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Glucose Deprivation and Immunostimulation Induced Death in Rat Primary Astrocytes is Mediated by Their Synergistic Effect on the Decrease in Cellular ATP Level

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Abstract – In this study we investigated whether ATP loss was involved in the potentiated death of immunostimulated rat primary astrocytes in glucose-deprived condition. Rat primary astrocytes immunostimulated with LPS plus IFN-γ for 48 h underwent death upon glucose deprivation, which dependent on the production of peroxynitrite. Intracellular ATP level was synergistically decreased by glucose deprivation in immunostimulated astrocytes but not in control cells, and the loss of ATP occurred well ahead of the LDH release. The synergistic cell death and ATP loss by immunostimulation and glucose deprivation were inhibited by iNOS inhibitor (L-NAME and L-NNA) or peroxynitrite decomposition catalyst (also a superoxide anion scavenger), Mn(III)tetrakis(N-methyl-4′-pyridyl)porphyrin (MnTMPyP). Exogenous addition of peroxynitrite generator, SIN-1 time-dependently induced ATP loss and cell death in the glucose-deprived astrocytes. Depletion of intracellular glutathione (GSH) and disruption of mitochondrial transmembrane potential (MTP) were also observed under same conditions. Supply cellular ATP by the addition of exogenous adenosine or ATP during glucose deprivation inhibited ATP depletion, GSH depletion, MTP disruption and cell death in SIN-1 treated or immunostimulated astrocytes. This study showed that perturbation in the regulation of intracellular ATP level in immunostimulated astrocytes might make them more vulnerable to energy challenging stimuli.

Keywords □ LPS, IFN-γ, glucose deprivation, peroxynitrite, ATP, astrocyte, GSH, MTP

INTRODUCTION

Activated glial cells play important roles in the pathogenesis of neurological diseases, such as Parkinson's disease, Alzheimers disease, multiple sclerosis and stroke (Le *et al.*, 2002; Hu *et al.*, 1997; Bo *et al.*, 1994; Rischke and Krieglstein, 1990). Nitric oxide (NO) and peroxynitrite ($ONOO^-$) produced by activated glial cells have been highlightened in neuronal injury occurred during a large number of pathologic conditions (Brown and Borutaite, 2002, Almeida *et al.*, 2001; Bal-Price and Brown, 2001).

NO and peroxynitrite have been reported to cause cell death mainly due to the depletion of cellular ATP through several mechanisms; 1) an inhibition of mitochondrial function via decreasing the activity of mitochondrial complex components (I, II, III, IV), 2) a shutdown of glycolytic pathway (Brooks *et al.*, 1999; Moss and Bates, 2001; Heales *et al.*, 1999). If cellular ATP level is decreased, mitochondrial function and a balance of antioxidative system including reduced glutathione (GSH) are destroyed, and then cell death is occurred (Nieminen et al., 1994; Almeida and Bolanos, 2001). It is well established that neurons appear particularly vulnerable to NO and peroxynitrite via a rapid depletion of cellular ATP. But astrocytes are more resistant to the action of those molecules than neurons because cellular ATP level is maintained via an activation of glycolysis (Almeida *et al.*, 2001). In fact, only when ATP level is severely decreased, cell death of astrocytes is occurred. In our previous reports, NO and peroxynitrite generated from immunostimulated astrocytes decreased ATP level without affecting cell viability (Shin *et al.*, 2001), but they made astrocytes more susceptible to the glucose deprivation-induced death (Shin *et al.*, 2002). Thus, we hypothesized that NO and peroxynitrite generated from immunostimulated astrocytes decreased ATP level, and glycolytic inhibition like glucose deprivation further decreased it, which eventually lead to the irreversible damage of astrocytes. In this study, we have investigated whether glucose deprivation and immunostimulation cause synergistic ATP loss and subsequent to death in rat primary astrocytes.

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MATERIALS AND METHODS

Materials

Interferon-γ (IFN-γ), glucose-free Dulbecco's modified Eagles medium (DMEM), DMEM/F12 and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). N^G-nitro-L-arginine (NNA) and 3-morpholinosydnonimine (SIN-1) were obtained from Research Biochemical International (Natick, MA). Mn(III)tetrakis(N-methyl-4′-pyridyl)porphyrin (MnT-MPyP) was purchased from Aldrich (St. Louis, MO). All other chemicals including lipopolysaccharide (LPS) and Nω-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO).

Primary rat astrocyte culture and glucose deprivation

Rat primary astrocytes were cultured from the prefrontal cortices of 2- to 4-day-old Sprague-Dawley rat pups as previously described (Shin et al., 2002). Briefly, cells were dissociated by mild trypsinization (10 min at 37°C with DMEM containing 0.1% trypsin) and passed through sterile nylon sieves (80 μm pore size) into DMEM containing 10% heat-inactivated FBS. Cells (5×10^4 cells/ml) were then plated onto poly-L-lysine (PLL, 20 μg/ml)-coated 75 cm^2 culture bottles and maintained for one week in DMEM supplemented with 10% FBS. Cells were then trypsinized, washed and re-plated onto 24 well culture plate in the above medium. Cells were used for the experiment 7 days later. The primary glial cell preparation used in this study contained about 95% of astrocytes and less than 5% of microglia as determined by cytochemical staining with antibodies against glial fibrillary acidic protein (for astrocytes, GFAP, Sigma, St. Louis, MO) and with biotin-conjugated isolectin B_4 (for microglia, Sigma, St. Louis, MO), respectively.

Rat primary astrocytes were pretreated with IFN-γ (100 U/ml) and LPS (1 μg/ml). Forty-eight hours later, cells were deprived of glucose. Alternatively, cells were treated with SIN-1 in the presence or absence of glucose.

Measurement of NO

NO production was determined by measuring nitrite, a stable oxidation product of NO, as described previously (Green *et al.*, 1990). In brief, nitrite levels were determined by adding the Greiss reagent (mixing equal volumes of 0.1% napthylethylenediamine dihydrochlroride and 1% sulfanilamide in 5% phosphoric acid) to the medium. After 10 min, the absorbance at 550 nm was determined using a spectrophotometer (Beckmann DU-650, Fullerton, CA).

Determination of peroxynitrite formation

Dihydrorhodamine (DHR) oxidation was widely used for the measurement of peroxynitrite formation (Ju *et al.*, 2000). At the end of treatment, glial cells were washed and incubated for 30 min in PBS containing DHR123 (20 μM) and the fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence microplate reader (Tecan, Austria).

Measurement of cell death

Cell death was assessed by morphological examination of cells using phase-contrast microscopy and quantified by measuring an amount of lactate dehydrogenase (LDH) released into the medium at various time points after starting glucose deprivation. The LDH amount corresponding to complete glial damage/death was measured in sister cultures treated with 0.1% Triton X-100 for 30 min at 37°C. Basal LDH levels (generally less than 10% of total LDH release) were determined in sister cultures subjected to sham wash with 5 mM glucose containing DMEM, and subtracted from the levels in experimental conditions to yield the LDH signal specific to experimental injury.

Measurement of ATP content

Intracellular ATP level was measured using luminescence detection kit (Molecular Probe, Eugene, OR). Briefly, cells were lysed with 10% trichloroacetic acid (TCA) and sonicated for 15 s on ice. The lysates were treated with 2 mM EDTA and 2 mg/ml BSA. After centrifugation, the supernatant was neutralized with 4 M KOH, and the ATP content was determined using luminescence detection kit (Molecular Probe, Eugene, OR).

Measurement of intracellular GSH

Cellular GSH level was measured using monochlorobimane (mBCl, Molecular Probe, Eugene, OR) as described previously (Ju *et al.*, 2000). Cells were washed with PBS, and incubated with 60 μM of mBCl for 20 min at 37°C. Cells were lysed with 0.2% Triton X-100 in PBS. Cellular GSH level was determined by assessment of the fluorescence intensity at excitation wavelength of 360 nm and emission wavelength of 465 nm using a fluorescence microplate reader (Tecan, Austria).

Measurement of mitochondrial transmembrane potential (MTP)

MTP was assessed with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimideazolocarbocyanine iodide (JC-1, Molecular Probe, Eugene, OR). Cells were incubated for 30 min at 37°C with

1 μM of JC-1, which was prepared in culture media. Depolarization of MTP was assessed by measuring the fluorescence intensity at excitation wavelength of 485 nm and emission wavelength of 530 nm (for monomer fluorescence) and 590 nm (for aggregate fluorescence) using a fluorescence microplate reader (Tecan, Austria). At each emission wavelength, autofluorescence (i.e., fluorescence of cells not loaded with JC-1) was subtracted from the JC-1 fluorescence. Data were expressed as the ratios of JC-1 aggregate fluorescence to monomer fluorescence (F_{590}/F_{530}). Alternatively, the fluorescence intensity at the same wavelength as above in cells was visualized on a confocal microscope (Leica, Germany) equipped with a krypton-argon laser (488 and 568 nm emission lines), using a 40×, 1.4 numerical aperture Plan Apo oil-immersion objective (Nikon). As a positive control for the complete mitochondrial depolarization, the potent mitochondrial uncoupler, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP, 5 μM) was applied at the end of the experiment.

Statistical Analysis

Data were expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Neuman-Keuls test as a *post-hoc* test and a *P*-value <0.05 was considered significant.

RESULTS

LPS (1 μg/ml) plus IFN-γ (100 U/ml) synergistically increased NO production from rat primary astrocytes (Fig. 1A). LPS alone but not IFN-γ slightly increased NO production (Fig. 1A). Glucose deprivation augmented LDH release from LPS plus IFN-γ-stimulated rat primary astrocytes (Fig. 1B). In other

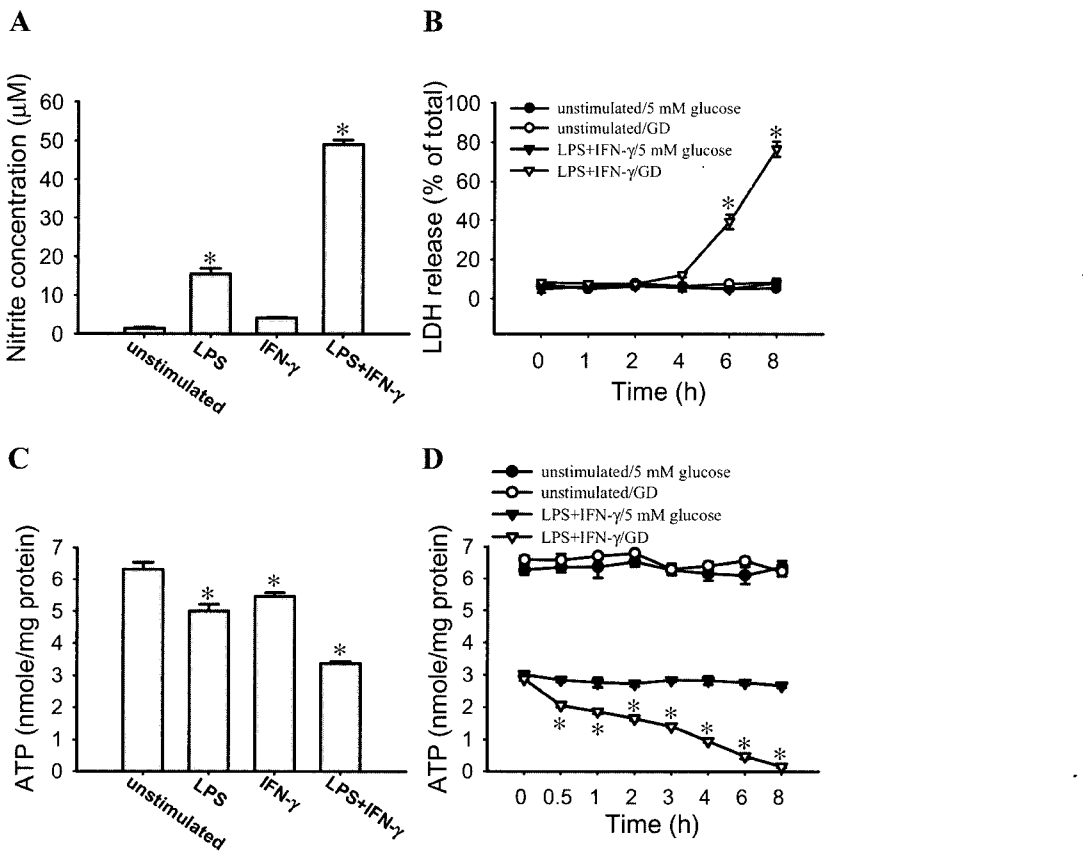

Fig. 1. ATP depletion and cell death in the immunostimulated rat primary astrocytes. Astrocytes were immunostimulated by LPS (1 mg/ml), IFN-γ (100 units/ml) or LPS + IFN-γ for 48 h. After incubation, the medium was removed for NO determination (A), and immunostimulated cells were deprived of glucose. An amount of LDH released (B) to the medium was determined at various time points after glucose-deprivation (GD). Alternatively, ATP levels were measured at the end of immunostimulation period (C). Immunostimulated astrocytes were further incubated in the absence and presence of 5 mM glucose, and intracellular ATP level was determined at various time points (D). Each data indicates the mean ± S.E.M. (n=4). *Significant difference from control (unstimulated for A & C; time zero for B & D) (P<0.05).

words, more than 80% of total cellular LDH was released from LPS plus IFN-γ-stimulated cells after 8 h of glucose deprivation (Fig. 1B). In contrast, glucose deprivation alone did not increase LDH release from unstimulated cells (Fig. 1B). In unstimulated cells, LDH release was not observed even after 24 h of glucose deprivation (data not shown). Immunostimulation with LPS plus IFN-γ decreased intracellular ATP level by 50%, which is coincident with our previous report (Fig. 1C, Shin *et al.*, 2001). LPS or IFN-γ alone showed only marginal effects, if any. The cellular ATP level is an important determinant for cell death including glial cells, neurons, and other peripheral cells (Almeida *et al.*, 2001; Beltran *et al.*, 2000; Shin *et al.*, 2002). The reduced ATP level in immunostimulated astrocytes implicates that mechanisms regulating intracellular energy level are impaired, which may restrict the resistance of astrocytes to energy-challenging stimuli. Therefore we tested whether stimuli inducing cellular energy crisis such as glucose deprivation causes cell death via synergistic depletion of cellular ATP in immunostimulated astrocytes. To determine whether potentiated death of immunostimulated astrocytes is closely related with the synergistic loss of intracellular ATP, we first investigated the time course of LDH release and ATP loss. Glucose deprivation did not change intracellular ATP level in unstimulated cells, while in LPS plus IFN-γ stimulated cells, it decreased ATP level by 80% at 4 h. In addition, 5 mM glucose supplementation in immunostimulated cells did not cause ATP depletion (Fig. 1D). The synergistic depletion of ATP from immunostimulated rat primary astrocytes was evident from 30 min after glucose deprivation (Fig. 1D), but cell death (LDH release) was first recognized at 6 h (Fig. 1B). It is noteworthy that loss of intracellular ATP occurred well ahead of cell death.

To test whether NO produced by immunostimulated astrocytes induces cell death and ATP depletion, inducible nitric oxide synthase (iNOS) inhibitor, NNA or L-NAME, was added at the start of immunostimulation. The synergistic cell death and ATP loss by glucose deprivation in immunostimulated cells were inhibited by the treatment of L-NAME (1 mM) or L-NNA (800 μM) (Fig. 2). And both of them diminished NO production by 70% (data not shown). Also, we determined DHR oxidation to measure peroxynitrite formation, and observed the increase by about 3 fold (297 ± 14% of control) in immunostimulated and glucose deprived cells. This was significantly inhibited by L-NAME or NNA treatment (Fig. 3A). In addition, 50 μM MnTMPyP (a cell permeable peroxynitrite decomposition catalyst and also a superoxide scavenger) blocked the glucose deprivation induced cell death and ATP depletion in

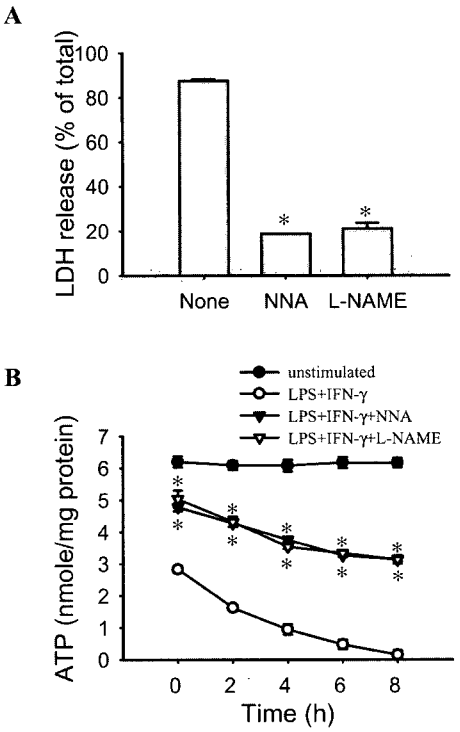

Fig. 2. Effects of iNOS inhibitors on the glucose deprivation (GD)-induced cell death and synergistic ATP depletion in immunostimulated rat primary astrocytes. Astrocytes were immunostimulated with LPS+IFN-γ in the absence and presence of NNA (800 μM) or L-NAME (1 mM). A. An amount of LDH released into the medium was determined at 8 h after glucose deprivation. B. ATP level was measured at various time points in glucose-deprived astrocytes. Data are mean ± S.E.M. (n=4). *Significant difference from each control immunostimulated and glucose-deprived astrocytes. ($P<0.05$).

the immunostimulated astrocytes (Fig. 3B, C). In contrast, superoxide dismutase (SOD) treatment, which cannot easily penetrate cell membrane, did not (Fig. 3A). These data suggest that peroxynitrite may be a major factor of ATP depletion and subsequent to cell death in our system. To further elucidate the mechanism of glial cell death we added peroxynitrite generator SIN-1 during glucose deprivation. We observed that in the absence of glucose, 50 μM SIN-1 significantly increased the amounts of LDH released into the medium at 4 h and decreased cellular ATP level at earlier time points (2 h) (Fig. 4). As in case of immunostimulated glial cells, cell death induced by SIN-1 and glucose deprivation was always preceded by the decrease of intracellular ATP level.

To investigate whether the synergistic depletion of intracellular ATP is related to the disruption of intracellular antioxidant defense mechanism, we determined the level of intracellular GSH. As shown in Fig. 5, glucose deprivation in immunostim-

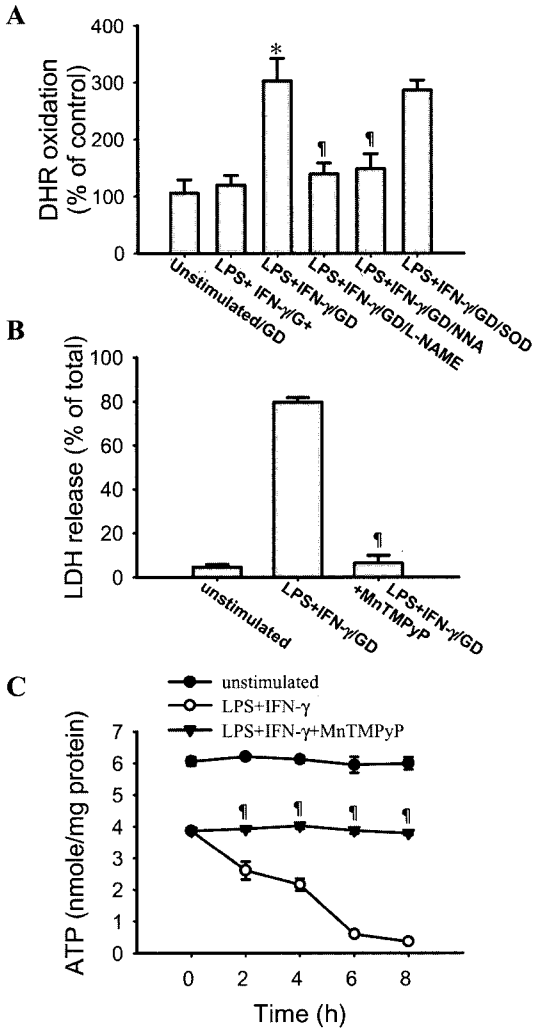

Fig. 3. Peroxynitrite-mediated cell death and synergistic ATP depletion in the glucose deprived (GD) and immunostimulated rat primary astrocytes. Immunostimulated rat primary astrocytes were deprived of glucose. Cells were treated with L-NAME or NNA during immunostimulation, and SOD was added to the medium during glucose deprivation. At 2 h after starting glucose deprivation, peroxynitrite formation was measured using DHR123 (A). Astrocytes were co-treated with MnTMPyP (50 μM) during immunostimulation period. An amount of LDH released into the medium was determined at 8 h after glucose deprivation (B). ATP level was measured at various time points in glucose-deprived astrocytes (C). Data are mean ± S.E.M. (n=4). *Significant difference from control (unstimulated/GD) ($P<0.05$). ¶ Significant difference from immunostimulated and glucose deprived astrocytes (LPS+ IFN-γ/GD) ($P<0.05$).

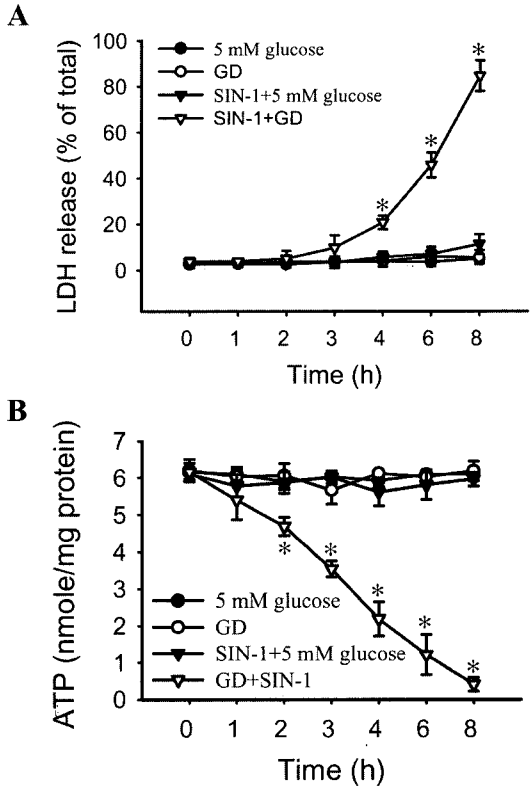

Fig. 4. SIN-1/ glucose deprivation (GD)-induced cell death and synergistic ATP depletion in rat primary astrocytes. Astrocytes were incubated for the indicated times with SIN-1 (50 μM) in the absence and presence of glucose (5 mM). An amount of LDH released into the medium (A) and ATP levels (B) were measured at various time points after glucose deprivation. Data are mean ± S.E.M. (n=4). *Significant difference from control (time zero) ($P<0.05$).

ulated (Fig. 5A) or SIN-1 treated (Fig. 5B) rat primary astrocytes rapidly depleted intracellular GSH level, which coincides with our previous reports (Ju *et al.*, 2000). Neither glucose deprivation alone nor treatment of SIN-1 or immunostimulation alone caused depletion of intracellular GSH level (Fig. 5A, B). We also investigated MTP disruption by glucose deprivation in immunostimulated or SIN-1 treated rat primary astrocytes (Fig. 5C, D). MTP was lost at 1.5 h in the immunostimulated and glucose deprived astrocytes (Fig. 5C), which was comparable to that observed after treatment of a potent mitochondrial uncoupler, FCCP (5 μM, data not shown). Furthermore, MTP was decreased as early as 1.5 h and completely disrupted at 2 h after treatment of SIN-1 and glucose deprivation (Fig. 5D). These results suggest that the synergistic cell death mediated by ATP depletion accompany, at least in part, the GSH depletion and MTP disruption.

Finally, we examined whether the maintenance of intracellular ATP level blocked the cell death induced by glucose deprivation and peroxynitrite. Exogenous addition of adenosine or ATP has been reported to furnish readily available intracellular ATP by providing glycolytic substrate from their ribose moiety in a variety of energetically challenged situations including

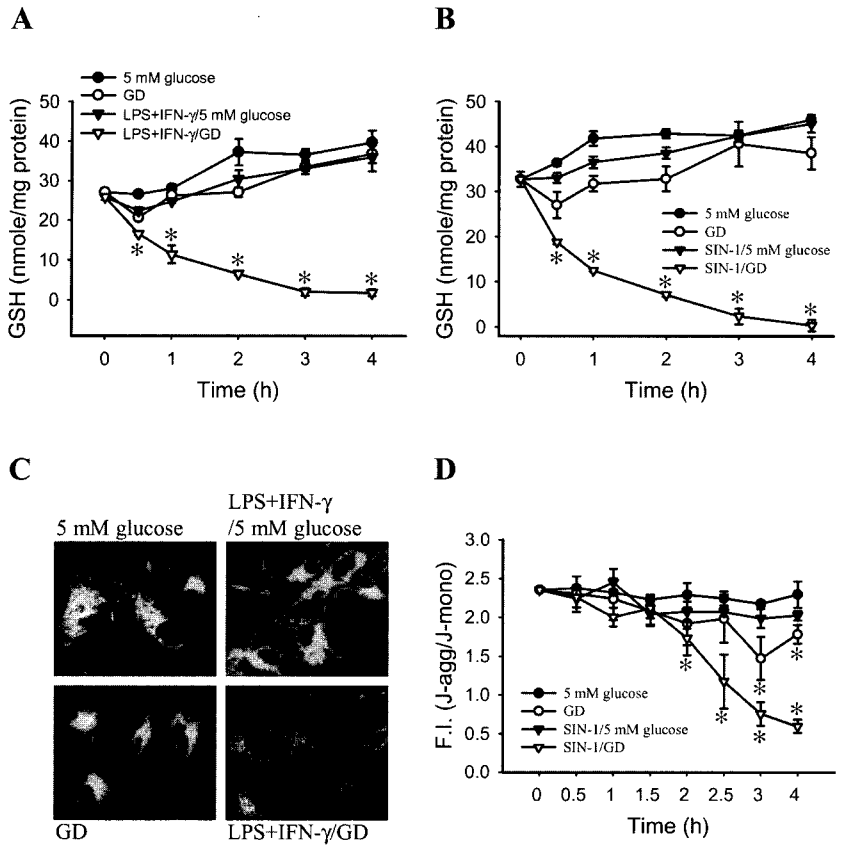

Fig. 5. Decrease of GSH level and MTP in immunostimulated and glucose deprived or SIN-1-treated and glucose deprived rat primary astrocytes. Immunostimulated (A,C) or SIN-1 treated (B,D) astrocytes were incubated for the indicated times in the absence and presence of glucose (5 mM). Intracellular GSH level (A,B) was measured at various time points after glucose deprivation. Immunostimulated cells were incubated with JC-1 (1 μg/ml) for 20 min, and then deprived of glucose for 2 h (C). Alternatively, unstimulated astrocytes were loaded with JC-1, and then incubated for the indicated times with SIN-1 (50 μM) in the absence and presence of glucose (5 mM) (D). Fluorescence intensity (green fluorescence for JC-1 monomers and red fluorescence for JC-1 aggregates) was measured using a confocal microscope (C) or the ratio of fluorescence intensity (JC-1 aggregates/JC-1 monomers) was measured using a fluorescence microplate reader at various time points after glucose deprivation (D). Data are mean ± S.E.M. (n=4). *Significant difference from control (time zero) ($P<0.05$).

chemical hypoxia model (Haun *et al.*, 1996; Jurkowitz *et al.*, 1998; Shin *et al.*, 2002). Exogenous addition of adenosine (50 μM) or ATP (50 μM) reversed the glucose deprivation induced-ATP depletion and -cell death both in immunostimulated and SIN-1 treated rat primary astrocytes (Table I). In addition, adenosine or ATP supplementation reversed GSH depletion and MTP disruption induced by peroxynitrite and glucose deprivation in rat primary astrocytes (Table I).

DISCUSSION

Previously, we reported that immunostimulated astrocytes were highly vulnerable to glucose deprivation, and that peroxynitrite was involved in the augmented cell death via the mechanisms including cellular GSH depletion and MTP disruption (Choi *et al.*, 2001; Ju *et al.*, 2000). Here, we demonstrated that in LPS plus IFN-γ-stimulated (immunostimulated) rat primary astrocytes, glucose deprivation synergistically depleted intracellular ATP via mechanism involving peroxynitrite generation, which consequently caused cell death.

Coincident with our previous report (Shin *et al.*, 2001), we observed that in immunostimulated astrocytes, intracellular ATP level was decreased up to 50% of control level without cell death (Fig. 1A, B). In such condition, astrocytes could still meet the cellular energy demand by increasing glycolysis coupled with lactate formation (Bolanos *et al.*, 1994). Since activation of glycolysis prevented further decrease of ATP, cell death did not occur. Although immunostimulated astrocytes are resistant to the relatively mild ATP depletion, an inhibition of glycolysis such as glucose deprivation can make ATP level to fall

Table I. Effects of adenosine and ATP supplementation. Rat primary astrocytes were immunostimulated with LPS + IFN-γ for 48 h and deprived of glucose in the absence and presence of adenosine (50 μM) or ATP (50 μM). Also the cells were treated with 50 μM SIN-1 during glucose deprivation (GD) in the absence and presence of adenosine or ATP. An amount of LDH released and ATP level were measured at 8 and 5 h after glucose deprivation, respectively. Intracellular GSH level and MTP were measured at 3 h after glucose deprivation. Each data indicates the mean ± S.E.M. (n=4). *Significant difference from control (G+, 5 mM glucose) (P<0.05). ¶ Significant difference from GD (P<0.05).

	Immunostimulated		SIN-1 treated			
	LDH (% of total)	ATP (nmole/mg proein)	LDH (% of total)	ATP (nmole/mg proein)	GSH (nmole/mg proein)	MTP (J-agg/J-mono)
G+	3.81 ± 2.14	3.31 ± 0.08	3.99 ± 1.68	5.73 ± 0.07	31.2 ± 6.29	2.84 ± 0.12
GD	82.31 ± 1.91*	0.49 ± 0.12*	70.33 ± 4.27*	1.88 ± 0.13*	2.76 ± 0.35*	0.9 ± 0.06*
GD+Adenosine	12.32 ± 2.43¶	3.28 ± 0.11¶	9.65 ± 3.04¶	5.82 ± 0.21¶	30.1 ± 3.04¶	2.34 ± 0.29¶
GD+ATP	11.99 ± 1.80¶	3.11 ± 0.05¶	10.07 ± 1.27¶	5.38 ± 0.05¶	28.9 ± 1.17¶	1.97 ± 0.13¶

below certain threshold level and induce death in these cells. In this case, a severe ATP loss is a major determinant for cell death. This could be supported by several findings in this study; 1) Immunostimulation only decreased ATP content up to 50% of untreated cells, but did not induce the death of astrocytes. 2) A further decline of cellular ATP level by glucose deprivation was already observed at early times (30 min), but cell death was shown at late times (6 h) when complete ATP loss (about 80% of control) was occurred. When intracellular ATP level was decreased to 10% of control, there was a near complete cell death (above 85% of control). 3) The preservation of intracellular ATP content by the addition of adenosine or ATP prevented cell death as previously reported by us (Shin *et al.*, 2002). Above results are well consistent with others' finding. Inability of ATP maintenance by the inhibition of glycolysis is one of main causes of cell death in neurons and glial cells (Almeida *et al.*, 2001). Moreover, moderate ATP fall disturbed intracellular ion homeostasis and membrane depolarization, but severe energy depletion expedited the collapse of ion gradients, loss of membrane potential, and eventually cell death (Yager *et al.*, 1994; Galeffi *et al.*, 2000). We observed a delay between ATP depletion and cell death, which was also observed by several other researchers (Jurkowitz *et al.*, 1998; Silver *et al.*, 1997). Combined together, these results suggest that severe and synergistic energy failure induced by ATP loss is one of mechanisms in the augmented death in immunostimulated and glucose deprived astrocytes.

A growing body of evidence indicates that NO and $ONOO^-$ are involved in cell death via cellular ATP loss (Bal-Price and Brown, 2001; Brorson *et al.*, 1999; Almeida *et al.*, 2001). Similar to others' findings, we showed that the synergistic cell death and ATP loss by immunostimulation was mediated by NO/ $ONOO^-$; 1) inhibition of NO production attenuated peroxynitrite formation, ATP loss and cell death. 2) peroxynitrite decomposition catalyst MnTMPyP, a cell permeable superoxide scavenger (Esteves *et al.*, 1998), completely inhibited ATP loss and cell death. 3), SIN-1, peroxynitrite generator, also induced ATP depletion followed by cell death in glucose deprived-astrocytes. Peroxynitrite, a highly reactive and toxic molecule, is produced from the reaction of NO and superoxide anion, and NO induced toxicity is mainly due to the peroxynitrite. (for see review, Torreilles *et al.*, 1999). Several researchers reported that NO/$ONOO^-$ adversely affected intracellular energy metabolism; inhibition of mitochondrial respiratory chain, an important source for ATP supply, aconitase involved in TCA cycle, and glycolysis (Anderson *et al.*, 1998; Jurkowitz *et al.*, 1998; Poderoso *et al.*, 1999; Sims and Anderson, 2002; Heals and Bolanos, 2002). In our previous and present study, we found that peroxynitrite formation was further increased in the combined condition of glucose deprivation and immunostimulation. Thus, the synergistic ATP depletion shown in this study may be due to the excessively generated peroxynitrite by glucose deprivation and immunostimulation.

An increase of peroxynitrite level in glucose deprived and immunostimulated astrocytes could be, in part, due to a decrease in cellular antioxidant capacity, such as GSH depletion. This was supported by our previous findings that GSH depletion potentiated glial cell death under the combined condition of glucose deprivation and immunostimulation or SIN-1 treatment (Ju *et al.*, 2000; Choi *et al.*, 2001), and GSH depletion was inhibited by MnTMPyP, a peroxynitrite scavenger (Choi *et al.*, 2001). Several researchers found that the intracellular synthesis of GSH required ATP, and sustained ATP depletion might limit intracellular free GSH concentration by preventing both the production and reduction of oxidized GSH, which eventually induced death of neurons and astrocytes

(Barker *et al.*, 1996). Although the relationship between ATP and GSH levels is not clear in this study, it is evident that ATP level is important for the maintenance of GSH level considering the prevention of GSH depletion by the addition of adenosine or ATP. Disruption of MTP has been shown to occur in various models of cell death (Kroemer *et al.*, 1997; Petit *et al.*, 1997; Brown and Borutaite, 2002), and peroxynitrite has been shown to induce a MTP disruption via inhibiting the mitochondrial respiratory chain (Poderoso *et al.*, 1999; Sims and Anderson, 2002; Heals and Bolanos, 2002). We also observed that MnTMPyP blocked the peroxynitrite-dependent depolarization of MTP (Choi *et al.*, 2001). Beltran *et al.* (2000) reported that ATP level was involved in the maintenance of mitochondrial membrane potential. Here, the initial decrease of cellular ATP (30 min) occurred ahead of mitochondrial depolarization (2 h) but complete disruption of MTP was appeared at earlier times than complete ATP loss. And preservation of cellular ATP level by the addition of adenosine or ATP inhibited MTP disruption in our condition. These results implicate that ATP loss deteriorates intracellular homeostatic mechanisms like GSH system and mitochondrial function especially in immunostimulated or SIN-1 treated and glucose deprived astrocytes.

In conclusion, we provided evidences that immunostimulated astrocytes rapidly underwent death during glucose deprivation through the synergistic ATP depletion. Energy failure was due to the production of NO and peroxynitrite, and it participated in a deterioration of antioxidative system and mitochondrial function. Thus, defining the importance of cellular ATP level in immunostimulated and glucose deprived astrocytes should provide the basis for the therapeutic strategies against ischemia and neurodegenerative diseases, in which conditions the immunostimulation of astrocytes and energy challenging situations can occur.

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