

Inhibition of NF- κ B Activation Increases Oxygen-Glucose Deprivation-Induced Cerebral Endothelial Cell Death

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Increasing evidences suggest that ischemia-induced vascular damage is an integral step in the cascade of the cellular and molecular events initiated by cerebral ischemia. In the present study, employing a mouse brain endothelioma-derived cell line, bEnd.3, and oxygen-glucose deprivation (OGD) as an *in vitro* stroke model, the role of nuclear factor kappa B (NF- κ B) activation during ischemic injury was investigated. OGD was found to activate NF- κ B and to induce bEnd.3 cell death in a time-dependent manner. OGD phosphorylated neither 32 Ser nor 42 Tyr of I κ B α . OGD did not change the amount of I κ B α . The extents of OGD-induced cell death after 8 h, 10 h, 12 h and 14 h of OGD were 10%, 35%, 60% and 85%, respectively. Reperfusion following OGD did not cause additional cell death, indicating no reperfusion injury after ischemic insult in cerebral endothelial cells. Three known as NF- κ B inhibitors, including pyrrolidine dithiocarbamate (PDTC) plus zinc, aspirin and caffeic acid phenethyl ester (CAPE), inhibited OGD-induced NF- κ B activation and increased OGD-induced bEnd.3 cell death in a dose dependent manner. There were no changes in the protein levels of bcl-2, bax and p53 which are modulated by NF- κ B activity. These results suggest that NF- κ B activation might be a protective mechanism for OGD-induced cell death in bEnd.3.

Key Words: Cerebral endothelial cell, Oxygen-glucose deprivation, Cell death, Nuclear factor kappa B

INTRODUCTION

A stroke occurs when the blood supply to the brain is disturbed in some way. As a result, brain cells are starved of oxygen, glucose and other nutrients. Some of the starved cells die and others are damaged, and neurologic deficits caused by stroke are mainly due to neuronal death and damage. However, there is increasing evidence that cerebral endothelial cells play important roles in the pathophysiology of stroke. First, ischemia induces the expression of leukocyte adhesion receptors such as P-selectin, intercellular adhesion molecule-1 (ICAM-1), and E-selectin on endothelium which mediates cerebral inflammation, thus exacerbating cerebral tissue injury (de Vries et al, 1997; del Zoppo & Hallenbeck, 2000). Breakdown of blood-brain barrier (BBB) is another pathophysiologically important event which occurs in ischemia on endothelium (Betz, 1996). This permeability barrier is formed by endothelial cell, inter-endothelial cell tight junction, basal lamina, and astrocyte end foot. BBB breakdown occurs due to loosening of inter-endothelial tight junctions, loss of basal lamina constituents and endothelial cell deaths, and leads to cerebral edema and hemorrhagic transformation which may be lethal events (Betz, 1996;

Hamann et al, 1999). Loss of basal lamina is the results of activation of plasminogen, secretion of matrix metalloprotease and release of granule enzymes from leukocytes (Sappino et al, 1993). However, there are few studies on the mechanism of cerebral endothelial cell death during cerebral ischemia.

Nuclear factor kappa B (NF- κ B) is originally identified as a transcription factor which stimulates the transcription of immunoglobulin kappa light chain in B lymphocytes (Sen & Baltimore, 1986). However, it is a ubiquitous transcription factor and has the important roles in immune responses and control of apoptosis (Baeuerle & Baltimore, 1996), and comprises homo- or hetero-dimer of p65, p50, p52, cRel and RelB. NF- κ B also acts as cell death factor or cell survival factor by transcriptional activation of various genes related to cell death and survival such as bcl-2 families, p53, and MnSOD (Mattson et al, 2000). In non-neuronal systems, NF- κ B is usually considered as a cell survival factor, but in neuronal system (Karin, 1998), especially in ischemic condition, remains the question of whether NF- κ B is a cell survival factor or a cell death factor (Clemens, 2000). Also, little is known about the role of NF- κ B in cerebral endothelial cell death induced by ischemia. In the present study, we sought to elucidate the

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ABBREVIATIONS: BBB, blood-brain barrier; NF- κ B, nuclear factor kappa B; OGD, oxygen-glucose deprivation; MnSOD, Manganese superoxide dismutase; PDTC, pyrrolidine dithiocarbamate; CAPE, caffeic acid phenethyl ester.

role of NF- κ B in cerebral endothelial cell, bEnd.3 cell, death by oxygen-glucose deprivation (OGD), an *in vitro* stroke model.

METHODS

Cell culture

A mouse cerebral endothelioma cell line, bEnd.3 was maintained in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37°C under a humidified atmosphere of 5% CO₂ in air.

OGD

Confluent bEnd.3 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) containing a mixed gas composed of 5% CO₂, 10% H₂, and 85% N₂ after the culture medium was replaced and washed three times with deoxygenated balanced salt solution containing no glucose (deoxyBSS₀, 116 mM NaCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 5.4 mM KCl, 1 mM NaH₂PO₄ and 26.2 mM NaHCO₃, pH 7.4). Reperfusion was performed by returning the cultures to normoxic incubator under 5% CO₂/95% air and by replacing the culture media with BSS_{5.5} (5.5 mM glucose, not deoxygenated).

MTT assay

MTT (Sigma, St. Louis, MO, USA) in DMEM was added at a concentration of 1 mg/ml. After two hours of incubation, the cultured cell was lysed with extraction solution (25% dimethylformamide, 10% SDS) and incubated for 12 hours. Absorbance was at 540 nm read in a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). In some experiments, reperfusion was performed for two hours in order to recover from energy failure caused by OGD.

Propidium iodine (PI) stain

Propidium iodine (Sigma, St. Louis, MO, USA) at 1 μ g/ml was added to the culture medium. After 30 min incubation, cells were examined under a fluorescence microscope.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method described previously (Lee et al, 1988) with modification (Xu et al, 1997). For EMSA, the following oligonucleotide with the NF- κ B consensus binding sequence was used: (5'-AGTTGAGGGGACTTTCCAGGC-3'). A mutant motif with G to C substitution (5'-AGTTGAGGCGACTTTCCAGGC-3') served as a control. Labeling of the oligonucleotide with γ -³²P-ATP and the EMSA method have previously been detailed (Xu et al, 1997). Reactions were started by addition of nuclear extracts and allowed for 30 min at room temperature. Samples were loaded on 4% polyacrylamide non-denaturing gel and electrophoresed for 2 h at 180V. The dried gel was exposed to Kodak XR5 film on intensifying screen for 10~20 h at -70°C. For competition assay, an unlabeled NF- κ B oligonucleotide at 100-fold excess was added. For supershift assay, 1 μ l of anti-p65 or anti-p50 antibodies (Santa Cruz Biotechnology, Santa Cruz,

CA, USA) was incubated with nuclear extract for 30 min in room temperature prior to binding reaction.

Western blot

Cells were rinsed with ice-cold PBS and harvested with a cell scraper. Cells were pelleted by centrifugation, and the cell pellet was resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride]. After centrifugation (15,000 rpm for 15 min at 4°C), equal amount of proteins from each supernatant were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Anti-p53 (Neomarker, Fremont, CA, USA), anti-bcl-2 and anti-bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used as primary antibodies, and corresponding horseradish peroxidase-conjugated mouse IgG and rabbit IgG were used as secondary antibodies. ECL reagent (Amersham, Bucking hamshire, UK) was used for antigen detection in all experiments.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 100 mM phosphate buffer, washed twice and permeabilized with 0.2% Triton X-100. Non-specific binding was blocked by 1% bovine serum albumin. Anti-p65 and biotinylated anti-rabbit antibodies were used as primary and secondary antibodies, respectively. Biotinylated peroxidase-avidin complex was added, and cells were finally stained with 0.5 mg/ml diaminobenzidine for 5 min.

RESULTS

OGD induced bEnd.3 cell death, but no reperfusion injury

Fig. 1 shows the phase contrast morphology (Fig. 1A, C) and propidium iodine staining of bEnd.3 cells exposed to BSS_{5.5} (control group) and OGD for 14 h. Control culture showed normal cell bodies, bright cell margin, and no cells stained by PI (Fig. 1B). However, 14-h OGD led to almost 100% cell death, confirmed by PI staining (Fig. 1D). The extents of OGD-induced cell death after 8 h, 10 h, 12 h and 14 h of OGD were 10%, 35%, 60% and 85%, respectively (Fig. 2A).

In many experiments using *in vitro* or *in vivo* ischemia, reperfusion exacerbated ischemic neuronal injury (Bulkley, 1987). This reperfusion injury can occur in clinical situations of stroke and limit the indications of revascularization therapy. Therefore, it was of importance to examine whether reperfusion exacerbated OGD-induced cell death in vascular endothelial cells. As shown in Fig. 2B, reperfusion following OGD did not further increase the extent of OGD induced death of bEnd.3 cells. Therefore, in the following experiments, we used OGD model without reperfusion instead of OGD/reperfusion paradigm.

OGD activated NF- κ B

The DNA binding activity of NF- κ B, assayed by EMSA, gradually increased up to 6 h of OGD (Fig. 3A), and the specificity of DNA binding was confirmed by competition assay (Fig. 3B). Unlabeled oligonucleotides of wild type and

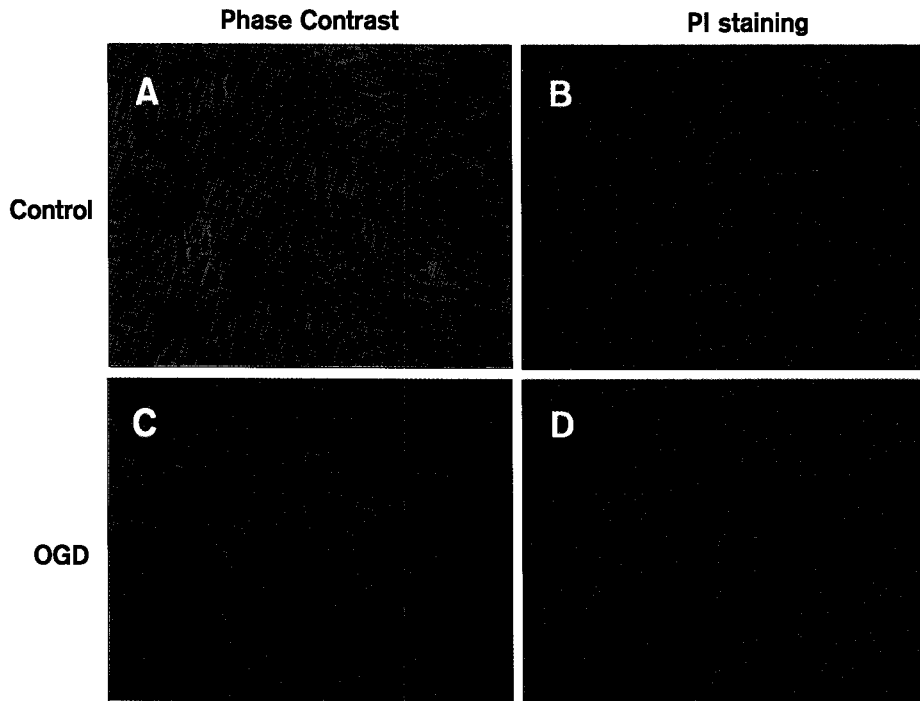


Fig. 1. OGD-induced bEnd.3 cell death. (A, C) Phase contrast image of control and 14-h OGD group. (B, D) Propidium iodine staining of control and 14-h OGD group.

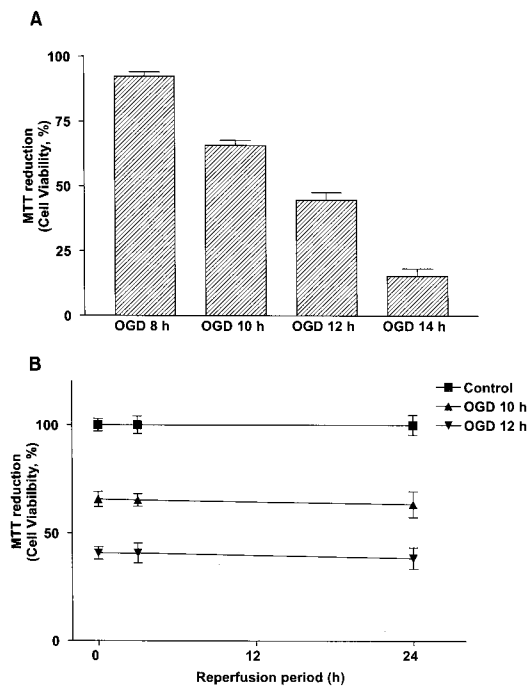


Fig. 2. Extent of OGD-induced bEnd.3 cell death. (A) bEnd.3 cells were treated with OGD for 8 h, 10 h, 12 h and 14 h. Subsequently, the extent of cell death was assayed by MTT method. (B) Reperfusion following 0 h-, 10 h- and 12 h-OGD was performed up to 24 h. The extent of cell death was assayed by MTT method.

mutant NF- κ B, AP-1 and SP-1 in 100-fold excess were used in binding reaction. The NF- κ B-DNA complex signal disappeared only by wild type oligonucleotide, indicating that the two bands were NF- κ B specific. Supershift assay showed that the two bands contained p50 and p65 subunits of NF- κ B (Fig. 3C). Immunocytochemistry was carried out with anti-p65 antibody to confirm nuclear translocation of NF- κ B by OGD. As seen in Fig. 3D and E, nuclear translocation of p65 by OGD was observed, providing another evidence of NF- κ B activation by OGD.

I κ B α was neither phosphorylated on 32Ser, nor degraded

To elucidate the mechanism of OGD-induced NF- κ B activation, we assayed the phosphorylation of I κ B α and subsequent degradation of I κ B α , which are considered as the mechanism of TNF α -induced NF- κ B activation. As shown in Fig. 4A, I κ B α was phosphorylated on 32Ser and degraded upon TNF α treatment, as expected. In the case of OGD-induced NF- κ B activation, however, I κ B α was neither phosphorylated on 32Ser nor degraded (Fig. 4B). It was reported that 42Tyr of I κ B α could be phosphorylated by hypoxia and in this circumstances, NF- κ B was activated without degradation of I κ B α (Imbert et al, 1996; Koong et al, 1994). Therefore, we assayed whether OGD could phosphorylate I κ B α on 42Tyr in endothelial cells. Cell lysates were immunoprecipitated with anti-I κ B α antibody and were immunoblotted with anti-phospho tyrosine antibody. In this experiment, OGD did not phosphorylate I κ B α on 42Tyr, while pervanadate, a tyrosine phosphatase inhibitor, did (Fig. 4C).

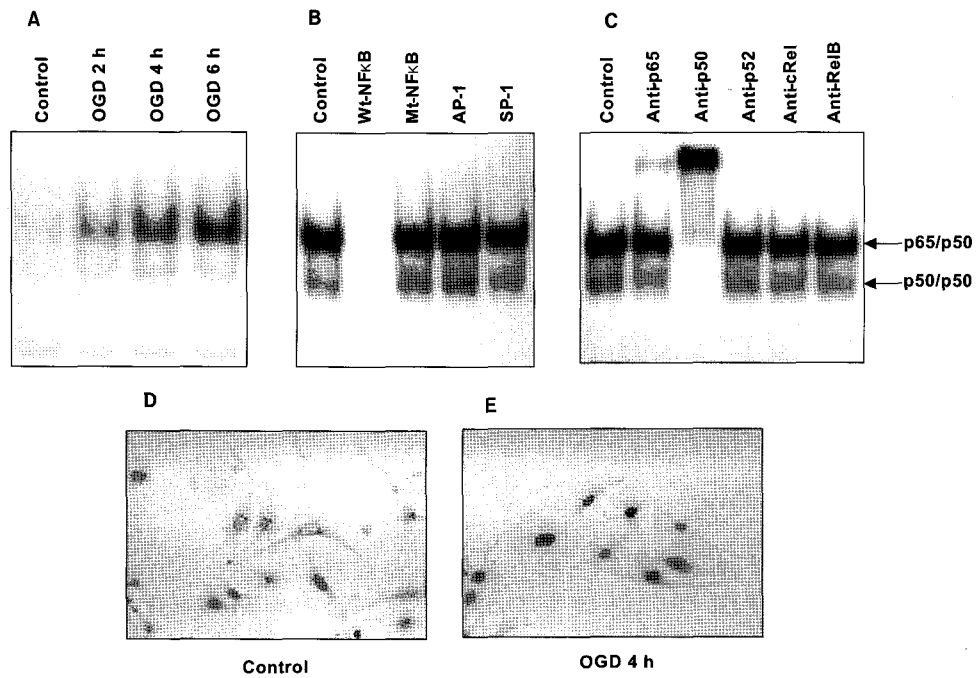


Fig. 3. OGD-induced NF- κ B activation. (A) Time course of OGD-induced NF- κ B activation. bEnd.3 cells were treated with OGD. At the time indicated, cells were washed twice with cold PBS and harvested. EMSA was performed as described in the text. (B) Competition assay. Various unlabeled oligonucleotides indicated above at 100-fold excess were added in the probe-DNA binding reactions. (C) Supershift assay. Supershift experiments were performed by adding anti-p65, anti-p50, anti-p52, anti-cRel and anti-RelB antibodies to the binding reactions. (D, E) Immunocytochemistry of OGD-induced nuclear translocation of p65. bEnd.3 cells were cultured on glass cover slip. After 4 h treatment with OGD, the cells were fixed and immunostained by anti-p65 antibody followed by biotinylated anti-rabbit antibody. The stained cultures were observed under light microscope.

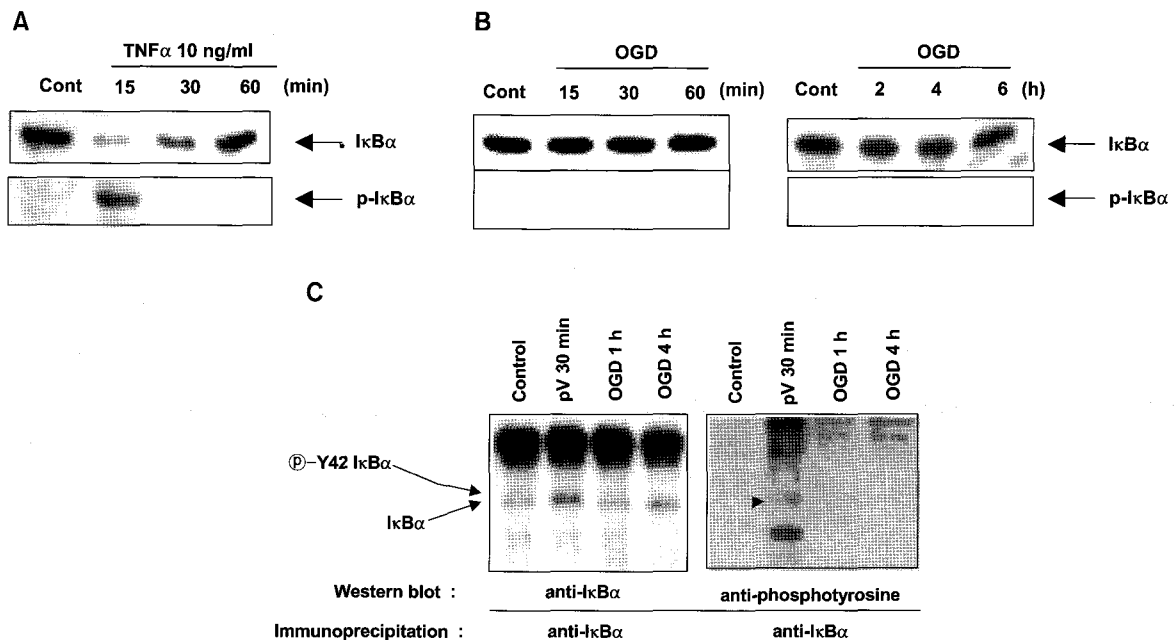


Fig. 4. I κ B α phosphorylation on 32Ser or 42Tyr and degradation by OGD. (A, B) Western blots with anti-I κ B α and anti-32 Ser-phospho-I κ B α antibodies. The treatments of TNF α and OGD were performed as indicated above. (C) Cells were treated as indicated above, harvested and lysed. Pervanadate (pV) treatment was performed by addition of 100 μ M sodium vanadate and 0.5 μ M hydrogen peroxide. The whole cell lysates were immuno precipitated with anti-I κ B α antibody and immunoblotted with anti-I κ B α antibody and anti-phosphotyrosine antibody. Arrow head shows I κ B α having phosphotyrosine.

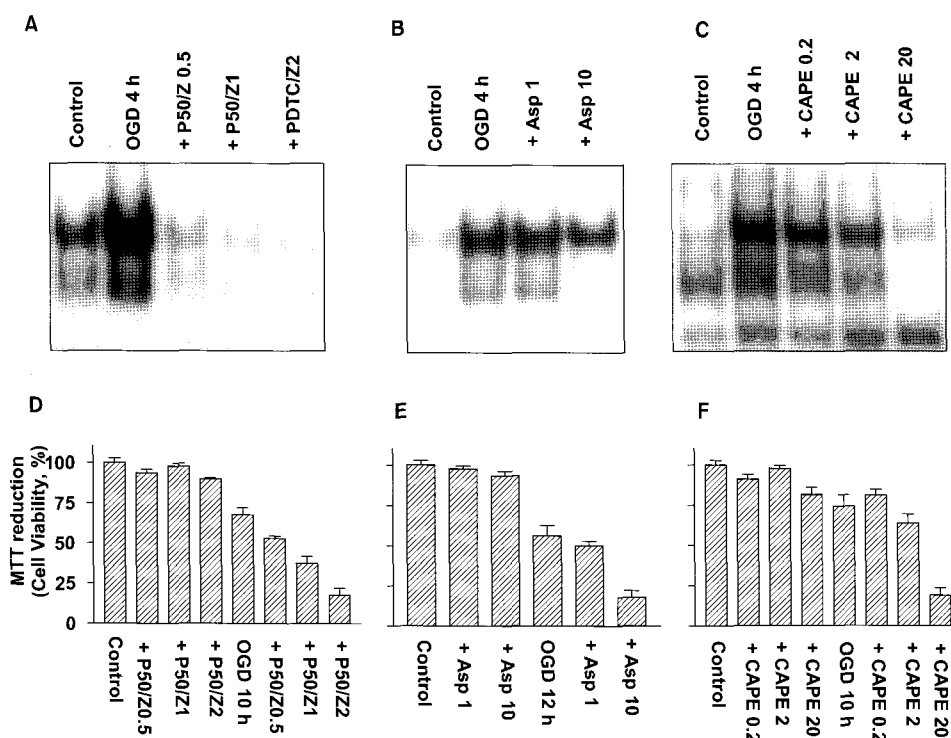


Fig. 5. Effects of PDTC plus zinc on OGD-induced NF- κ B activation and cell death. (A, B, C) EMSA of NF- κ B. OGD was carried out with or without following drugs: 50 μ M PDTC plus 0.5 to 1 μ M zinc sulfate (P50/Z0.5, P50/Z1), 1 to 10 mM aspirin (Asp1, 10) and 0.2 to 20 μ M CAPE (CAPE 0.2, 2, 20). After treatments, nuclear proteins were extracted and EMSA was performed. (D, E, F) MTT assay. After the treatments as indicated above, the extent of cell death was assayed by MTT method.

Effects of three NF- κ B inhibitors on OGD-induced NF- κ B activation and bEnd.3 cell death

PDTC is a well-known inhibitor of NF- κ B, and we recently reported that PDTC inhibition of NF- κ B activation required zinc (Kim et al, 1999). Therefore, we used PDTC plus zinc as a NF- κ B inhibitor, and Fig. 5A and D show that PDTC plus zinc inhibited NF- κ B activation caused by OGD and exacerbated OGD-induced bEnd.3 death. The mechanism of aspirin, an another NF- κ B inhibitor, is thought to be competitive inhibition against ATP binding to I κ B kinase (IKK) (Kopp and Ghosh, 1994). As seen in Fig. 5B and E, aspirin treatment inhibited NF- κ B activation caused by OGD and increased OGD-induced bEnd.3 cell death. Caffeic acid phenethyl ester (CAPE) is antioxidant and has also been shown to be a potent inhibitor of NF- κ B (Natarajan et al, 1996). The treatment of endothelial cells with CAPE also showed inhibition of NF- κ B activation and augmentation of cell death (Fig. 5C, F). These results imply that OGD-induced NF- κ B activation of bEnd.3 cells would be a cell survival factor and that elimination of this factor led to exacerbation of cell death.

OGD did not change the amounts of bcl-2, bax and p53

To elucidate the mechanism of NF- κ B to act as a cell survival factor, we observed the changes in protein levels of bcl-2, bax and p53, which are well-known cell survival/death factors and whose expressions are regulated

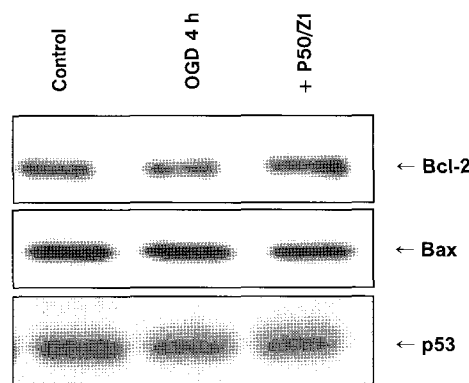


Fig. 6. Effects of PDTC plus zinc on the amounts of bcl-2, bax and p53. OGD was performed for 4 h with or without 50 μ M PDTC plus 1 μ M zinc sulfate (P50/Z1). After treatments, cells were washed with cold PBS and lysed. The whole cell lysates were analyzed for bcl-2, bax and p53 abundance by Western blot.

by NF- κ B activity. However, OGD and OGD with PDTC plus zinc did not change the level of any of the three factors (Fig. 6).

DISCUSSION

Cerebral endothelial cell dysfunction plays important

roles in the pathophysiology of stroke. In the present study, we focused on cerebral endothelial cell death and the role of NF- κ B activation using *in vitro* model, OGD. The results showed that NF- κ B was activated in response to OGD. NF- κ B activation by various stimuli, including TNF α , usually occurred within 1 h and was terminated within 2 hrs after stimulation (Schmitz & Baeuerle, 1995). However, NF- κ B activation induced by OGD in the present experiment increased gradually up to 6 h. This phenomenon suggested that OGD might have a mechanism of NF- κ B activation different from those of typical stimulators. Therefore, the mechanism of OGD-induced NF- κ B activation was investigated in more detail. As shown above, NF- κ B was activated by OGD without degradation of I κ B α , following phosphorylation on 32Ser which was considered to be a typical mechanism of NF- κ B activation by TNF α . Earlier studies showed that hypoxia activated NF- κ B through dissociation of I κ B α from NF- κ B without degradation which was due to phosphorylation of I κ B α on 42Tyr (Koong et al, 1994; Imbert et al, 1996). We assayed I κ B α phosphorylation on 42Tyr, however, could not observe the 42Tyr phosphorylation by OGD. Since several studies suggested that I κ B β was involved in long-lasting activation NF- κ B (Johnson et al, 1996; Phillips & Ghosh, 1997), the possibility of I κ B β involvement in NF- κ B activation by OGD remains to be studied.

When NF- κ B activation was inhibited by three inhibitors, PDTC plus zinc, aspirin and CAPE, OGD-induced cell death of bEnd.3 increased, suggesting that OGD-induced NF- κ B activation could be a cell survival factor. Many genes responsive to NF- κ B activation play roles of cell survival or death factors. Cell survival factors include bcl-2, bcl-xL, MnSOD and IAPs, and cell death factors include p53, bax, iNOS and leukocyte adhesion molecules (Clemens, 2000). It was reported that hypoxia-induced endothelial apoptosis occurred through NF- κ B-mediated bcl-2 suppression which implied NF- κ B as a cell death factor (Matsushita et al, 2000). In the present study, however, aortic endothelial cells were used instead of cerebral microvascular endothelial cells which have unique features. In contrast, there are several reports that bcl-2 was upregulated by NF- κ B activation (Heckman et al, 2002; Poulaki et al, 2002). However, in the present study, we could not observe changes in the amounts of bcl-2, bax and p53 by OGD. Other possible molecules remain to be investigated.

Inducible nitric oxide synthase (iNOS) has been shown to be upregulated by OGD and resulting increase of nitric oxide induces cerebral endothelial cell death (Xu et al, 2000). iNOS is upregulated by NF- κ B activation (Bereta et al, 1995). These considerations suggest that NF- κ B might be a cell death factor. Nitric oxide cytotoxicity is due to peroxynitrite formed from superoxide (Pryor & Squadrito, 1995; Squadrito and Pryor, 1995), however, several studies showed that iNOS could play a role of cell survival factor (Kim, 1997; Suschek et al, 1999). Therefore, the question of whether iNOS could act as a cell survival factor in OGD-induced bEnd.3 cell death is another topic to be studied.

Aspirin may be used to prevent recurrence of stroke (Carolei et al, 1986; Grotta et al, 1985; Joseph et al, 1992). Clinically, aspirin is expected to act as an antiplatelet agent via inhibition of cyclooxygenase (Pedersen and FitzGerald, 1984). However, it is also important to consider that aspirin may act as a NF- κ B inhibitor. Based on our data together

with findings by others, this property of aspirin may cause a disastrous event; hemorrhagic transformation through exacerbation of cerebral endothelial cell death caused by ischemia. Hemorrhagic transformation may outweigh suggested neuroprotective effects of aspirin (Grilli et al, 1996). Therefore, it is very important to consider that administration of aspirin in order to prevent stroke recurrence may be harmful in certain conditions.

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REFERENCES

- Baeuerle PA, Baltimore D. NF-kappa B: ten years after. *Cell* 87: 13–20, 1996
- Bereta J, Cohen MC, Bereta M. Stimulatory effect of ouabain on VCAM-1 and iNOS expression in murine endothelial cells: involvement of NF-kappa B. *FEBS Lett* 377: 21–25, 1995
- Betz AL. Alterations in cerebral endothelial cell function in ischemia. *Adv Neurol* 71: 301–311, 1996
- Bulkley GB. Free radical-mediated reperfusion injury: a selective review. *Br J Cancer Suppl* 8: 66–73, 1987
- Carolei A, Prencipe M, Fiorelli M, Fieschi C. Severity of stroke and aspirin. *Neurology* 36: 1010–1011, 1986
- Clemens JA. Cerebral ischemia: gene activation, neuronal injury, and the protective role of antioxidants. *Free Radic Biol Med* 28: 1526–1531, 2000
- de Vries HE, Kuiper J, de Boer AG, Van Berkel TJ, Breimer DD. The blood-brain barrier in neuroinflammatory diseases. *Pharmacol Rev* 49: 143–155, 1997
- del Zoppo GJ, Hallenbeck JM. Advances in the vascular pathophysiology of ischemic stroke. *Thromb Res* 98: 73–81, 2000
- Grilli M, Pizzi M, Memo M, Spano P. Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* 274: 1383–1385, 1996
- Grotta JC, Lemak NA, Gary H, Fields WS, Vital D. Does platelet antiaggregant therapy lessen the severity of stroke? *Neurology* 35: 632–636, 1985
- Guarini L, Su ZZ, Zucker S, Lin J, Grunberger D, Fisher PB. Growth inhibition and modulation of antigenic phenotype in human melanoma and glioblastoma multiforme cells by caffeic acid phenethyl ester (CAPE). *Cell Mol Biol* 38: 513–527, 1992
- Hamann GF, del Zoppo GJ, von Kummer R. Hemorrhagic transformation of cerebral infarction-possible mechanisms. *Thromb Haemost* 82 Suppl 1: 92–94, 1999
- Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. *Oncogene* 21: 3898–3908, 2002
- Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 86: 787–798, 1996
- Johnson DR, Douglas I, Jahnke A, Ghosh S, Pober JS. A sustained reduction in I kappa B-beta may contribute to persistent NF-kappaB activation in human endothelial cells. *J Biol Chem* 271: 16317–16322, 1996
- Joseph R, Han E, Tsering C, Grunfeld S, Welch KM. Platelet activity and stroke severity. *J Neurol Sci* 108: 1–6, 1992
- Karin M. The NF-kappa B activation pathway: its regulation and role in inflammation and cell survival. *Cancer J Sci Am* 4 Suppl 1: S92–S99, 1998
- Kim CH, Ahn YS. Nitric oxide prevents the bovine cerebral endothelial cell death induced by serum-deprivation. *Korean J Physiol Pharmacol* 1: 515–521, 1997

- Kim CH, Kim JH, Hsu CY, Ahn YS. Zinc is required in pyrrolidine dithiocarbamate inhibition of NF-kappaB activation. *FEBS Lett* 449: 28–32, 1999
- Koong AC, Chen EY, Mivechi NF, Denko NC, Stambrook P, Giaccia AJ. Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res* 54: 5273–5279, 1994
- Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 265: 956–959, 1994
- Lee KA, Bindereif A, Green MR. A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal Tech* 5: 22–31, 1988
- Matsushita H, Morishita R, Nata T, Aoki M, Nakagami H, Taniyama Y, Yamamoto K, Higaki J, Yasufumi K, Ogihara T. Hypoxia-induced endothelial apoptosis through nuclear factor-kappaB (NF-kappaB)-mediated bcl-2 suppression: in vivo evidence of the importance of NF-kappaB in endothelial cell regulation. *Circ Res* 86: 974–981, 2000
- Mattson MP, Culmsee C, Yu Z, Camandola S. Roles of nuclear factor kappaB in neuronal survival and plasticity. *J Neurochem* 74: 443–456, 2000
- Natarajan K, Singh S, Burke TR, Jr., Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc Natl Acad Sci USA* 93: 9090–9095, 1996
- Pedersen AK, FitzGerald GA. Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. *N Engl J Med* 311: 1206–1211, 1984
- Phillips RJ, Ghosh S. Regulation of IkappaB beta in WEHI 231 mature B cells. *Mol Cell Biol* 17: 4390–4396, 1997
- Poulaki V, Mitsiades CS, Jousseen AM, Lappas A, Kirchhof B, Mitsiades N. Constitutive Nuclear Factor-kappaB Activity Is Crucial for Human Retinoblastoma Cell Viability. *Am J Pathol* 161: 2229–2240, 2002
- Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* 268: L699–L722, 1995
- Sappino AP, Madani R, Huarte J, Belin D, Kiss JZ, Wohlwend A, Vassalli JD. Extracellular proteolysis in the adult murine brain. *J Clin Invest* 92: 679–685, 1993
- Schmitz ML, Baeuerle PA. Multi-step activation of NF-kappa B/Rel transcription factors. *Immunobiology* 193: 116–127, 1995
- Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46: 705–716, 1986
- Squadrito GL, Pryor WA. The formation of peroxynitrite in vivo from nitric oxide and superoxide. *Chem Biol Interact* 96: 203–206, 1995
- Suschek CV, Krischel V, Bruch-Gerharz D, Berendji D, Krutmann J, Kroncke KD, Kolb-Bachofen V. Nitric oxide fully protects against UVA-induced apoptosis in tight correlation with Bcl-2 up-regulation. *J Biol Chem* 274: 6130–6137, 1999
- Xu J, He L, Ahmed SH, Chen SW, Goldberg MP, Beckman JS, Hsu CY. Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells. *Stroke* 31: 1744–1751, 2000
- Xu J, Wu Y, He L, Yang Y, Moore SA, Hsu CY. Regulation of cytokine-induced iNOS expression by a hairpin oligonucleotide in murine cerebral endothelial cells. *Biochem Biophys Res Commun* 235: 394–397, 1997