Inhibitors of Inducible Nitric Oxide Synthase Prevent Damage to Human Lens Epithelial Cells Induced by Interferon-Gamma and Lipopolysaccharide

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We previously found that Ca2+ concentrations, inducible nitric oxide synthase (iNOS) mRNA, and protein expression in lenses of the Shumiya cataract rat (SCR) increase with the development of cataracts. In this study, we investigated the change in Ca2+-ATPase activities and ATP levels in the human lens epithelial cell line SRA 01/04 (HLE cells) with the stimulation of interferon-gamma (IFN-γ) and lipopolysaccharide (LPS). Expression levels of iNOS mRNA in HLE cells, which were determined using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR methods, increased during stimulation with IFN-γ (1000 IU) and LPS (100 ng/ml). NO release from HLE cells, expressed as the sum of NO2- and NO3- levels, increased with the increase in iNOS expression levels. Ca2+-ATPase activities increased and ATP levels decreased in HLE cells stimulated with the combination of IFN-γ and LPS. Furthermore, both diethyldithiocarbamate and aminoguanidine attenuated the increase in Ca2+-ATPase activities and the decrease in ATP levels. These results suggest that excessive production of NO may cause mitochondrial damage, resulting in an increased Ca2+ concentration in the lens. The increase in Ca2+ concentration in the lens may increase Ca2+-ATPase activities.

Key words inducible nitric oxide synthase; nitric oxide; ATP; Ca2+-ATPase; human lens epithelial cell line SRA 01/04

MATERIALS AND METHODS

Cell Culture and Treatments The HLE cell line SRA 01/04 was donated by Professor Ibaraki (Jichi Medical School Hospital, Ibaraki, Japan). HLE cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and gentamicin 10 μg/ml. Cells were incubated under humidified air containing 5% CO2 at 37 °C. Each treatment was carried out at a time when the cells were 80% confluent, usually on the third day after seeding (0.4×104 cells/cm2). The culture medium was changed every other day and then 1 h before each experiment. In the experiment, HLE cells were pre-treated with IFN-γ for 1 h followed by coincubation with LPS. For treatment with DDC or AG, HLE cells were pre-treated with IFN-γ for 1 h, followed by coincubation with LPS. DDC 10 μM or AG 250 μM were added 6 h before the...
collection of cells.

**RNA Preparation** Total cellular RNA in HLE cells was extracted and purified using the RNeasy Min Kit and RNease-Free DNase Set (Qiagen, Tokyo, Japan). The purity and concentrations of RNA were determined spectrophotometrically at 260 nm (nucleic acids), 280 nm (proteins), and 320 nm (background), respectively. Samples with a ratio of OD260/OD280 values greater than 1.8 were used in this experiment.

**Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** The RT reaction was performed using the RNA PCR kit (AWV Ver 2.1, Takara Bio Inc., Tokyo, Japan). One microgram of total RNA was mixed with 3 μl of Tris–HCl 10 mM buffer (pH 8.3) containing MgCl₂ 5 mM and KCl 50 mM. The following components were then added to give a final volume of 10 μl: RNAse inhibitor 1 unit/μl, deoxyxynucleotide triphosphates 10 mM, reverse transcriptase 2.5 units/μl, and oligo dT-adaptor primer 0.125 μM. The RT reaction was carried out at 42 °C for 15 min, followed by 5 min at 95 °C. The PCR reactions were performed by adding 10 μl of cDNA to 40 μl of the reaction mixture containing MgCl₂ 3.125 mM, Tris–HCl 12.5 mM, pH 8.3, 1.563 units of Taq DNA polymerase, and iNOS- or glyceraldehydes-3-phosphate dehydrogenase (GAPDH)-specific primers 25 pmol. The specific primers were: 5’-CCAGTGACAGAGGTCTTCG-3’ and 5’-TGCCATTTTGGAATGCCG-3’ for iNOS; and 5’-CAGGAGCGAGA-3’ and 5’-CCACACCTCAGGAGC-3’ for GAPDH. The conditions for PCR were: 94 °C for 2 min, 35 cycles of 94 °C for 30 s (denaturing), 65 °C for 30 s (annealing), and 72 °C for 1 min (extension) for amplifying 600-bp (iNOS) and 750-bp (GAPDH) products. Finally, the extension of products was performed at 72 °C for 10 min. The PCR products were separated on a 1.5% agarose gel, visualized using ethidium bromide, and then photographed with an ImageMaster-CL (Amersham Biosciences Corp., Piscataway, NJ, U.S.A.).

**Quantitative Real-Time RT-PCR** The composition and conditions of the RT reaction were the same as those described for the semiquantitative RT-PCR method. The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer’s instructions (Roche, Tokyo, Japan). Briefly, 2 μl of cDNA was mixed with 2 μl of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart Taq DNA Polymerase, reaction buffer, MgCl₂, SYBR Green I dye, and deoxyxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 μl containing iNOS- or GAPDH-specific primers 10 pmol. The following primers were used: 5’-CCAGAGAGAAGAGAGATTTCCACTTGAA-3’ and 5’-TGATTTTCTGTCTCTGTCGCA-3’ for iNOS; and 5’-TGCCACCAACTGCTTAGCC-3’ and 5’-GGCATGGGACTGTGGTATGAGA-3’ for GAPDH. The conditions for PCR were: 95 °C for 10 min, 50 cycles of 95 °C for 10 s (denaturing), 60 °C for 10 s (annealing), and 72 °C for 5 s (extension). Quantities of PCR production were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche, Tokyo, Japan). After the PCR reactions were finished, dissociation curves of the PCR products were generated using the program of LightCycler Software Version 4.0 to detect nonspecific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and iNOS were used to calculate the expression levels of iNOS mRNA in HLE cells.

**Measurement of NO Release** HLE cells with or without each treatment were cultured in medium, which was then collected in a tube. A concentric microdialysis probe (A-1-20-05, 5-mm length; Eicom, Kyoto, Japan) was placed in the medium and perfused with Ringer’s solution (NaCl 140 mM, KCl 4 mM, CaCl₂ 1.26 mM, and MgCl₂ 1.15 mM, pH 7.4) at a constant flow rate of 2 μl/min using a micro syringe pump (ESP-64, Eicom). NO₂⁻ and NO₃⁻ in the medium were separated using a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm, Eicom), and NO₂⁻ was reduced to NO₃⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). NO₃⁻ was mixed with a Griess reagent to form a purple azo dye in a reaction coil and placed in a column oven that was set at 35 °C. The absorbance of the color product dye at 540 nm was determined with a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase consisted of 10% methanol containing NaCl–NH₄Cl 0.15 M and 4Na-EDTA 0.5 g/l, and was delivered by a pump at a rate of 0.33 ml/min. The Griess reagent, which was 1.25% HCl and sulfanilamide 5 g/l with N-naphthylethlenediamine 0.25 g/l was delivered at a rate of 0.1 ml/min. In this paper, NO amounts express the total NO metabolite level, which is the sum of the NO₂⁻ and NO₃⁻ levels.

**Measurement of ATPase Activity** HLE cells in 75-cm² flasks were washed with ice-cold Ca²⁺, Mg²⁺-free buffer (NaCl 145 mM, KCl 5 mM, NaHCO₃ 5 mM, HEPES 15 mM, Tris 8 mM, EDTA 0.5 mM, pH 7.4, 290 mMOSm) and harvested using a cell scraper (Iwaki Co., Ltd., Tokyo, Japan). The collected cells were homogenized in 600 μl of hypotonic buffer (mannitol 100 mM, HEPES 7.5 mM, Tris base 6.25 mM, pH 7.4). Unbroken cells were pelleted at low speed (1500 rpm, 10 min), and the supernatant obtained was assayed for ATPase activity, as assessed by Pi liberated from ATP. Ca²⁺-ATPase activity was calculated as the difference in the phosphate liberation measured in the presence or absence of Ca²⁺ 0.1 mM. Na⁺/K⁺-ATPase activities were calculated as the difference in the phosphate liberation measured in the presence or absence of ouabain 1 mM.

**Measurement of ATP** HLE cells in 25-cm² flasks were washed with ice-cold phosphate-buffered saline (PBS; Gibco, Tokyo, Japan) and harvested with a cell scraper (Iwaki Co., Ltd.). The cells collected were homogenized in 200 μl of HEPES/KOH buffer 10 mmol/l (pH 7.4). Unbroken cells were pelleted in a centrifuge (15000 rpm, 15 min), and the resultant supernatant was assayed for ATP levels. ATP levels were determined using a Sigma ATP Bioluminescent Assay Kit (Sigma Chemical, Tokyo, Japan) and a luminometer AB-2200 (Atto Corporation, Tokyo, Japan) according to the manufacturers’ instructions.

**Measurement of Protein** Protein levels in the HLE cells were determined according to the method of Bradford, using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with bovine serum albumin as the standard.

**Statistical Analysis** All values are expressed as mean± standard error of the mean (S.E.). Significance of the mean
differences in each experiment was analyzed using Student’s
*t*-test, and a *p* value of less than 0.05 was considered signifi-
cant.

RESULTS

Increase in iNOS mRNA Expression and NO Release from HLE Cells Stimulated with a Combination of IFN-γ and LPS Figure 1 shows the expression of iNOS mRNA in HLE cells stimulated with or without IFN-γ and LPS de-
tected by the semiquantitative RT-PCR method. iNOS mRNA
was not detected in unstimulated HLE cells. Stimulation with
IFN-γ or LPS alone had a negligible effect on iNOS mRNA
induction. On the contrary, stimulation with a combination of
IFN-γ and LPS of HLE cells induced iNOS mRNA expres-
sion. In addition, stimulus with LPS 100 ng/ml induced greater
iNOS mRNA expression than that with LPS 10 ng/ml. Figure 2
shows the expression of iNOS mRNA in HLE cells deter-
mined using the quantitative real-time RT-PCR method. Ex-
pression levels of iNOS mRNA in the HLE cells increased
over time when incubated with a combination of IFN-γ and
LPS.

Figure 3 shows NO release from HLE cells stimulated with a
combination of IFN-γ and LPS. NO release into the medium
was found to increase from HLE cells with the increase in
iNOS mRNA levels. In addition, both DDC and AG signifi-
cantly attenuated the increase of NO release from HLE cells.

Changes in ATPase Activity in HLE Cells Stimulated with a Combination of IFN-γ and LPS Figure 4 shows Ca²⁺-ATPase activities in HLE cells stimulated with the
combination of IFN-γ and LPS. The Ca²⁺-ATPase activities
increased after stimulation with the combination of IFN-γ
and LPS and reached a plateau at 12 h. Both DDC and AG
significantly attenuated the increase in Ca²⁺-ATPase activi-
ties.

Na⁺/K⁺-ATPase activities in HLE cells stimulated with a
combination of IFN-γ and LPS were also measured. In con-
trast to the result on Ca²⁺-ATPase activity, Na⁺/K⁺-ATPase
activities decreased at 6 h and there was no significant differ-
ence in the HLE cells from 12 to 24 h after stimulation with
the combination of IFN-γ and LPS (Fig. 5).

Changes in ATP Levels in HLE Cells Stimulated with a
Combination of IFN-γ and LPS Figure 6 shows changes in
ATP levels in HLE cells stimulated with a combination of
IFN-γ and LPS. ATP levels in HLE cells decreased with in-
cubation time using the combination of IFN-γ and LPS and
markedly decreased 6—24 h after the stimulation. Both DDC
and AG significantly attenuated the decrease in the ATP lev-
els.

DISCUSSION

Many factors have been implicated in cataract develop-
ment. In particular, the increase in Ca²⁺ concentration found
in numerous cataractous lenses is considered important in cataract development. We previously reported that excessive production of NO by iNOS may cause an increased Ca$^{2+}$ concentration in lenses. The present study demonstrates that iNOS mRNA levels are elevated and excessive amounts of NO are released from HLE cells stimulated with a combination of IFN-$\gamma$ and LPS. The excessive production of NO by iNOS increases Ca$^{2+}$-ATPase activity and decreases ATP levels in HLE cells. Both DDC and AG have the ability to attenuate these changes in Ca$^{2+}$-ATPase activities and ATP levels in HLE cells treated with those stimuli. Our present study using HLE cells partly supports those in vivo results.

The results of this study show that stimulation with IFN-$\gamma$ or LPS alone resulted in negligible levels of iNOS mRNA in HLE cells. However, stimulation with a combination of IFN-$\gamma$ and LPS resulted in elevated iNOS mRNA levels and NO release into the medium from HLE cells. It has been reported that LPS activates transcription nuclear factor kappa B (NF-$\kappa$B) and regulates iNOS gene expression levels in several cell types, and putative NF-$\kappa$B binding sites ($\kappa$B site) are present in mouse, rat, and human iNOS promoter regions. Two positions on the murine macrophage iNOS gene promoter region containing a $\kappa$B site and IFN-$\gamma$-activated site (GAS) are necessary for iNOS induction in response to LPS/IFN-$\gamma$. Therefore, the coinubcation of both IFN-$\gamma$ and LPS is needed for excessive amounts of iNOS mRNA production compared with the stimulation by IFN-$\gamma$ or LPS alone. On the other hand, NO release was detected even in unstimulated HLE cells. NO release in unstimulated HLE cells may be made up of NO$_2$ or NO$_3$, which are present under normal conditions, since the level was not changed by treatment with DDC or AG alone (nontreatment, 1.38$\pm$0.14; DDC, 1.34$\pm$0.26; AG, 1.40$\pm$0.20 mmol/10$^6$ cells, mean$\pm$S.E. of four to seven samples, HLE cells were treated with DDC or AG for 6 h).

Ca$^{2+}$-ATPase plays a central role in Ca$^{2+}$ transport and the maintenance of low internal Ca$^{2+}$ concentration using intracellular ATP. In this study, stimulation with the combination of IFN-$\gamma$ and LPS caused an increase in Ca$^{2+}$-ATPase activities and decrease in ATP levels in HLE cells. Both DDC and AG attenuated the increase in Ca$^{2+}$-ATPase activities and the decrease in ATP levels. The increase in Ca$^{2+}$-ATPase activity does not reflect a generalized increase in ATPase, since Na$^+/K^+$-ATPase activity in HLE cells did not increase. In addition, Bartlett et al. reported that the increase in Ca$^{2+}$ concentration in lens cells increases Ca$^{2+}$-ATPase activities. Taking these findings together, we hypothesize that the excessive production of NO by iNOS in HLE cells may cause mitochondrial damage resulting in decreased ATP production. The decrease in ATP levels may lead to a loss of cellular ATP. In this study, stimulation with the combination of IFN-$\gamma$ and LPS significantly decreased, but only slightly in comparison with the decreases at other incubation times. It is a possibility that the decrease in Na$^+/K^+$-ATPase activities at 6 h may be caused by another mechanism not involved in the iNOS induction system.

Further studies are needed to elucidate the precise mechanisms of the decreasing ATP level and collapse of Ca$^{2+}$ regulation by excessive production of NO. We are now in the progress of investigating iNOS protein expression, Ca$^{2+}$ regulation, and mitochondrial respiration in HLE cells. The findings may provide significant information that can be used in designing further studies to develop potent anticataract drugs.

REFERENCES