Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2009 | 108 | 475–485 doi: 10.1111/j.1471-4159.2008.05783.x

NF-κB p50/RelA and c-Rel-containing dimers: opposite regulators of neuron vulnerability to ischaemia

Ilenia Sarnico,* Annamaria Lanzillotta,* Flora Boroni,* Marina Benarese,* Manuela Alghisi,* Markus Schwaninger,† Ioana Inta,† Leontino Battistin,‡ PierFranco Spano*,§ and Marina Pizzi*,§

*Division of Pharmacology and Experimental Therapeutics, Department of Biomedical Sciences & Biotechnologies, School of Medicine, University of Brescia, Brescia, Italy

†Department of Neurology, University of Heidelberg, Heidelberg, Germany

‡Istituto Ricovero e Cura a Carattere Scientifico, S. Camillo Hospital, Venice, Italy

§National Institute of Neuroscience, Turin, Italy

Abstract

Diverse nuclear factor-κB subunits mediate opposite effects of extracellular signals on neuron survival. While RelA is activated by neurotoxic agents, c-Rel drives neuroprotective effects. In brain ischaemia RelA and p50 factors rapidly activate, but how they associate with c-Rel to form active dimers and contribute to the changes in diverse dimer activation for neuron susceptibility is unknown. We show that in both cortical neurons exposed to oxygen glucose deprivation (OGD) and mice subjected to brain ischaemia, activation of p50/RelA was associated with inhibition of c-Rel/RelA dimer and no change p50/c-Rel. Targeting c-Rel and RelA expression revealed that c-Rel dimers reduced while p50/RelA enhanced neuronal susceptibility to anoxia. Activation of p50/RelA complex is known to induce the pro-apoptotic *Bim* and *Noxa* genes. We now show that c-Rel-containing dimers, p50/c-Rel and RelA/c-Rel, but not p50/RelA, promoted Bcl-xL transcription. Accordingly, the OGD exposure induced *Bim*, but reduced Bcl-xL promoter activity and decreased the content of endogenous Bcl-xL protein. These findings demonstrate that within the same neuronal cell, the balance between activation of p50/RelA and c-Rel-containing complexes fine tunes the threshold of neuron vulnerability to the ischaemic insult. Selective targeting of different dimers will unravel new approaches to limit ischaemia-associated apoptosis.

Keywords: Bcl-xL, brain ischaemia, c-Rel, primary cortical neurons, RelA, SK-N-SH.

J. Neurochem. (2009) **108**, 475–485.

Nuclear factor-κB (NF-κB) transcription factor acts in the nervous system as a pleiotropic regulator of physiological processes associated with neurodevelopment (Bakalkin *et al.* 1993; Bhakar *et al.* 2002), synaptic activity and memory formation (O'Neill and Kaltschmidt 1997; Meffert *et al.* 2003; Levenson *et al.* 2004; Meffert and Baltimore 2005; O'Riordan *et al.* 2006; Ahn *et al.* 2008). However, NF-κB activation is also involved in the pathophysiology of neurological diseases associated with neurodegeneration (Clemens *et al.* 1997; O'Neill and Kaltschmidt 1997; Schneider *et al.* 1999), where a dual role of NF-κB as regulator of apoptosis has been demonstrated and widely discussed (Grilli *et al.* 1996; Kaltschmidt *et al.* 1999; Qin *et al.* 1999; Yu *et al.* 1999; Mattson and Camandola 2001; Pizzi and Spano 2006). Work over the last 10 years has shown that NF-κB is an inflammation and apoptosis regulator contributing to the subacute pathogenesis of post-ischaemic injury (Schneider *et al.* 1999; Nurmi *et al.* 2004; Herrmann *et al.* 2005; Crack *et al.* 2006). Cerebral ischaemia rapidly activates NF-κB in both neurons and glial cells. However, experiments in mice expressing a super-repressor form of IκBα in neurons or carrying a neuron-targeted deletion of IκB kinase 2 gave compelling evidence that NF-κB activation in neurons rather than in glia plays a pivotal role in the ischaemic damage (Zhang *et al.* 2005).

Received August 8, 2008; revised manuscript received November 6, 2008; accepted November 6, 2008.

Address correspondence and reprint requests to Marina Pizzi, Division of Pharmacology, Department of Biomedical Sciences & Biotechnologies, Viale Europa, 11, Brescia 25123, Italy.
E-mail: pizzi@med.unibs.it

Abbreviations used: BH3, Bcl2 homology 3; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; LF 2000, Lipofectamine 2000; MCAO, middle cerebral artery occlusion; mGlu5, metabotropic glutamate type 5; NF-κB, nuclear factor-κB; OGD, oxygen glucose deprivation; RA, retinoic acid; SDS, sodium dodecylsulfate; siRNA, small interfering RNA.

Nuclear factor-κB is a dimeric transcription factor that can be formed by the assembly of five diverse proteins, p50, p52, RelA (p65), RelB and c-Rel which, in combination, specify the dimer trancriptional activity (Hoffmann *et al.* 2003). By dissecting the different NF-κB components activated after brief exposure to pro- or anti-apoptotic stimuli, recent research has shown that the diverse NF-κB complexes are involved in neurodegeneration in different ways. While aberrantly activated p50/RelA dimers contribute to the apoptotic program induced by glutamate or β-amyloid, the c-Rel-containing dimers, p50/c-Rel and RelA/c-Rel, activated by interleukin-1β, S100B, agonists at metabotropic glutamate type 5 (mGlu5) receptor and leptin, increase the resistance of injured neuronal cells (Pizzi *et al.* 2002b, 2005; Kögel *et al.* 2004; Valerio *et al.* 2008). To analyse which subunit of NF-κB is involved in stroke, focal cerebral ischaemia was induced in mice with selective deletion of p50 (Schneider *et al.* 1999), p52 and c-Rel or with a conditional deletion of RelA (Inta *et al.* 2006). These studies showed that only mice with p50 or RelA deficiencies have a reduced infarct size when exposed to middle cerebral artery occlusion (MCAO).

To examine the reciprocal assembly of RelA, p50 and c-Rel subunits to form the NF-κB active dimers in response to ischaemia, together with the role of diverse complexes in modulating the neuronal vulnerability, we studied primary cortical cells exposed to oxygen glucose deprivation (OGD) and mice subjected to permanent MCAO. Our results indicate that the nuclear content of p50/RelA complex increases in neurons exposed to OGD as well as in ischaemic cortices of mice subjected to MCAO, while the level of RelA/c-Rel dimers decreases. No change was produced in p50/c-Rel content. The p50/RelA and c-Rel-containing dimers exert opposite modulation of the threshold of neuron vulnerability by diversely affecting the transcription of Bcl-2 family genes, respectively *Bim* and *Bcl-xL*, which play a major role as apoptosis regulators in brain ischaemia (Gillardon *et al.* 1996; Graham and Chen 2001; Cao *et al.* 2002; Inta *et al.* 2006).

By focusing on the specific role of different NF-κB components in neuronal cell survival, this study suggests that NF-κB selective inducers of c-Rel dimers as well as specific inhibitors of aberrantly activated p50/RelA complexes should have much higher beneficial effects in treating ischaemia than the general blockers of NF-κB pathway tested to date.

Material and methods

Cell culture

Primary cultures of mouse cortical neurons

C57BL/6 mice were purchased from Charles River (Calco, Italy). Fifteen-day embryonic mice were harvested with caesarean section from anaesthetized pregnant dams. Cerebral cortices were isolated and dissociated by manual dispersion with a fire-polished Pasteur pipette. Cells were plated at a density of 1.0×10^5 cells/cm^2 in 21-cm^2 culture dishes (Nunc, Langenselbold, Germany) for Western blot and ELISA analyses, and in 2-cm^2 tissues culture dishes for the viability studies. Cells were plated at the density of 1–1.5×10^5 cells/cm^2 in 8-cm^2 tissues culture dishes for transfection experiments. Culture dishes were coated with 10 μg/mL poly-L-lysine. The cells were plated in Neurobasal medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2% B27 (Invitrogen Corp.), 0.5 mM L-glutamine (EuroClone, Siziano, Italy) and 50 U/mL penicillin/streptomicin (Euroclone). Three days after plating, 50% of the medium was changed with fresh medium and, subsequently, 50% of the medium was changed twice a week until 11 days *in vitro* (DIV).

SK-N-SH cell culture

The human SK-N-SH neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA) and was grown at 37°C in a humidified atmosphere of 5% CO_2, 95% O_2 in Dulbecco's modified Eagle's medium (DMEM) medium (Euroclone), supplemented with fetal calf serum (Euroclone), 4 mM glutamine and 100 U/mL penicillin/streptomicin (DMEM+). When exposed to retinoic acid (RA), the human SK-N-SH cells underwent mitotic arrest and differentiated to a neuronal phenotype. For differentiation, SK-N-SH cells were incubated in DMEM+ containing 50 μM RA (Sigma, St. Louis, MO, USA) for 12–15 days, as previously described (Pizzi *et al.* 2002a). The cells were plated at a density of 2.5×10^5 cells in 8-cm^2 culture dishes (Nunc) for both transfection experiments and viability studies and 1.5×10^6 cells were plated in 21-cm^2 culture dishes for western blot analyses.

Cerebral ischaemia models

Middle cerebral artery occlusion

As *in vivo* model of permanent focal cerebral ischaemia, a distal MCAO was performed in mice, as previously described (Inta *et al.* 2006). Briefly, C57BL/6 mice ($n = 8$) were anaesthetized with i.p. injection of 150 μL of 2.5% Avertin (Tribromoethanol, Aldrich Chemical Co., Milwaukee, WI, USA) per 10 g of body weight. A skin incision was made between the ear and the orbit on the left side. The parotid gland and the temporal muscle were removed. The stem of the middle cerebral artery was occluded by microbipolar coagulation (Erbe, Tübingen, Germany), maintaining the body at a temperature of 37°C. The procedure was carried out under the microscope so that the occlusion of the middle cerebral artery was directly observed. For laser Doppler validation of the drop of cerebral blood flow, the probe (P415-205; Perimed, Piscataway, NJ, USA) was placed 3 mm lateral and 6 mm posterior to the bregma. Relative perfusion units were determined (Periflux 4001; Perimed).

In a separate cohort of animals, the femoral artery was cannulated to measure pH, pCO_2 and pO_2, emoglobine, glucose and mean arterial blood pressure, 15 min before and 15 min into MCAO. The physiological parameters were superimposable to those previously reported (Zhang *et al.* 2005; Inta *et al.* 2006). After 48 h, the mice were deeply re-anaesthetized with Avertin and perfused intracardially with Ringer's solution. Measurements of the infarct size were performed as previously described (Herrmann *et al.* 2003). A separate group of mice ($n = 4$) was killed 4 h after MCAO occlusion, and the cerebral cortices were rapidly dissected for NF-κB studies.

Oxygen glucose deprivation

Oxygen glucose deprivation was performed on 11 DIV cortical neurons and in differentiated SK-N-SH cells. Cells were incubated with warm deoxygenated glucose-free balanced salt solution [(containing in mM): 5.36 KCl, 116.35 NaCl, 0.81 $MgSO_4$ and 1.01 NaH_2PO_4) and transferred to an air-tight chamber fluxed with an anaerobic gas mixture (95% N_2 and 5% CO_2) for 10 min to remove oxygen. The oxygen concentration was <0.4% throughout the OGD period, as assessed by an oxygen analyzer (Servomex 580A, Taylor Servomex, Edenbridge, UK). Cortical neurons were exposed to OGD at 37°C for 3 h. At the end of anoxic incubation, cortical neurons were allowed to recover in Neurobasal medium containing 0.4% B27 supplement under normoxic conditions for 2, 4 and 6 h before protein extraction or for 2, 6 and 24 h for the cell death evaluation.

SK-N-SH neuronal cells were exposed to OGD for 3, 15 or 24 h. Thereafter, they were replaced in fresh DMEM without serum for 4 or 24 h before protein extraction or for 24 h for evaluation of cell viability. Control cell cultures were incubated with balanced salt solution containing 5.5 mM glucose and bubbled with an aerobic gas (95% CO_2 and 5% O_2) in a normal aerated incubator for the same periods. Neuronal injury was evaluated by measuring the amount of lactate dehydrogenase (LDH) released into the culture using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI, USA). Duplicate LDH measurements were made on OGD experiments, run in triplicate, from at least three different cell cultures. LDH release was calculated relative to releasable LDH obtained by incubating the cells for 30 min with 1% Triton X-100 at the end of each experiment. A second measure of cell viability was always made by detecting the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to purple formazan (data not shown).

To evaluate apoptosis, the release of cytochrome *c* was measured 2 and 6 h after OGD by immunoblot analysis of cytosolic extracts. Terminal deoxynucleotidyltransferase-mediated dUTP-nick-end labelling was performed using the kit purchased by Roche Molecular Biochemicals (Indianapolis, IN, USA) according to the manufacturer's instructions.

Transfection with silencing RNA

Double-stranded small interfering RNA (siRNAs) corresponding to homologous sequences of mouse c-Rel and RelA genes were designed with 5′-phosphate, 3′-hydroxyl and two base overhangs on each strand. They were synthesized by Qiagen (Valencia, CA, USA). The following gene-specific sequences were used successfully: c-Rel siRNA sense, 5′-CCGUGCUCCAAAUACUGCA-3′ and antisense, 3′-UGCAGUAUUUGGAGCACGG-5′; RelA siRNA sense, 5′-CUCUGAGAUCCUGCUUCCA-3′ and antisense, 3′-UGGAAGCAGGAUCUCAGAG-5′. RelA siRNA from Santa Cruz Biotechnology, Inc (sc-29411; Santa Cruz, CA, USA) was also used. As a negative control (non-siRNA), the following sequences were used: sense, 5′-UUCUCCGAACGUGUCACGU-3′ and antisense, 3′-ACGUGACACGUUCGGAGAA-5′. The siRNAs were dissolved in buffer [100 mM CH_3COOK, 30 mM HEPES–KOH and 2 mM $(CH_3COO)_2Mg$, pH 7.4] to a final concentration of 20 μM, heated at 90°C for 60 s and incubated at 37°C for 60 min, before use, to disrupt any higher aggregates that formed during the synthesis. Cell transfection was carried out using RNAiFect Transfection Reagent (Qiagen). For every 2-cm^2 dishes, c-Rel siRNA from Qiagen (0.8 μg per well) or a mixture of RelA siRNA from Qiagen (0.4 μg per well) together with 26.5 nM RelA siRNA from Santa Cruz Biotechnology was formulated with 6 μL of RNAiFect Transfection Reagent, at 22°C for 10 min. To transfect cortical cultures, the transfection complex was diluted in 300 μL of Neurobasal/B27 medium and added directly to the cells. It was replaced with fresh Neurobasal/B27 3 h later for c-Rel siRNA and 24 h later for RelA siRNA. Treatment with control fluorescein-conjugated siRNA showed a transfection efficiency of about 80% (data not shown). Time-course studies of c-Rel and RelA silencing showed that both proteins were selectively reduced from 24 h up to 72 h, after the transfection. The OGD experiments were performed 48 h after the siRNA treatment.

Transfection with expression plasmids

Experiments of transfection with NF-κB expression plasmids were performed in neuronally differentiated SK-N-SH cells because of higher transfection efficiency obtained in these cells when compared with cortical neurons (about 50%, data not shown). Transfection was carried out according to the manufacturer's instructions with Lipofectamine 2000 Reagent (LF 2000; Invitrogen Corp.). The day before transfection, neuronal SK-N-SH cells were incubated with normal growth medium containing serum and without antibiotics. Cells were transfected with expression plasmids encoding c-Rel (pSG-c-Rel), p50 (pSG-p50) and RelA (pSG-RelA), or with the empty expression vector, pSG5, as a negative control. Plasmids were kindly provided by P. Jalinot (Crenon *et al.* 1993). For each cell well, 1 μg of DNA was diluted into 50 μL of Opti-MEM (Invitrogen Corp.) and 3 μL of LF 2000 Reagent (Invitrogen Corp.) into 50 μL of Opti-MEM. The two solutions were mixed and incubated for 20 min at 22°C to form the transfection complex. After washing the cells with serum-free medium, the transfection complex was added to the cells at a final volume of 1 mL in DMEM without serum and antibiotics. Cells were incubated at 37°C under an atmosphere of 5% CO_2, 95% air for 24 h, before undergoing the OGD experiments.

Reporter gene assays

For reporter fusion gene experiments, we used (i) the Bcl-xL promoter luciferase and Bcl-xL ΔκB luciferase, carrying a mutation of the κB site, produced by Ron Hay (University of Dundee, UK) and kindly provided by Dr Perkins (University of Dundee, UK) (Rocha *et al.* 2003); (ii) Bim promoter luciferase and Bim-ΔκB-luciferase, carrying a mutation in κB site (Inta *et al.* 2006). After 11 DIV, cortical neurons were transfected using LF 2000 with 1 μg/mL Bim or Bcl-xL plasmids. Twenty-four hours after the transfection, cells were exposed to 3 h of OGD and additional 4 h of re-oxygenation. Differentiated SK-N-SH neuronal cells were co-transfected with 1 μg/mL Bcl-xL luciferase reporter plasmid together with 1 μg of pSG-c-Rel, pSG-RelA and pSG-p50 plasmids singularly or in combination. Control cells were co-transfected with 1 μg/mL Bcl-xL luciferase plasmid and with 1 μg/mL empty expression vector pSG5. SK-N-SH cells were maintained in the transfection mixture for 24 h and incubated for additional 6 or 24 h in fresh medium. To normalize the transfection efficiency, 0.05 μg per well *Renilla* luciferase (phRLTK) control plasmid (Promega) was used, and firefly and *Renilla* luciferase were measured using Dual Luciferase Reporter Assay (Promega). All experiments were

performed for at least three times before calculating means and standard errors.

Western blot assay

Mouse cortical neurons and SK-N-SH neuronal cells were harvested in 100 μL of lysis buffer (pH 6.9) containing 1 mM methyl sulphonyl fluoride, 1 μg/mL leupeptin, 5 μg/mL aprotinin and 1 μg/mL pepstatin. The suspension was sonicated for 30 s at full power and centrifuged at 11 000 *g* for 20 min at 4°C. All proteins present in the supernatant (25 μg proteins/sample) were suspended in the sample loading buffer [62.5 mM Tris–HCl, 1% sodium dodecyl-sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue, pH 7.5] and resolved by 10% SDS–polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically onto nitrocellulose membrane. Immunodetection was performed by incubating the membrane overnight at 4°C, with the following primary antibodies: polyclonal anti-c-Rel antibody (2 μg/mL, sc-71; Santa Cruz Biotechnology, Inc.), polyclonal anti-RelA antibody (1 μg/mL, sc-372; Santa Cruz Biotechnology, Inc.), polyclonal anti-Bcl-xL antibody (2 μg/mL, sc-7195; Santa Cruz Biotechnology, Inc.) and anti-β-tubulin antibody (1 : 1500; Neo-Markers, Fremont, CA, USA). The immunoreaction was revealed by 1-hour incubation at 37°C with secondary antibodies coupled to horseradish peroxidase (1 : 1500; Santa Cruz Biotechnology, Inc.) and chemoluminescence detection was carried out using enhanced chemiluminescence western blotting reagents (GE Healthcare, Chalfont St Giles, UK). Quantification of immunoblots was performed by densitometric scanning of exposed film using GelPro Analyser software (Media Cybernetics, Bethesda, MD, USA).

Analysis of cytochrome *c* in cytosolic extracts was performed as previously described (Movsesyan *et al.* 2004; Pizzi *et al.* 2005). Briefly, cells were resuspended in 100 μL of lysis buffer (1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.96 mM $Na_2HPO_4{\cdot}7H_2O$, 80 mM KCl, 250 mM sucrose, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotinin, 1 μM pepstatin and 0.1 mg/mL digitonin, pH 7.4). The lysate was centrifuged at 15 000 *g* for 15 min at 4°C. Twenty-five micrograms of protein of the resulting supernatant, representing the cytosolic fraction, was analysed by immunoblotting. The cytochrome *c* was detected with the monoclonal anticytochrome *c* antibody (0.4 μg/mL, sc13156; Santa Cruz Biotechnology, Inc.).

Co-immunoprecipitation studies

Co-immunoprecipitation studies to identify NF-κB dimer composition were carried out in nuclear extracts (Pizzi *et al.* 2005) from primary cortical neurons exposed to 3 h of OGD and from cortices of mice dissected 4 h after the onset of MCAO (Inta *et al.* 2006). Fifty micrograms of nuclear extracts was diluted in RIPA buffer [pH 8, Tris–HCl, 10 mM NaCl, 140 mM 0.5% (v/v) Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA); 1 mM sodium orthovanadate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail] and incubated at 4°C overnight with 4 μg/mL of corresponding antibodies. The antibodies used for immunoprecipitation were goat anti-c-Rel (sc-71G; Santa Cruz Biotechnology, Inc.), goat anti-RelA (sc-372G; Santa Cruz Biotechnology, Inc.) and normal goat IgG (Chemicon Corp, Billerica, MA, USA). After incubation, 25 μL of protein A/G Plus-Agarose (sc-2003; Santa Cruz Biotechnology, Inc.) was added to the reaction mixture and rotated for 2 h at 4°C. Immunoprecipitates were collected by centrifuging at 770 *g* for 5 min and washed with the RIPA buffer as recommended by protein A/G Plus-Agarose manufacturer. The procedure was repeated four times. After the final wash, all proteins that adhered to the protein A/G beads were detached by boiling the beads in loading buffer. Samples were rapidly centrifuged to pellet the agarose beads and supernatants were analysed by SDS–polyacrylamide gel electrophoresis. Co-immunoprecipitated proteins were detected by western blotting using the following antibodies: rabbit anti-c-Rel (2 μg/mL, sc-71; Santa Cruz Biotechnology, Inc.), rabbit anti-p50 (1 : 500 Abcam, Cambridge, UK) and rabbit anti-RelA (1 μg/mL, sc-372; Santa Cruz Biotechnology, Inc.). Data from densitometry analysis have been expressed as the ratio of ischaemic to control templates. Equal amounts of nuclear proteins were used for immunoprecipitation, as confirmed by western blot analysis of β-actin in 20 μg of nuclear extracts (data not shown).

Statistics

Columns represent the means ± SEM of at least four values. Data describing cell survival were analysed by Kruskal–Wallis non-parametric ANOVA with adjustment for multiple comparisons. $p \leq 0.05$ was considered as significant.

Results

NF-κB activation in neurons exposed to oxygen glucose deprivation

The primary cultures of mouse cortical neurons at 11 DIV were exposed to OGD for 3 h. Apoptosis and necrosis were evaluated 2, 6 and 24 h later, to check the timing of diverse cell death in response to the anoxic insult. In line with previous evidence, the necrosis of neuronal cells appeared to be secondary to apoptosis (Bonfoco *et al.* 1995). The cytochrome *c* was detected in the cytosol already 2 h after the OGD; at 6 h, the cytosolic amount of cytocrome *c* increased and most cells showed to be terminal deoxynucleotidyltransferase-mediated dUTP-nick-end labelling-positive (Fig. 1a and b), in the absence of any detectable LDH in the medium. A significant increase of LDH release was measured 24 h after the OGD exposure (Fig. 1c). *In vivo* brain ischaemia was performed by permanent occlusion of the MCAO in mice (Herrmann *et al.* 2003). MCAO produced a specific cortical lesion, as evaluated 48 h after the onset of ischaemia (Fig. 1d).

It has been shown that, as an early response to OGD or brain ischaemia, NF-κB RelA rapidly activates in neurons and contributes to the post-ischaemic neurodegenerative process (Herrmann *et al.* 2005; Zhang *et al.* 2005; Inta *et al.*2006). To examine the specific assembly of RelA with p50 or c-Rel in brain ischaemia, we investigated the reciprocal association of RelA, p50 and c-Rel factors to form active dimers in primary neurons exposed to 3 h OGD or in cortices of mice exposed to MCAO. Nuclear extracts

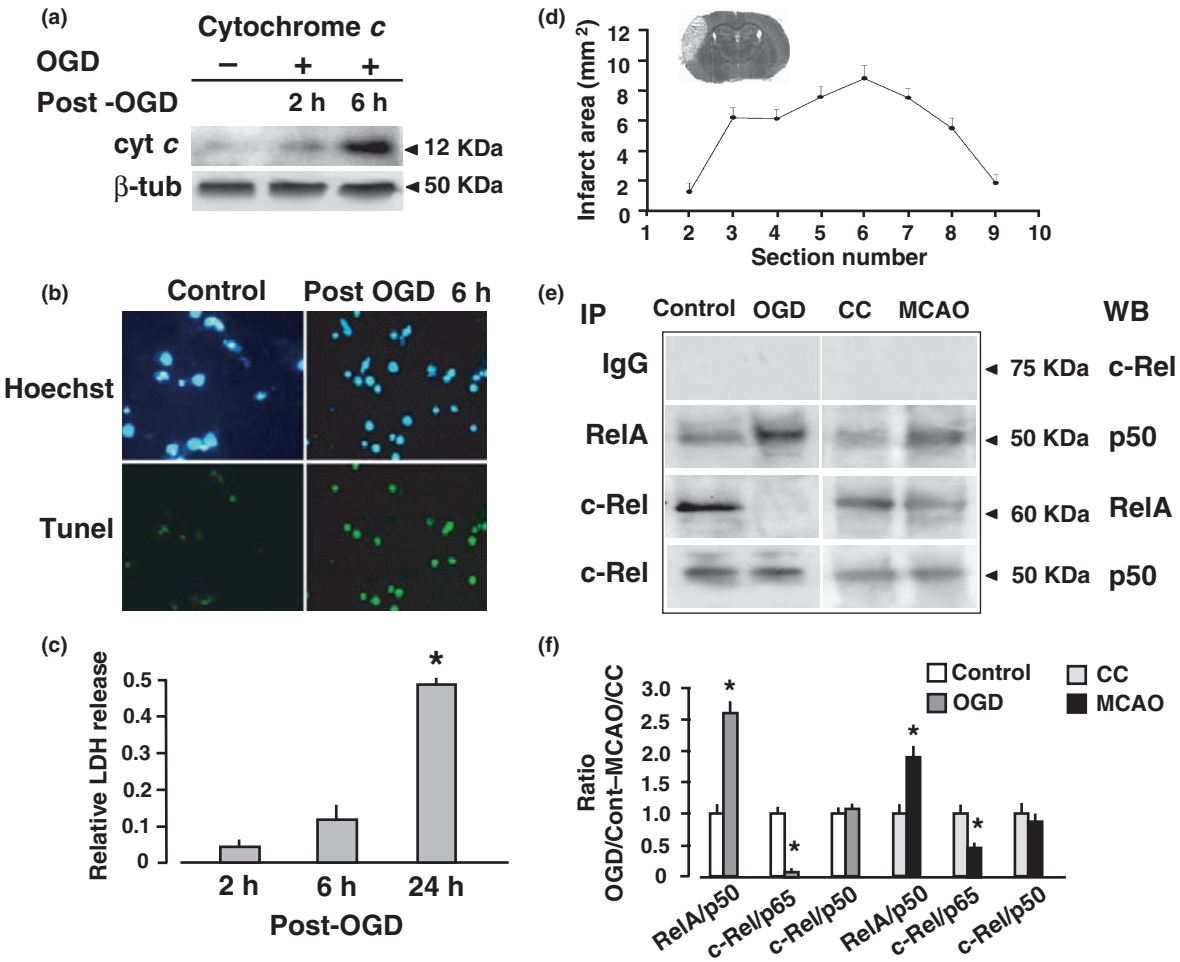

Fig. 1 Cell death and nuclear factor-κB (NF-κB) activation in primary cortical cells and cortices of mice exposed to middle cerebral artery occlusion (MCAO). (a,b) Primary cortical neurons were exposed to 3 h of oxygen glucose deprivation (OGD). Apoptotic cells were evaluated 2 and 6 h later by western blot analysis of cytochrome *c* in the cytosol and by TUNEL staining. Neurons underwent apoptosis during the initial hours after the ischaemic insult which was followed by delayed necrosis. (c) After the OGD exposure for 2, 6 and 24 h, neurons were evaluated for cell viability by lactate dehydrogenase assay. Bars represent the means ± SEM of at least three separate experiments run in triplicate (*$p \leq 0.05$ vs. the corresponding control value). (d) Distribution of infarct on coronal sections from rostral to caudal brain in male C57BL/6 mice exposed to distal permanent occlusion of the MCAO. Values are means ± SEM ($n = 8$). A representative brain coronal section is shown. (e) Representative pictures of co-immunoprecipitation analysis of NF-κB dimers in 50 μg of nuclear extracts of primary cortical neurons exposed to OGD or cortices from mice subjected to MCAO. OGD enhanced the nuclear translocation of p50/RelA dimers. The nuclear content of RelA/c-Rel complexes decreased while p50/c-Rel remained unchanged. A similar pattern of activation was present in the ischaemic cortices 4 h after permanent MCAO. (f) Data from densitometry analysis of immunoblots are expressed as ratio of OGD ($n = 3$) to controls ($n = 3$) or ratio of MCAO ($n = 4$) to contro-lateral cortex (CC, $n = 4$). Values are expressed as means ± SEM (*$p \leq 0.05$ vs. the corresponding control value). TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-nick-end labelling; IP, immunoprecipitation; WB, Western Blot; β-tub, β-tubulin.

were immunoprecipitated using antibodies against c-Rel or RelA. The western blot analysis of co-immunoprecipitated NF-κB factors revealed high activation of p50/RelA dimer but a reduction of RelA/c-Rel complex in OGD extracts. No significant change was detected in the nuclear content of p50/c-Rel dimer (Fig. 1e and f). Results from densitometry analysis of the diverse dimers activated in neurons by OGD have been expressed as ratio of OGD and control templates (Fig. 1f). The analyses of the NF-κB complexes activated in cortices of mice exposed to brain ischaemia were performed 4 h after the permanent artery occlusion. This time interval was chosen as the inhibition of NF-κB signalling was only effective up to 4.5 h after the onset of MCAO (Herrmann *et al.* 2005). As observed in cortical neurons exposed to OGD, the ischaemic cortical templates showed a relevant induction of p50/RelA, together with a decrease of RelA/c-Rel and no modification of p50/c-Rel dimers (Fig. 1e and f).

Opposing regulation of neuronal vulnerability by c-Rel and RelA silencing

To investigate the specific role of p50/RelA and c-Rel-containing dimers in anoxic injury, we produced a post-transcriptional silencing of RelA and c-Rel. Double-stranded siRNAs targeting c-Rel and RelA were added to 8 DIV cortical cells, as described in Material and methods. The knockdown of c-Rel and RelA proteins was verified by western blot analysis of cell extracts (Fig. 2a and b). The treatments did not affect *per se* the neuron viability. The c-Rel- and RelA-silenced neurons were exposed to OGD. As shown in Fig. 2a, the c-Rel knockdown made cells more vulnerable to the ischaemic insult when compared with cells treated with control non-siRNA. On the contrary, silencing RelA significantly reduced cortical neuron vulnerability to OGD exposure (Fig. 2b). The opposite regulation of neuronal survival obtained by targeting c-Rel and RelA was mirrored by effects produced by over-expressing the two NF-κB factors in neuronally differentiated SK-N-SH cells. Under exposure to RA, SK-N-SH neuroblastoma cells switch from a non-neuronal to a neuronal phenotype, expressing antigenic changes typical of post-mitotic neurons together with functional NMDA receptors and vulnerability to excitotoxic injuries (Pizzi *et al.* 2002b) and OGD. The neuronal cultures were transiently transfected with expression plasmids coding for human c-Rel (pSG-c-Rel), RelA (pSG-RelA) or with control pSG5 empty vector. Twenty-four hours after transfection, the over-expression of c-Rel and RelA was verified

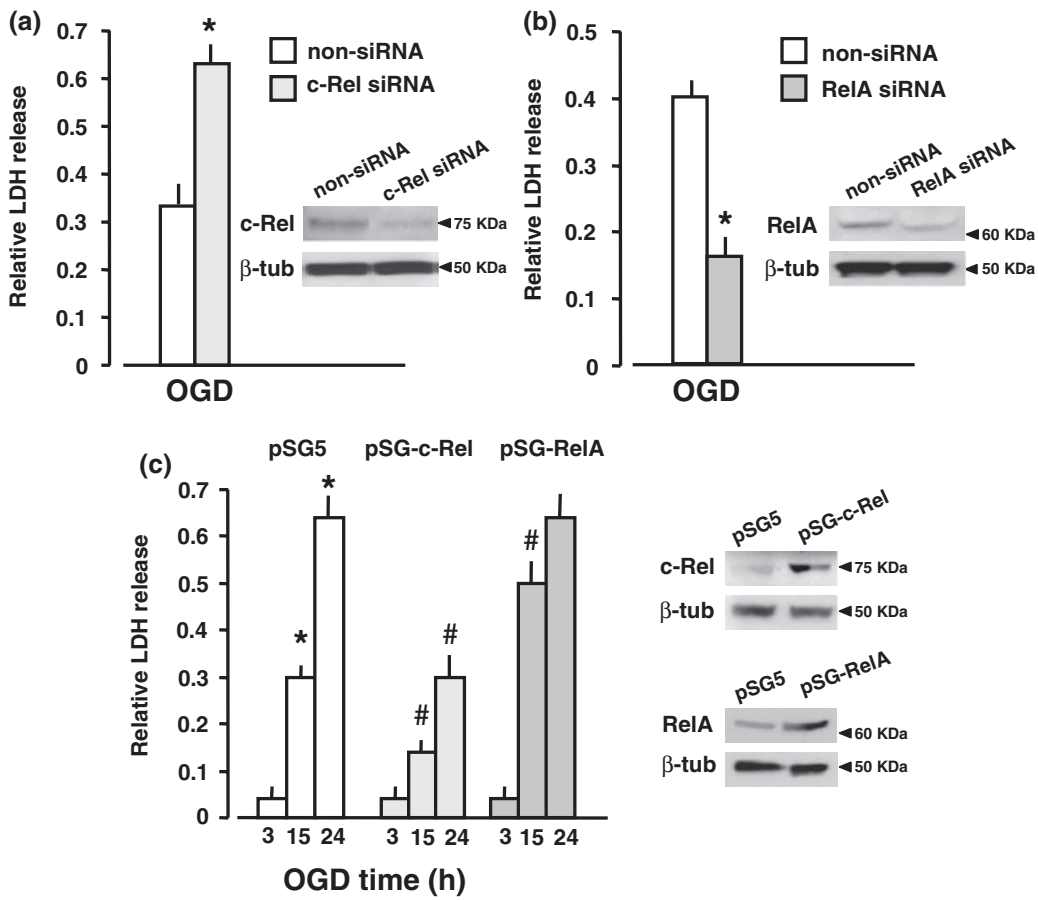

Fig. 2 Opposing regulation of neuronal vulnerability by c-Rel and RelA proteins. (a,b) Primary cortical neurons were transfected with small interfering RNA (siRNA) cognate to c-Rel gene (c-Rel siRNA), RelA gene (RelA siRNA) or with a control non-specific siRNA (non-siRNA). The silencing of c-Rel and RelA expression was evaluated in cell extracts by western blot analysis. c-Rel knockdown made cells more vulnerable to the ischaemic insult, while RelA silencing significantly reduced cortical neuron vulnerability to oxygen glucose deprivation (OGD). Values are mean ± SEM of three experiments run in triplicate (*$p \leq 0.05$ vs. the corresponding non-siRNA-treated cells). (c) Neuronal SK-N-SH cells were transfected with pSG-c-Rel and pSG-RelA plasmids or with a pSG5 vector for 24 h. Cell extracts immunoblotted with c-Rel and RelA antibodies revealed higher c-Rel and RelA expression in cells transfected with the relative plasmids. Cell survival was measured in transfected SK-N-SH cells exposed to 3, 15 or 24 h of OGD. OGD toxicity was prevented by c-Rel over-expression, while over-expressing RelA significantly increased cell loss. Similar results were obtained in three separate experiments run in triplicate (*$p \leq 0.05$ vs. the corresponding control value, #$p \leq 0.05$ vs. corresponding pSG5-treated cells). β-tub, β-tubulin.

by immunoblot analysis in total cell extracts (Fig. 2c). The cells were then exposed to OGD for 3, 15 and 24 h. The cell viability was measured by LDH (Fig. 2c) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown), 24 h later. The cells transfected with the empty vector showed a time-dependent vulnerability to OGD, with significant cell death at 15 and 24 h. Over-expressing c-Rel significantly reduced the cell vulnerability to OGD at 15 and 24 h, while over-expressing RelA significantly augmented the cell loss. The increased susceptibility of RelA over-expressing cells was only evident after 15 h of OGD, a condition producing a submaximal effect in control cells (Fig. 2c).

Pro-apoptotic and anti-apoptotic gene expression: effects of RelA and c-Rel

It has been recently demonstrated that during cerebral ischaemia, the promoter activation and the mRNA expression of pro-apoptotic genes, *Bim* and *Noxa*, depend on RelA activation (Inta *et al.* 2006). Here, we investigated the specific contribution of different NF-κB dimers to the anoxia-induced apoptotic program by studying the response of Bcl-xL promoter to OGD. Neurons were transfected with Bim- and Bcl-xL promoter-luciferase plasmids directing luciferase expression, or with Bcl-xL ΔκB and Bim ΔκB carrying a mutation in the sequence of NF-κB binding site. As expected, OGD induced a twofold increase in the activity of Bim promoter (Fig. 3). No modification was observed in the Bim ΔκB plasmid, demonstrating that changes in the Bim transcription were NF-κB-specific. Conversely, the Bcl-xL promoter activity was partially reduced (−35%) by OGD exposure (Fig. 3). Mutation in the NF-κB binding site of Bcl-xL promoter diminished the basal activity (−28%) and the responsiveness to OGD (Fig. 3). We then checked the modification of Bcl-xL level in cortical neurons 4 h after OGD exposure. Western blot analysis revealed a reduction of endogenous anti-apoptotic protein in cells exposed to OGD. The decrement was more evident in c-Rel-silenced neurons, while it recovered in RelA-knockdown cells (Fig. 4a). To confirm the relation between c-Rel induction and Bcl-xL gene expression in ischaemia, we checked the effect of c-Rel activation in SK-N-SH neuronal cells. Intracellular levels of Bcl-xL protein was reduced in control SK-N-SH cells exposed to 15 h OGD and maintained for additional 4 h in normoxic conditions. The Bcl-xL expression totally recovered in SK-N-SH cells over-expressing c-Rel (Fig. 4b). Finally, to evaluate the dependency of Bcl-xL transcription on specific NF-κB dimers, we investigated the effects of c-Rel, RelA and p50 expression plasmids on Bcl-xL promoter activity. Bcl-xL promoter was strongly induced by c-Rel over-expression in neuronal SK-N-SH cells at 6 and 24 h after transfection (Fig. 5a). Likewise c-Rel homodimers, the c-Rel heterodimers, p50/c-Rel and RelA/c-Rel, efficiently induced the Bcl-xL promoter in a κB-dependent

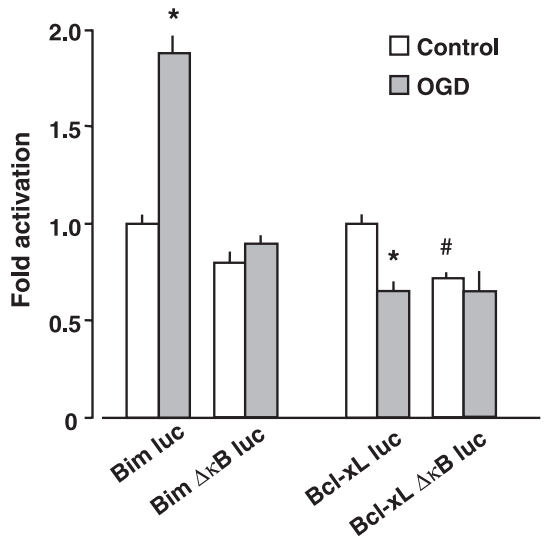

Fig. 3 Oxygen glucose deprivation (ODG)-induced nuclear factor-κB (NF-κB) activation promotes *Bim* but not *Bcl-xL* transcription. Primary cortical neurons were transfected with *Bim* or *Bcl-xL* luciferase reporter plasmid or with *Bim* and *Bcl-xL* ΔκB luciferase reporter plasmids for 24 h and then exposed for 3 h to OGD. Four hours later, luciferase activity was measured. The OGD exposure significantly induced Bim and decreased *Bcl-xL* promoter activity. Mutation of NF-κB binding sites reduced the luciferase expression. Values are mean ± SEM of three experiments run in triplicate (*$p \leq 0.05$ vs. the corresponding control value; #$p \leq 0.05$ vs. corresponding wild-type luciferase reporter plasmid).

manner (Fig. 5b). On the contrary, the RelA homodimers as well as the p50/RelA did not significantly modify the Bcl-xL promoter activity (Fig. 5a and b).

Discussion

Our data show that NF-κB dimer composition discriminates NF-κB transcriptional activity on pro-apoptotic and anti-apoptotic genes affecting neuronal survival. While p50/RelA dimer contributes to the pathogenesis of post-ischaemic injury, activation of c-Rel-containing dimers increases neuron resistance to ischaemia.

Previous studies demonstrated that neuronal cell fate can rely on the recruitment of different NF-κB subunits in response to pro-apoptotic or neuroprotective agents. While glutamate or β-amyloid mainly activate p50 and RelA subunits, neuroprotective interleukin-1β, mGlu5 receptor agonists, and leptin activate c-Rel, besides p50 and RelA proteins. The c-Rel dimers induced by neuroprotective agents are responsible for the elicited anti-apoptotic effects (Pizzi *et al.* 2002b, 2005; Valerio *et al.* 2008). This study overcomes the issue of different NF-κB activation in response to distinct extracellular stimuli to examine the role of NF-κB dimers in modulating gene transcription and intrinsic neuron vulnerability to ischaemia. It has been shown that nuclear

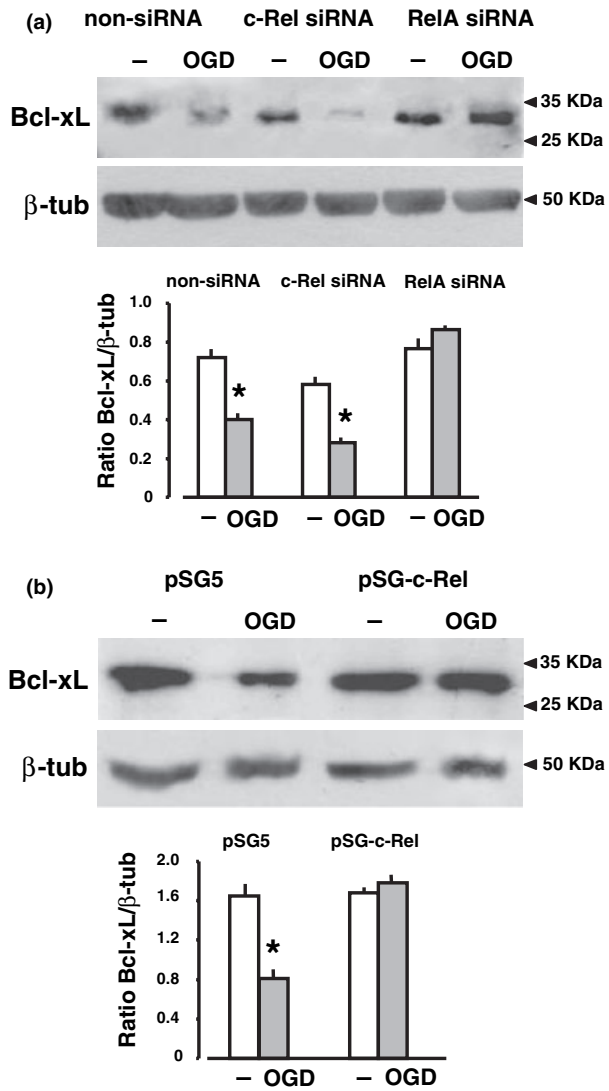

Fig. 4 Modulation of Bcl-xL level during oxygen glucose deprivation (OGD) by RelA and c-Rel. (a) Western blot analysis of Bcl-xL in cell extracts of cortical neurons treated with c-Rel silencing RNA (siRNA), RelA siRNA or control non-siRNA and exposed to OGD. After 4 h of re-oxygenation, protein extracts were prepared and immunoblotted with Bcl-xL or β-tubulin (β-tub) antibodies. (b) SK-N-SH neuronal cells were transfected with pSG-c-Rel or with control pSG5 vector and then exposed to 15 h of OGD. Protein cell extracts were prepared 4 h later and immunoblotted with Bcl-xL or β-tubulin antibodies. Data from densitometry analyses of immunoblots are expressed as ratio of Bcl-xL to β-tubulin. Columns are expressed as means ± SEM of three separate experiments (*$p \leq 0.05$ vs. the corresponding control, normoxic value).

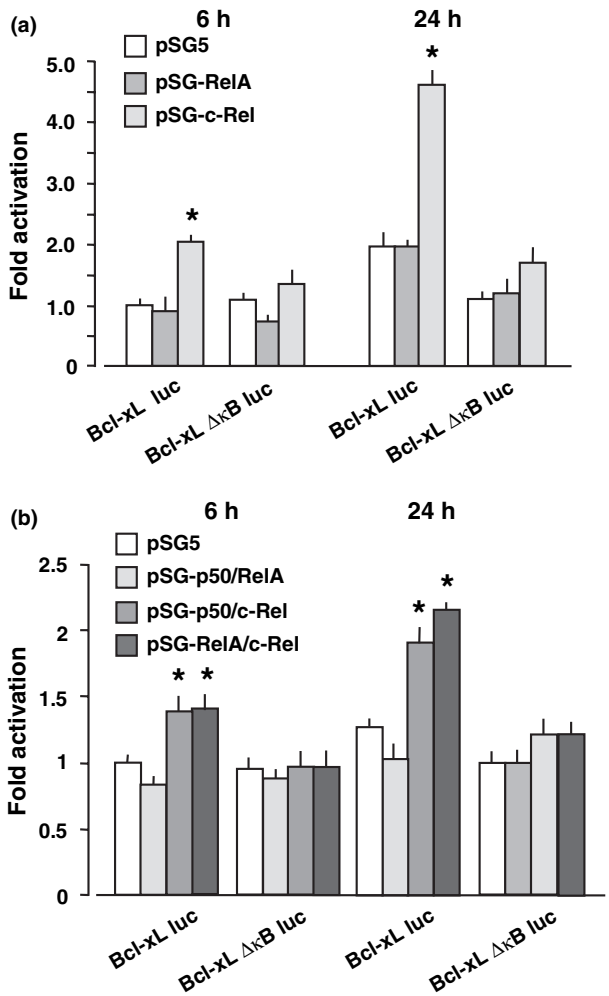

Fig. 5 c-Rel-containing dimers but not RelA/RelA or p50/RelA dimers stimulate *Bcl-xL* transcription. SK-N-SH neuronal cells were co-transfected with *Bcl-xL* or *Bcl-xL* ΔκB luciferase reporter plasmids together with pSG-c-Rel and pSG-RelA, or a combination of pSG-p50, and pSG-RelA and pSG-Rel expression plasmids. Control cells were transfected with empty pSG5 vector. The luciferase activity was measured after 6 and 24 h. Mutation of nuclear factor-κB binding site blocked the luciferase expression. (a) The c-Rel, but not RelA over-expression, stimulated the activity of *Bcl-xL* promoter. (b) Co-transfection with p50/c-Rel and RelA/c-Rel, but not p50/RelA, was able to activate *Bcl-xL* promoter. Values are mean ± SEM of three experiments run in triplicate (*$p \leq 0.05$ vs. the corresponding control value).

translocation of NF-κB subunits is induced in the ischaemic hemisphere of mice exposed to MCAO, with a prevalent activation of RelA and p50 subunits (Crack *et al.* 2006; Inta *et al.* 2006). Here, we investigated how these NF-κB subunits dimerize among them and with c-Rel protein, in mice exposed to MCAO and in primary cortical neurons exposed to OGD. We observed a similar pattern of NF-κB activation in MCAO ischaemic tissues (Inta *et al.* 2006) and primary cortical neurons undergoing OGD-induced apoptosis. Specifically, the analysis of NF-κB complexes in both *in vivo* and *in vitro* experimental settings revealed major activation of the p50/RelA dimer and inhibition of c-Rel-containing complexes. To investigate the precise contribution of p50/RelA and c-Rel-containing dimers to neuronal

vulnerability, we targeted c-Rel and RelA in primary cortical neurons. The c-Rel knockdown made neuronal cells more vulnerable to OGD. Conversely, the RelA silencing significantly reduced the neuronal cell death. The indication of opposite roles played by c-Rel and RelA in OGD, anti-apoptotic, the former, and pro-apoptotic, the latter, was confirmed by over-expressing the two subunits in neuronal cells. The SK-N-SH cells over-expressing c-Rel appeared to be more resistant to OGD, while cells over-expressing RelA were more susceptible. The pivotal role of p50/RelA in the pathogenesis of ischaemic injury is in line with studies demonstrating that brain infarct size after MCAO is reduced in mice carrying a conditional, brain-specific deletion of RelA ($RelA^{CNSKO}$) as well as in p50 knockout mice (Schneider *et al.* 1999), but not in mice carrying a germline deficiency of p52 or c-Rel subunits (Inta *et al.* 2006; Ridder and Schwaninger 2008). Contrary to what we found in cells acutely silenced for c-Rel protein, cortical neurons prepared from *c-Rel*−/− mice did not show any enhanced vulnerability to OGD (data not shown), neither were *c-Rel*−/− mice more susceptible to MCAO (Inta *et al.* 2006). This suggests that while acute c-Rel knockdown unmasks the c-Rel participation in anti-apoptotic programs, the congenital lack of c-Rel during brain development and maturation may be overridden by other NF-κB factors which recombine to guarantee brain cell resistance to the acute oxidative stress.

We then investigated the target genes and transcriptional activity of diverse NF-κB dimers during ischaemia. Both pro- and anti-apoptotic genes are involved in NF-κB regulation of neuron survival. Among these, the Bcl-2 family anti-apoptotic member, *Bcl-xL*, and the *Bcl-2* homology 3 (BH3)-only pro-apoptotic genes, *Bim* and *Noxa* (Cao *et al.* 2002). It has been shown that *Bim* and *Noxa* are significantly induced 6 h after MCAO and their up-regulation is abolished in $RelA^{CNSKO}$ mice. Experiments of reporter fusion genes confirm that *Bim* and *Noxa* promoters are under the transcriptional control of RelA (Inta *et al.* 2006). With regard to the anti-apoptotic *Bcl-xL*, it has been found to be transcriptionally regulated by c-Rel in non-neuronal cells (Chen *et al.* 2000; Kirito *et al.* 2002; Banerjee *et al.* 2008). In neurons, the activation of c-Rel by mGlu5 receptors promotes expression of *Bcl-xL* that is ultimately responsible for protective effects against β-amyloid-induced apoptosis (Pizzi *et al.* 2005). To investigate the relationship between NF-κB activity and anti-apoptotic genes in ischaemia, we checked the expression of endogenous Bcl-xL in cortical neurons exposed to OGD. The intracellular amount of Bcl-xL decreased in cells exposed to the ischaemic insult. Bcl-xL level recovered in cells silenced for RelA, but further decreased in c-Rel-silenced neurons. The knockdown of c-Rel also reduced the basal Bcl-xL content, an event possibly responsible for the increased cell susceptibility of c-Rel-silenced neurons to anoxia. By studying the reporter fusion gene, we demonstrated that in neuronal cells, the anti-apoptotic *Bcl-xL* is transcriptionally activated by c-Rel homo- and heterodimers, but not by p50/RelA complex. Despite the ability of p50/RelA to bind the *Bcl-xL* promoter, RelA has been found to act as activator or repressor of *Bcl-xL* transcription in cancer cells, as a result of cell type and applied stimulus (Dong *et al.* 2002; Campbell *et al.* 2004). Our results indicate that in neuronal cells the dimerization of RelA with p50 does not activate or repress *Bcl-xL* promoter, but coupling with c-Rel switches RelA activity to a stimulatory mode. In line with this evidence, the activity of *Bcl-xL* reporter plasmid decreased in cortical cells exposed to OGD, where endogenous RelA/c-Rel diminished and p50/RelA prevailed, while *Bim* reporter plasmid was highly stimulated in a κB-specific manner. It is conceivable that the decrease of endogenous Bcl-xL protein, which we observed 4 h after OGD, is the result of both reduced transcription and increased cleavage of Bcl-xL. The Bcl-xL degradation to form pro-apoptotic ΔBcl-xL fragments is induced in brain ischaemia already 1 h after the ischaemic injury (Miyawaki *et al.* 2008) and is strictly dependent on the release of cytochrome *c* and activation of caspase 9 cascade (Chen *et al.* 2007; Miyawaki *et al.* 2008). Recent evidence has shown that Bim, as other BH3-only proteins, can contribute to the cytochrome *c* release and apoptosis by directly activating pro-apoptotic Bax and Bak (Kuwana *et al.* 2005) or by displacing Bax and Bak from association with Bcl-xL/Bcl-2 prosurvival homologues (Puthalakath and Strasser 2002; Willis *et al.* 2007; Zhang *et al.* 2008). Reducing p50/RelA activation by limiting Bim expression and cytochrome *c* release, could also limit Bcl-xL degradation after OGD, as suggested by the recovery of Bcl-xL in RelA-silenced neurons. Thus, a change in the balance between pro-apototic BH3-only proteins and Bcl-2 prosurvival factors, in response to transcriptional signals, can be the determinant of cell death or survival (Puthalakath and Strasser 2002). In tune with this model, the c-Rel-dependent increase of Bcl-xL protein, by leading to the formation of Bim/Bcl-xL complexes and inactivation of Bim, can contribute to the lipopolysaccharide-induced survival of B cells (Banerjee *et al.* 2008).

It has been proposed that opposite effects of NF-κB as a regulator of neuronal survival may rely on the different type of cells where activation occurs. According to this concept, NF-κB is neuroprotective when activated in neurons and neurotoxic when induced in glial cells (Mattson and Meffert 2006). Our results demonstrate that, within the same neuronal cell, unbalanced activation of NF-κB p50/RelA dimer over c-Rel-containing complexes contributes to cell death secondary to the ischaemic insult. While p50/RelA acts as transcriptional inducer of pro-apoptotic *Bim* and *Noxa* (Inta *et al.* 2006), c-Rel dimers specifically promote transcription of *Bcl-xL* gene. Changes in the nuclear content of c-Rel dimers strongly affect the threshold of neuron vulnerability to brain ischaemia, in line with evidence that c-Rel activation *in vivo* is area-specific and correlates with

increased Bcl-xL expression and neuronal resistance to hypoxia (Qiu *et al.* 2001).

Finally, it has been shown that RelA subunit acts as an inducer of anti-apoptotic gene expression in tumour cells, but under certain circumstances it can act as a repressor of the same target genes. This functional switch of NF-κB to a repressor form results from site-specific phosphorylation of RelA and association with co-repressor complexes endowed with histone deacetylase activity rather than co-activators endowed with histone acetyltransferase activity (Campbell and Perkins 2004; Rocha *et al.* 2005). This additional level of NF-κB regulation might also modulate target gene specificity for diverse NF-κB dimers in neuronal cells, with relevant implications for pathological conditions. Identifying the molecular mechanisms shaping functional activation and transcriptional activity of diverse NF-κB dimers in neuronal cells will unravel new potential targets to achieve selective manipulation of NF-κB complexes to treat neurological diseases associated with apoptotic cell death.

Acknowledgements

This work was supported by grants from Italian Ministry of Education, University and Scientific Research – PRIN 2004, 2005 and 2006; Center of Study and Research on Ageing, Brescia; MIUR Center of Excellence for Innovative Diagnostics and Therapeutics (IDET) of Brescia University and Rotary Club Rodengo Abbazia, Brescia.

References

Ahn H. J., Hernandez C. M., Levenson J. M., Lubin F. D., Liou H. C. and Sweatt J. D. (2008) c-Rel, an NF-kappaB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation. *Learn Mem.* **15**, 539–549.

Bakalkin G. Y., Yakovleva T. and Terenius L. (1993) NF-kappa B-like factors in the murine brain. Developmentally-regulated and tissue-specific expression. *Brain Res. Mol. Brain Res.* **20**, 137–146.

Banerjee A., Grumont R., Gugasyan R., White C., Strasser A. and Gerondakis S. (2008) NF-{kappa}B1 and c-Rel cooperate to promote the survival of TLR4 activated B cells by neutralizing Bim via distinct mechanisms. *Blood* [Epub ahead of print] doi: 10.1182/blood-2007-10-120832.

Bhakar A. L., Tannis L. L., Zeindler C., Russo M. P., Jobin C., Park D. S., MacPherson S. and Barker P. A. (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. *J. Neurosci.* **22**, 8466–8475.

Bonfoco E., Krainc D., Ankarcrona M., Nicotera P. and Lipton S. A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl Acad. Sci. USA* **92**, 7162–7166.

Campbell K. J. and Perkins N. D. (2004) Reprogramming RelA. *Cell Cycle* **3**, 869–872 (Review).

Campbell K. J., Rocha S. and Perkins N. D. (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. *Mol. Cell* **13**, 853–865.

Cao G., Pei W., Ge H., Liang Q., Luo Y., Sharp F. R., Lu A., Ran R., Graham S. H. and Chen J. (2002) In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J. Neurosci.* **22**, 5423–5431.

Chen C., Edelstein L. C. and Gelinas C. (2000) The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol. Cell. Biol.* **20**, 2687–2695.

Chen M., Guerrero A. D., Huang L., Shabier Z., Pan M., Tan T. H. and Wang J. (2007) Caspase-9-induced mitochondrial disruption through cleavage of anti-apoptotic BCL-2 family members. *J. Biol. Chem.* **282**, 33888–33895.

Clemens J. A., Stephenson D. T., Smalstig E. B., Dixon E. P. and Little S. P. (1997) Global ischemia activates nuclear factor-kappa B in forebrain neurons of rats. *Stroke* **28**, 1073–1080.

Crack P. J., Taylor J. M., Ali U., Mansell A. and Hertzog P. J. (2006) Potential contribution of NF-kappaB in neuronal cell death in the glutathione peroxidase-1 knockout mouse in response to ischemia-reperfusion injury. *Stroke* **37**, 1533–1538.

Crenon I., Beraud C., Simard P., Montagne J., Veschambre P. and Jalinot P. (1993) The transcriptionally active factors mediating the effect of the HTLV-I Tax transactivator on the IL-2R alpha kappa B enhancer include the product of the c-rel proto-oncogene. *Oncogene* **8**, 867–875.

Dong Q. G., Sclabas G. M., Fujioka S. *et al.* (2002) The function of multiple IkappaB: NF-kappaB complexes in the resistance of cancer cells to taxol-induced apoptosis. *Oncogene* **21**, 6510–6519.

Gillardon F., Lenz C., Waschke K. F., Krajewski S., Reed J. C., Zimmermann M. and Kuschinsky W. (1996) Altered expression of Bcl-2, Bcl-X, Bax, and c-Fos colocalizes with DNA fragmentation and ischemic cell damage following middle cerebral artery occlusion in rats. *Brain Res. Mol. Brain Res.* **40**, 254–260.

Graham S. H. and Chen J. (2001) Programmed cell death in cerebral ischemia. *J. Cereb. Blood Flow Metab.* **21**, 99–109.

Grilli M., Pizzi M., Memo M. and Spano P. (1996) Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* **274**, 1383–1385.

Herrmann O., Tarabin V., Suzuki S. *et al.* (2003) Regulation of body temperature and neuroprotection by endogenous interleukin-6 in cerebral ischemia. *J. Cereb. Blood Flow Metab.* **23**, 406–415.

Herrmann O., Baumann B., de Lorenzi R. *et al.* (2005) IKK mediates ischemia-induced neuronal death. *Nat. Med.* **11**, 1322–1329.

Hoffmann A., Leung T. H. and Baltimore D. (2003) Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. *EMBO J.* **22**, 5530–5539.

Inta I., Paxian S., Zhang W. *et al.* (2006) Bim and Noxa are candidates to mediate the deleterious effect of the NF-κB subunit RelA in cerebral ischemia. *J. Neurosci.* **26**, 12896–12903.

Kaltschmidt B., Uherek M., Wellmann H., Volk B. and Kaltschmidt C. (1999) Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. *Proc. Natl Acad. Sci. USA* **96**, 9409–9414.

Kirito K., Watanabe T., Sawada K., Endo H., Ozawa K. and Komatsu N. (2002) Thrombopoietin regulates Bcl-xL gene expression through Stat5 and phosphatidylinositol 3-kinase activation pathways. *J. Biol. Chem.* **277**, 8329–8337.

Kögel D., Peters M., König H. G., Hashemi S. M., Bui N. T., Arolt V., Rothermundt M. and Prehn J. H. (2004) S100B potently activates p65/c-Rel transcriptional complexes in hippocampal neurons: clinical implications for the role of S100B in excitotoxic brain injury. *Neuroscience* **127**, 913–920.

Kuwana T., Bouchier-Hayes L., Chipuk J. E., Bonzon C., Sullivan B. A., Green D. R. and Newmeyer D. D. (2005) BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol. Cell* **17**, 525–535.

Levenson J. M., Choi S., Lee S. Y., Cao Y. A., Ahn H. J., Worley K. C., Pizzi M., Liou H. C. and Sweatt J. D. (2004) A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel. *J. Neurosci.* **24**, 3933–3943.

Mattson M. P. and Camandola S. (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J. Clin. Invest.* **107**, 247–254.

Mattson M. P. and Meffert M. K. (2006) Roles for NF-kappaB in nerve cell survival, plasticity, and disease. *Cell Death Differ.* **13**, 852–860.

Meffert M. K. and Baltimore D. (2005) Physiological functions for brain NF-κB. *Trends Neurosci.* **28**, 37–43.

Meffert M. K., Chang J. M., Wiltgen B. J., Fanselow M. S. and Baltimore D. (2003) NF-kappa B functions in synaptic signaling and behavior. *Nat. Neurosci.* **6**, 1072–1078.

Miyawaki T., Mashiko T., Ofengeim D., Flannery R. J., Noh K. M., Fujisawa S., Bonanni L., Bennett M. V., Zukin R. S. and Jonas E. A. (2008) Ischemic preconditioning blocks BAD translocation, Bcl-xL cleavage, and large channel activity in mitochondria of postischemic hippocampal neurons. *Proc. Natl Acad. Sci. USA* **105**, 4892–4897.

Movsesyan V. A., Stoica B. A. and Faden A. I. (2004) MGLuR5 activation reduces beta-amyloid-induced cell death in primary neuronal cultures and attenuates translocation of cytochrome c and apoptosis-inducing factor. *J. Neurochem.* **89**, 1528–1536.

Nurmi A., Lindsberg P. J., Koistinaho M., Zhang W., Juettler E., Karjalainen-Lindsberg M. L., Weih F., Frank N., Schwaninger M. and Koistinaho J. (2004) Nuclear factor-kappaB contributes to infarction after permanent focal ischemia. *Stroke* **35**, 987–991.

O'Neill L. A. and Kaltschmidt C. (1997) NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci.* **20**, 252–258.

O'Riordan K. J., Huang I. C., Pizzi M. *et al.* (2006) Regulation of nuclear factor kappaB in the hippocampus by group I metabotropic glutamate receptors. *J. Neurosci.* **26**, 4870–4879.

Pizzi M. and Spano P. (2006) Distinct roles of diverse nuclear factor-kappaB complexes in neuropathological mechanisms. *Eur. J. Pharmacol.* **545**, 22–28. Review.

Pizzi M., Boroni F., Bianchetti A., Moraitis C., Sarnico I., Benarese M., Goffi F., Valerio A. and Spano P. (2002a) Expression of functional NR1/NR2B-type NMDA receptors in neuronally differentiated SK-N-SH human cell line. *Eur. J. Neurosci.* **16**, 2342–2350.

Pizzi M., Goffi F., Boroni F., Benarese M., Perkins S. E., Liou H. C. and Spano P. (2002b) Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta. *J. Biol. Chem.* **277**, 20717–20723.

Pizzi M., Sarnico I., Boroni F., Benarese M., Steimberg N., Mazzoleni G., Dietz G. P., Bähr M., Liou H. C. and Spano P. F. (2005) NF-κB factor c-Rel mediates neuroprotection elicited by mGlu5 receptor agonists against amyloid β-peptide toxicity. *Cell Death Differ.* **12**, 761–772.

Puthalakath H. and Strasser A. (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ.* **9**, 505–512.

Qin Z. H., Chen R. W., Wang Y., Nakai M., Chuang D. M. and Chase T. N. (1999) Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. *J. Neurosci.* **19**, 4023–4033.

Qiu J., Grafe M. R., Schmura S. M., Glasgow J. N., Kent T. A., Rassin D. K. and Perez-Polo J. R. (2001) Differential NF-kappa B regulation of bcl-x gene expression in hippocampus and basal forebrain in response to hypoxia. *J. Neurosci. Res.* **64**, 223–234.

Ridder D. A. and Schwaninger M. (2008) NF-kappaB signaling in cerebral ischemia. *Neuroscience* [Epub ahead of print] doi: 10.1016/j.neuroscience.2008.07.007.

Rocha S., Campbell K. J. and Perkins N. D. (2003) p53- and Mdm2-independent repression of NF-kappa B transactivation by the ARF tumor suppressor. *Mol. Cell* **12**, 15–25.

Rocha S., Garrett M. D., Campbell K. J., Schumm K. and Perkins N. D. (2005) Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *EMBO J.* **24**, 1157–1169.

Schneider A., Martin-Villalba A., Weih F., Vogel J., Wirth T. and Schwaninger M. (1999) NF-kappaB is activated and promotes cell death in focal cerebral ischemia. *Nat. Med.* **5**, 554–559.

Valerio A., Dossena M., Bertolotti P. *et al.* (2008) Leptin is induced in the ischemic cerebral cortex and exerts neuroprotection through NF-κB/c-Rel-dependent transcription. *Stroke* [Epub ahead of print] doi: 10.1161/STROKEAHA.108.528588.

Willis S. N., Fletcher J. I., Kaufmann T. *et al.* (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315**, 856–859.

Yu Z., Zhou D., Bruce-Keller A. J., Kindy M. S. and Mattson M. P. (1999) Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. *J. Neurosci.* **19**, 8856–8865.

Zhang W., Potrovita I., Tarabin V., Herrmann O., Beer V., Weih F., Schneider A. and Schwaninger M. (2005) Neuronal activation of NF-kappaB contributes to cell death in cerebral ischemia. *J. Cereb. Blood Flow Metab.* **25**, 30–40.

Zhang L., Xing D. and Chen M. (2008) Bim(L) displacing Bcl-x(L) promotes Bax translocation during TNFalpha-induced apoptosis. *Apoptosis* **13**, 950–958.

