unsaturated ketones that are highly reactive with nucleophilic amino acids such as cysteine, lysine and histidine in lens proteins (Korlimbinis et al., 2007b, Staniszewska and Nagaraj, 2007, Staniszewska and Nagaraj, 2005). Such reactions produce deep yellow to brown proteins with extensively crosslinked structures. Although the chemical nature of these crosslinked structures is largely unknown, a few non-crosslinking adducts are known to be primarily Michael adducts (Hood et al., 1999, Garner et al., 2000, Staniszewska and Nagaraj, 2007, Staniszewska and Nagaraj, 2005).

An age-associated increase in the formation of Michael adducts of Kyn with lysine and histidine residues has been detected in human lenses from patients over 50 years old (Korlimbinis et al., 2007b). GSH in the lens reverses Michael adducts (Parker et al., 2007), which can be UV sensitizers and, upon sensitization, can produce reactive oxygen species (ROS) (Parker et al., 2004). In cataractous human lenses, Michael adducts are present at relatively low concentrations, possibly due to their further degradation (Korlimbinis et al., 2007a).

IDO is induced in cells by IFN-γ (Taylor and Feng, 1991). This induction appears to play a role in the suppression of adaptive T-cell-mediated immunity in inflammation and maternal tolerance of the fetus (King and Thomas, 2007). In the lens, IDO is present in the anterior epithelium and is known to be induced by IFN-γ (Takikawa et al., 1999). In inflammatory conditions such as uveitis, increases in IFN-γ levels are seen in the aqueous humor (Ongkosuwito et al., 1998, Lacomba et al., 2000). Thus, in chronic inflammation, lens IDO may be induced. In fact, chronic uveitis is a risk factor for cataract development in humans (Durrani et al., 2004, Hooper et al., 1990, Skarin et al., 2009, Azar and Martin, 2004).

Previous studies have shown that IFN-γ induces apoptosis in lens epithelial cells (Awasthi and Wagner, 2004, Egwuagu et al., 2006). In addition, transgenic mice overexpressing IFN-γ in the lens have been reported to develop morphological abnormalities and cataracts (Egwuagu et al., 1994). A single nucleotide polymorphism in the IFN-γ receptor (IFNGR1), which increases the transcription of IFNGR1, also promotes cataract formation in humans (Matsuda et al., 2007). These studies suggest that IFN-γ mediates deleterious effects in the lens, but the molecular mechanisms underlying these effects are currently unknown.

The study presented here investigated the effects of IFN-γ on kynurenine-mediated apoptosis in human lens epithelial cells. Our results demonstrate that IFN-γ strongly induces IDO expression through the JAK-STAT1 pathway, which increases the levels of both

intracellular and extracellular kynurenines, and that the production of 3OHKyn induces apoptosis in human lens epithelial cells.

2. Methods

2.1. Cell Culture and treatment

Immortalized human lens epithelial cells (HLE-B3 cells, from Dr. Usha Andley, Washington University, St. Louis, MO) were cultured in Eagle's minimal essential medium (MEM, Mediatech, Inc., VA) with 20% (v/v) fetal bovine serum (FBS), 50 μg/ml gentamicin, and 2 mM L-glutamine. Cells between passages 5 and 8 at ~50% confluence were treated with recombinant human IFN-γ (Invitrogen, CA) (1-100 U/ml) for 2 days (unless otherwise mentioned) without replacing the medium. In some cases, cells were cultured with 20 μM 1-methyl-DL-tryptophan (MT, Sigma) or 50 μM 3,4-dimethoxy-N-[4-(3-nitrophenyl)-2-thiazolyl]benzenesulfonamide (Ro61-8048, Tocris Bioscience, MO) to block the activities of IDO and kynurenine 3-hydroxylase, respectively. To block JAK-STAT1 signaling, cells were cultured with 25 μM fludarabine (Sigma), an inhibitor of cytokine induced STAT1 phosphorylation (Torella et al., 2007). To study the effects of kynurenines on apoptosis, cells were weaned into serum-free medium (SFM) and exposed to Nfk, Kyn and 3OHKyn for 2 days. The kynurenines were dissolved in 25 μl DMSO and then diluted in 10 ml SFM to obtain the desired final concentration. SFM was essential in order to reduce the reaction of the kynurenines with serum proteins and to facilitate their effects on the cells. In these experiments, cells grown in serum-containing medium (SCM) and SFM with DMSO (2.5 μl/ml) served as negative controls. To inhibit caspase activity, cells were treated with a caspase family inhibitor, Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK) (BioVision, CA) at 2 μM for 2 days.

2.2. Protein extraction

Trypsinized cells were suspended in PBS and centrifuged twice to avoid contamination with the culture media. Cells were lysed using the Mammalian Protein Extraction Reagent (MPER, Pierce, Thermo Scientific, IL) and a phosphatase inhibitor cocktail (Sigma, MO) according to the manufacturer's protocol. Soluble proteins were collected from the supernatant. Protein concentrations were quantified using a BCA Protein Assay Kit (Thermo Scientific, IL).

2.3. Cell fractionation

To determine the translocation of cytochrome c, subcellular fractionation was performed using the Qproteome Cell Compartment Kit (Qiagen, Valencia, CA) according to manufacturer's protocol.

2.4. Determination of IDO activity

IDO activity was measured by HPLC as described previously (Mailankot et al., 2009). Briefly, cell lysates were added to a reaction mixture (50 mM sodium phosphate buffer, pH 6.5, 20 mM ascorbic acid sodium salt, 200 μg/mL bovine pancreatic catalase, 10 μM methylene blue and 400 μM L-tryptophan) and incubated at 37°C for 1 hr. After stopping the reaction with 30% trichloroacetic acid, the samples were incubated at 65°C for 15 min to convert Nfk to Kyn. To ensure that the Kyn measured in the samples was due to IDO activity, the samples were incubated with 20 μM MT, a competitive inhibitor of IDO, and in some cases, the samples were incubated to 65°C for 10 min to denature the enzyme.

2.5. Determination of kynurenines and tryptophan

Kynurenines and tryptophan were measured as previously described (Mailankot et al., 2009). To avoid contamination from the cells, the culture medium was centrifuged to pellet the cells, and only the supernatant was lyophilized. Kynurenines in the cells and lyophilized media were extracted with ethanol and analyzed by HPLC using authentic standards.

2.6. SDS-PAGE and western blotting

Cell lysates with a total of 75 μg of protein were resolved using SDS-PAGE (15% gel), electrophoretically transferred to a nitrocellulose membrane and incubated with a monoclonal antibody against one of the following: cytochrome c (Stressgen, MI), IDO, GAPDH (Millipore, MA) (diluted 1:1,000 in 5% NFDM/PBST), phospho-JAK1 (pJAK1, Tyr1022/1023), phospho-JAK2 (pJAK2, Tyr 1007/1008) or phospho-STAT1 (pSTAT1, Tyr701) (Cell Signaling, MA, diluted 1:1,000 in 5% BSA/TBST). Appropriate secondary antibodies conjugated to HRP (Promega, Madison, WI, diluted 1:2,500 in 5% NFDM/PBST) were used, and protein bands were detected using an Enhanced Chemiluminescence Detection Kit (Pierce Chemicals, Rockford, IL).

2.7. Determination of protein modifications

An ELISA was performed as described previously (Mailankot et al., 2009) with modifications. Briefly, microplate wells were coated with cell lysate (1 μg protein) in 0.05 M carbonate buffer (pH 9.5) at a concentration of 1 μg and blocked with 5% NFDM/PBST. The wells were then incubated with a mouse anti-Kyn mAb (Staniszewska and Nagaraj, 2007) (diluted 1:1000 in PBS) and a mouse anti-3OH Kyn mAb (Staniszewska and Nagaraj, 2005) (diluted 1:50 in PBS), followed by incubation with a goat anti-mouse IgG conjugated to HRP (diluted 1: 5,000 in PBS). Enzyme activity was assessed by adding 3,3′,5,5′-tetramethylbenzidine and reading the signal at 450 nm. The results are expressed as relative absorbance. In some cases, the primary antibody incubated with kynurenine modified RNase A was used as a negative control. In others wells, only the secondary antibody was added, or the wells were coated with BSA instead of cell lysate as an additional negative control.

2.8. Immunocytochemistry

Cells were cultured in 24-well plates, and staining was performed as previously described (Mailankot et al., 2008) with slight modifications. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 80% methanol. After blocking with 3% NFDM/1% BSA, the wells were incubated with a monoclonal antibody against either Kyn-modified proteins or 3OHKyn-modified proteins (Staniszewska and Nagaraj, 2005, Staniszewska and Nagaraj, 2007) for 1 hr at RT. A goat anti-mouse IgG conjugated to Oregon green (diluted 1:400 in PBS) was used as the secondary antibody, and the cells were permanently mounted with DAPI/Vectashield. In some cases, the primary antibody was pre-incubated with Kyn- or 3OHKyn-modified RNase A as a negative control, and in others cases, the cells were incubated with only the secondary antibody.

2.9. Detection of Apoptosis

Cells grown in 96-well plates at ~50% confluence were treated as indicated in the figure legends. The numbers of apoptotic and viable cells in randomly selected areas were determined by Hoechst staining. A total of ~500 cells from randomly selected areas in the well were counted for each group. The percentage of apoptotic cells relative to total number of cells was calculated.

2.10. Estimation of protein-free thiols

The concentration of protein-free thiols (GSH and cysteine) in the cells was determined as previously described (Cui and Lou, 1993). A cell lysate sample of 10 μl was mixed with 10 μl of 5,5′-dithiobis(2-nitrobenzoic acid) (2.0 mg per 2.5 ml methanol). The volume was then adjusted to 200 μl with 1 M Tris-HCl buffer (pH 8.2) containing 0.02 M EDTA. The absorbance of the reaction product was measured at 412 nm.

2.11. Determination of caspase-3 activity

Activity was determined in a buffer containing 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose, pH 7.2. DEVD-AFC was used as the substrate for caspase-3. The samples were read at excitation and emission wavelengths of 400 nm and 505 nm, respectively, using a Spectramax Gemini XPS spectrofluorometer (Molecular Devices, CA).

2.12. ROS Imaging

HLE-B3 cells were grown in 96-well plates, and when they reached ~50% confluence, they were treated overnight with IFN-γ with or without MT, RO 61-8048 and 3OHKyn as indicated in the figure legends. ROS were detected using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, CA). In brief, the cells were incubated for 5 min with CM-H_2DCFDA (5 μg/ml) in Hanks balanced salt solution. DCF fluorescence was observed under an Olympus BX60 microscope, and images were acquired with an attached digital camera (Spot RT Slider; Diagnostic instruments, Slider software, version 3.5.5). Identical settings were used for all samples.

3. Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test (Statview 5.0 software; SAS Institute, Inc., Cary, NC). Results with $p<0.05$ were considered significant.

4. Results

We first determined the effects of various concentrations of IFN-γ on the IDO activity of HLE-B3 cells. IDO activity was assessed by measuring the quantity of Kyn, the product of the reaction. From the basal level, an 100- and 200-fold increase in IDO activity was detected with IFN-γ concentrations of 5 and 10 U/ml, respectively. A further 2-fold increase in IFN-γ resulted in only a meager increase (220-fold from the basal level) in enzyme activity. The highest enzyme activity was detected between 40-80 U/ml IFN-γ, at which point the enzyme activity remained almost the same, and further increases in IFN-γ reduced the enzyme activity (Fig. 1A). Heat treatment and incubation with MT completely abolished enzyme activity in IFN-γ-treated (5 U/ml) cells (data not shown), confirming that the measured Kyn level was due to IDO activity. Because of this rapid induction, we evaluated IDO induction at lower concentrations. An IFN-γ concentration of 1-4 U/ml induced IDO activity (Fig. 1B), but this activity could be completely blocked by co-treatment with 20 μM MT, further confirming that the Kyn levels measured in these samples were due to IDO activity. We then determined IDO protein levels by western blotting. As seen in Fig. 1C, IDO protein levels increased with increasing concentrations of IFN-γ, even at IFN-γ concentrations above 80 U/ml where IDO activity declined (Fig. 1A). It is possible that the antibody is not sensitive enough to detect IDO at IFN-γ concentrations <5 U/ml. As the IFN-γ concentration of 1-4 U/ml (0.5-2.0 ng/ml) was within the range of that seen in the aqueous humor with uveitis (Takase et al., 2006), we used this concentration to study apoptosis. In

order to study the signaling pathway involved in IFN-γ-mediated IDO expression, we used HLEB3 cells treated with 5 U/ml IFN-γ.

It has been reported that in bone marrow-derived dendritic cells, IDO induction occurs through the activation of the JAK1/2/STAT1 pathway (Jeong et al., 2009). Thus, it is likely that a similar mechanism occurs in HLE-B3 cells. To test this possibility, we treated HLE-B3 cells with 5 U/ml IFN-γ with or without fludarabine, an inhibitor of cytokine-induced STAT1 activation (Torella et al., 2007, Frank et al., 1999), and determined the phosphorylation profile of JAK1, JAK2 and STAT1. pJAK1 and pJAK2 was detected in all cells, but pSTAT1 was only detected in IFN-γ–treated cells (Fig. 2). Fludarabine at 25 μM completely blocked IFN-γ–induced STAT1 phosphorylation and IDO expression. These results indicate that IFN-γ-mediated IDO expression occurs through the JAK/STAT1 pathway in HLE-B3 cells.

Next, we determined the intracellular concentrations of three kynurenines, Nfk, Kyn and 3OHKyn, in HLE-B3 cells treated with 1-4 U/ml IFN-γ. As can be seen in Fig. 3A, the synthesis of all three kynurenines increased with increasing concentrations of IFN-γ. The levels of 3OHKyn were higher than those of the other two kynurenines at all concentrations of IFN-γ tested. For example, with 4 U/ml IFN-γ, the 3OHKyn level was 6-fold and 2-fold higher than the level of Nfk and Kyn, respectively. None of the kynurenines were detected in control cells (no IFN-γ). This suggests that Nfk is effectively converted to Kyn and then to 3OHKyn in HLE-B3 cells. We also measured kynurenines in the HLE-B3 cell culture medium after treatment with IFN-γ. At 4 U/ml of IFN-γ, we found 3OHKyn to be the dominant kynurenine in the culture medium (Fig. 3B), with a concentration that was nearly 90-fold and 12-fold higher than the concentrations of Nfk and Kyn, respectively (Fig. 3C). In culture medium of control cells (no IFN-γ small quantities of Kyn and 3OHKyn were detected (0.16 and 1.4 nmoles/ml respectively). We also noticed that an IFN-γ dose-dependent decrease in L-tryptophan content of the culture medium (Fig. 3D). Based on these data, 3OHKyn content in the culture medium was 3.5% of tryptophan in the absence of IFN-γ and 25% with 4 units of IFN-γ after 48 hrs of treatment. The tryptophan utilized during this time (with 4 units of IFN-γ was ~15 nmoles/ml and 3OHKyn produced as ~9 nmoles/ml. Together these results suggest that kynurenines formed in the cell are secreted into the local environment (the culture medium in this case).

We have previously shown that extracellular kynurenines can modify intracellular proteins in HLE-B3 cells (Staniszewska and Nagaraj, 2007, Staniszewska and Nagaraj, 2005). Since kynurenines were present both in cells and in the culture medium of IFN-γ-treated HLE-B3 cells, we sought to determine whether IFN-γ treatment results in kynurenine modification of proteins. By ELISA, we detected a significantly higher OD for Kyn (Fig. 4A) and 3OHKyn (Fig. 4B) modifications in IFN-γ treated cells than in control cells (no IFN-γ treatment). Immunocytochemistry also showed Kyn (Fig. 4C) and 3OHKyn (Fig. 4D) modifications in IFN-γtreated cells. Control cells (no IFN-γ treatment) were negative for Kyn and 3OHKyn immunostaining. Figs. 4C and D show Kyn and 3OHKyn protein modifications in cells treated with 1 U/ml IFN-γ. Even after repeated attempts, cells in the other treatment groups were lost during the initial washing, possibly due to the cytotoxicity of IFN-γ. No immunoreactivity was detected when the primary antibody was pre-incubated with Kyn- or 3OHKyn-modified RNase A or in wells incubated with only the secondary antibody. Taken together, these results imply that IFN-γ-mediated induction of IDO results in kynurenine modification of proteins in HLE-B3 cells.

We then determined whether IFN-γ-mediated apoptosis in HLE-B3 cells was mediated by IDO. Cells were treated with 1-4 U/ml of IFN-γ for 2 days and apoptosis was measured by Hoechst staining. Nearly 25% of the cells were apoptotic after treatment with 1 U/ml IFN-γ,

and this increased to 27% in cells treated with 4 U/ml IFN-γ (Fig. 5A). Treatment with 20 μM MT significantly inhibited IFN-γ-induced apoptosis at all concentrations. To determine if apoptosis occurred as a result of caspase activation, we measured the activity of caspase-3 and found that it increased from the basal value of ~1 AFU to ~100 AFU in cells treated with IFN-γ (Fig. 5B). The activity of caspase-3 remained at the basal level in MT-treated cells. A caspase family inhibitor (Z-VAD-FMK) blocked IFN-γ (4 U/ml)-induced apoptosis in HLE-B3 cells (Fig. 5C). Taken together, these results suggest that IFN-γ-induced apoptosis is due to IDO activation and mediated by caspases. We then tested whether apoptosis was occurring through the mitochondrial pathway by measuring cytochrome c in the cytosolic fraction of HLE-B3 cells treated with IFN-γ as cytochrome c is released from mitochondria and enters the cytosol during mitochondria-mediated apoptosis. Cytochrome c was detected in the cytosolic fraction of IFN-γtreated cells, but not in control cells (no IFN-γ treatment) or MT-treated cells (Fig. 5D), suggesting that IFN-γ-induced activation of IDO causes apoptosis through the mitochondrial pathway. To determine whether exogenous kynurenines could cause apoptosis in HLE-B3 cells, we incubated cells with 50 μM each of Nfk, Kyn and 3OHKyn for 2 days. Neither Nfk nor Kyn caused apoptosis as the percentage of apoptosis in these groups was comparable to groups exposed to SFM alone. 3OHKyn caused massive levels of apoptosis, with almost all cells being apoptotic after 2 days (Fig. 6A). These results suggest that among the kynurenines, 3OHKyn is pro-apoptotic, and IFN-γ induced apoptosis could be due to 3OHKyn formation following the activation of IDO. In cells, the conversion of Kyn to 3OHKyn is catalyzed by kynurenine 3-hydroxylase. To verify that the observed IFN-γ-induced apoptosis was due to 3OHKyn formation, we incubated cells with Ro61-8048, a competitive inhibitor of kynurenine 3-hydroxylase. IFN-γ-induced apoptosis was significantly reduced by Ro61-8048 (Fig. 6B). To confirm the effect of Ro61-8048 on kynurenine 3-hydroxylase, we determined the 3OHKyn level in treated cells, and no 3OHKyn was detected (Fig. 6C). We then determined whether exogenous 3OHKyn caused apoptosis in HLE-B3 cells and whether or not it was dependent on the concentration of 3OHKyn. The percentage of apoptotic cells increased by 2.5 fold with 20 μM 3OHKyn and further increased by 11, 19 and 30 fold with 30, 40 and 50 μM 3OHKyn, respectively. Treatment with Z-VAD-FMK blocked 3OHKyn-mediated apoptosis (Fig. 6D). Neither MT (20 μM) nor Ro61-8048 (50 μM) inhibited apoptosis caused by exogenous 3OHKyn (data not shown). We also detected cytochrome c in the cytosolic fraction of cells treated with 3OHKyn (Fig. 6E). These data confirm that increased formation of 3OHKyn in IFN-γ treated HLE-B3 cells leads to mitochondria-mediated apoptosis.

Deaminated 3OHKyn reacts spontaneously with GSH, and this reaction may prevent the reaction of 3OHKyn with proteins (Taylor et al., 2002). There was roughly a 9% reduction in protein-free thiols (-SH) in cells treated with 1 U/ml IFN-γ, and this decreased further by ~21% in cells treated with 4 U/ml IFN-γ (Fig. 7A). In cells treated with MT and Ro61-8048, the level of protein-free thiols was comparable to that of control cells (no IFN-γ treatment). We detected high ROS levels in cells treated with IFN-γ, but not in the presence of either MT or Ro61-8048 (Fig. 7B). High ROS levels were also seen in cells treated with 25 and 50 μM 3OHKyn (Fig. 7C). Furthermore, exogenous 3OHKyn (25 and 50 μM) caused a reduction in the protein-free thiol content (Fig. 7D). These data suggest that IDO activation leads to the depletion of protein-free thiols and the generation of ROS in HLE-B3 cells and that such changes are due to the formation of 3OHKyn.

5. Discussion

The objective of the present study was to determine whether the deleterious effects of IFN-γ in the lens are due to IDO-mediated kynurenine formation. The major findings were the following: 1) in human lens epithelial cells, IDO is inducible by IFN-γin a dose-dependent

manner; 2) IFN-γ-mediated IDO induction is dependent on JAK-STAT1 signaling; 3) the induction of IDO by IFN-γ results in the production of kynurenines and protein modification; 4) kynurenines formed inside cells are secreted into the culture medium; 5) the formation of 3OHKyn causes H_2O_2 generation in cells and 6) IFN-γ-induced apoptosis is mediated by 3OHKyn.

IFN-γ has been shown to induce IDO in several cell types (King and Thomas, 2007) and is produced by inflammatory cells in response to immune activation (Billiau and Matthys, 2009). Such activation occurs in infectious, autoimmune and malignant diseases (Wirleitner et al., 2003). In the lens, however, such induction is not well-documented, although one previous study showed IDO upregulation by IFN-γ in lens epithelial cells (Takikawa et al., 1999). Inflammatory conditions in the eye such as chronic uveitis are accompanied by high levels of IFN-γ in the aqueous humor (Sijssens et al., 2007, Takase et al., 2006). These conditions could produce IDO-mediated negative effects on the lens. In fact, chronic uveitis is considered to be a risk factor for cataract formation (Paroli et al., 2009, Lim et al., 2009). It could very well be that under these conditions, IFN-γ-mediated IDO activation and the formation of kynurenines play a role in cataract formation. Supporting this notion are our previous observations that IDO overexpression in the mouse lens causes high levels of kynurenines, which then lead to extensive protein modification by kynurenine and cell cycle arrest in lens epithelial cells (Mailankot et al., 2008), apoptosis in undifferentiated fiber cells and cataract formation (Mailankot et al., 2009). Furthermore, other studies have shown that IFN-γ causes apoptosis in cultured lens epithelial cells and in transgenic animals overexpressing IFN-γ in the lens (Awasthi and Wagner, 2004, Egwuagu et al., 1994). Taken together, these finding suggest that the induction of IDO by IFN-γ in lens epithelial cells causes kynurenine-mediated damage, possibly leading to cataract formation in chronic inflammatory uveitis.

The interaction of IFN-γ with its cell surface receptor leads to the activation of JAK1 and JAK2 (phosphorylation), which in turn phosphorylate and activate the transcription factor STAT1. Phosphorylated STAT1 forms a homodimer and translocates to the nucleus where it binds to GAS (gamma interferon activation site) to initiate the transcription of target genes (Decker et al., 1997, Kanno et al., 2005). We observed activation of JAK1, JAK2 and STAT1 in IFN-γ treated cells. Fludarabine, an inhibitor of cytokine-induced STAT1 phosphorylation, blocked the IDO expression induced by IFN-γ. Thus, it is likely that IFN-γ-induced IDO expression occurs via a GAS element.

A notable finding in our study was that IFN-γ enhanced the production of 3OHKyn, which then induced the apoptosis of HLE-B3 cells. This result is supported by our finding that a specific inhibitor of kynurenine 3-hydroxylase prevented IFN-γ-induced apoptosis. Apoptosis did not result from tryptophan depletion (due to the increased IDO activity) as the tryptophan content in the medium was only slightly reduced in cells treated with IFN-γ. This reduction is similar to our findings in transgenic mouse lens epithelial cells overexpressing IDO that accumulated Kyn without a drastic reduction in tryptophan content in the medium (Mailankot et al., 2008). In addition, the apoptosis induced by IFN-γ was blocked by the inhibition of the kynurenine 3-hydroxylase, ruling out tryptophan depletion as a cause of apoptosis.

In this study, we observed the release of cytochrome c into the cytosol from mitochondria in cells treated with IFN-γ. These result demonstrate that IFN-γ-induced apoptosis occurs via the intrinsic pathway. 3OHKyn autoxidizes easily and forms H_2O_2 (Goldstein et al., 2000, Korlimbinis et al., 2006). H_2O_2 can then form highly reactive hydroxyl radicals through the Fenton reaction, and these can cause cellular damage and induce apoptosis. We detected ROS in cells treated with IFN-γ, but treatment with MT and Ro61-8048 blocked ROS

generation. In neuronal cells, 3OHKyn-mediated apoptosis is attenuated by catalase (Okuda et al., 1996), suggesting that 3OHKyn-mediated toxicity is primarily mediated by H_2O_2. The loss of protein-free thiols in cells treated with IFN-γ could further increase oxidative stress. Moreover, treating cells with 3OHKyn mirrored the IFN-γ-mediated loss of protein-free thiols, generation of ROS and cytosolic release of cytochrome c. The loss of protein-free thiols could also promote protein modification by kynurenines (Parker et al., 2007), and proteins modified by 3OHKyn (Stutchbury and Truscott, 1993, Staniszewska and Nagaraj, 2005, Aquilina et al., 2000) may cause apoptosis by yet unknown mechanisms.

In summary, our study demonstrates that IFN-γ–mediated toxicity is due to the upregulation of IDO and the subsequent formation of 3OHKyn, which is accompanied by an increase in oxidative stress (Fig. 8) in lens epithelial cells. This finding could have implications for inflammation-associated cataracts in humans and pathologies in other tissues as well, especially those in which the induction of IDO and inflammation coexist, including kidney disease (Schefold et al., 2009) and Alzheimer's disease (Takikawa, 2005,Yamada et al., 2009).

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Abbreviations

IDO	indoleamine 2,3-dioxygenase
MT	1-methyl-DL-tryptophan
IFN-γ	interferon-gamma
Nfk	*N*-formylkynurenine
Kyn	kynurenine
3OHKyn	3-hydroxykynurenine
3OHKynG	3OHKyn glucoside
HLE	human lens epithelial cells
JAK	janus kinase
STAT	signal transducer and activator of transcription
Z-VAD-FMK	Z-Val-Ala-Asp (OMe)-fluoromethylketone
ROS	reactive oxygen species
CM-H_2DCFDA	5-(and-6)-chloromethyl-2′, 7′-dichlorodihydro-fluorescein diacetate

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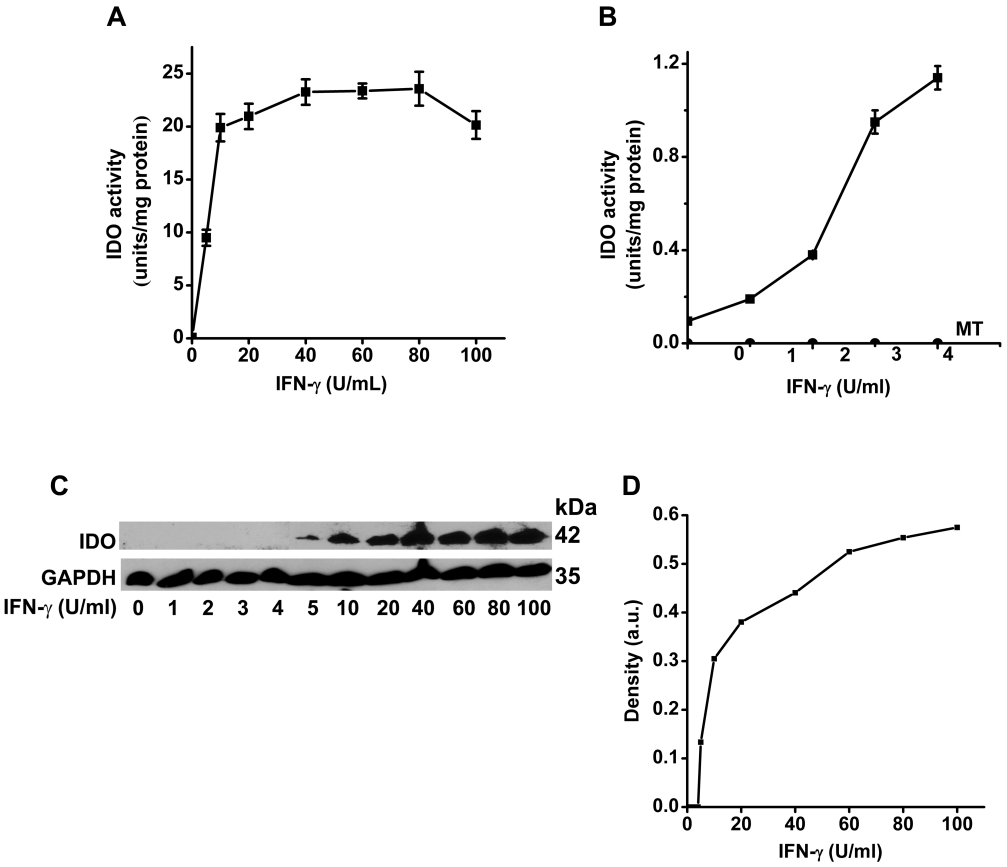

Fig. 1. IFN-γ induces IDO activity in a dose-dependent manner in HLE-B3 cells

Cells were incubated with 5-100 U/ml IFN-γ for 2 days, and IDO activity was measured **(A)**. Since maximum induction was achieved rapidly, the dose was reduced to 1-4 U/ml **(B)**. MT at 20 μM completely inhibited IDO activity in cells treated with 1-4 U/ml IFN-γ. The results are expressed as the mean ± SD from three independent experiments. IFN-γ enhanced the synthesis of IDO in a dose-dependent manner as seen by western blot analysis **(C)** in cells treated with various concentrations of IFN-γ. The blot was initially probed for IDO, then subsequently stripped and reprobed for GAPDH (loading control). Western blots are representative of two independent experiments. The band intensities were quantified by scanning densitometry and expressed relative to GAPDH, the loading control **(D).** 1 unit of IDO activity is equal to 1 nmole of Kyn produced/mg protein/hr.

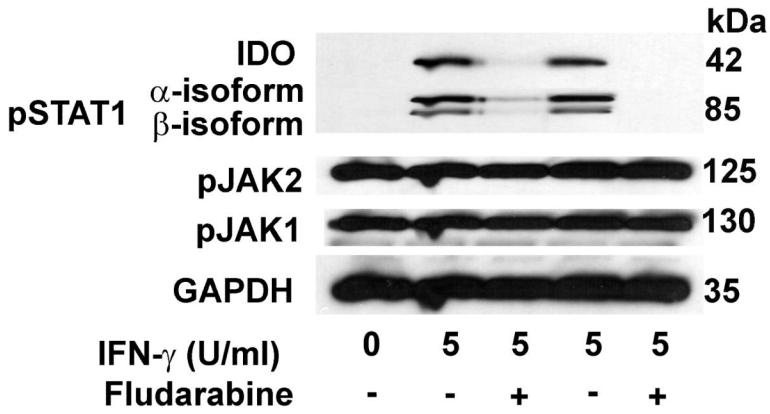

Fig. 2. Activation of JAK/STAT signaling by IFN-γ in HLE-B3 cells

HLE-B3 cells were treated with 5 U/ml IFN-γ for 2 days with or without fludarabine, a specific inhibitor of STAT1 phosphorylation. Western blotting was performed using monoclonal antibodies against pJAK1, pJAK2, pSTAT1 and IDO. GAPDH was used as a loading control. The western blots shown are representative of two independent experiments.

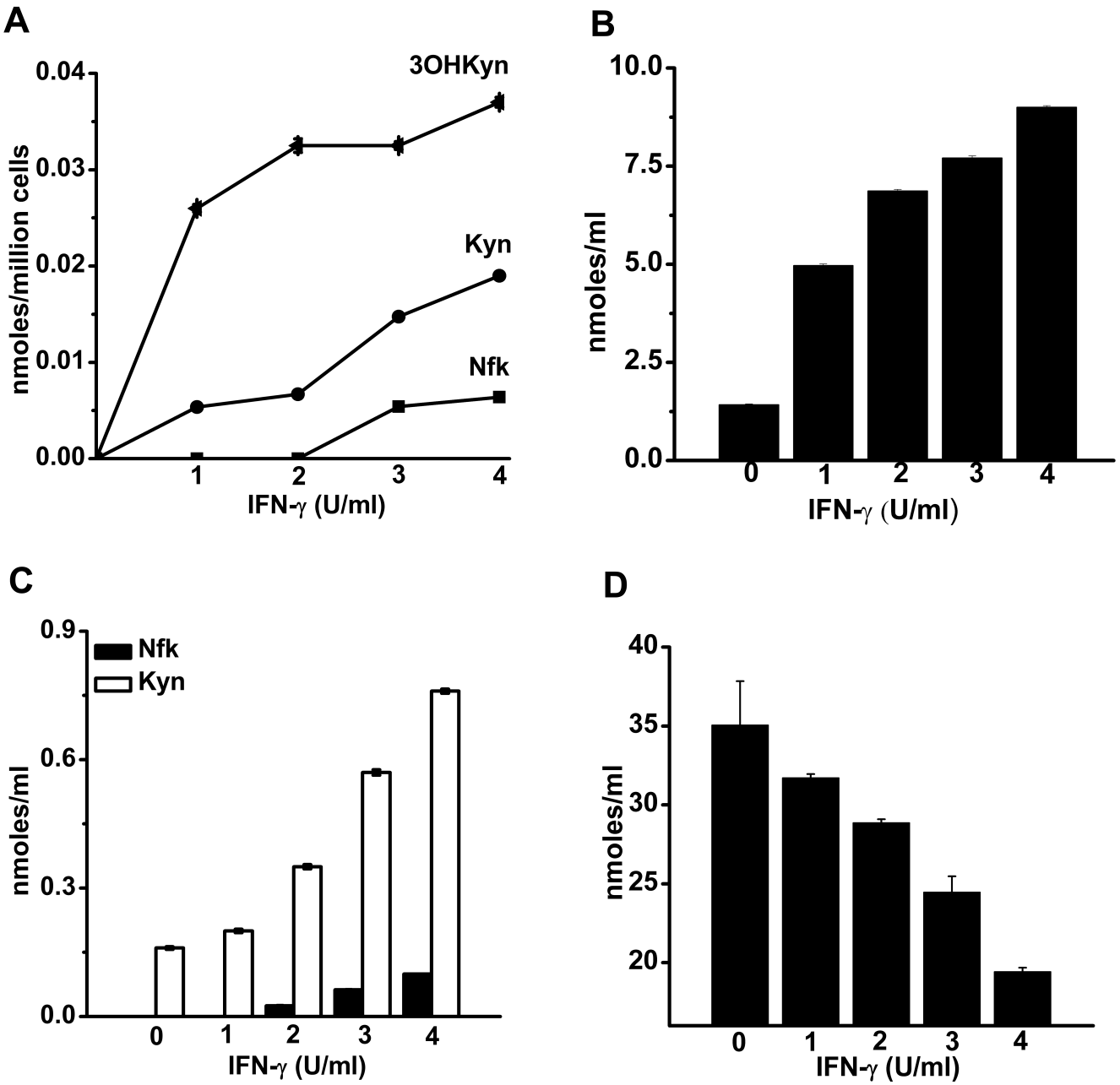

Fig. 3. Formation of kynurenines in IFN-γ treated HLE-B3 cells

Cells were treated with the indicated concentrations of IFN-γ for 2 days. Kynurenine levels in the cells **(A)** and culture medium **(B** and **C)** and tryptophan in the culture medium **(D)** were determined by HPLC**.** 3OHKyn content in the culture medium is shown in **B** and Nfk and Kyn contents are shown in **C**. Data are mean ± SD of three independent measurements.

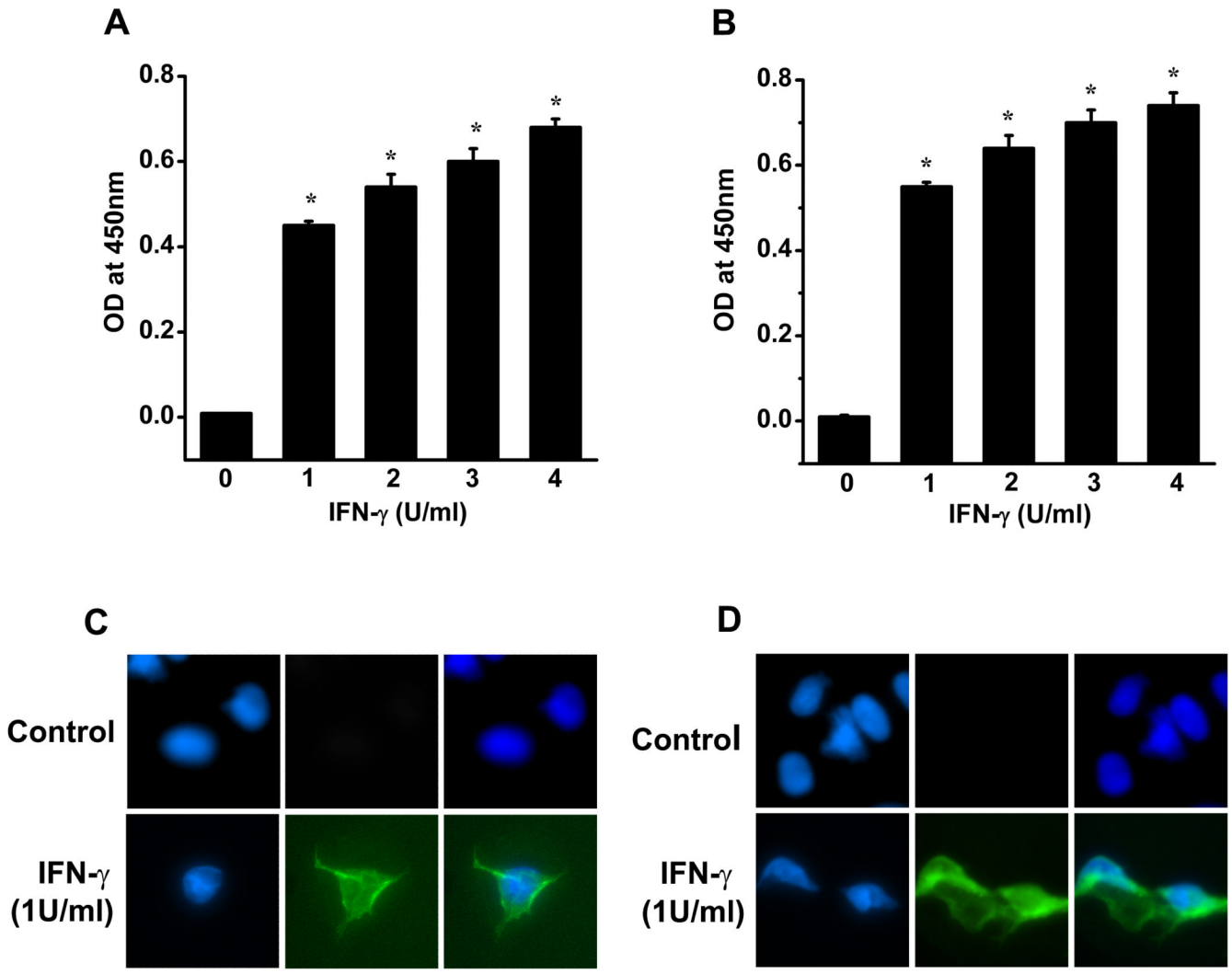

Fig. 4. IFN-γ induces protein modification by kynurenines in HLE-B3 cells

Cells were treated with the indicated concentrations of IFN-γ for 2 days. Modifications were detected by ELISA using specific monoclonal antibodies. The induction of IDO by IFN-γ resulted in both Kyn **(A)** and 3OHKyn **(B)** modifications. The results are the mean ± SD of three independent measurements. Asterisks indicate $p<0.001$ compared with control. Immunocytochemistry was performed to localize Kyn **(C)** and 3OHKyn **(D)** modifications using mouse anti-Kyn and 3OHKyn monoclonal primary antibodies and an Oregon green-conjugated goat anti-mouse IgG secondary antibody. Cells were counterstained with a nuclear stain, DAPI (blue) (From left; DAPI, Kyn or 3OHKyn modifications and merge). Images are representative of three independent experiments.

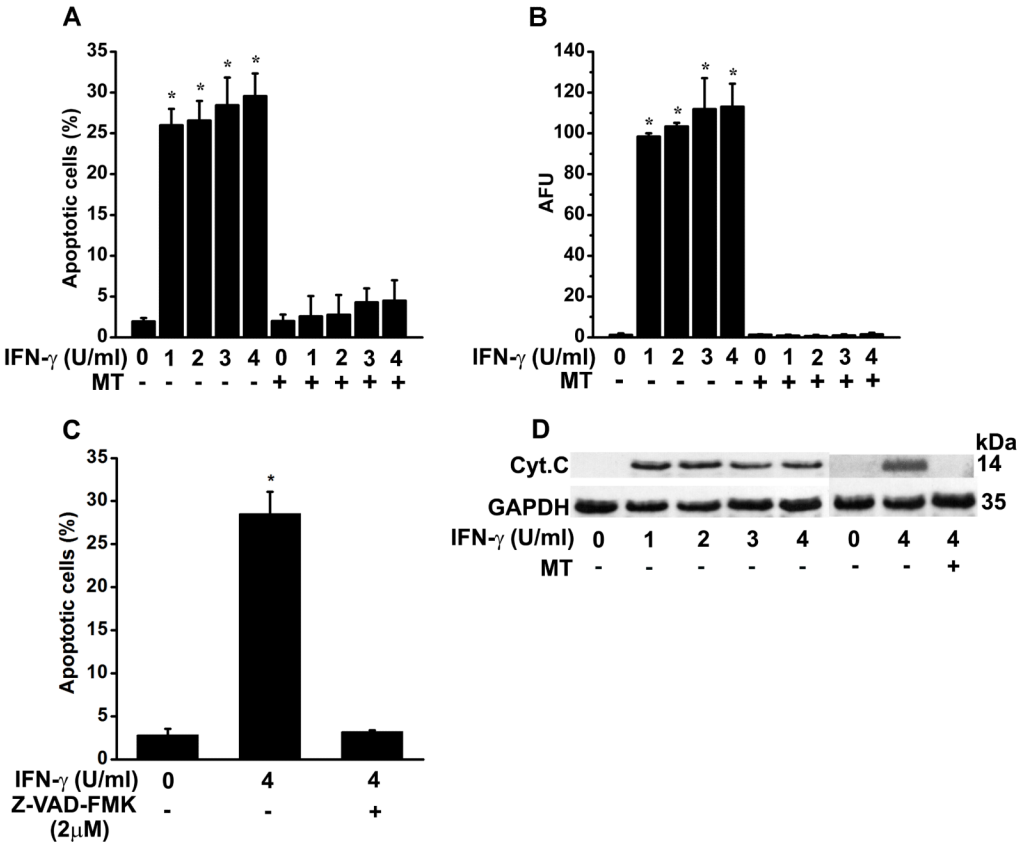

Fig. 5. IFN-γ induces apoptosis in HLE-B3 cells

Cells were treated with the indicated concentration of IFN-γ for 2 days, and apoptosis was measured by Hoechst staining **(A)**. The induction of apoptosis was accompanied by elevated caspase-3 activity **(B)**. The results are the mean ± SD of three independent measurements. MT at 20 μM completely inhibited IFN-γ-induced apoptosis and the elevation of caspase-3 activity. A caspase family inhibitor, Z-VAD-FMK inhibited IFN-γ (4 U/ml)-induced apoptosis **(C)**. Apoptosis occurred via the intrinsic pathway as shown by the release of cytochrome c into the cytosol, which was blocked by treatment with MT (**D**). Cytochrome c was detected in cytosolic protein fractions using a monoclonal antibody. GAPDH was used as a loading control. The western blot shown is representative of two independent experiments. Asterisks indicate $p<0.0001$ compared with control. AFU=Arbitrary fluorescence units.

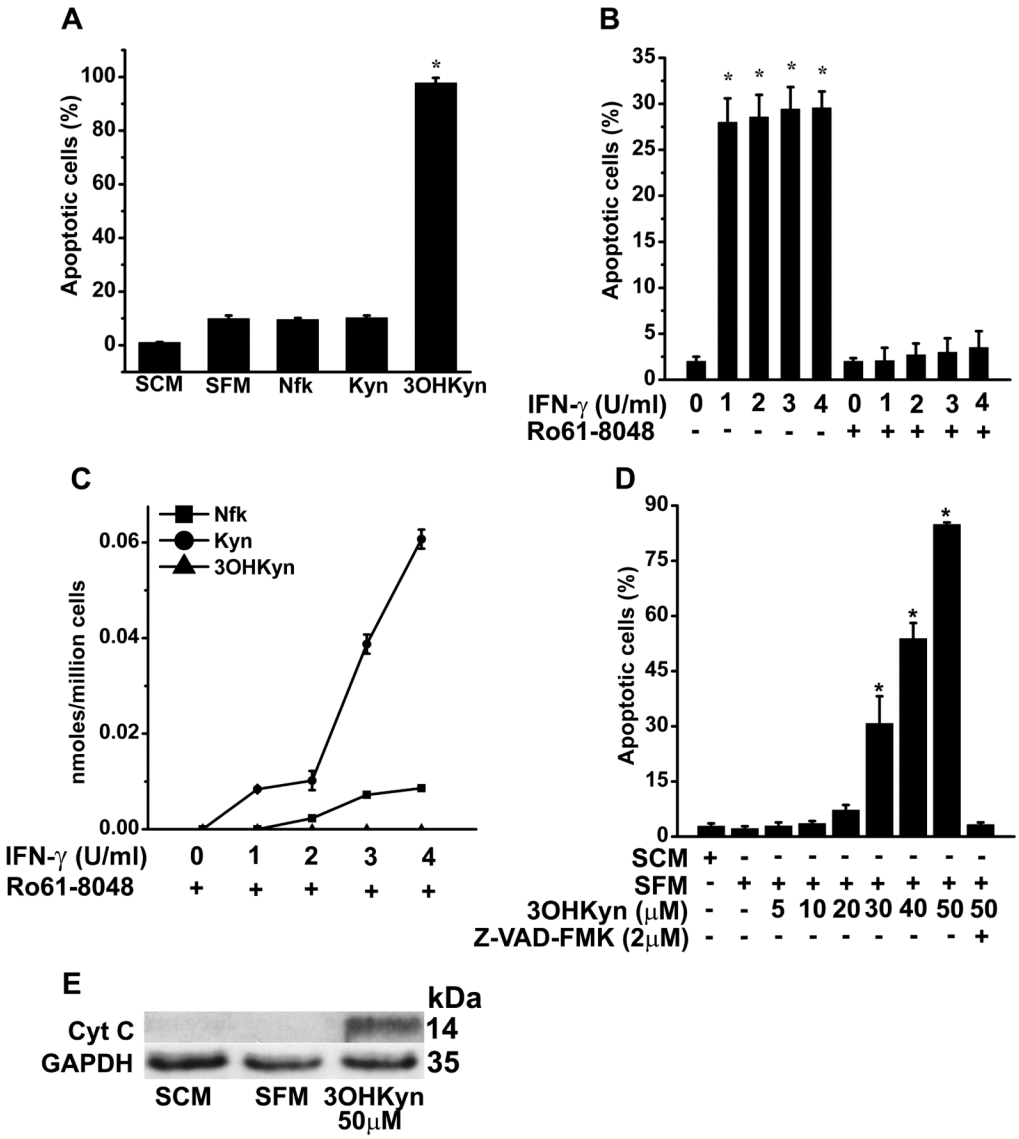

Fig. 6. 3OHKyn is the strongest apoptosis-inducing kynurenine

HLE-B3 cells were treated with 50 μM each of Nfk, Kyn and 3OHKyn for 2 days in serum free medium (SFM), and apoptosis was analyzed by Hoechst staining **(A)**. Cells grown in serum-containing medium (SCM) and SFM serve as controls. 3OHKyn treatment resulted in more apoptotic cells than treatment with the other kynurenines. IFN-γ-induced apoptosis was completely blocked by treatment with the kynurenine 3-hydroxylase inhibitor Ro61-8048 (**B**), and no 3OHKyn was detected in Ro61-8048-treated cells **(C)**. Exogenous 3OHKyn dose-dependently induced apoptosis, which was blocked by the caspase family inhibitor, Z-VAD-FMK (**D**). Exogenous 3OHKyn induced cytochrome c release in to the cytosol **(E)**. Each bar represents the mean ± SD of three independent experiments. The western blot shown is representative of two independent experiments. Asterisks indicate $p<0.0001$ compared with control.

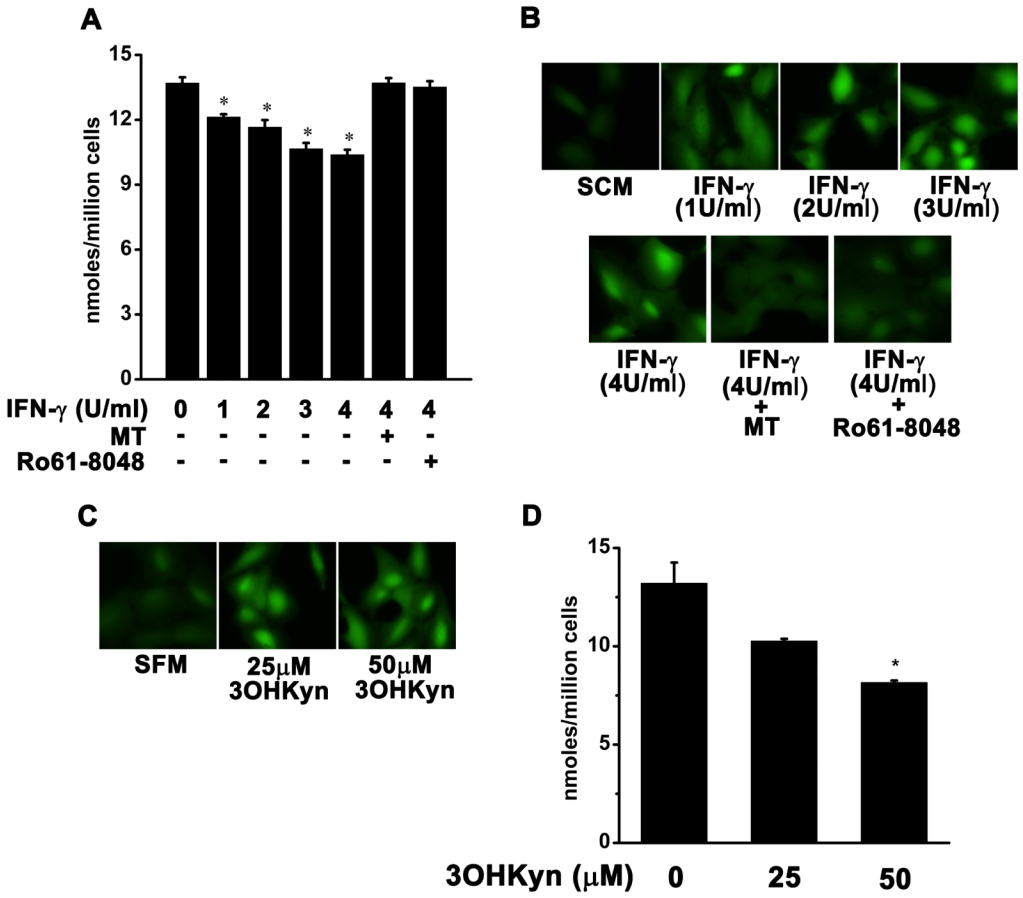

Fig. 7. 3OHKyn formation reduces protein-free thiols (-SH) and increases ROS content in HLE-B3 cells

HLE-B3 cells were treated with the indicated concentrations of IFN-γ +/− MT or Ro61-8048. Protein-free -SH **(A and D)** were evaluated using 5,5′-dithiobis(2-nitrobenzoic acid), and ROS **(B and C)** were detected using CM-H_2DCFDA. MT and Ro61-8048 completely blocked IFN-γ-induced -SH depletion **(A)** and ROS generation **(B)**. Exogenous 3OHKyn induced -SH depletion **(D)** and ROS generation **(C)**. Each bar represents the mean ± SD of three independent experiments. The images shown are representative of three independent experiments. Asterisks indicate $p<0.05$ compared with control.

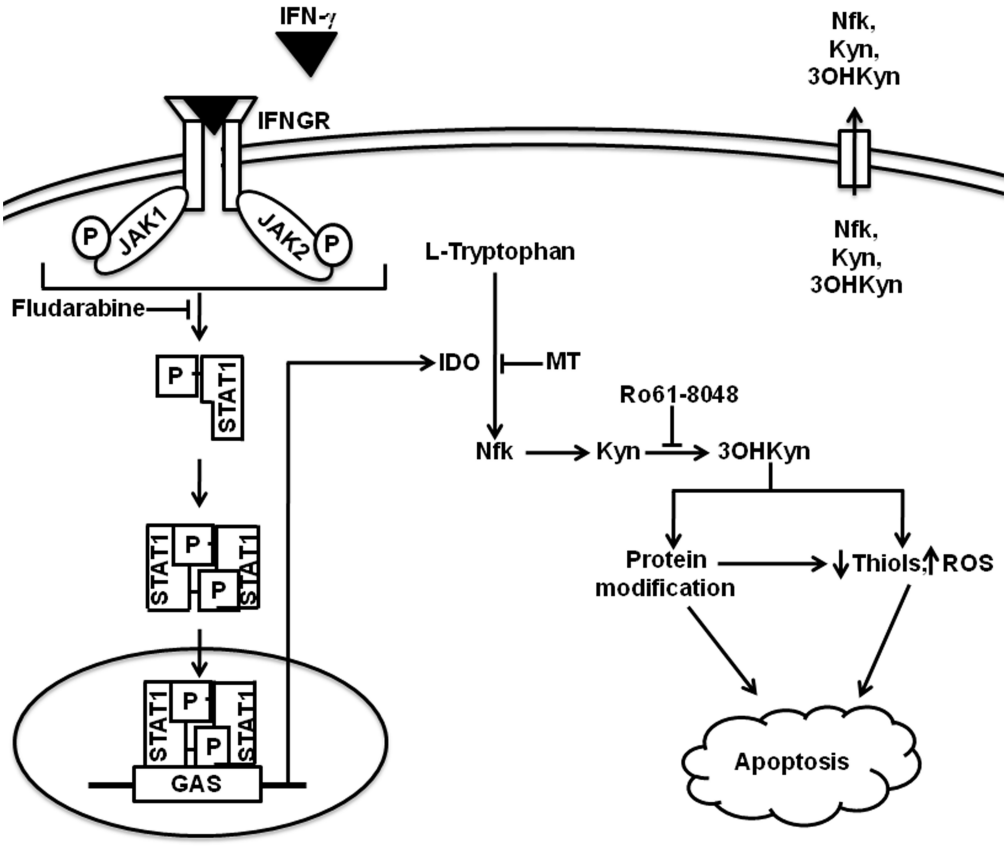

Fig. 8. A conceptual view of the mechanism of IFN-γ-induced apoptosis

IFN-γ induces IDO via JAK-STAT1 signaling, which catalyzes the first step in the kynurenine pathway. This pathway produces 3OHKyn, which reacts and chemically modifies proteins and also generates ROS, resulting in apoptosis.