

## Nitric Oxide Prevents the Bovine Cerebral Endothelial Cell Death Induced by Serum-Deprivation

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Endothelial cells play a central role in the inflammatory processes, and activation of nuclear factor kappa B (NF- $\kappa$ B) is a key component in that inflammatory processes. Previously, we reported that tumor necrosis factor alpha (TNF  $\alpha$ ) had protective effect on cell death induced by serum deprivation and this protection was related to NF- $\kappa$ B activation. Inducible nitric oxide synthase (iNOS) is a member of the molecules which transcription is regulated mainly by NF- $\kappa$ B. And the role of nitric oxide (NO) generated by iNOS on cell viability is still controversial. To elucidate the mechanism of TNF  $\alpha$  and NF- $\kappa$ B activation on cell death protection, we investigate the effect of NO on the cell death induced by serum-deprivation in bovine cerebral endothelial cells in this study. Addition of TNF  $\alpha$ , which are inducer of iNOS, prevented serum-deprivation induced cell death. Increased expression of iNOS was confirmed indirectly by nitrite measurement. When selective iNOS inhibitors were treated, the protective effect of TNF  $\alpha$  on cell death was partially blocked, suggesting that iNOS expression was involved in controlling cell death. Exogenously added NO substrate (L-arginine) and NO donors (sodium nitroprusside and S-nitroso-N-acetylpenicillamine) also inhibited the cell death induced by serum deprivation. These results suggest that NO has protective effect on bovine cerebral endothelial cell death induced by serum-deprivation and that iNOS is one of the possible target molecules by which NF- $\kappa$ B exerts its cytoprotective effect.

Key Words: Endothelial cell death, iNOS, NO, NF- $\kappa$ B, TNF  $\alpha$

### INTRODUCTION

Endothelial cells play a central role in the inflammatory processes and activation of nuclear factor kappa B (NF- $\kappa$ B) is a key component in that inflammatory processes (Pahl et al, 1996).

NF- $\kappa$ B activation directs transcription of a large number of molecules, so it is one of the most important regulatory mechanism within cell. Recently, NF- $\kappa$ B's ability to block cell death process is reported, and it is now considered one of the cell survival

mechanism. Several clues to the protective role of NF- $\kappa$ B come from the observations that pyrrolidine dithiocarbamate and N <sup>$\alpha$</sup> -Tosyl-Phe-Chloromethylketone (TPCK) induced apoptosis through inhibition of NF- $\kappa$ B (Wu et al, 1996), and that NF- $\kappa$ B knockout mouse (Beg and Baltimore, 1996), super-repressor I $\kappa$ B (Wang et al, 1996) and transdominant-negative mutant of I $\kappa$ B (Van Antwerp et al, 1996)-expressed cultured cells were more reluctant to cell death mechanism. Beg et al, (1996) also reported that mice lacking RelA lead to embryonic lethality. We also reported previously that NF- $\kappa$ B activation in bovine cerebral endothelial cells by tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ) and phorbol 12-myristate 13-acetate (PMA) prevented cell death induced by serum deprivation (Ahn et al, 1996). Among variety of molecules

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regulated by NF- $\kappa$ B, however, which of these are involved with cytoprotective action is largely unknown. To introduce more concrete evidence that NF- $\kappa$ B act as cell survival factor, it is essential to elucidate cytoprotective molecules responsible for it.

Knowing that inducible nitric oxide synthase (iNOS) is present ubiquitously in various cell types in addition to macrophage, much attention have been focused on the potential function of this highly reactive molecule. But, the regulatory effects of this molecule on cell function is still in question. Particularly, the effect of iNOS on cell viability is quite controversial. Many researchers reported that nitric oxide, which is produced by iNOS, promote cell death (Lu L et al, 1996; Melkova et al, 1997), but others reported contradictory results (Chun et al, 1995). Three isoforms of NOS-endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS)-have been identified. Among these, the activation process of iNOS is regulated mainly in transcriptional level (Xie et al, 1994; Lorsbach et al, 1993). And NF- $\kappa$ B is known to have crucial role in transcriptional regulation of iNOS.

It is assumed that nitric oxide plays a complex role in regulating cell viability. In primary cultured cortical neuron, NO mediated the glutamate toxicity (Dawson et al, 1991). Depending on NO concentration, necrosis or apoptosis could be induced by NO (Bonfoco et al, 1995). Under certain circumstances, NO mediated the TNF $\alpha$  cytotoxicity in bovine aortic endothelial cells (Estrada et al, 1992). In contrast, in various cell types, NO generation prevented apoptosis (Beauvais et al, 1995; Chun et al, 1995; Genaro et al, 1995). NO had also protective role in damage caused by reactive oxygen species (Wink et al, 1993). Recently, Kim et al (1997) reported that NO had protective effect in hepatocyte by inducing heat shock protein 70. So it has been discussed that NO effect on cell viability is dependent on its concentration, cellular redox state, cell types and interacting cell regulatory mechanism.

Therefore, under the hypothesis that the protective effect of TNF $\alpha$  on the serum-deprivation cell death (Ahn et al, 1996) is mediated via nitric oxide (NO) produced by NF- $\kappa$ B activation and iNOS induction, we examined the effects of NO on bovine cerebral endothelial cell death induced by serum-deprivation in this study.

## METHODS

### *Materials*

Aminoguanidine was purchased from RBI (Natick, MA, USA). 2-Amino-5,6-dihydro-6-methyl-4H-1,3 thiazine (AMT) was obtained from Tocris Cookson (Lanford, Bristol, UK). TNF $\alpha$ , sodium nitroprusside, pyruvic acid, S-nitroso-N-acetylpenicillamine, sulfanilic acid,  $\beta$ -nicotinamide adenine dinucleotide (reduced form), N-(1-naphthyl)-ethylenediamine, sodium nitrite, triton X-100, D-arginine and L-arginine were products of Sigma Chemical Co. (St Louis, MO). Cell culture products were purchased from GIBCO (Staley Road, Grand Island, NY).

### *Cell culture*

Bovine cerebral endothelial cells (BCECs) were prepared and characterized as previously described (DeBault et al, 1979, 1981; Abbott et al, 1991) with modification. Briefly, bovine brains from freshly slaughtered adult animals were immediately placed in ice-cold Hank's balanced salt solution (HBSS) containing antibiotics. The meninges and superficial blood vessels were removed, and the brain gray matter was disrupted in a loose Dounce homogenizer. Then the homogenates were filtered sequentially through 300  $\mu$ m and 80  $\mu$ m nylon meshes. The resulting microvessels were treated with collagenase B (4 mg/ml) for 2 hours and 25% bovine serum albumin was added. The pellets were resuspended in HBSS and loaded over 50% of percoll and centrifuged. The second band was collected and washed prior to plating onto tissue culture dishes precoated with collagen.

BCECs migrating from vessels were pooled to form a culture of proliferating endothelium that was maintained in Dulbecco's modified eagle medium (DME) with 10% fetal calf serum (FCS) and endothelial growth supplements. Purity of BCECs was determined by positive immunocytochemical detection of Factor VIII and vimentin, but absent expression of fibronectin,  $\alpha$ -actinin and glial fibrillary acidic protein. BCECs identity and culture purity were established by light microscopic appearance, thrombomodulin activity, uptake of acetylated low density lipoprotein and Griffonia simplicifolia agglutinin cytofluorimetry. The cell cultures contained more than 95% BCECs, based on composition of Factor

VIII and vimentin positive cells. BCECs of passage 4~12, which still express functional bradykinin receptors based on agoinst-induced calcium influx, and phosphoinositide turnover, were plated on 10cm<sup>2</sup> culture dishes, maintained in DME with 10% FCS.

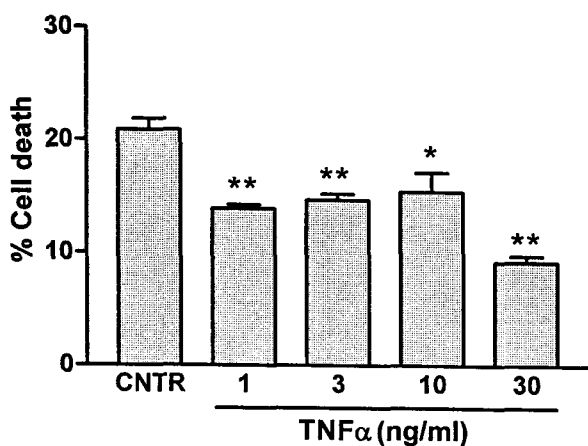
#### Cell death assessment

Quantitation of cell death is done by measuring released lactate dehydrogenase (LDH) from membrane-damaged cells. Assessment is performed on the base of death ratio, released LDH in media for 20 hours from serum-deprived or drug treated cells per total LDH measured after cells were completely destroyed by 1% triton X-100. LDH released from cells was reacted with pyruvic acid and  $\beta$ -nicotinamide adenine dinucleotide, reduced form. The extent of BCEC death as % of total cell death was defined by the following formula:

$$\% \text{ cell death} = \frac{\text{LDH released from damaged cells into the media}}{\text{Total LDH after lysis of cells with triton X-100}}$$

#### Nitrite measurement

Nitric oxide is measured indirectly by using Griess



**Fig. 1.** Effect of TNF $\alpha$  on serum-deprivation induced BCECs death. TNF $\alpha$  was treated for 1 hour and then cell death assessment was performed 19 hours later. Extent of cell death was designated as ratio of released LDH in media and total LDH measured after when cells were completely destroyed by 1% triton X-100. Values are means  $\pm$  SE. \* $P$ <0.05, \*\* $P$ <0.01 compared to serum-deprivation control (CNTR)

reaction (Follett and Ratcliff, 1963). 4 M HCl was added to supernatant and after ten minutes, 2 mg/ml sulfanilic acid and 1 mg/ml N-(1-naphtyl)-ethylenediamine were added. After incubation for 30 min, absorbance was measured in spectrophotometer in the wavelength of 550 nm. Absorbance of a sample was compared with a standard sodium nitrite.

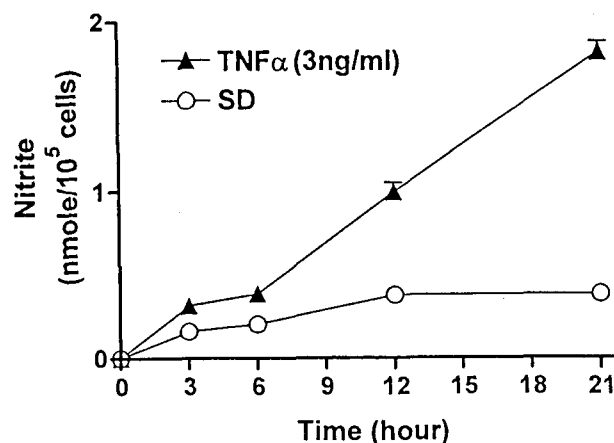
#### Statistical analysis

All values were expressed as means  $\pm$  standard errors of means. Analysis of variance was used to assess the differences between multiple groups. If the F statistic was significant, the mean values obtained from each group were then compared by Scheffe's method. Differences were considered to be statistically significant when P values were less than 0.05.

## RESULT

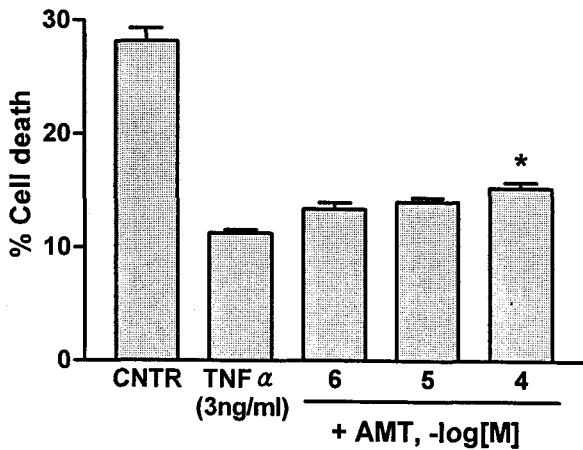
#### Effect of TNF $\alpha$ on cell death induced by serum-deprivation

When serum deprivation was continued to 20 hours, cell death ratio amounted to 20~30%. In contrast, when 10% FCS was present, cell death ratio up to 20 hours was below 10% in preliminary experiment. This value in serum condition is in part due

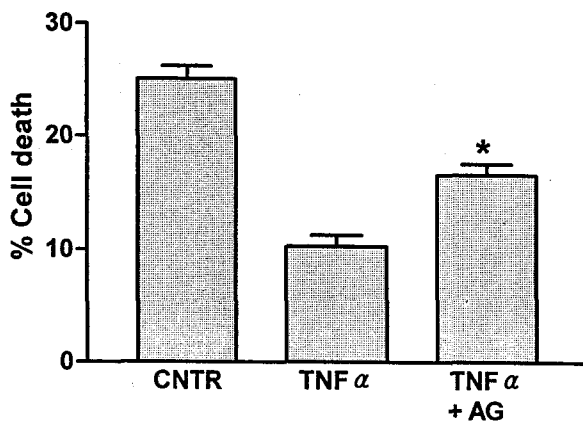


**Fig. 2.** Nitrite formation by TNF $\alpha$ . BCECs were incubated with TNF $\alpha$  in media up to 21 hours. At the time indicated, nitrite was determined in supernatant using Griess reaction. Values are means  $\pm$  SE. SD; serum-deprivation

to LDH originally present in FCS irrespective of cell membrane destruction.  $TNF\alpha$  was treated for 1 hour in the serum-deprived media and then the media were changed with fresh serum-deprived media. Cell death was assessed 20 hours later. Cell death ratios were decreased to 10~15% by  $TNF\alpha$  treatment in the range of 1~30 ng/ml concentration compared with 20~30% death ratios in serum-deprived condition (Fig. 1).



**Fig. 3.** Effect of AMT on reduction of BCECs death treated with  $TNF\alpha$ . 2-Amino-5,6-dihydro-6-methyl-4H-1,3 thiazine (AMT) was added after 1 hour treatment of  $TNF\alpha$ . Cell death assessment was done at 20 hours. Values are means  $\pm$  SE. \* $P$ <0.05 compared to serum-deprivation control (CNTR)

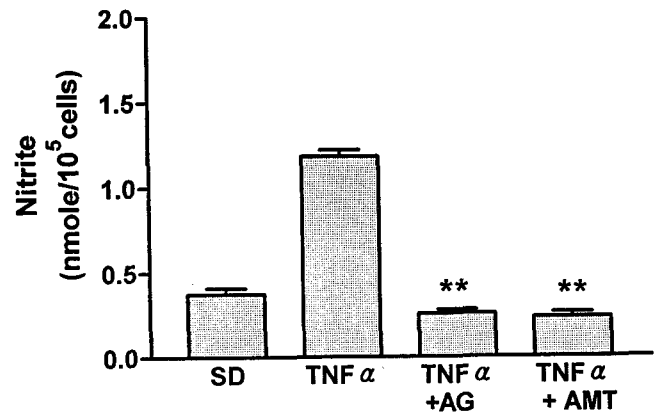


**Fig. 4.** Effect of aminoguanidine on  $TNF\alpha$ -rescued BCECs death. Aminoguanidine was added after 1 hour treatment of  $TNF\alpha$ . Cell death assessment was done at 20 hours. Values are means  $\pm$  SE. \* $P$ <0.05 compared to serum-deprivation control (CNTR)

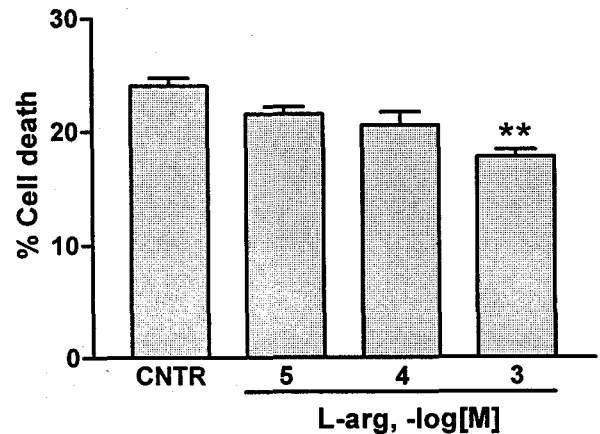
When  $TNF\alpha$  was treated in the serum-deprived media for 21 hour, nitrite formation was significantly increased compared to that of serum deprivation control (Fig. 2).

*Effect of iNOS inhibitors and NO substrate*

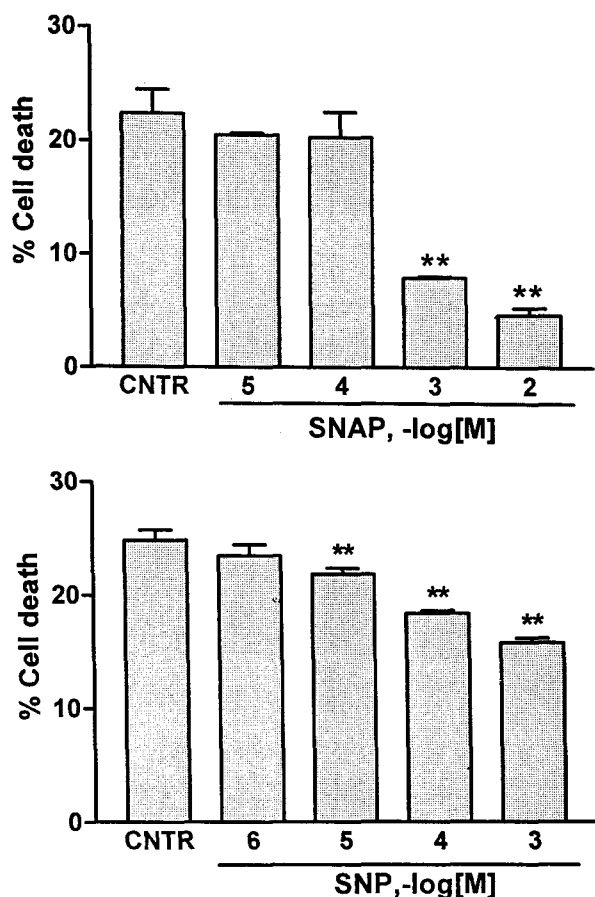
iNOS inhibitors were added to changing fresh media after 1 hour treatment of  $TNF\alpha$ . Among NOS



**Fig. 5.** Effect of iNOS inhibitors on production of NO by  $TNF\alpha$ . After 1 hour  $TNF\alpha$  treatment, iNOS inhibitors were added to changing serum-deprived media. Nitrite in media was measured spectrophotometrically at 20 hours. Values are means  $\pm$  SE.



**Fig. 6.** Effect of L-arginine on serum-deprivation induced cell death. L-arginine was treated for 20 hours, and then cell death assessment was performed. Values are means  $\pm$  SE. \*\* $P$ <0.01 compared to serum-deprivation control (CNTR)

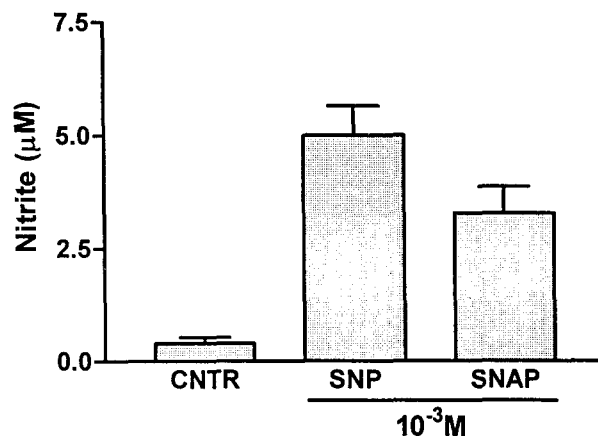


**Fig. 7.** Effect of NO donors on serum-deprivation induced cell death. After 20 hours incubation with sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP) of various concentration, cell death was assessed. Values are means  $\pm$  SE. \* $P < 0.05$ , \*\* $P < 0.01$  compared to serum-deprivation control (CNTR).

inhibitors, iNOS selective inhibitors, aminoguanidine or AMT, were used. Twenty hours later from TNF  $\alpha$  treatment, so as to 19 hours later after addition of iNOS inhibitor, cell death assessment was performed. AMT reversed the cell death ratio dose-dependently which would have been reduced by TNF  $\alpha$  (Fig. 3). Aminoguanidine also reversed the effect of TNF  $\alpha$  on cell death (Fig. 4). However, reversal effect of aminoguanidine ( $10^{-4}$ M) and AMT ( $10^{-4}$ M) was partial.

After iNOS selective inhibitors, aminoguanidine or AMT, were added to changing media after 1 hour stimulation, nitrite formation was near completely inhibited by these agents up to 20 hours (Fig. 5).

Furthermore, we were also able to see that L-arginine, iNOS substrate, decreased the cell death ratio



**Fig. 8.** Nitrite formation from NO donors at same concentration. Sodium nitroprusside (SNP) or S-nitroso-N-acetylpenicillamine (SNAP) was incubated in DMEM media for 20 hours, and then nitrite in supernatant was determined. Values are means  $\pm$  SE. CNTR; serum-deprivation control

when the concentration was increased up to  $10^{-3}$ M (Fig. 6). Seeing that D-arginine never reduced the cell death ratio in endothelial cells, the possible intrinsic effect of L-arginine was excluded (data not shown).

#### *Effect of NO donors on serum-deprivation induced cell death*

NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP), were added into the media to see the effect of NO on bovine cerebral endothelial cell death induced by serum-deprivation

Treatment of SNP or SNAP for 20 hours suppressed the cell death induced by serum-deprivation in a dose-dependent manner up to  $10^{-3}$ M (Fig. 7). When we checked the amount of nitrite formation after 20 hours of SNP or SNAP treatments, both increased nitrite concentration about 24 and 36 folds respectively compared to control (Fig. 8).

## DISCUSSION

In bovine cerebral endothelial cells, we showed that NO had protective effect on cell death induced by serum deprivation and induction of iNOS by NF- $\kappa$ B activation is, at least, a part of cell protection mechanism of TNF  $\alpha$ .

Both cytoprotective and cytotoxic effect were reported for TNF  $\alpha$ . The possibility is discussed that role of TNF  $\alpha$  in cell viability is determined upon signal pathway predominantly regulated by it and type of cell used. Although many TNF  $\alpha$  related receptor family and pathway were elucidated, the effect of TNF  $\alpha$  on cell viability was not clearly demonstrated yet. In the previous report, we showed that TNF  $\alpha$  and PMA prevented serum-deprivation induced cell death (Ahn et al, 1996) and NF- $\kappa$ B activation could be a cell protective mechanism rather than eliciting apoptotic pathway. However, target molecule that is responsible for cytoprotective effect of NF- $\kappa$ B activation was not identified in that study.

iNOS is one of the molecules whose transcription is regulated mainly by NF- $\kappa$ B. Therefore, the role of NO produced by iNOS induction was examined in this study on serum-deprivation apoptotic model of bovine cerebral endothelial cells. TNF  $\alpha$  was treated only for 1 hour and media were changed 3 times with serum depleted media with very gentle manner, because washing itself may increase the cell death ratio.

At first to ascertain that iNOS was really induced to produce large amount of NO, we measured the nitrite, a metabolite of NO which is commonly used as indirect index of NO production. We could see that nitrite was increased continuously up to 21 hours after TNF  $\alpha$  treatment.

Addition of iNOS inhibitors, aminoguanidine and AMT, increased cell death compared with non-treated control. Many iNOS inhibitors have drawback that their action mechanism is unclear, so in some cases iNOS inhibitors act as substrate of iNOS rather than as inhibitor. In our experiments, iNOS inhibitors were treated after 1 hour incubation of TNF  $\alpha$ . And NO production seem to be almost completely suppressed by iNOS inhibitors when nitrite, reflecting NO production, was measured. Possibility that intrinsic activity of iNOS inhibitors may affect cell viability was excluded, seeing that iNOS inhibitors themselves didn't change cell death rate compared with serum deprivation control (data not shown).

Addition of nitric oxide donor, sodium nitroprusside, to serum deprived media diminished the cell death compared to serum-deprivation control. It occurs as dose dependent manner. Because sodium nitroprusside contain cyanide in its structure, possible toxic effect of it should take into consideration. Therefore, another nitric oxide donor, S-nitroso-N-acetylp-

icillamine, which is different in structure from sodium nitroprusside, was used. In this case, cell death was also blocked. This suggest that NO released from NO donor has protective role in serum-deprivation induced cell death process. Similar results with NO donors were reported in neuron (Farinelli et al, 1996), eosinophil (Beauvais et al, 1995), and B lymphocytes (Genaro et al, 1995).

In this study, iNOS inhibitors partially reversed the protective effect of TNF  $\alpha$  in bovine cerebral endothelial cells. Similar result was reported by Chun *et al* (1995) that the protective effect of IL-1 $\beta$  as ovarian survival factor is partially mediated via NO. Therefore, iNOS expression and subsequent production of nitric oxide seemed to be partially responsible for protective effect in this paradigm.

Although NF- $\kappa$ B is regarded as cell survival factor, controversy is still present on the role of NF- $\kappa$ B. This is mainly due to the fact that which molecule mediate it's cell protective or cytotoxic effect is unknown. In our previous report, NF- $\kappa$ B activation is well correlated with cell viability and inhibition of NF- $\kappa$ B activation lead to cell death (Ahn et al, 1996). Moreover, in this study, NO under the regulation of NF- $\kappa$ B activation and iNOS induction has partial protective effect on serum-deprivation induced cell death. This result suggest that iNOS induction may be one of the cell protective mechanism. Since iNOS inhibitors have partial reversal action on effect of TNF  $\alpha$  on serum deprivation, other mechanisms for cell protective effect of these agents may be present possibly in the up- or down- stream of NF- $\kappa$ B activation. So investigation of other mechanisms are crucial in understanding the full context of cell survival signaling in conjunction with NF- $\kappa$ B activation.

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