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"MOLECULAR MECHANISMS OF NCX1 AND NCX3-INDUCED NEUROPROTECTION IN ANOXIC BRAIN PRECONDITIONOING"

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SUMMARY

INTRODUCTION

The term *ischemia* (Greek *iskhein* to keep back + *haema* or *hema* blood) means a restriction in blood supply to a bodily organ or tissues. Interruption of blood flow to the brain, or part(s) of the brain, is known as cerebral ischemia or stroke. There are two different kinds of stroke: haemorrhagic or ischemic. Hemorrhagic stroke is due to the rupture of a cerebral artery because of the increase of blood pressure; ischemic stroke, that is far more common, is due to a deficit of cerebral blood flow.

The stroke is a medical emergency, which can cause permanent neurological damage, complications, and death. Stroke causes 9% of all deaths around the world, is the third most common cause of death after ischemic heart disease and cancer, and may soon become the leading cause of death worldwide. It is the leading cause of adult disability, since 76% of people in the United States and Europe survive their stroke (American Heart Association; 2008).

Representing stroke one of the three leading causes of death and the major cause of long-term disability makes it and its therapy, one of the major challenges facing contemporary medicine. In order to reduce the consequences of this disease, and to develop therapeutic strategies aimed to reduce neuronal damage, the medical research has to conduct experiments to highlight the cellular, biochemical and molecular mechanisms involved in

neuronal damage. Nowadays, there are no effective therapeutic protocols able to induce significant improvement of ischemia-induced brain damage, and all clinical trials conducted in the eighties and nineties were ineffective in the clinical treatment of stroke. The only FDA-approved treatment for acute cerebral ischemia is rTPA, which has demonstrated to cause a improvement in long-term outcomes. However, only a small percentage of patients with ischemic stroke are treated with rTPA due to its narrow therapeutic window and contraindications to thrombolytic therapy.

Thrombolytic therapy does not directly interfere with the ischemic cascade, but changes the cellular and molecular environment that induces it. Neuronal damage often continues with restored blood flow to areas. New therapies based on the understanding of the complex interactions of the ischemic cascade may eventually increase the therapeutic window for salvage of the penumbra and provide direct neuroprotection. Combination treatment with a neuroprotective drug or multiple drugs and thrombolytic therapy might theoretically improve stroke outcomes, but this hypothesis requires confirmation in clinical trials. Potentially, patients at risk of ischemic stroke could receive prophylactic treatment with a neuroprotective drug, either extending the window for thrombolytic efficacy or decreasing the damaged area. The prevention of oxidative damage with novel free radical trapping agents may be effective in improving outcome by reducing disability caused by ischemic stroke. Such agents may be efficacious both as a stand-alone

treatment in view of the efficacy seen in models of permanent ischemia in several species, as well as when used as adjuncts to thrombolytic therapy. A combination treatment approach may, in addition to reducing the damage due to ischemia, also reduce reperfusion injury, which may be caused by the deleterious effects of oxygen radical species produced when oxygenation is restored to the ischemic brain (Weinberger, 2006).

PHYSIOPATHOLOGY OF ISCHEMIC DAMAGE

The ischemic process is due to a complex series of pathophysiological events triggered by a decreased supply of oxygen and glucose in the cerebral tissue which requires, for a smooth running, large amounts of these two molecules. The cerebral energy production, given the scarcity of glucose deposits and other macromolecules available for anaerobic pathways of ATP production, depends almost entirely on the good operating of the mitochondrial oxidative chain.

The main pathogenetic mechanism involved in the genesis of ischemic injury seems to be excitotoxicity, peri-infarct depolarization, oxidative stress, inflammation, intracellular ionic dysregulation. In fact, altered Ca^{2+} dysregulation, triggering Ca^{2+}-dependent bio-polymer degradation and mitochondrial and bioenergetic failure, ultimately culminates in the activation of reactions leading to necrotic/apoptotic cell death. The ischemic cell death is due to changes directly related to the inhibition of oxidative phosphorylation. These modifications include pH reduction, ATP decrease, production of free radicals from the mitochondrial chain, increase in $[Na^+]_i$. The energetic depletion determines the block of Na^+/K^+ ATPase and Ca^{2+} ATPase lying to a reduction of neuronal and glial membrane potential (Katsura et al., 1994) (Figure 1).

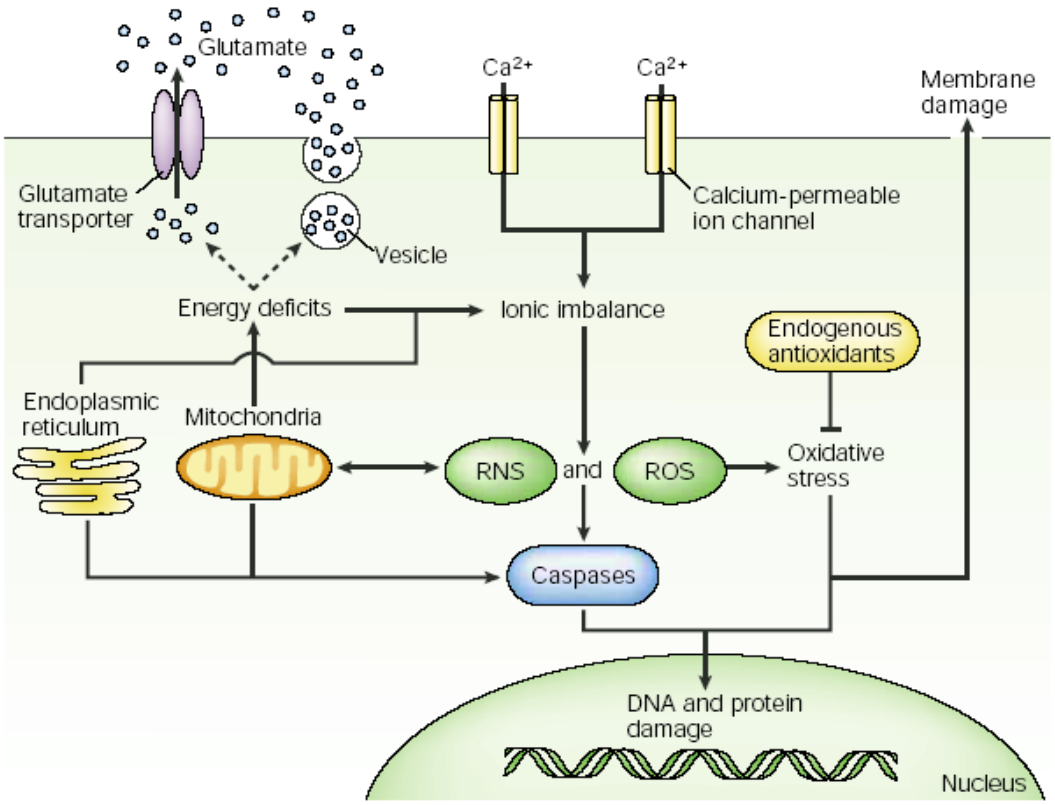


Figure 1: Physiopathology of ischemic damage

This kind of depolarization, called “anoxic depolarization” as it occurs some minutes after the stimulus anoxic/ischemic, determines both the activation of voltage gated Ca^{2+} channels (VOCC) and an inversion in the operation mode of the Na^{+} depending transporters, such as the Na^{+}/Ca^{2+} exchanger activated in *reverse mode* of operation. These modifications determine a massive entrance of Ca^{2+} into the cell with a consequent increase of its concentration, critical for the genesis and perpetuation of the ischemic damage. Indeed, this ion is able to activate enzyme and metabolic pathways potentially

detrimental, to induce the release of neurotransmitter such as glutamate, and to inhibit oxidative mitochondrial chain.

Glutamate is the most common excitatory neurotransmitter in the central nervous system and many functions of the brain occur through the release of this mediator in the synapses. Therefore, an alteration of glutamatergic neurotransmission seems to be responsible for different diseases, including them the ischemic neuronal death. In neurons, glutamate is stored in synaptic vesicles by a vesicular transmitter which uses, as energy source, a H^+ gradient created by the vesicular proton pump. This excitatory neurotransmitter is released from the presynaptical terminal after the Ca^{2+} entrance and acts both on presynaptic and postsynaptic receptors, which are ionotropic as AMPA, NMDA e kainate, and metabotropic.

The glutamate extracellular concentrations briskly rise during acute brain injury since its presynaptical Na^+ dependent transporter is blocked by the anoxic depolarization and consequent rising of $[Na^+]_i$ induced by ischemic insult.

The metabotropic glutamate receptors are coupled to a Gq protein and their stimulation activate phospholipase-C that, through the production of inositol triphosphate (IP3), mobilizes calcium from intracellular stores contributing to further increasing on its concentrations. Therefore, there is a continuous "*release to release*" of glutamate, followed by entry of Ca^{2+} and Na^+ into the cell and consequent damage and cell death.

These findings have led to the elaboration of the paradigm of glutamate excitotoxicity that explained ischemic neuronal cell death as a mere consequence of Na^+ and Ca^{2+} influx through glutamate receptors. Although this theory has been guiding basic research in the field of neurodegeneration for almost 3 decades, more recently it has become the object of serious criticism and reassessment. What has aroused such skepticism among researchers has been the fact that although first, second, and third generation glutamate receptor antagonists have long yielded promising results in animal models of brain ischemia, they have failed to elicit a neuroprotective action in stroke and traumatic brain injury in humans. Therefore, the theory of excitotoxicity, though a fascinating paradigm, can only explain some of the events occurring in the acute phase of anoxic insult but cannot be seen as a major target for developing new therapeutic avenues for brain ischemia.

Increasing evidence suggests that there may exist mechanisms of neurotoxicity that operate independently of, or in parallel with, excitotoxicity. Indeed, during ischemia, neuronal calcium channels and transporters (including NCX, TRM2, TRPM7, ASICS, CaV1.2 and hemichannels) as well as glutamate receptors (NMDAR, AMPAR, KAR) are overactivated. The increased activity of plasma membrane Ca^{2+} channels can then trigger the entry of Ca^{2+} into the cytosol of neurons, leading to larger than usual increases in the cytosolic calcium concentration. Ca^{2+} ions may gain access

to the neuronal cytoplasm via ion channels or Ca^{2+} transport systems, or through the release of Ca^{2+} ions from intracellular stores (Figure 2) (Szydlowska et al., 2009).

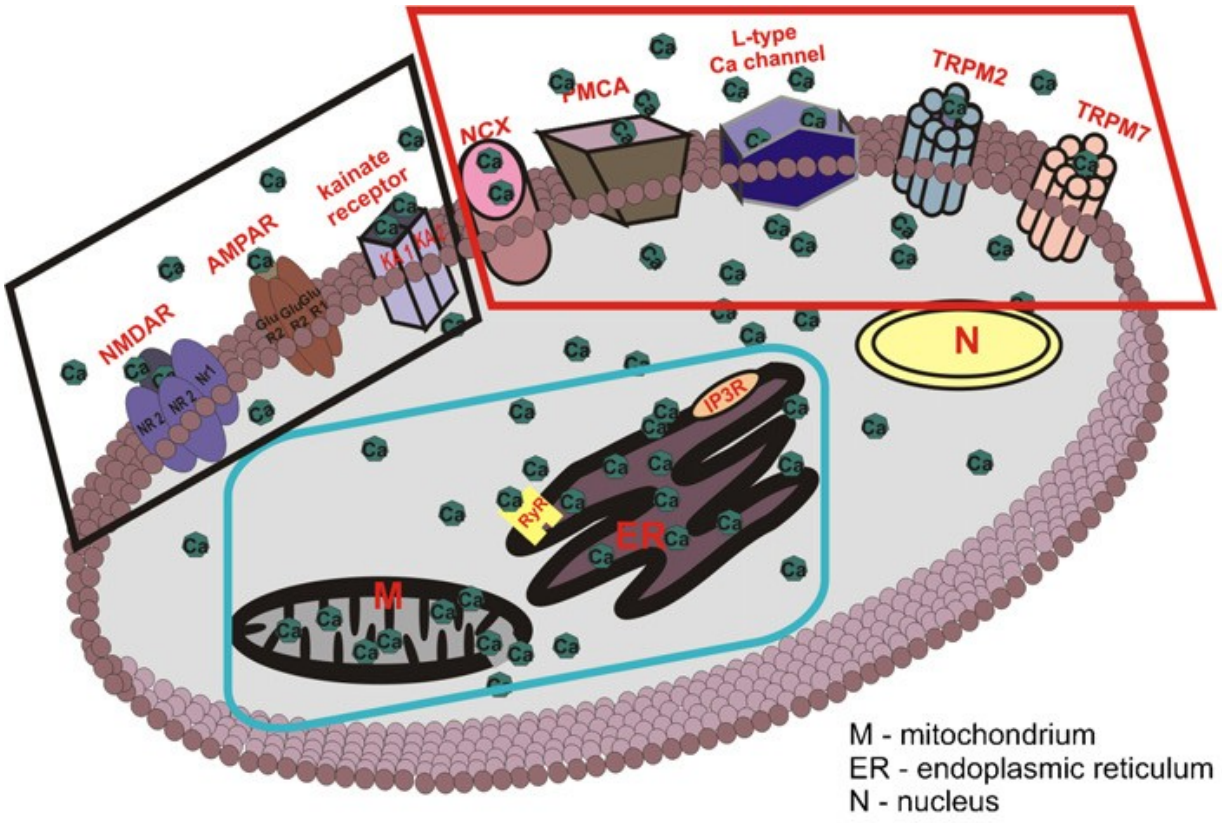


Figure 2: Routes of calcium entry into neurons after ischemic injury

Depletion of calcium ions from the endoplasmic reticulum (ER) has been suggested as an initial signal for ER dysfunction in ischemic neurons (Paschen et al., 1999). Many studies indicate that a strong release of calcium ions from ER is associated with damage to cells, including damage to neurons after ischemia (Pisani et al., 2000). Chen et al. reported that dysregulation of Ca^{2+}ER homeostasis following ischemia involves two phases: accumulation of Ca^{2+} in ER stores and subsequent release of Ca^{2+} from ER following ischemia/reoxygenation (REOX) (Chen et al., 2008). NKCC1 and NCXrev are involved in Ca^{2+} ER dysregulation, which includes overload of Ca^{2+} into ER during oxygen and glucose deprivation (OGD) and release of Ca^{2+} from ER during REOX (Chen et al., 2008). Ca^{2+} ER release is largely mediated by IP3R activation during early REOX (Bruno et al., 2001). Inhibition of NKCC1 during OGD prevented Ca^{2+} ER accumulation. This occurs because NKCC1-mediated Na^{+} flux may trigger Ca^{2+} influx through NCXrev and contributes to Ca^{2+} ER loading (Chen et al., 2008). It has been suggested that IP3R-mediated ER Ca^{2+} release can enter the adjacent mitochondria and trigger cytochrome c release (Rizzuto et al., 2006).

Consistent with an elevation of NCX activity and in accordance with normal cytosolic calcium concentrations, Sirabella et al. found that during OGD an increased refilling of Ca^{2+} into ER occurred (Sirabella et al., 2009). NCX1 upregulation occurred following OGD and may play a fundamental role in ER Ca^{2+} refilling thus preventing ER stress and cell death during OGD.

As well as endoplasmic reticulum, mitochondria sense and shape cytosolic Ca^{2+} signals by taking up and subsequently releasing Ca^{2+} ions during physiological and pathological Ca^{2+} elevations.

Mitochondria contain two membranes, an outer membrane permeable to solutes and an inner membrane impermeable to solutes that harbours the respiratory chain complexes. The respiratory chain pumps protons against their concentration gradient from the matrix of the mitochondrion into the inter-membrane space, generating an electrochemical gradient in the form of a negative inner membrane potential and of a pH gradient, the matrix being more alkaline than the cytosol (Bernardi, 1999; Poburko et al., 2011). The electrical and chemical components of the proton-motive force add up to energize the back-flux of protons down their electrochemical gradient across the ATP synthase, the enzyme that generates ATP. The negative mitochondrial membrane potential ($\Delta\Psi m$) used to drive the entry of protons also favors the entry of calcium, a divalent cation, into the mitochondrial matrix. As a result, mitochondria can accumulate large amounts of calcium through a Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter (MCU) (Kirichok et al., 2004; Nicholls et al., 2000). The MCU has a relatively low Ca^{2+} affinity, Kd~10 mM in permeabilized cells (Bernardi, 1999), but Ca^{2+} uptake can be readily detected in intact cells because a significant fraction of mitochondria are located close to calcium release or calcium entry channels and therefore exposed to microdomains of high calcium concentrations

(Giacomello et al., 2010; Rizzuto et al., 1993; Pacher et al., 2002). Electrophysiological recordings of mitoplasts, small vesicles of inner mitochondrial membrane, revealed that the MCU is a highly Ca^{2+}-selective inward-rectifying ion channel (Kirichok et al., 2004).

Instead, Ca^{2+} efflux from mitochondria is catalyzed by antiporters that drive Ca^{2+} out of the mitochondrial matrix in exchange with Na^{+} or H^{+} (Nicholls and Crompton, 1980). Two types of exchangers have been functionally characterized in the 1970s, the Na^{+}/Ca^{2+} and the H^{+}/Ca^{2+} exchangers; the former is particularly abundant in excitable tissues (Carafoli, 2003). These two pathway have been defined Na^{+}-independent pathway for Ca^{2+} efflux ("NICE") and Na^{+}-dependent pathway for Ca^{2+} efflux ("NCE") respectively. They have different kinetic of activation and calcium affinity (Harris, 1979; Lehninger et al., 1978; Ramachandran and Bygrave, 1978). Another mechanism responsible of mitochondrial calcium efflux is represented by mPTP.

Although the Na-dependent Ca^{2+} efflux is characteristic of excitable cells, recent observations indicate that the mitochondrial Na^{+}–Ca^{+} exchanger (mNCX) plays and important role in the control of $[Ca^{2+}]_c$ also in non-excitable cells, since the mNCX inhibitor CGP37157 blunts oscillations induced by G-protein coupled agonists (Hernández-SanMiguel 2006). Located in the inner mitochondrial membrane, the mNCX mediates the efflux of Ca^{2+} from the mitochondria coupled to the influx of Na^{+}, with a

stoichiometry of $3Na^+/2Ca^{2+}$ (Pfeiffer et al., 2001; Saris and Carafoli, 2005). This implies that the exchanger is electrogenic because it transports an excess of positive charge into the matrix. In respiring mitochondria, the relative membrane depolarization, caused by the activity of the exchanger, is compensated by proton pumping through the respiratory chain. As for most ion transporters of the inner mitochondrial membrane, identity of these proteins has been long searched. Only recently has the NCLX exchanger, a member of the NCX family, been hypothesized to be expressed and localized in the inner membrane of mitochondria (IMM) (Palty et al., 2010). This Li^+ sensitive protein localized within the cristae, is both phylogenetically and functionally distinct from NCX and NCKX family members (Palty et al., 2010). These authors also showed that NCLX participates to the mitochondrial Na^+/Ca^{2+} exchanger activity. However, the molecular mechanisms involved in NCLX expression, transport, localization, as well as its role in mitochondrial activity regulation still remain unidentified. On the other hand, the analysis of mitochondrial DNA does not reveal the presence of genes encoding for proteins belonging to the family of the Na^+/Ca^{2+}exchanger, suggesting that OMM and the IMM do not possess their own endogenous proteins operating as a Ca^{2+}efflux/influx pathway. Therefore, it is conceivable that a member of the NCX family, coded by nuclear DNA, might be present on mitochondria, as it occurs for the majority of mitochondrial proteins (Stojanovski, 2003). Indeed, 90% of mitochondrial proteins are coded by nuclear genes,

synthesized in the cytosol, and, subsequently, imported into mitochondria through protein translocation machineries of the outer and inner membranes (Stojanovski, 2003). More recently, our group provided evidence that the nuclear encoded NCX3 is the only isoform of the Na^+/Ca^{2+}-exchanger localized within the OMM, where it plays a relevant role in the control of mitochondrial Ca^{2+} homeostasis both under basal and under hypoxic conditions. This novel finding is not in contrast with the results of Palty et al, since it possible to hypothesize that the Na^+/Ca^{2+} exchanger in mitochondria requires two consecutive steps. The first, operated by the Na^+-sensitive NCLX, mediates Ca^{2+} transport from the matrix to the intermembrane space, and the second, operated by mNCX3, that promotes Ca^{2+} efflux from the intermembrane space to the cytosol. This interpretation is in line with the recent physiological role attributed to the OMM in the control of mitochondrial Ca^{2+} cycling. Indeed, although the outer surface of the membrane is not a passive permeable membrane, it does constitute a permeability barrier not only to Ca^{2+} influx but also to Ca^{2+} efflux (Szabadkai and Duchen, 2008). On the other hand, evidence that mNCX3 is involved in Ca^{2+} extrusion is that the Ca^{2+} lowering effect found in cells transfected with NCX3 was completely prevented by the benzothiazepine compound CGP-37157, which is considered a selective mitochondrial Na^+/Ca^{2+}exchanger inhibitor (Szabadkai and Duchen, 2008).

The Na^+-independent Ca^{2+} efflux is the main mitochondrial Ca^{2+} efflux system in non-excitable cells and since no specific cations have been found to be exchanged with Ca^{2+} it is believed to be a Ca^{2+}–H^+ exchanger (Saris et al., 2005). This transport mechanism requires transmembrane potential, since it is not observed in non-energized mitochondria, thus indicating that it is not an electroneutral passive $1Ca^{2+}$–$2H^+$ exchanger (Gunter et al., 1991). Indeed, this system is able to extrude Ca^{2+} against a gradient that is much higher than predicted from thermodynamics for an electroneutral H^+/Ca^{2+} exchanger, which indicates that it uses a component of the electrochemical gradient for its activity. A characteristic of this transporter is that it saturates at low calcium loads and its kinetics is extremely slow (Bernardi, 1999). This emphasizes a feature of the mitochondrial Ca^{2+} machinery: it is equipped with high Vmax uptake transport systems coupled to slow and easily saturable release systems, increasing the risk of Ca^{2+} overload.

As discussed above, cytosolic Ca^{2+} elevations are rapidly transmitted to the mitochondrial matrix, where they amplify the activity of Krebs cycle enzymes and of the ATP synthase, thereby increasing the production of ATP (Denton et al., 2009; Jouaville et al., 1999). During physiological Ca^{2+} elevations, the boost of ATP enables PMCA to extrude the cytosolic calcium and to sustain neuronal activity. During ischemia however, the levels of oxygen and glucose drop rapidly, impairing the production of ATP by mitochondria and by cytosolic glycolysis. As a result, ATP dependent calcium extrusion

mechanisms progressively come to a halt because the intracellular reservoir of ATP is depleted by the continuous activity of the Na^+/K^+ ATPases. The importance of the Na^+/K^+ ATPases in “stealing “ ATP from PMCA could be directly demonstrated as PMCA activity, which collapsed during metabolic depletion, could be rescued by inhibition of the Na^+/K^+ ATPases (Castro et al., 2006). PMCA inhibition amplifies the cytosolic calcium elevations that are transmitted to the mitochondrial matrix, and can then triggers a vicious sequence of mitochondrial calcium overload, mitochondrial dysfunction, release of mitochondrial pro-apotpotic factors, and the activation of death signals (Starkov et al., 2004; Duchen, 2004; Kristian et al., 1998). Mitochondria located near the plasma membrane (subplasmalemmal mitochondria) are more exposed to calcium overload due to their proximity to plasma membrane voltage-sensitive calcium channels and to the functionally incapacitated PMCA (Frieden et al., 2002; Frieden et al., 2005; Jousset et al., 2007). Intracellular mitochondria are also at risk however, and calcium release from the endoplasmic reticulum has been associated to ischemia induced-cell damage (Paschen et al., 1999; Pisani et al., 2000; Chen et al., 2008). Mitochondria are embedded within sheets of endoplasmic reticulum and the two organelles are maintained in very close proximity by linker proteins (Csordas et al., 2010; Csordas et al., 2006). Because of this proximity, the release of calcium ions through IP3 receptor of the endoplasmic reticulum readily triggers an entry of calcium in adjacent

mitochondria (Giacomello et al., 2010; Rizzuto et al., 1993). Thus, neuronal mitochondria are exposed both to Ca^{2+} ions entering across membrane channels and to Ca^{2+} released from endoplasmic reticulum Ca^{2+} stores.

Currently, it is unclear whether the mitochondrial matrix Ca^{2+} elevations occurring during ischemia are causally related to the neuronal cell death that occurs after cerebral ischemia. The best established link between mitochondrial Ca^{2+} and cellular toxicity is the opening of a Ca^{2+}-activated channel located in the inner mitochondrial membrane, the permeability transition pore (PTP) (Halestrap, 2009; Rasola et al., 2007, Zoratti et al., 2010), responsible for the so-called mitochondrial permeability transition (MPT). The MPT (Zoratti et al., 1995) is a phenomenon induced by high levels of matrix Ca^{2+} accumulation and oxidative stress, responsible for a sudden increase in the mitochondrial inner membrane permeability to solutes with molecular masses up to 1500 Da. The PTP is a Ca^{2+}, ROS (reactive oxygen species), voltage-dependent and Csa sensitive high- conductance channel, located in the inner mitochondrial membrane. Despite the great interest generated by this channel, which has been extensively characterized at the pharmacological and biophysical level, the molecular identity of the PTP is not known. The classical model envisions a supramolecular complex spanning the double membrane system of mitochondria, localized at contact sites (Brdiczka et al., 1998). Proteins of all mitochondrial compartments have been proposed to be part of the PTP (Halestrap, 2009; Rasola et al., 2007;

Zoratti et al., 2005), in particular cyclophilin D (CypD) in the mitochondrial matrix, the adenine nucleotide translocator in the inner membrane and mitochondrial porin VDAC in the outer membrane. Surprisingly, genetic studies have demonstrated that MPT can still be observed in mitochondria devoid of each of these proteins (Basso et al., 2005; Kokoszka et al., 2004; Krauskopf et al., 2006). Some other proteins, including inter-membrane and cytosolic proteins and even the proapoptotic Bcl-2 family protein Bax, have been proposed to be part of the PTP under particular conditions (Marzo et al., 1998; Narita et al., 1998), but Ca^{2+}-dependent MPT was shown to be independent of Bax (De Marchi et al., 2005). Although the proteins responsible for this important mitochondrial process have not yet been identified, the generation of CypD-knockout mice has now established an unquestionable role for CypD in facilitating PTP opening (Basso et al., 2005; Baines et al., 2005; Nagakawa et al., 2005; Schinzel et al., 2005; De Marchi et al., 2006). The biophysical properties (conductance, voltage-dependence, selectivity) of PTP were indistinguishable in mitochondria isolated from isogenic wild-type and engineered mice lacking CypD (De Marchi et al., 2006), but CsA only inhibited the PTP in wild-type mice (Basso et al., 2005; De Marchi et al., 2006), demonstrating that CypD represents the target for PTP inhibition by CsA. That the permeability transition is triggered by an elevation in the free Ca^{2+} concentration within the mitochondrial matrix was discovered early (Haworth et al., 1979; Crompton et al., 1988; Petronilli et al.,

1993). Chelation of matrix Ca^{2+} induces a rapid closure of PTP and divalent cations such as $Mg^{2+,}$ Mn^{2+}, Ba^{2+} and Sr^{2+}, instead of inducing PTP opening, can act as inhibitors of Ca^{2+} trigger sites (Bernardi et al., 1992). The relationship between MPT and cerebral ischemia inferred from the effects of CsA was nicely confirmed in CypD-knockout mice, by measuring the infarct size after cerebral ischemia/reperfusion injury induced by the occlusion of the middle cerebral artery (Schinzel et al., 2005). In this study, Schinzel and colleagues demonstrated that isolated, CypD-deficent mitochondria showed an increased capacity to retain calcium and were resistant to Ca^{2+}-induced MPT in swelling experiments. When they induced ischemia/reperfusion, a dramatic decrease in infarct size (62%) was recorded in the brains of CypD deficient mice, suggesting an essential role for CypD in cell death in the brain. A correlation between gene dosage and the extent of injury was elegantly established, by recording a partial protection in heterozygous mice (37% of reduction in infarct size). These data proved that conditions required for the activation of PTP were present during ischemia/reperfusion, as suggested by earlier pharmacological studies (Shiga et al., 1992; Khaspekov et al., 1999; Matsumoto et al., 1999). Although the causal relationship between mitochondrial Ca^{2+} accumulation and PTP opening is well established, and despite the fact that MPT invariably leads to neuronal cell death, these relationships do not necessarily imply that matrix Ca^{2+} accumulation is directly responsible for the injuries related to cerebral

ischemia. The group of Lemasters, for example, has proposed that mitochondrial Ca^{2+} overload is a consequence, rather than a cause, of the bioenergetic failure that follows MPT onset. In this view, the mitochondrial Ca^{2+} elevation is only a signature of diseased mitochondria and is not involved in the induction of the MPT, which occurs after reperfusion (Kim et al., 2006). In this study of adult rat myocytes, ROS but not Ca^{2+} overload has been suggested to trigger pH- and MPT-dependent death after ischemia/reperfusion. Another important parameter to take into account is the timing of the PTP opening during ischemia/reperfusion. In the heart, there is a broad consensus that during ischemia the factors favoring PTP opening (increased matrix Ca^{2+} and depolarization) are balanced by PTP antagonists (intracellular acidosis, high levels of Mg^{2+} and ADP) that prevent PTP opening during ischemia (Di Lisa et al., 2011). Upon reperfusion, oxygen and substrate supplies are restored to the tissue, mitochondria re-energize, take up the Ca^{2+} that has accumulated in the cytosol during ischemia, and produce a burst in ROS. The combination of these factors provides ideal conditions for triggering PTP opening (Halestrap et al., 2010; Di Lisa et al., 2011). Direct methods to asses PTP opening in intact hearts support the concept that PTP is more likely to open upon reperfusion (Griffiths et al., 1995; Di Lisa et al., 2001). Whether the same sequence of events also occurs in ischemic brain is not known, and further studies are needed to determine the precise timing of the PTP opening during cerebral ischemia.

The recent identification of the proteins involved in mitochondrial Ca^{2+} uptake and release provides new opportunities to study the role of mitochondrial Ca^{2+} in neuronal death during cerebral ischemia. The role of calcium in MPT activation and cell death can now be directly tested by modulating the expression levels of mitochondrial transport proteins. Targeting the proteins that control the fluxes of Ca^{2+} should reveal whether altered mitochondrial Ca^{2+} handling is causally related to ischemic neuronal death, and can potentially increase the repertoire of therapeutic tools to treat ischemic brain diseases.

It is not yet clear which sources of Ca^{2+} and which pathways are involved, however the defective cross-talk between intracellular stores is presumed in the etiopathogenesis of the injury (Bano D., 2007).

The activation of intracellular second messengers Ca^{2+}-related, the increase of reactive oxygen species, but also the same hypoxia, trigger the expression of many pro-inflammatory genes that induce synthesis of transcription factors such as the nuclear factor κB (NFκB) (O'Neill L.A. et al., 1997) and the hypoxia inducible factor (HIF) (Ruscher, K. et al., 1998). Moreover, the inflammation mediators, such as platelet activating factor (PAF), tumoral necrosis factor (TNF-α) and the interleukin-1, are produced by impaired neurons and glial cells. The activation of inflammatory process after stroke can induce many consequences and seems to be related to the apoptosis as well. Antibodies against adhesion molecules, as well as limits in the

development of inflammatory process, are able to reduce the number of cell dead in ischemic brain (Chopp, M. et al., 1996).

In light of what has been said so far it is clear that the pathogenesis of ischemic injury is complex and depends on multifactorial events and that intracellular ionic homeostasis alterations, impaired mitochondrial function and the production of free radicals play a major role leading to neuronal death consequent to anoxic damage.

This complexity of events explains the great discrepancy between the increasing number of cerebral ischemic accidents and the difficulty with which, unfortunately, are made studies in the development of effective treatments able to inhibit or slow the neuronal death following an ischemic episode. Hence the urgent need to identify new potential targets for the development of therapeutic strategies able to defend the ischemic brain.

In this context, one approach that has received particular attention in recent years has been an important aspect of the ischemic process referred as ischemic tolerance. This phenomenon, also known as ischemic preconditioning, is a sub-lethal anoxic insult that increases the tissue tolerance to a subsequent and potentially lethal ischemia. The cerebral ischemic preconditioning is very important because give us the possibility to study the endogenous mechanisms activated in neurons to promote cell survival after this sublethal stimulus, and thus to develop new therapeutic

strategies more selective for the treatment of the ischemic disease. The great interest in the cerebral ischemic preconditioning and in the tolerance evoked by itself, also comes from the similarity of this phenomenon with some clinical situations encountered in the human brain. Indeed, the tolerance evoked by brief ischemic episodes reminds the transient ischemic attacks (TIAs) that don't cause structural damage but appear to protect against a subsequent "stroke" (Weih et al., 1999a; Moncayo et al., 2000) and in clinical practice that precede a subsequent stroke.

ISCHEMIC PRECONDITIONING

Ischemic preconditioning or ischemic tolerance of the brain, heart, and other organs refers to a natural adaptive process that can be induced by a variety of sublethal insults (e.g., transient hypoxia, spreading depression, oxidative stres, hyperthermia, heat shock), and which increases the tissue tolerance to a subsequent, potentially lethal ischemia. This adaptive cytoprotection is a fundamental capability of living cells, allowing them to survive exposure to potentially recurrent stressors. Clearly identified in the heart by Murry et al. in 1986, preconditioning and subsequent ischemic tolerance were then demonstrated in the brain (Kitagawa et al., 1990), and attracted rapidly the interest of clinical and basic neuroscientists for several reasons. First, this biological process became widely recognized as a pertinent and effective experimental probe to understand how the brain protects itself against ischemia, thereby providing an innovative approach for the discovery of novel cerebroprotective strategies. Second, retrospective case-control studies showed a clinical correlate of the phenomenon discovered experimentally.

Preconditioning induces two different time windows of tolerance. The first phase, named “rapid preconditioning”, occurs rapidly after the stimulus and is characterized by post-translational and metabolic modifications, ionic channel activation but is not dependent on the synthesis of new proteins; the second phase, named “delayed preconditioning”, corresponds to a robust state of

tolerance that is usually detectable 24 h after preconditioning induction, peaks at 3 days, fades after 7 days and requires the synthesis of new proteins. In the brain, conversely in the heart, the tolerance induced by ischemic preconditioning is generally of the delayed type (Kitagawa et al., 1990; Liu et al., 1992; Nishi et al., 1993; Gidday et al., 1994; Goldberg et al., 1998).

The induction of ischemic tolerance is accompanied by substantial changes in gene expression, suggesting that preconditioning stimulates a fundamental genomic reprogramming of cells that confers cytoprotection and survival (Stenzel-Poore et al., 2007). The genomic response after ischemic preconditioning is a signature of the complex interplay of multiple signalling pathways. These highly specialized pathways in different cell types of the brain seem to refine the cellular and systemic response to combat the noxious stimulus. Hundreds of genes are either upregulated or downregulated in response to ischaemic preconditioning stimuli (Bernaudin et al., 2002; Stenzel-Poore et al., 2003; Tang et al., 2006). Changes in gene expression differ between harmful ischemia and ischemic preconditioning. Preconditioning seems to attenuate the response to ischemia (Stenzel-Poore et al., 2003) and the tolerance induced by it changes the expression of genes involved in the suppression of metabolic pathways, immune responses, ion-channel activity, and blood coagulation (Stenzel-Poore et al., 2003). Gene expression is regulated by transcription factors but also depends on

epigenetic mechanisms such as DNA methylation and histone modification, which modify the chromatin structure that controls access of transcription factors to regulatory loci. Inhibitions of DNA methylation and increased histone acetylation have neuroprotective effects in experimental models of stroke (Endres et al., 2000).

EXPERIMENTAL MODELS OF ISCHEMIC PRECONDITIONING

Starting from these assumptions many subsequent studies, both *in vivo* and *in vitro*, have confirmed the presence of the ischemic brain tolerance, and, in order to clarify the intracellular mechanisms underlying this phenomenon, several experimental models have been developed to reproduce *in vitro* and *in vivo* this condition.

In vivo models

A large number of studies in animal model of cerebral ischemia have shown that brief periods of non-lethal focal or global ischemia may have a protective effect against further episodes of brain ischemia.

About three decades ago, was proposed a model in which Mongolian gerbils were subjected to bilateral carotid occlusion for 5 min followed by processing of their brains for light and electron microscopy. The authors reported that there occurred rapid ischemic changes in the CA4 region, with development of slower abnormalities in the CA2 area and even still slower changes in the CA1 region (Kirino et al., 1982). Using that model, others groups (Kitagawa et al., 1990) showed that exposure to 2 min of ischemia caused depletion of high-energy phosphate compounds and perturbation of protein synthesis without any evidence of neuronal death. In addition, they found that single episodes of 2-min ischemia were able to provide protection against cell death

caused by 5 min ischemic events performed 1 or 2 days after the pretreatment. Moreover, two 2-min ischemic pretreatments performed one day apart provided complete protection against cell death induced by 5 minutes of ischemia (Kitagawa et al., 1990). The preconditioning with 2 min of ischemia prevented damage to the hippocampal CA1 area following 3 min of ischemia induced 3 days later (Kato et al., 1992). In addition, the pretreatment with unilateral occlusion of the middle cerebral artery (MCA) could induce ischemic tolerance in the hippocampal CA1 neurones of gerbils (Miyashita et al., 1994). Moreover, two minutes of preconditioning ischemia also protected gerbil hippocampal neurons against damage caused by a 3.5 min ischemia performed 3 days later (Ohtsuki et al., 1996). Because previous investigators had focused their attention on younger animals and because most ischemic strokes occur in older individuals, other groups (Dowden et al., 1999) used 18- to 20-month-old gerbils to test if they could observe preconditioning-induced protection in these animals. They found that two episodes of global ischemia, each lasting 1.5-minutes, separated by 24 hours protected animals who underwent a 5-minute occlusion of both carotid arteries 72 hours later. They also reported normal microtubule-associated protein-2 expression in the hippocampal CA1 region of preconditioned animals in contrast to the complete loss of microtubule-associated protein-2 staining observed in ischemic animals.

The ischemic preconditioning was reproduced using rats to test if sub-lethal ischemia could protect against neuronal death caused by subsequent lethal ischemic insults (Liu et al., 1992). The forebrain ischemia for 3 min was able to protect against hippocampal CA1 neuronal damage caused by 6 and 8 min of ischemia but not against damage caused by 10 min of ischemia (Liu et al., 1992). Other groups (Simon et al., 1993) also showed that two brief periods of global cerebral ischemia, separated by 24 h of normoxia, which did not cause significant cell death in the brain, were able to significantly reduce the size of an infarct caused by permanent MCA occlusion. It was also reported that global ischemia can provide substantial protection against strokes caused by MCA occlusion in the rat brain (Matsushima et al., 1995). Moreover, in another very interesting study, the possibility that ischemic preconditioning applied only 30 min before a lethal insult could still offer protection against histological damage in the rat brain was assessed. In that study, the conditioning ischemic insult lasted 2 min. After only 30 min of reperfusion, the rats were subjected to 10-min of ischemia. This procedure caused a significant degrees of protection in the hippocampal CA1 subfield and in the cortex (Perez-Pinzon et al., 1997).

In vitro models

Cell culture systems have also been used extensively to investigate the effects of preconditioning. The model of combined oxygen and glucose deprivation (OGD) that recapitulates the cascades of changes that occur during ischemia-induced injuries in the mammalian brain (Goldberg et al., 1993; Martin et al., 1994) has been largely used to investigate the potential bases of preconditioning-induced neuroprotection. In that model, transient deprivation of rodent cortical cell cultures of both oxygen and glucose causes neuronal swelling, followed by severe neuronal degeneration over a period of several hours even when the cells were returned to normal media (Goldberg et al., 1993). Acute and delayed injuries were inhibited by combined removal of extracellular Ca^{2+} in conjunction with Na^{+} or Cl^{-} substitution. The toxic effects of OGD appear to be mediated by excitotoxic damage because it causes a large increase in the levels of extracellular glutamate and because N-methyl-D-aspartate (NMDA) receptor antagonists provided protection against both the early and the late damage caused by OGD. Different groups used the OGD model to study the effects of preconditioning in vitro. They found that cellular damage was significantly reduced in cells pre-exposed to different short times (from 5 minutes to 90 minutes) of OGD with an interval of 24-48 hours before a second exposure to 3 hours of OGD. Interestingly, it was also reported that the preconditioned cultures were not protected against NMDA, kainate-, or glutamate-induced neuronal death (Grabb et al., 1999).

This is in contrast with a study in which it was reported that subjecting rat cortical cultures to a preconditioning OGD paradigm provided cross-tolerance to NMDA-induced cell death. It was also reported that NMDA receptor blockade during preconditioning by OGD eliminated tolerance (Tauskela et al., 2001). Another study comparing various preconditioning manipulations to OGD found that cycloheximide, heat stress, MK801 were as protective as OGD preconditioning in both acute and delayed models of ischemia-induced neuronal death (Meloni et al., 2002). Similar results have been reported using hippocampal slice cultures (Hassen et al., 2004), after combined deprivation of glucose and amino acids (Gaspar et al., 2006), and after exposure to neuronal cultures to various agents including anesthetic agents and diazoxide (Bickler et al., 2005; Kis et al., 2003), mild hypoxic insults (Khaspekov et al., 1998), as well as stimulation of adenosine receptors (Heurteaux et al., 1995). Interestingly, the OGD model was reproduced in organotypic cultures in which provided protection against cell death and the transient loss of evoked potential responses (Badaut et al., 2005).

Other models of ischemic preconditioning

In addition to ischemia preconditioning, there are other paradigms that have been shown to protect against ischemic injuries. These include spreading depression, hyperoxia and oxidative stress, prolonged hypoperfusion, and, as alluded to above, hyperthermia or heat shock. Therefore, one stressor can

promote ‘cross-tolerance’ to another. The sheer variety of stimuli capable of inducing an ischemia-resistant phenotype in the brain indicates that the signalling pathways activated by these different triggers converge downstream on some common, fundamental mechanisms that ultimately account for the protection. Many exogenously delivered chemical preconditioning agents (for example, inflammatory cytokines, anaesthetics and metabolic inhibitors) can also induce ischemic tolerance, raising the hope that in the future it will be possible to pharmacologically activate these distal pathways in the human brain (Gidday, 2006). The efficacy of cross-tolerance, relative to the classic ischemic preconditioning (i.e., brief, sublethal cerebral ischemia, with subsequent test ischemia that would damage the naıve brain) may be more modest, and it appears to vary with the nature and intensity of the first challenge. The window of ischemic tolerance may be also shifted or different; for example, the “tolerizing” effect or LPS needed more time to be manifest, starting at 48 h after LPS injection and reaching full potential at 72 h as described in the study of Tasaki et al. (Tasaki et al., 1997). Cross-tolerance is an important feature, but potentially misleading by suggesting that different preconditioning stimuli result ultimately in similar mechanisms of tolerance. That may be only partially true, and dependent on the nature of the stimuli considered. Comparison of the genomic profiles of brain responses to ischemia from mice preconditioned with either brief ischemia or low-dose LPS revealed that a substantial subset of the

differentially-expressed genes were unique to each preconditioning stimulus (Stenzel-Poore et al., 2007). This suggests that the nature of the preconditioning stimulus may determine a specific neuroprotective phenotype. When a specific variable (gene expression, level of a specific protein, etc.) is found to be altered in animals subjected to both preconditioning stimulus and test insult, this change may be causally related to the mechanism of protection, and this interpretation is often favored by authors. However, as the fundamental nature of preconditioning is to protect the brain against a subsequent insult, an alternative and valid interpretation may be that the identified change simply reflects, or is an epiphenomenon of, the reduced damage.

INTRACELLULAR MECHANISMS ACTIVATED BY ISCHEMIC PRECONDITIONING

Recently, three phases temporally consecutives of the preconditioning phenomenon have been identified: induction, transduction, and tolerance.

To induce tolerance, the preconditioning stimulus must be recognized by molecular sensors as a sign of something potentially much more severe to come. So far, numerous types of sensor have been identified, including neurotransmitter, neuromodulator, cytokine and toll-like receptors (Kariko et al., 2004) as well as ion channels and redox-sensitive enzymes. In turn, these sensors activate transduction pathways that initiate the adaptive response. Although dependent in part on the nature of the preconditioning stimulus, members of these transduction pathways for which there is strong general support include mitogen-activated protein kinases (MAPKs) and their phosphorylated Ras, Raf, MEK and ERK subfamilies (Dawson et al., 2000; Gonzalez-Zulueta et al., 2000; Jones et al., 2004), mitochondrial ATP-sensitive K+ (KATP) channels (Heurteaux et al., 1995; Yoshida et al., 2004) Akt (also known as protein kinase B) (Yano et al., 2001; Wick et al., 2001; Hashiguchi et al., 2004) and the protein kinase C-ε isoform (Raval et al., 2003). The possibility that the nitric oxide-based adaptive response to hypoxia in Drosophila (Wingrove et al., 1999) is evolutionarily conserved suggests that this multifunctional modulator might be a logical choice as an autocrine and paracrine mediator of preconditioning stress. Indeed,

pharmacological and genetic evidence supporting the involvement of nitric oxide in the transduction process is continuing to mount. Given the redox sensitivity of many kinases and transcription factors, reactive oxygen species might also serve as transducers (Puisieux et al., 2004; Zhang et al., 2004; Ravati et al., 2001). Adenosine, another prototypical paracrine mediator and 'retaliatory metabolite', the production of which is linked to ATP degradation, seems integral to tolerance induction in some models (Heurteaux et al., 1995; Yoshida et al., 2004; Plamondon et al., 1999; Nakamura et al., 2002). Finally, caspases might be essential induction catalysts, given that cyclic AMP responsive element-binding protein (CREB), the p50 and p65 subunits of nuclear factor-κB (NF-κB), and protein kinase C and other kinases are caspase substrates (McLaughlin et al., 2003; Algeciras-Schimnich et al., 2002). Notably, some of the aforementioned molecular transducers and signalling intermediates also serve as post-ischaemic effectors of the ischaemia-tolerant phenotype (Dirnagl et al., 2003).

Role of nitric oxide

Nitric oxide (NO) is synthesized from L-arginine and oxygen by the oxidoreductase nitric oxide synthase, an enzyme present in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible or 'immunological' NOS (iNOS) (Alderton et al. 2001). NO exerts its biological effects by reacting with oxygen, superoxide, or transitional metal centres

(Gross & Wolin, 1995; Pacher et al. 2007). The cytoprotective effects of NO typically occur through direct post-translational modification of proteins (S-nitrosylation), through activation of the cGMP second messenger system and through reaction with superoxide to form peroxynitrite. S-Nitrosylation, produced by the direct reaction of NO with cysteine thiol groups, has been linked to neuroprotection through inhibition of NMDA receceptor activity (Lei et al. 1992; Lipton et al. 1993; Kim et al. 1999). In addition, S-nitrosylation may confer neuroprotection by inhibition of apoptosis, particularly through inactivation of caspases, proteases that degrade key intracellular proteins (Melino et al. 1997; Budihardjo et al. 1999; Zhou et al. 2005). S-Nitrosylation also leads to activation of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway (Yun et al. 1998), which triggers the expression of prosurvival genes in cortical neurons. S-Nitrosylation can also play a role in gene expression through modification of transcription factors involved in neuronal survival, such as CREB (cyclic adenosine monophosphate response element binding; Lonze & Ginty, 2002). Recently, a study in cortical neurons found that the brain-derived neurotrophic factor (BDNF), well known for its role in cell survival, induced CREB binding to target DNA (Riccio et al. 2006). These effects of NO on CREB-DNA binding are mediated by S-nitrosylation of histone deacetylase 2 (HDAC2) (Riccio et al. 2006). In addition, S-nitrosylation of the p50 subunit negatively regulates the pro-inflammatory transcription factor NF-κB (nuclear

factor κ light chain enhancer of activated B cells), possibly contributing to an anti-inflammatory environment (Colasanti & Persichini, 2000). A main biological target of NO is the haem-containing enzymesoluble guanylyl cyclase (sGC), which catalyses the conversion of GTP to cGMP. Numerous studies suggest that NO-dependent elevations in cGMP and consequent activation of protein kinase G (PKG) inhibit cell death and activate prosurvival pathways. A role for NO in the expression of specific neuroplasticity-associated proteins, including BDNF, was recently demonstrated in cortical neurons and in the whisker barrel cortex, an effect that involves cGMP/PKG and ERK signalling (Gallo & Iadecola, 2011). Similarly, cGMP improves synaptic function and CREB activation in a mouse model of Alzheimer's disease (Puzzo et al. 2009). A glimpse into the cellular mechanisms of NO protection downstream of PKG is provided by studies in cardiomyocytes. Following ischaemia–reperfusion, NO-dependent PKG activity leads to activation of protein kinase Cε, which phosphorylates mitochondrial ATP-sensitive K+ (mitoKATP) channels (Costa & Garlid, 2008). The subsequent opening of the channels results in cytoprotection by inhibiting the mitochondrial transition pore (Costa & Garlid, 2008). Opening of mitoKATP channels is also associated with neuroprotection through mechanisms related to stabilization of mitochondrial function and prevention of apoptosis (Liu et al. 2002; Mayanagi et al. 2006). NO rapidly reacts with superoxide to form peroxynitrite (Beckman et al. 1990). Besides its well-established its toxic

potential at high concentrations (Calabrese et al. 2009), at low concentrations peroxynitrite can act as a signalling molecule (Liaudet et al. 2009). For instance, in the vasculature, peroxynitrite is able to induce vascular relaxation and inhibition of platelet aggregation (Liu et al. 1994; Moro et al. 1994). Peroxynitrite protects cardiomyocytes from ischaemia–reperfusion injury (Lefer et al. 1997), an effect associated with inhibition of leukocyte adhesion to endothelial cells. In addition, peroxynitrite leads to redox modulation of proteins within signalling cascades that mediate neuroprotection (Liaudet et al. 2009). For example, peroxynitrite activates ERK and Akt signalling in different cells promoting their survival (Jope et al. 2000; Pesse et al. 2005; Li et al. 2006). These observations suggest that NO has powerful protective effects that involve not only pro-survival signalling pathways, but also blood flow and vascular inflammatory events that play a prominent role in ischaemic brain injury. These aspects of NO biology are critical for understanding the protective effect that NO exerts in the context of ischaemic preconditioning. Indeed, NO has been implicated both in early and late preconditioning in the newborn as well in the adult brain. NO derived from eNOS and nNOS is essential for induction of tolerance in models of early preconditioning. For example, the lesion produced by neocortical injection of the glutamate receptor agonist NMDA was markedly reduced 1 h after administration of the pro-inflammatory mediator LPS in mice (Orio et al. 2007). The effect was not abolished by the protein synthesis inhibitor

anisomycin, attesting to the fact that the protection did not require new protein synthesis, a hallmark of early preconditioning. The protection conferred by LPS was abolished by inhibitors of nNOS, and could be reconstituted by neocortical injection of a NO donor. Furthermore, the tolerance was not observed in nNOS or eNOS null mice, but was preserved in iNOS null mice and in mice treated with the iNOS inhibitor aminoguanidine (AG) (Orio et al. 2007). In agreement with LPS preconditioning in NMDA lesions, the early tolerance to focal ischaemic injury produced by a transient episode of focal cerebral ischaemia was not observed in eNOS or nNOS null mice (Atochin et al. 2003), suggesting that eNOS and nNOS-derived NO is also involved in other preconditioning and injury modalities. These observations implicate eNOS and nNOS, but not iNOS, as sources of NO. There is evidence that the mechanisms by which NO induces early tolerance involve the second messenger cGMP. The mechanisms by which cGMP leads to tissue protection may involve PKG and mitoKATP channels. These observations, collectively, indicate that NO is critical for the establishment of ischaemic tolerance induced by transient ischaemia or by the proinflammatory mediator LPS, an effect involving activation of sGC and cGMP Production. Mounting evidence suggests that NO is also involved in delayed preconditioning. In hypoxic preconditioning in a neonatal rat model of ischaemic–hypoxic damage, the protection is abolished by the non-selective NOS inhibitor nitro-L-arginine, but not by the nNOS inhibitor 7-nitroindazole

or the iNOS inhibitor AG implicating eNOS as the source of NO. Similar results were obtained in another model of preconditioning in neonatal rats (Lin et al. 2010). With some exceptions (Hashiguchi et al. 2004;Vellimana et al. 2011), the majority of studies on the role of NO in delayed preconditioning in the adult have indicated that the source of NO is iNOS. Studies in adult mice have revealed a key role of iNOS in the preconditioning produced by LPS or transient forebrain ischaemia. iNOS null mice do not develop tolerance to focal ischaemia or NMDA lesions after treatment with LPS or transient forebrain ischaemia (Cho et al. 2005; Kawano et al. 2007; Kunz et al. 2007). Similarly, AG prevents the development of tolerance in these models. The preconditioning is associated with an increase in mitochondrial resistance to calcium-mediated depolarization (Cho et al. 2005), supporting the involvement of mitochondria also in the mechanisms of delayed tolerance. There is increasing evidence that preconditioning stimuli, in addition to changes in gene expression and mitochondrial function, also improve cerebrovascular function (Gidday, 2006). Therefore, the mechanisms of ischaemic tolerance may also include vascular effects that lead to an improvement of cerebral perfusion and blood–brain barrier function.

Moreover, is well known that NO generated by nNOS activates the G-protein Ras (Lander et al., 1995; Yun et al., 1998; Kolch et al., 2000). Ras, in turns also stimulates the PI3 kinase/Akt pathway. This signaling pathway is now largely

recognized as one of the most relevant pathways in regulating neuronal survival (Song et al., 2005). Indeed, it has various functions: 1) it regulates glucose metabolism; 2) it inactivates the mitochondrial death pathway (i.e., BAD and caspase 9) and FOXO pathway; 3) it activates CREB phosphorylation, which regulates the expression of genes critical for survival, such as those encoding for cytokines and brain derived neurotrophic factor; and 4) it activates the antiapoptotic factor nuclear factor-κB through the activation of IkB kinase complex (Fukunaga and Kawano, 2003). In addition, Akt regulates Ca^{2+} homeostasis through the potentiation of L-type voltage-gated calcium channels (Blair et al., 1999), sarco(endo)plasmatic Ca^{2+} ATPase (Kim et al., 2003), and intracellular ligand-gated ion channels (Barac et al., 2005), thus enhancing neuronal survival. Moreover, it has been demonstrated that the overexpression of constitutively active Akt1, the most highly diffused and expressed isoform in the brain (Hanada et al., 2004; Hui et al., 2005), induces an up-regulation of NCX1 and NCX3. Indeed, the overexpression of NCX1 and NCX3 isoforms induced by this serine-threonine kinase was only partially responsible for the 80% reduction of hypoxia induced cell death by Akt1-positive mutants, because siRNA-NCX1 and siRNA-NCX3 reduced this neuroprotective effect to values of 26 and 28%, respectively. This result suggested that Akt1, besides activating several neuroprotective pathways, produces an overexpression of NCX1 and NCX3 that may contribute to the reduction of hypoxia-induced cell death through an

improvement of intracellular Ca^{2+} and Na^{+} dysregulation. Together, these findings indicate that NCX1 and NCX3 constitute novel and additional targets of Akt neuroprotective action.

Overall, these downstream effector mechanisms of Akt1 result in a program of cell survival.

Role of inflammatory cytokines

Inflammatory cytokines are known to have an important role in acute stroke. Cytokines such as interleukin-1 (IL-1), and TNF-α are important mediators of the inflammatory reactions seen in cerebral ischemia (Kariko et al., 2004). TNF-α is one of the pro-inflammatory cytokines and is expressed in the ischemic brain. The effects of pretreatment with TNF administered intracisternally in mice that were subjected to MCAO 48 h later were studied (Nawashiro et al., 1997). A significant reduction in infarct size was noted in mice pretreated with TNF at the dose of 0.5 microgram/mouse. Immunohistochemical analysis of brains subjected to 24 h of MCAO revealed a significant decrease in CD11b immunoreactivity after TNF pretreatment compared with the MCAO control. Therefore, TNF induces significant protection against ischemic brain injury and is likely to be involved in the signaling pathways that regulate ischemic tolerance. Liu et al (Liu et al., 2000) demonstrated that preconditioning of rat cortical neurons with mild hypoxia protected them from hypoxia and oxygen and glucose deprivation

(OGD) injury 24 h later (50% protection). Interestingly, TNF-α pretreatment could be substituted for hypoxic preconditioning (HP). HP was attenuated by TNF-α-neutralizing antibody. The role of IL-1 in the induction of tolerance to global ischemia in Mongolian gerbils was also demonstrated (Ohtsuki et al., 1996). Arterial IL-1α and IL-1β became elevated between 1 and 3 days after a 2-min ischemic exposure. Recombinant human IL-1 receptor antagonist (IL-1ra) blocked ischemic tolerance induction by 2-min preconditioning ischemia. The possible mechanisms of IL-1 action include release of arachidonic acid, enhancement of NMDA activation and stimulation of nitric oxide synthase (Huang et al., 2006).

Role of heat shock proteins (HSPs)

Although most protein synthesis is inhibited after cerebral ischemia, the remaining active protein synthesis may have an important role in maintaining cell viability. HSPs are molecular chaperones and among the proteins synthesized during ischemia. HSPs are expressed both constitutively (cognate proteins) and under stressful conditions (as inducible forms). In addition to heat shock, a variety of stressful situations including environmental (ultraviolet radiation or heavy metals), pathological (infections or malignancies), or physiological (growth factors or cell differentiation) stimuli induce a marked increase in HSPs synthesis, known as the stress response (Tsan et al., 2004). Inducible HSPs are thought to assist the

maintenance of cellular integrity and viability by preventing protein denaturation and improper polypeptide aggregation during exposure to physiochemical insults (Ravati et al., 2001).

HSP70 is the most abundant HSP found in cells. It is expressed constitutively and is only mildly inducible. Many researchers have shown the extensive links between HSP70 overexpression and tolerance in ischemic brain injury. Some of them have reported an increase in HSP70 expression in an *in vivo* model of preconditioning (McLaughlin et al., 2003). Furthermore, HSP70 messenger RNA (mRNA) was compared in the cases of a 15-min ischemia 2 days after sham treatment and a 15-min ischemia 2 days after 10-min preconditioning (Sakurai et al., 1998). The authors reported that HSP70 mRNA in the motor neurons was strong at 8 h after preconditioning with 10-min ischemia, mild at 1 or 2 days, and not observed at 7 days after preconditioning. Others reported that thermal preconditioning with 44^{o}C body temperature protected rat cerebellar granule neurons by modulating HSP70 expression. HSP70 mRNA was detected after thermal preconditioning at 30, 60, and 90 min and increased gradually with time, whereas HSP70 antisense oligodeoxynucleotides inhibited the protective effects of thermal preconditioning against apoptosis (Chen et al., 2004). All these results implicate that HSP70 is an important player in the preconditioning process.

The underlying mechanisms of ischemic brain tolerance HSP70-mediated are not only related to its important functions in protein refolding and

transport. Emerging evidence suggests that the HSP70 family is also capable of binding and sequestering activated caspases, such as Apaf and AIF (Yenari et al., 1999). Overexpression of HSP70 inhibits the activation of NF-κB, which is persistently activated during ischemia and appears to promote apoptotic cell death. On the contrary, the deletion of the HSP70 gene notably increases cytochrome *c* release into the cytoplasm and subsequent caspase-3 activation, thereby exacerbating apoptosis and increasing infarction volume after focal cerebral ischemia (Lee et al., 2004). Furthermore, HSP70 expression is regulated by transcription factors, the activity of which is increased by ERK phosphorylation (Minet et al., 2000), a process which has been implicated in preconditioning.

Role of the anti-apoptotic protein Bcl-2

The protein Bcl-2 is an anti-apoptotic protein that resides in the outer mitochondrial membranes and the membranes of the endoplasmic reticulum. Overexpression of Bcl-2 is known to block the release of cytochrome *c*, which contributes to a signaling pathway leading to apoptosis. Indeed, two minutes of bilateral carotid artery occlusion in gerbils produced an increase in Bcl-2 at 30 h and peaked at 96 h, suggesting that the expression of Bcl-2 is involved in ischemic preconditioning (Kato et al., 1991). In addition, 20 min of transient focal ischemia, representing a sub-lethal insult, produced ischemic tolerance (attenuated infarction volume) (Shimizu et al., 2001). The results of Western

blot analysis from tolerant caudate-putamen demonstrated an increase in Bcl-2 expression 3–7 days after preconditioning. Immunocytochemical examination also found that Bcl-2 was expressed in cells with both neuronal and non-neuronal morphology in striatum. Bcl-2 antisense oligodeoxynucleotides (ODNs) treatment reduced expression of Bcl-2 in the striatum and blocked the induction of tolerance by preconditioning ischemia. Therefore, Bcl-2 appears to be a major determinant in this model of induced tolerance to focal ischemia. The transcription factors driving the induction of Bcl-2 during tolerance include CREB, indeed it has been described a cAMP-responsive element in Bcl2 promoter. As mentioned above, CREB is regulated by NMDA receptor activation, and phosphorylated robustly in the penumbral region of the preconditioned rats (Nakajima et al., 2002). Moreover, multiple protein kinases can activate transcription via the CRE, by phosphorylation of CREB, further implicating that the interrelation of these different molecular regulatory pathways could be involved in ischemic preconditioning.

Role of adenosine A_1 receptors and ATP-sensitive K^+ channels

In 1995, was demonstrated the essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in ischemic brain preconditioning. Adenosine is an endogenous neuroprotectant that can inhibit the release of excitatory amino acids. When ischemia occurs, adenosine can increase conspicuously. Adenosine inhibits synaptic transmission, decreases K+-

stimulated glutamate release, and inhibits presynaptic calcium fluxes via adenosine A1 receptors. The response to calcium influx is also important for the development of protection against ischemia, because calcium influx has been linked to the production of reactive oxygen species and the initiation of a number of signaling cascades leading to cell damage (Kristian et al., 1998). Many reports have shown that preconditioning-induced neuroprotection is dependent on adenosine A1 receptors. In rats, ischemic preconditioning increased adenosine A1 receptor immunoreactivity in the hippocampal CA1 region at days 1, 3, and 7 after preconditioning induction, within the window of ischemic tolerance (Zhou et al., 2004). Some unspecific and specific adenosine A1 receptor antagonists abolished the ischemic tolerance. Moreover, was demonstrated that the tolerance isoflurane-induced against focal cerebral ischemia model was attenuated by adenosine A1 receptor antagonists in a rat middle cerebral artery occlusion (MCAO) (Liu et al., 2006). Activation of the ATP-sensitive potassium channels (K+ ATP channels) also has a role in ischemic brain preconditioning. Blockade of the K+ ATP channels abolished preconditioning and the protection afforded by adenosine and R-PIA (an adenosine A1 receptor agonist). By contrast, a K+ ATP channel opener (RP-52891, aprikalim) induced ischemic tolerance. Meanwhile, recent evidence showed that transient infusion of the K+ ATP channel antagonist sulfonylurea tolbutamide prior to ischemia could block preconditioning induced protection after forebrain ischemia, whereas

pinacidil, a K+ ATP channel agonist, can emulate this phenomenon in hippocampal slices (Pérez-Pinzón et al., 1999). Although the precise K+ ATP channels involved in the tolerance remain undefined, two K+ ATP channels have been described recently. One of these channels resides in the mitochondrial inner membranes; the other resides in the plasma membranes. The mitochondrial K+ ATP (mK+ATP) has been suggested to be the key channel involved in ischemic preconditioning, because the mK+ ATP blocker 5-hydorxydecanoate (5-HD) prevented BIP-induced neuroprotection. It has been hypothesized that opening the mK+ ATP channels may depolarize mitochondrial membrane potential and promote an increase in the electron transport chain rate and thus increase ATP production (Schultz et al., 1999).

Role of opioid receptors

There are three types of opioid receptor: δ, κ, μ opioid receptors. The activation of opioid receptors is neuroprotective when the body encounters ischemia, hypoxia and cold. Zhang and collaborators showed that the δ opioid receptors were involved in hypoxia preconditioning in cultured rat cortical neurons (Zhang et al., 2001). They observed that δ opioid receptor activation protected cortical neurons from hypoxia injury, whereas the δ opioid receptor antagonist naloxone blocked such protection. In addition, another group found that morphine (an agonist for δ, κ, μ opioid receptors) and Tan-67 (a selective δ receptor agonist) induced a delayed

preconditioning effect both in vivo and in vitro. The morphine preconditioning-induced neuroprotection was inhibited by β-funaltrexamine, a μ-opioid receptor antagonist, but not by 7-benzylidenenaltrexone, a δ-receptor antagonist, or norbinaltorphimine, a κ-receptor antagonist. The Tan-67 preconditioning-induced neuroprotection was inhibited by 7-benzylidenenaltrexone. These results suggest that the delayed phase of morphine preconditioning may involve μ opioid receptors and δ opioid receptors. Morphine and Tan-67 may activate a shared intracellular signalling pathway to induce the delayed preconditioning effects in the brain (Zhao et al., 2006).

Role of the ubiquitin-proteasome pathway

There are two protein degradation systems in mammalian cells, the autophagy/lysosomal pathway and the ubiquitinproteasomal pathway. The ubiquitin-proteasomal pathway has been studied in cerebral ischemia and ischemic preconditioning. In rat forebrain ischemia models, transient cerebral ischemia followed by reperfusion leads to delayed selective neuronal death in hippocampal CA1 pyramidal neurons. Under electron microscopy (EM), visible protein aggregates progressively accumulate in some CA1 neurons and accumulation of the aggregates seem to occur primarily in neurons destined to undergo delayed neuronal death after brain ischemia.

Further evidence (Yoneda et al., 2004; De Gracia et al., 2007) showed that the endoplasmic reticulum (ER), mitochondria and cytoplasm all respond to the accumulation of unfolded proteins by compartment-specific signaling pathways to participate in neuronal injury, whereas ubiquintin-proteasome as well as beneficial chaperones function to prevent protein aggregation and assist in protein folding (Ghaemmaghami et al., 2003; Hebert et al., 2007). Furthermore, by utilizing a rat transient cerebral ischemic preconditioning model, was demonstrated that ischemic preconditioning significantly reduced protein aggregation in CA1 neurons after ischemia. Biochemical analyses revealed that ischemic preconditioning decreased accumulation of ubiquitin-conjugated proteins (ubi-proteins) and reduced free ubiquitin depletion after brain ischemia. Ischemic preconditioning also reduced redistribution of heat shock cognate protein 70 and Hdj1 (HSP40) from cytosolic fraction to protein aggregate-containing fraction after brain ischemia (Liu et al., 2005). Indeed, the proteasome comprises multiple protein subunits and degrades cytosolic proteins as well as misfolded proteins that fail to pass protein quality control in the ER (Wu et al., 2006). Misfolded proteins in the ER are recognized by ER-specific E3 ligases that mediate polyubiquitination of the misfolded protein on the cytosolic side of the ER and are subsequently degraded by the proteasome (Chen et al., 2008). Proteasome degradation of ubi-proteins is strictly ATP-dependent. Ubi-proteins serve as signals to activate heat shock transcription factors to induce expression of molecular chaperones (Voellmy

et al., 2004), which then shield hydrophobic surfaces of proteins in non-native states, thereby blocking their aggregation. Major cellular chaperones are ATPases and assist protein folding through numerous cycles of binding and release of unfolded protein substrates by hydrolysis of ATP. Therefore, ubiquitin–proteasome and molecular chaperons may be concerned with the neuroprotection of ischemic preconditioning and may together prevent protein aggregation in lethal ischemia.

Role of autophagy/lysosomal pathway

Recently, the roles of autophagy and the lysosomal pathway in cerebral ischemia have attracted intensive attention. Studies have reported the activation of autophagy following ischemic insults, but the contribution of autophagy to neuronal death/ survival is still under debate (Adhami et al., 2006; Carloni et al., 2008; Koike et al., 2008; Rami et al., 2008; Balduini et al., 2009). In an earlier study, we found profound activation of autophagy and lysosomes after permanent middle cerebral artery occlusion (pMCAO) and an autophagic mechanism may contribute to ischemic neuronal injury (Wen et al., 2008). However, autophagy is a double-edged sword (Shintani et al., 2004). Although massive autophagy is associated with cell death through excessive self-digestion and degradation of cellular constitutions, activation of autophagy can also protect the neurons by degrading unfolded proteins and damaged organelles. A study carried out in primary cultured

cardiomyocytes (Dosenko et al., 2006) showed that inhibition of autophagy with 3-methyladenine (3-MA, an autophagy inhibitor) during anoxia-reoxygenation caused an increase in the number of necrotic cells and a decrease of the living cell population. Thus, the process of autophagy during anoxia-reoxygenation was proposed to provide some protective effects. In addition, a recent in vitro study in PC12 cells also showed that autophagy was associated with neuroprotective effects induced by IPC (Park et al., 2009). Inhibition of autophagy, especially during reperfusion or lethal oxygen-glucose deprivation periods, ameliorated the neuroprotective effects of IPC, and also attenuated HSP70 upregulation induced by IPC. Furthermore, our recent observations showed that autophagy was also induced in a rat ischemic preconditioning model. Pretreatment with 3-MA before the onset of ischemic preconditioning completely suppressed the neuroprotective effects of preconditioning. These results strongly supported the hypothesis that induction of autophagy in ischemic preconditioning may play a part in protecting against the sequential lethal cerebral ischemia (Sheng et al, manuscript submitted).

Role of hypoxia inducible factor-1 (HIF-1)

Activation of hypoxia-inducible factor-1 (HIF-1) has been proposed as a crucial event in the regulation of hypoxia-inducible gene expression in mammalian cells (Semenza, 2000). HIF-1 is a member of the basic helix-

loop-helix family of transcription factors and requires the dimerization of HIF-1α and HIF-1β subunits for its activity (Wang and Semenza, 1995). Hypoxia-inducible factor-1 heterodimer binds to a specific DNA consensus sequence in the enhancer and promoter regions of many hypoxiainducible genes (Ebert et al., 1996; Semenza et al., 1994, 1996). Under normoxic conditions, HIF-1α protein is negatively regulated by ubiquitination and proteasomal degradation (Huang et al., 1998; Kallio et al., 1999) such that constitutive levels of HIF-1α protein are generally low or undetectable (Wiener et al., 1996; Bergeron et al., 1999*b*, 2000). During hypoxia, HIF-1α expression and concomitant HIF-1 DNA binding activity are markedly increased in several cell lines (Wang and Semenza, 1995; Wang et al., 1995; Jiang et al., 1996*a*) and primary neuronal cultures (Ruscher et al., 1998). In the brain, increased HIF-1α expression has been observed in rats exposed to systemic hypoxia (Wiener et al., 1996; Bergeron et al., 2000) or subjected to global (Jin et al., 2000) and focal ischemia (Bergeron et al., 1999*b*; Marti et al., 2000). Interestingly, *in vitro* exposure to the divalent metal cobalt chloride (CoCl2) or the iron chelator desferrioxamine during normoxic conditions triggers transcriptional changes that mimic a hypoxic response that is characterized by increased HIF-1α and HIF-1β expression, up-regulation of HIF-1 binding activity, and consequent HIF-1 target gene expression (Ebert et al., 1996; Semenza et al., 1994, 1996; Zaman et al., 1999). The hypoxia-mimetic effect of these compounds may involve the interaction with a ferroprotein oxygen sensor. Recent evidence

has shown that HIF-1α and HIF-1β mRNA and protein are induced after preconditioning with hypoxia or $CoCl_2$ in neonatal rats, suggesting that HIF-1–regulated gene expression may be involved in the hypoxia-induced tolerance to ischemic injury in immature brain (Bergeron et al., 2000).

Recently, we demonstrated that the gene coding for the ubiquitous Na^+/Ca^{2+} exchanger NCX1 protein can be included in the family of genes activated by the transcription factor HIF-1. This regulation assumes particular relevance in light of the recently emerged role of HIF-1 and NCX1 in several pathophysiological aspects of cerebral ischemia. In fact, neuron specific knocking-out of HIF-1α exacerbates brain damage after tMCAO, suggesting its neuroprotective action (Baranova et al., 2007). Similarly, NCX1 knocking-down by oligodeoxynucleotide antisense strategy increases infarct damage after permanent middle cerebral artery occlusion (Pignataro et al., 2004).

Interestingly, ncx1 transcript levels have been demonstrated to be up-regulated in regions surrounding the ischemic core (Boscia et al., 2006). Therefore, HIF-1-dependent up-regulation of ncx1 expression provides an additional mechanism involved in the pro-survival role attributed to HIF-1.

Cellular ionic homeostasis and energy metabolism

As ischemic preconditioning activates intracellular biological responses prior to a potential lethal insult, it is expected that an increase of energy metabolism or a latency in anoxic depolarization after the onset of ischemic insult might represent the mechanisms by which organs strengthen their tolerance when exposed to a sublethal insult. In this regard, several experiments have been performed, both in vivo and in vitro, in order to demonstrate that a reduction in energy demand and in the activity of ion channels represent determinant factors for ischemic tolerance (Stenzel-Poore et al., 2003). In fact, impairment in voltage-gated potassium channels has been observed in cortical neurons exposed to brief non injurious oxygen and glucose deprivation. Similarly, in vivo experiments demonstrated that ischemic preconditioning prevented the inhibition of Na^+/K^+-ATPase activity after brain ischemia in hippocampal and cortical neurons of rats exposed to global forebrain ischemia (De Sonza et al., 2000). In the last years, it has been shown that some integral plasma-membrane proteins, involved in the control of Ca^{2+} and Na^+ ion influx or efflux and, therefore, responsible for maintaining the homeostasis of these two cations, might function as crucial players in the brain ischemic process (Pignataro et al., 2004). These proteins, by regulating Na^+ and Ca^{2+} homeostasis may represent more suitable molecular targets for therapeutic intervention. Indeed, during ischemic preconditioning, the results of in vivo experiments in gerbils showed

an increase in Ca^{2+}-ATPase activity and an enhancement in mitochondrial calcium sequestration in CA1 hippocampal neurons after preconditioning (Ohta et al., 1996). In line with this result, intracellular calcium imaging performed in hippocampal neurons of preconditioned gerbils showed that the increase in $[Ca^{2+}]_i$ occurring after anoxic and aglycemic episodes was markedly inhibited in the ischemic tolerant animals (Shimazaki et al., 1998). The molecular mechanisms underlying this effect are still under investigation. A possible explanation could be increased expression of the Ca^{2+}-ATPase isoform 1 [plasma membrane calcium ATPase 1 (PMCA-1)] as recently demonstrated by Kato et al. (Kato et al., 2005). Also, mitochondria co-operate in the regulation of intracellular Ca^{2+} homeostasis, both in physiological and in pathological conditions. Although mitochondria have a high capacity for Ca^{2+} sequestration, excessive amounts of Ca^{2+}, as it occurs during ischemia, impair mitochondrial function. This induces a massive uncoupling of oxidative phosphorylation and reduction of mitochondrial membrane potential with consequent mitochondrial permeability transitional pore (MPTP) opening and reversal of the action of ATPase, which in turn hydrolyses, rather than synthesizes, ATP. As a consequence of ATP depletion and increased permeability of the inner mitochondrial membrane, cell death occurs. Studies performed both in the heart and in the brain suggested that the inhibition of MPTP opening and its signalling cascade represent crucial events responsible for cytoprotection observed in ischemic preconditioning

(Halestrap et al., 2007; Dirnagl et al., 2008). The molecular mechanisms underlining these effects are still the objects of investigation. Nitrite and protein kinases have been proposed as possible MPTP regulators (Zhao et al., 2006; Shiva et al., 2007).

The hypothesis that a modulation of the expression and activity of the sodium calcium exchanger (NCX) might play a role in the regulation of calcium and sodium homeostasis during ischemic tolerance cannot be ruled out. Indeed, the Na^+/Ca^{2+} exchanger, in parallel with selective ion channels and ATP dependent pumps, maintains the physiological cytosolic concentrations of these ions (Blaustein and Lederer, 1999). In the brain, unlike other tissues, this exchanger is present in 3 different gene products, named NCX1, NCX2, and NCX3, with a distinct distribution pattern in different brain regions (Canitano et al., 2003). Under physiological conditions, its primary role is to extrude Ca^{2+} through a *forward mode* of operation in response to a depolarization or to an increase in intracellular Ca^{2+} concentrations coupled to receptor stimulation. However, during hypoxic conditions, owing to the compromission of the 2 plasma-membrane ATP-dependent pumps Na^+/K^+ ATPase and Ca^{2+} ATPase, NCX assumes a relevant role in controlling the intracellular homeostasis of these two cations since is able to operate in the *reverse mode*, thus extruding Na^+ ions while promoting Ca^{2+} influx (Annunziato et al., 2004). Although this reverse mode of operation in the early phase of anoxia does undoubtedly elicit an increase in $[Ca^{2+}]_i$, its effect

could be beneficial for neurons because it contributes to decrease $[Na^+]_i$ overload, a phenomenon which would otherwise lead to cell swelling and, thus, sudden necrotic neuronal death. Conversely, in the later phase of neuronal anoxia, when $[Ca^{2+}]_i$ overload takes place, NCX working in the forward mode can contribute to the lowering of Ca^{2+} concentrations, and thus can protect neurons from Ca^{2+} intracellular overload, neurotoxicity and subsequent cell death. A new emerging concept is that NCX1, NCX2, and NCX3 may exert different roles during in vitro and in vivo anoxic conditions. Indeed, in cells singly and stably transfected with NCX3, this isoform contributes more significantly to the maintenance of $[Ca^{2+}]_i$ homeostasis during experimental conditions mimicking ischemia, thereby preventing mitochondrial $\Delta\Psi$ collapse and cell death (Secondo et al., 2007). In addition, in *in vivo* experiments, the selective knocking down of NCX1 and NCX3, but not of NCX2, by antisense oligodeoxynucleotide strategy, (Pignataro et al., 2004) or the disruption of the *ncx3* gene, renders the brain more susceptible to the ischemic insult (Molinaro et al., 2008). Moreover, the induction of permanent middle cerebral artery occlusion in rats, correlates with NCX1 mRNA upregulation in the peri-infarct area thus suggesting the possibility that this isoform could be a new druggable target for the treatment of cerebral ischemia. In line with this hypothesis, we recently demonstrated that NCX1 transcript and protein were up-regulated by ischemic cerebral preconditioning. This effect was mediated by the transcriptional factor HIF1 a

and was accompanied by a relevant neuroprotective effect (Valsecchi et al., 2011).

AIM OF THE STUDY

Since several players are involved in the complex molecular events implicated in ischemic tolerance, the aim of this study was to identify whether the sodium-calcium exchanger might play a role in the neuroprotection induced by cerebral ischemic preconditioning through the regulation of intracellular ionic homeostasis.

To address this issue a model of hypoxia/reoxygenation was set up in vitro by exposing cortical neurons to 3 hours of combined oxygen and glucose deprivation (OGD) followed by subsequent 24 hr exposure to normoxic conditions (reoxygenation), which represented the severe insult. The sub-lethal preconditioning stimulus was reproduced by exposing neurons to 30 minutes of OGD, 24 hours before the lethal insult.

After these treatments, experiments were conducted to detect the rate of neuronal survival by using propidium iodide (PI) and fluorescein diacetate (FDA) assay. The amount of cell death was determined measuring the ratio between the number of PI positive- dead cells compared to the total number of cells (PI and FDA positive cells) (Wei et al. 2000). The results showed that the preconditioning stimulus did not impair neuronal survival and prevented neuronal death induced by OGD/reoxygenation. Once demonstrated the neuroprotective effect of ischemic preconditioning, a further aim of the study was to investigate the molecular and cellular events involved in the regulation

of this phenomenon. In particular, the attention was addressed to the role of Na^+/Ca^{2+} exchanger (NCX). Therefore, the expression and activity of the three NCX isoforms was evaluated by Western Blot analysis, confocal microscopy and single cell microfluorimerty.

The results obtained demonstrated that NCX1 and NCX3 protein expression increased in neurons exposed to the sub-lethal insult within 48 hours, an effect that was observed even when ischemic preconditioning was applied to neurons exposed to OGD/reoxygenation, suggesting the possibility that this effect could be positively correlated with the development of tolerance. Interestingly, the rise in NCXs protein expression occurring during IPC was related to the over-activity of the nNOS synthase and NO production which in turn stimulated Akt activation. Indeed, the treatment with L-NAME and LY294002, well known inhibitors of nNOS and PI3 kinase respectively prevented NCX3 IPC-induced overexpression. Conversely, the effect of IPC on NCX1 expression was counteracted by LY294002 pretreatment, but was not affected by L-NAME. On the other hand, the activation of Akt observed after IPC was inhibited by the treatment with L-NAME, thus supporting the hypothesis that PI3K/akt/NO pathway could play a role in the regulation of NCX1 and NCX3 protein expression in the IPC phase.

To understand the functional implication of NCXs protein expression in the IPC-induced neuroprotection, the activity of the exchanger was evaluated by single cell microfluorimetry. The results showed an increase in NCX activity

in the reverse mode of operation in neurons exposed to IPC that was still detected in preconditioned neurons exposed to OGD/Reoxygenation. This raised activity did not affect cytosolic calcium concentration but promoted ER-calcium refilling. Interestingly, the treatment with siRNA against NCX1 and L-NAME abolished this effect, suggesting the importance of this isoform in the regulation of ER calcium content in a NO dependent manner. Conversely, the treatment with siRNA against NCX3 induced an increase in ER calcium content detectable 30 minutes, 24, and 48 hrs after the IPC stimulus. However, as a reduction in NCX activity was observed after the treatment with siNCX3 as well, the role of NCX3 as possible player in the regulation of mitochondrial calcium concentration ($[Ca2+]_m$) during IPC was also investigated. To address this issue, $[Ca2+]_m$ was measured, by confocal microscpy and single cell microfluorimetry, in neurons treated with siNCX3 and exposed to IPC and IPC followed by OGD/reoxygenation. The results obtained with this experimental approach suggested that NCX3 during IPC contributes to the regulation of $[Ca^{2+}]_m$ which results lower compared with that observed in control neurons or in neurons exposed to OGD/reoxygenation. This effect was more pronounced 48 hrs after IPC stimulus and was not affected by the treatment of neurons with siRNA against NCX1. Moreover, during IPC, the treatment of neurons with CGP37157 slightly modifies NCX3 activity on mitochondria inducing an increase in mitochondrial calcium concentration. Interestingly, this effect was

related to an impairment of mitochondrial oxidative capacity during IPC whereas, in the absence of the mNCX inhibitor this sub-lethal insult did not affect mitochondrial function.

Finally, to correlate the role of the NCX isoforms 1 and 3 with the neuroprotection occurring in the ischemic brain tolerance, we assessed neuronal survival in the presence of siRNAs against NCX1 and NCX3. These treatments resulted in an increase of neuronal death after preconditioning insult whereas such stimulation in the absence of treatment did not affect cell viability.

MATERIALS AND METHODS

Primary culture of cortical neurons

Cortical neurons were prepared from brains of 16-day-old Wistar rat embryos (Charles River) and used at 7 to 10 days in vitro for protein expression analysis. Briefly, dissection and dissociation were performed in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing glucose (30 mmol/L). Tissues were incubated with papain for 10 minutes at 37°C and dissociated by trituration in Earl's balanced salt solution containing DNAse (0.16 U/mL), bovine serum albumin (10 mg/mL), and ovomucoid (10 mg/mL). Cells, plated in plastic Petri dishes 100 mm diameter with a confluence of $15x10^6$ (Falcon; Becton-Dickinson) precoated with poly-D-lysine (20 μg/mL), were grown in MEM/F12 containing glucose, 5% of deactivated fetal bovine serum, and 5% horse serum, glutamine (2 mmol/L), penicillin (50 U/mL), and streptomycin (50 μg/mL; Invitrogen). To carry out single cell videoimaging and confocal microscopy, cultures of cortical neurons were prepared as described previously (Abramov et al., 2007) with modifications, from Wistar rat pups 2–4 days postpartum. The tissue was minced and trypsinized (0.1% for 15 min at 37°C), triturated, and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and 2mM L-glutamine. Cells were plated at concentration of

$1.8x10^6$ on 25-mm glass coverslips. Cultures were maintained at 37°C in a humidified atmosphere of 5%CO2 and 95% air, fed twice a week, and maintained for a minimum of 10 days before experimental use.

Combined oxygen and glucose deprivation (OGD)

In cortical neurons, OGD insult was reproduced in vitro by exposing cells to a medium previously saturated with 95% N2 and 5% CO2 for 20 min and containing (in mmol/L) 116 NaCl, 5.4 KCl, 0.8 MgSO4, 26.2 NaHCO3, 1 NaH2PO4, 1.8 CaCl2, 0.01 glycine, and 0.001 wt/vol phenol red (Scorziello et al., 2004). Hypoxic conditions were maintained using a hypoxia chamber (Billups Rothemberg Inc; temperature 37°C, atmosphere 5% CO2, and 95% N2) for 3 hours. Then, following their removal from the hypoxic chamber, they were challenged with a culture medium containing glucose and previously saturated with a mixture of 95% O2 and 5% CO2 for 10 min. Thus, reoxygenation (Rx) was achieved by returning neurons to normoxic conditions (5% CO2 and 95% air) for 24 h.

Instead, the preconditioning stimulus was reproduced by exposing neurons to 30 minutes of OGD.

Western Blot

Protein samples (50 μg) were analyzed on 8% sodium dodecyl sulfate polyacrilamide gel with 5% sodium dodecyl sulfate stacking gel (SDS-PAGE) and electrotransferred onto Hybond ECL nitrocellulose paper (Amersham). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 (TBS-T; 2 mmol/l Tris–HCl, 50 mmol/l NaCl, pH 7.5) for 2 h at RT and subsequently incubated overnight at 4 °C in the blocked buffer with the 1:1000 antibody for NCX1 (polyclonal rabbit antibody, Swant); 1:5000 antibody for NCX3 (polyclonal rabbit antibody, Philipson laboratory); 1:1000 antibody for Akt and pAKT (polyclonal rabbit antibody); 1:1000 antibody for β-actin (monoclonal antibody, Sigma). The membranes were washed with 0.1% Tween-20 and incubated with the secondary antibodies for 1 h (1:5000 NCX3 and 1:1000 NCX1; Amersham). Immunoreactive bands were detected with the ECL (Amersham). The optical density of the bands was determined by Chemi Doc Imaging System (Biorad).

Imaging mitochondrial Ca^{2+} and mitochondrial membrane potential

Mitochondrial calcium concentration ($[Ca^{2+}]_m$) was assessed using the fluorescent dye X-Rhod1. Cells were loaded with X-Rhod1 0.2 μM for 15 min in a medium containing 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes. The pH was adjusted to 7.35 with NaOH. At the end of the incubation, cells were washed

3 times in the same medium. An increase of mitochondrial localized intensity of fluorescence was indicative of mitochondrial Ca^{2+} overload.

Mitochondrial membrane potential was assessed using the fluorescent dye tetramethyl rhodamine ethyl ester (TMRE) in the "redistribution mode". Cells were loaded with TMRE (20 nM) for 30 min in the above described medium. At the end of the incubation, cells were washed in the same medium containing TMRE (20 nM) and allowed to equilibrate. A decline of mitochondria-localized intensity of fluorescence was indicative of mitochondrial membrane depolarization.

Confocal images were obtained using a Zeiss inverted 510 confocal laser scanning microscopy and a 63X oil immersion objective. The illumination intensity of 543 Xenon laser, used to excite X-Rhod-1 fluorescence, was kept to a minimum of 0.5% of laser output to avoid phototoxicity.

$[Ca^{2+}]_i$ measurment

$[Ca^{2+}]_i$ was measured by single cell computer-assisted videoimaging (Secondo et al., 2007). Briefly, cells, grown on glass coverslips, were loaded with 5 μM Fura-2 acetoxymethyl ester (Fura-2AM) for 30 min at room temperature in normal Krebs solution containing (in mM): 5.5 KCl, 160 NaCl, 1.2 MgCl2, 1.5 CaCl2, 10 glucose, and 10 Hepes–NaOH, pH 7.4. At the end of the Fura-2AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto a Zeiss

Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40X oil objective lens. The experiments were performed as previously reported (Secondo et al., 2007). The amount of Ca^{2+} extruded in the cytoplasm upon FCCP exposure was measured as $[Ca^{2+}]_i$ increase. This release is widely considered as an index of mitochondrial Ca^{2+} efflux.

Determination of cell death: Propidium iodide/Fluorescein assay

Neuronal death was evaluated by measuring the ratio between dead and living cells. To quantify cell death after the experimental procedures, the cells were washed with normal Krebs and double stained with 36μM Fluorescein Diacetate (FDA) and 7μM Propidium Iodide (PI) for 5 min at 37°C in a phosphate buffer solution. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 nm and 546 nm (Secondo *et al* 2007). PI- and FDA-positive cells were counted in three representative high power fields of independent cultures and cell death was determined by the ratio of the number of PI positive cells/PI + FDA-stained positive cells (Wei *et al* 2000).

Determination of mitochondrial function: MTT assay

Neuronal survival was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) (Hansen et al. 1989; Amoroso et al. 1999). The assay was based on the red-ox ability of living mitochondria to

convert dissolved MTT into insoluble formazan. Briefly, after treatments, the medium was removed and cells were incubated in 2 mL of MTT solution (0.5 mg/mL) for 1 h in a humidified 5% CO2 incubator at 37_C. To stop incubation, MTT solution was removed and 1 Ml dimethyl sulfoxide was added to solubilize the formazan product. The absorbance was monitored at 540 nm with a Perkin-Elmer LS 55 luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, England). The data are expressed as percentage of cell viability compared to sham-treated cultures.

Small interfering RNA against NCX1 AND NCX3

The mammalian expression vector, pSUPER.retro.puro (Oligo-Engine), was used to express siRNA against NCX1 (siRNA-NCX1; NM_019268) in cortical neurons. To prepare siRNA-NCX1, a 60-base oligonucleotide and another oligonucleotide with the complementary sequence were annealed and inserted into pSUPER.retro.puro as previously reported by Formisano et al. A mismatch sequence cloned in the same vector was used as an experimental control. To express siRNA against NCX3, the gene-specific insert contained a 19-nucleotide sequence corresponding to the coding region +124 to +142 relative to the first nucleotide of the start codon of rat NCX3 (GenBank accession no. U53420), whose specificity was verified by BLAST, was used. A mismatch sequence cloned in the same vector and pSUPER.retro.puro vector itself were used as experimental controls.

After 72 hours' plating, cortical neurons were transfected with siRNA-NCX1, siRNA-NCX3 or siRNA mismatch in OPTIMEM by means of lipofectamine LTX (Invitrogen). After 5 hours, it was replaced with fresh medium.

Materials

All the reagents were purchased from Sigma Chemicals (Milan Italy), unless otherwise specified. LY294002 was supplied by Calbiochem (Inalco, Milan, Italy). L-NAME (500 lmol/L) and PD98059 (25 lmol/L) were present in the medium 30 min before exposure to IPC and during the IPC phase.

Statistical analysis

Western blot analyses were repeated three times. All the other experiments were performed in triplicate and repeated at least three times. Data were expressed as mean ± SEM values and as percentages of basal values. Statistical significance among the means was determined by the ANOVA followed by the Newman–Keuls test. A p-value <0.05 was considered statistically significant.

RESULTS

Effect of ischemic preconditioning on neuronal survival of cortical neurons exposed to OGD/REOXYGENATION

The exposure of cortical neurons to 30 minutes of OGD, a condition that is comparable to a sublethal ischemic insult and commonly defined as ischemic preconditioning (IPC), did not affect neuronal survival. 3 hours of OGD followed by 24 hours of reoxygenation, representing the lethal insult, caused an increase in neuronal death. However, this effect was thwarted when the cells were pre-exposed, 24 hours before the lethal insult, to IPC followed by 24 hours of reoxygenation. (Figure 1).

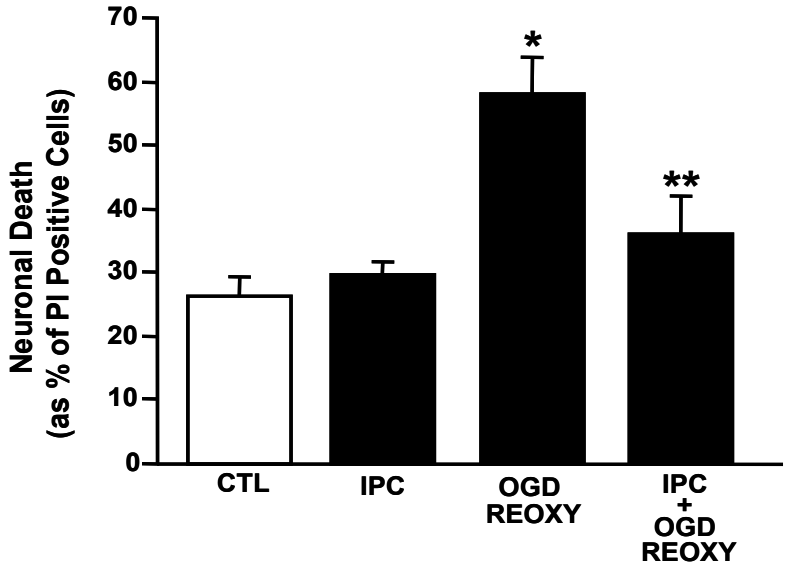


Figure 1: Effect of ischemic preconditioning on neuronal survival of cortical neurons exposed to OGD/REOXY: cortical neurons (12 DIV) were exposed to IPC (30 min di OGD) followed by 24 hrs of normoxia, and then to 3 hrs of OGD followed by 24 hrs of reoxygenation. Cell viability was measured by propidium iodide and fluorescein diacetate assay at the end of reoxygenation. *P<0.05 vs CTL and IPC. **P<0.05 vs OGD/Reoxy.

NCX1 and NCX3 protein expression after ischemic preconditioning in cortical neurons exposed to OGD/REOXYGENATION

The exposure of cortical neurons to IPC followed by 48 hours of reoxygenation determined a significant increase in NCX1 (Figure 2A) and NCX3 (Figure 2B) protein expression. The same increase was observed in neurons exposed to 3 hrs of OGD preceded, 24 hours before, by a sublethal insult followed by 24 hours of normoxia. These results create the conditions to investigate first the molecular mechanisms underlying the NCX1 and NCX3 increased expression, and to understand if this increase is positively related to the neuroprotection induced by cerebral ischemic preconditioning.

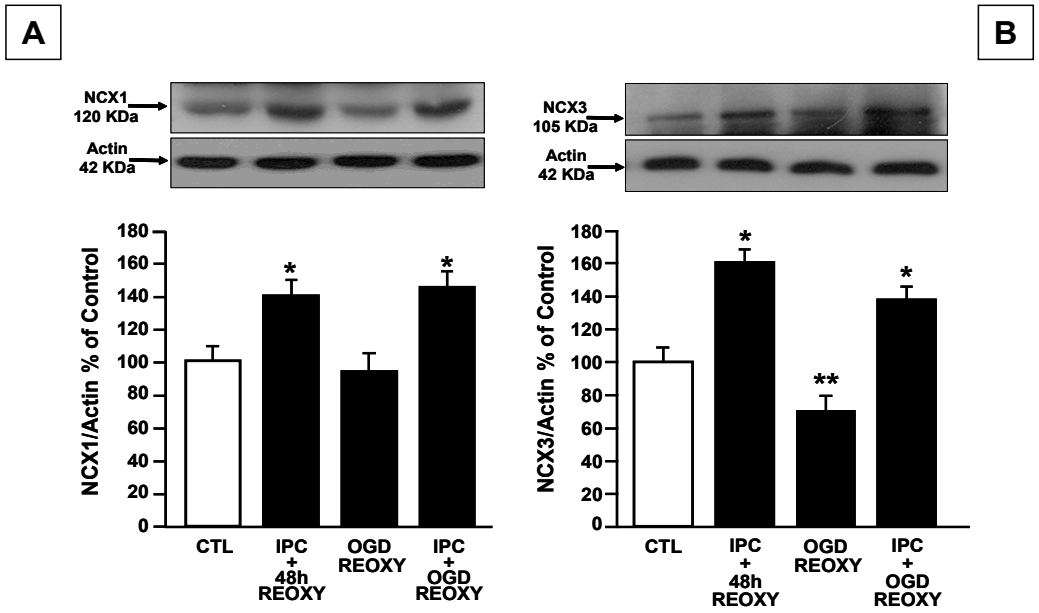


Figure 2: NCX1 e NCX3 expression after IPC in cortical neurons exposed to OGD/REOXY: cortical neurons exposed to IPC followed by 24 hrs of normoxia, and to 3 hrs of OGD followed by 24 hrs of reoxygenation. Protein expression was evaluated by western blotting at the end of reoxygenation (A) NCX1 expression. (B) NCX3 expression. *P<0.05 Vs CTL and OGD/REOXY.

Akt activation in cortical neurons exposed to IPC

The exposure of cortical neurons to IPC yielded a significant increase in phosphorylated, therefore activated, Akt expression. In order to understand if the biochemical mechanisms responsible for this rise were related to the increased nitric oxide (NO) production induced by IPC, Akt activation was explored after the treatment with L-NAME, a well known NOS inhibitor (Knepper and Kurylo 1998). The results demonstrated that this treatment was able to induce a significant decrease in pAkt expression (Figure 3), suggesting a role of NO in the regulation of Akt activity in ischemic tolerance.

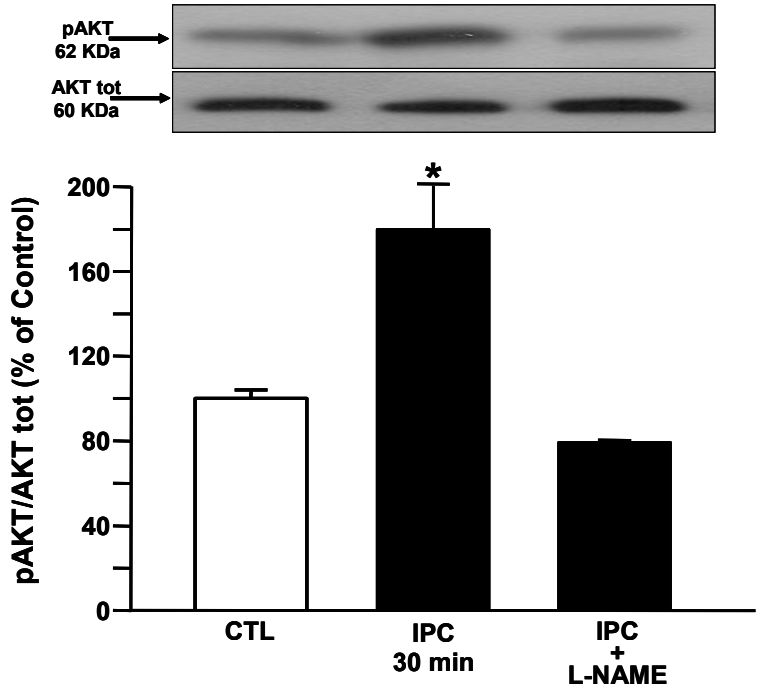


Figure 3: Effect of IPC and NOS inhibition on Akt activation: cortical neurons (12 DIV) were exposed to IPC (30 min OGD) with and without L-NAME. Protein expression was evaluated by western blotting at the end of IPC stimulus. *P<0.05 Vs CTL and IPC+L-NAME.

NCX1 and NCX3 expression after LY294002 and L-NAME treatment in cortical neurons exposed to IPC

To correlate the increase in the expression of NCX1 and NCX3 observed in cortical neurons exposed to IPC with the rise in NO production and the activation of Ras/IP3/Akt pathway, the expression of the above mentioned proteins was investigated in cortical neurons treated with LY294002 (8μmol/L), a well known IP3-kinase inhibitor, and L-NAME (500 μmol/L) before the exposure to a subletlal insult. The data confirmed that LY294002 was able to reduce significantly the NCX1 expression, whereas L-NAME did not affect its expression (Figura 4A). Instead, both LY294002 and L-NAME were able to reduce NCX3 expression (Figura 4B), suggesting that the two different isoforms of NCX might be target of the transductional Ras/IP3/Akt pathway activated by NO, probably through different mechanisms.

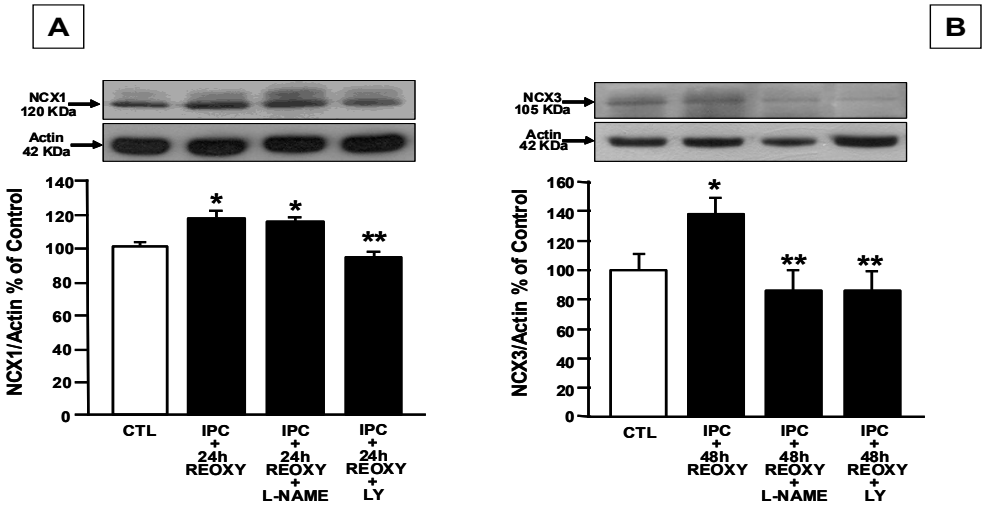
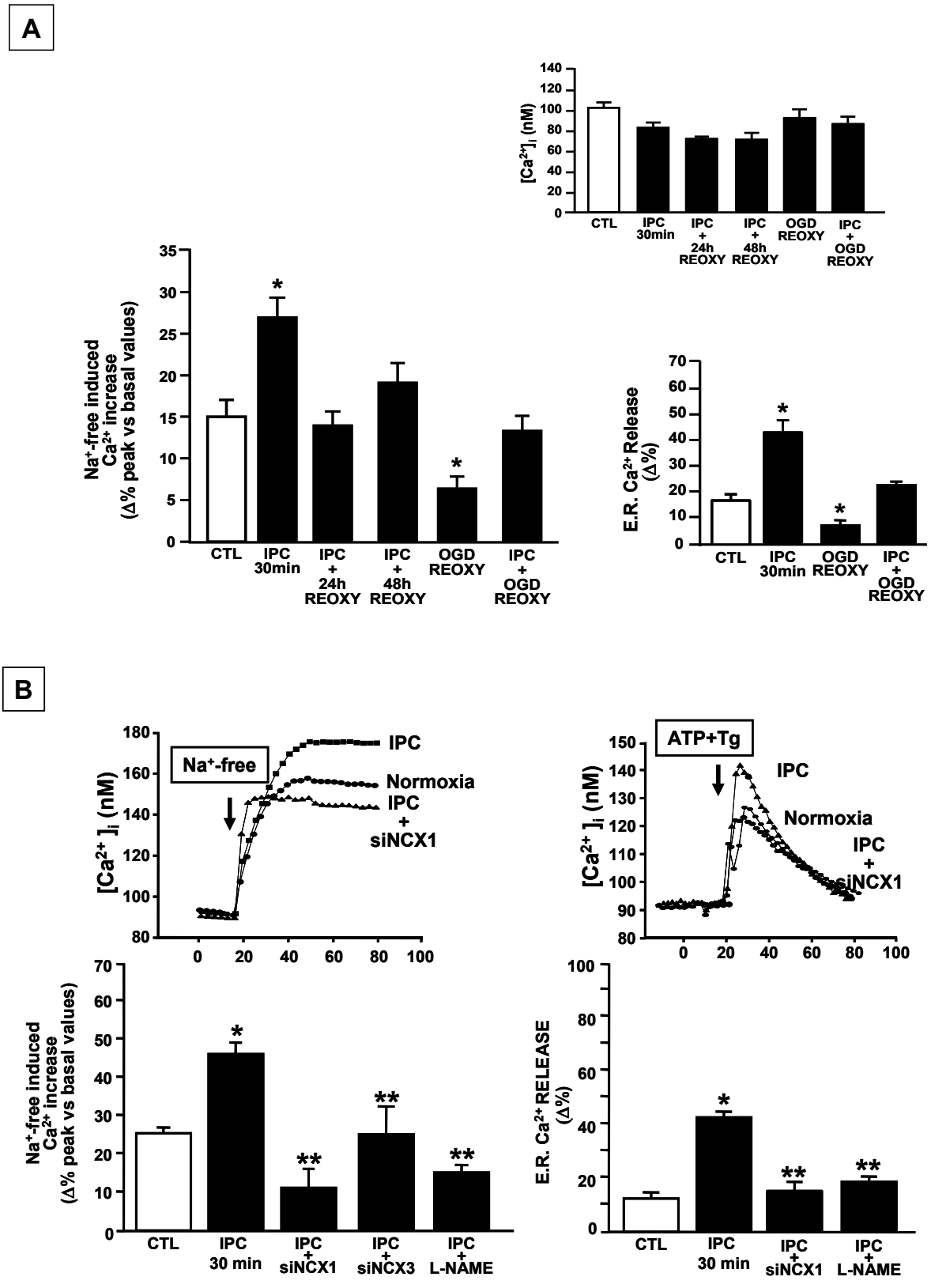


Figure 4: NCX1 and NCX3 expression after the treatment with LY294002 and L-NAME: cortical neurons exposed to IPC and treated with LY294002 and L-NAME. Protein expression was evaluated by western blotting.(A) NCX1 expression. (B) NCX3 expression. *P<0.05 Vs CTL; **P<0.05 Vs IPC.

NCX activity after ischemic preconditioning in cortical neurons exposed to OGD/REOXYGENATION

Parallel studies have been conducted to assess NCX activity during ischemic tolerance. The experiments were carried out by using single cell microfluorimetry with the fluorescent probe Fura-2AM. The results obtained demonstrated an early increase in NCX activity in the reverse mode of operation in cortical neurons exposed to IPC and IPC followed by OGD/Reoxygenaton. Since this increased activity did not affect $[Ca^{2+}]_i$, it has been hypothesized that the increase in NCX activity observed during IPC could be related with endoplasmic reticulum (ER) Ca^{2+} refilling. To this aim, cortical neurons exposed to 30 min of IPC were loaded with Fura-2AM and stimulated with a solution containing thapsigargin to induce a massive calcium release from intracellular IP3 sensitive calcium stores. By this approach, it was possible to verify that the calcium content in the ER is higher in cortical neurons exposed to IPC compared to non preconditioned neurons (Figure 5A). Since it has been demonstrated that NCX1 plays a role in ER Ca^{2+} refilling and this is considered a protective mechanism able to prevent apoptotic death associated to ER stress (Sirabella et al. 2009), the hypothesis that NCX1 might regulate the entry of calcium into endoplasmic reticulum during the preconditioning was investigated. To verify this hypothesis the experiments described above were performed in presence of siRNA against NCX1 and NCX3. The results demonstrated that only the

siNCX1 was able to prevent ER Ca^{2+} refilling during ischemic preconditioning. Moreover, the same effect was observed after the pretreatment of neurons with L-NAME (Figure 5B), thus suggesting again that NCX1 has a crucial role to prevent neuronal death during ischemic preconditioning by stimulating ER calcium refilling, probably in a NO dependent manner. The involvement of NCX1 in the regulation of ER calcium content is an early event since it was detectable within 24hr by the IPC stimulus time in which the amount of calcium into ER is higher when compared with that observed in control neurons. Conversely, 48 hrs after IPC the amount of calcium into the ER was similar to that observed in the control cells. Interestingly, siRNA against NCX3 induced an increase in ER calcium content both after 30 minutes and after 24 and 48 hrs after the IPC stimulus. In these conditions it is possible to speculate that the impairment of NCX3 activity working in the forward mode of operation, leads to the increase of intracellular calcium content, further contributing to NCX1-induced ER calcium refilling. The treatment of cortical neurons with CGP37157, the inhibitor of the mitochondrial sodium calcium exchanger, during IPC further supports this hypothesis. Indeed, in these experimental conditions the amount of calcium content in the ER was comparable to that observed in the untreated cells, thus excluding the involvement of NCX3 in the regulation of ER calcium refilling (Figure 5C).


A
[Ca2+]i (nM)
CTL
IPC 30min
IPC + 24h REOXY
IPC + 48h REOXY
OGD REOXY
IPC + OGD REOXY
Na+-free induced Ca2+ increase (Δ% peak vs basal values)
E.R. Ca2+ Release (Δ%)
B
Na+-free
IPC
Normoxia
IPC + siNCX1
ATP+Tg
IPC 30 min
IPC + siNCX1
IPC + siNCX3
IPC + L-NAME
E.R. Ca2+ RELEASE (Δ%)

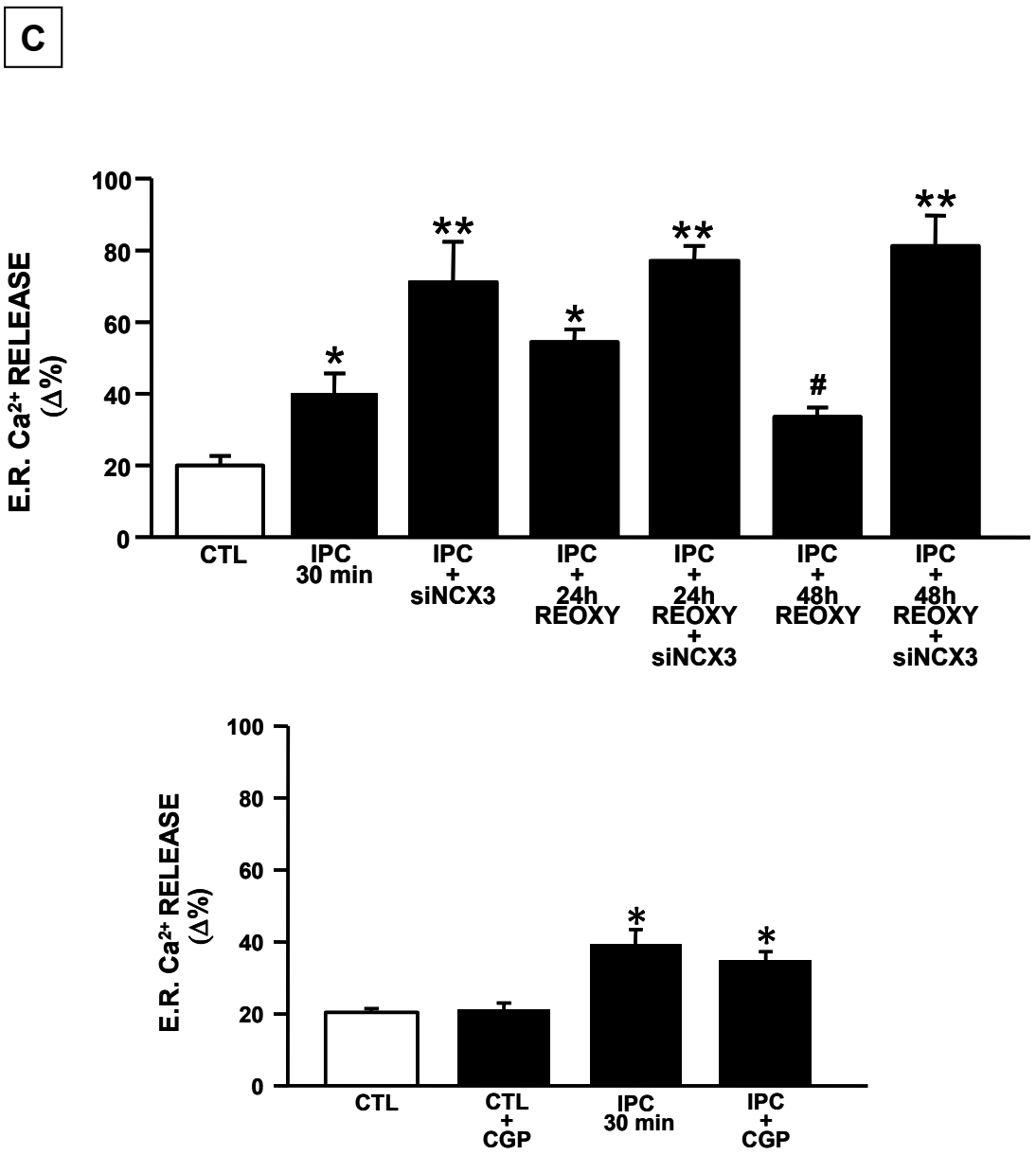
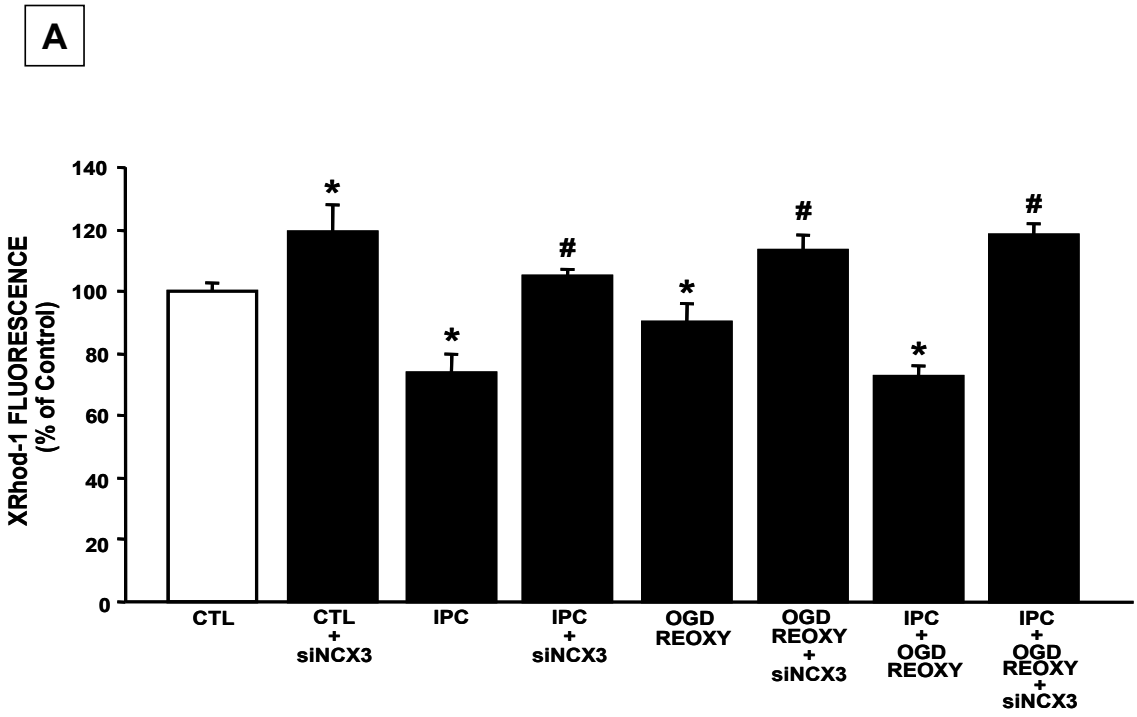


Figure 5: Effect of IPC on NCX activity and ER Ca^{2+} refilling: (A) cortical neurons exposed to IPC, followed by 24 and 48 hrs of normoxia and to 3 hrs of OGD followed by 24 hrs of Reoxygenation. $[Ca^{2+}]_i$, NCX activity and ER calcium release, after tapsigarghin stimulation, were evaluate at the end of the treatments. *P<0.05 Vs CTL; (B) effect of siNCX1and L-NAME on NCX activity and Ca^{2+} release from ER. *P<0.05 Vs CTL; **p<0.05 Vs IPC 30 min. (C) effect of siNCX3 and CGP on Ca^{2+} release from ER. *P<0.05 Vs CTL; **p<0.05 Vs not silenced neurons; **p<0.005 Vs IPC+24h reoxy and IPC+48h reoxy.

$[Ca^{2+}]_m$ in cortical neurons treated with siRNA against NCX3 and exposed to OGD/REOXYGENATION

Since the previous experiments demonstrated that NCX3 knocking down impairs NCX activation during IPC and stimulate ER calcium refilling, the next experiment was addressed to understand how NCX3 could contribute to this phenomenon. On the basis of data obtained from our research group showing that NCX3 is the only isoform present on mitochondria where it plays a key role in the mitochondrial calcium handling, and considering that mitochondria work in concert with ER in the regulation of intracellular calcium homeostasis, we performed experiments aimed to evaluate the contribution of NCX3 in the regulation of mitochondrial calcium concentration in cortical neurons exposed to ischemic preconditioning. The results showed an increase in mitochondrial calcium concentrations after the silencing of NCX3, whereas the exposure of neurons to the only IPC stimulus was able to reduce significantly the calcium levels, even when exposed to 3 hours of OGD followed by 24 hours of reoxygenation (Figure 6A). These data, obtained by using confocal microscopy and the fluorescent probe X-Rhod1, were confirmed with single cell microfluorimetry experiments. To this aim, neurons exposed to IPC were loaded with Fura 2 and then treated with FCCP uncoupler (250 nM) to induce mitochondrial depolarization and Ca^{2+} extrusion. The amount of Ca^{2+} extruded in the cytoplasm is considered an index of mitochondrial Ca^{2+} content. The results obtained with this

experimental approach suggested that NCX3 during IPC contributes to the regulation of $[Ca^{2+}]_m$ which results lower when compared with that observed in control neurons or in neurons exposed to OGD/reoxygenation (Figure 6B). Interestingly, this effect was not affected by the treatment of neurons with siRNA against NCX1 (Figure 6C). To further support the hypothesis that NCX3 on the outer mitochondrial membrane participate to mitochondrial calcium handling during IPC, neurons were treated with CGP37157 during IPC and mitochondrial calcium concentrations was measured by confocal microscopy. The results of this experiments demonstrated that CGP37157 significantly affects NCX3 activity inducing an increase in mitochondrial calcium concentration (Figure 6C).



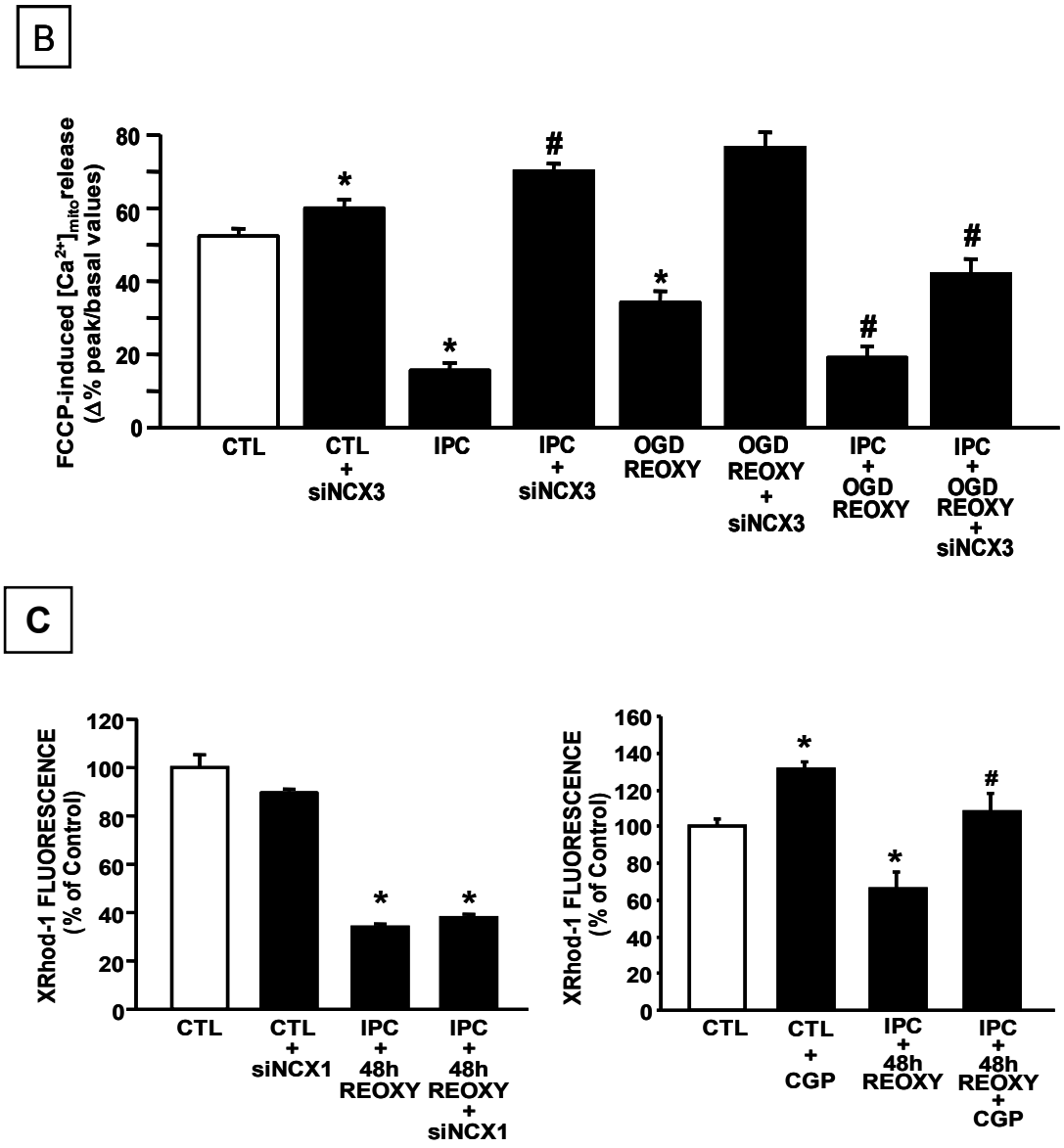


Figure 6: $[Ca^{2+}]_m$ in cortical neurons treated with siNCX3 and exposed to IPC/OGD followed by reoxygenation: (A) $[Ca^{2+}]_m$ evaluated by using confocal microscopy with the fluorescent probe X-Rhod1 in neurons (12 DIV) exposed to IPC, followed by 24 and 48 hrs of normoxia and to 3 hrs of OGD followed by 24 hrs of reoxygenation. (B) $[Ca^{2+}]$m evaluated by using single cell microfluorimetry with the probe Fura-2AM after the treatment with FCCP in cortical neurons exposed to IPC, followed by 24 e 48 hrs of normoxia and then to 3 hrs of OGD followed by 24 hrs of reoxy. $[Ca^{2+}]_m$ were measured at the end of the treatmens. *P<0.05 Vs CTL; # P<0.05 Vs its not treated CTL. (C) effect of siNCX3 and CGP on $[Ca^{2+}]_m$ in neurons exposed to IPC followed by 48 hrs of normoxia and treated with siNCX1 and CGP. *P<0.05 Vs CTL; # P<0.05 Vs IPC+48h reoxy.

Mitochondrial function in cortical neurons treated with CGP and exposed to IPC followed by OGD/REOXYGENATION

To understand whether the variations observed in mitochondrial calcium concentrations could be responsible for the neuroprotection induced by cerebral ischemic preconditioning, cell viability was assessed in terms of mitochondrial function after treatment with CGP, in cortical neurons exposed to IPC. The data showed a worsening in the mitochondrial function in neurons exposed to IPC insult after this treatment, whereas the sublethal insult, in the absence of CGP, did not affect mitochondrial function. Moreover, the significant improvement in mitochondrial activity observed in preconditioned neurons exposed to OGD followed by reoxygenation was abolished by treatment of cells with CGP (Figure 7). To rule out the possibility that CGP effect might be related to a modification of mitochondrial membrane potential and not to a real inhibition of mitochondrial exchanger, mitochondrial membrane potential was measured by using TMRE fluorescent probe in CGP-treated neurons exposed to IPC and IPC followed by OGD/Rexoygenation. The results demonstrated that CGP did not affect mitochondrial membrane (Figure 7, inset).

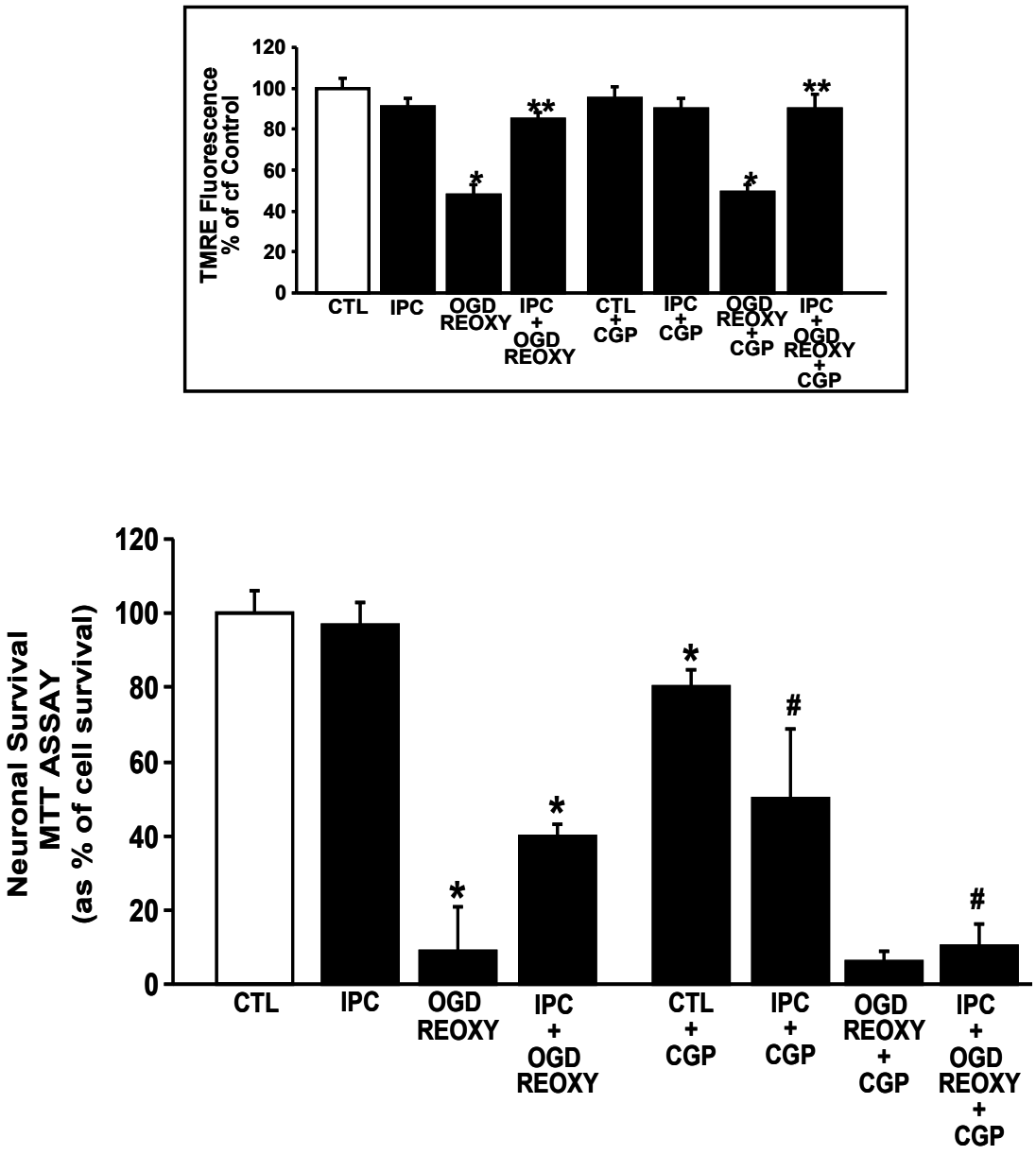


Figure 7: Mitochondrial function in cortical neurons treated with CGP and to IPC followed by OGD/Reoxygenation: cortical neurons exposed to PC followed by OGD/REOXY after the treatment with CGP. Mitochondrial function was measured by using MTT assay at the end of reoxygenation. *P<0.05 Vs CTL e IPC; # P<0.05 Vs no CGP treatment. (7 Inset) Mitochondrial membrane potential in cortical neurons exposed to IPC/OGD reoxygenation after the treatment with CGP *P<0.05 Vs CTL; **P<0.05 Vs OGD reoxy.

Effect of ischemic preconditioning on neuronal viability of cortical neurons treated with siRNA against NCX1 and NCX3 and exposed to OGD/REOXYGENATION

To further support the hypothesis that both NCX1 and NCX3, by regulating ER and mitochondrial calcium content, may be among the effectors responsible for the tolerance induced by cerebral ischemic preconditioning, neuronal viability was evaluated after the silencing of the two Na^+/Ca^{2+} exchanger isoforms. The data obtained demostrated that after these treatments there was a significant increase in cell death in cortical neurons exposed to the only IPC stimulus, whereas this sublethal insult did not affect neuronal survival but rather was able to protect neurons from death induced by OGD/Reoxygenation. Moreover, the treatment with siRNA against both NCX1 and NCX3 prevented the neuroprotective effect of ischemic preconditioning in cortical neurons exposed to a subsequent OGD followed by 24 hours of reoxygenation (Figure 8).

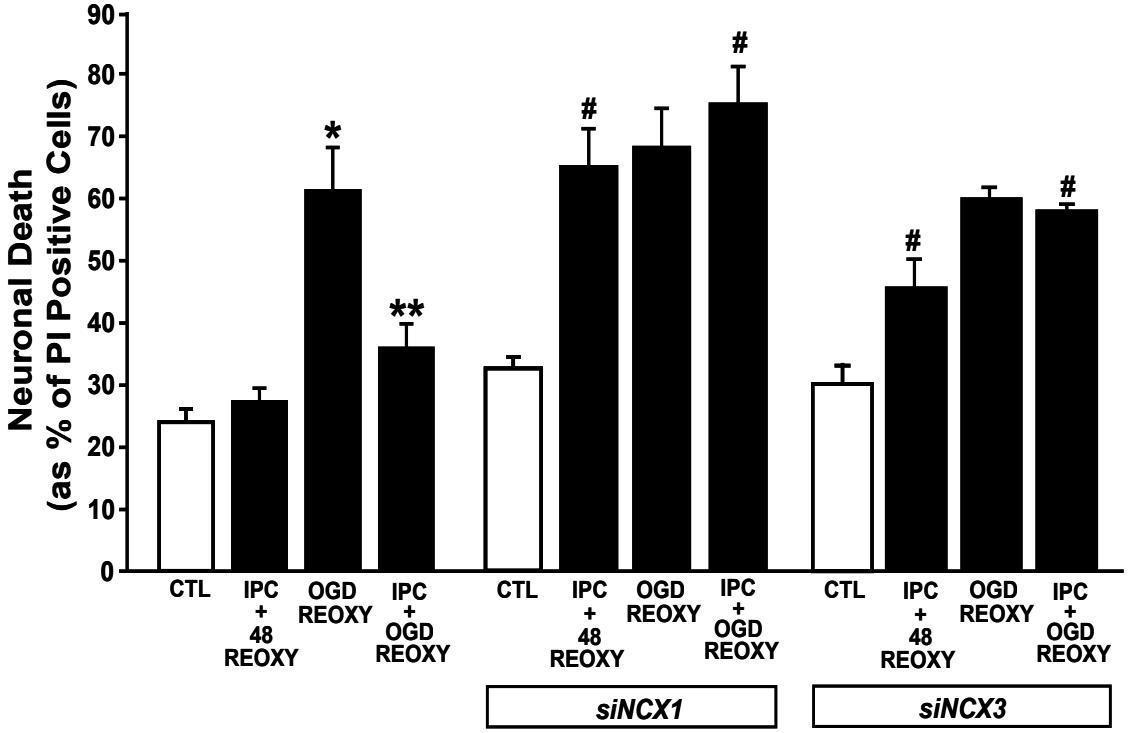


Figure 8: Effect of IPC on neuronal servival of cortical neurons treated with siNCX1 and siNCX3and exposed to OGD/Reoxy: cortical neurons (12 DIV) were expesed to IPC (30 min di OGD) followed by 24 hrs of normoxia, and then to 3 hrs of OGD followed by 24 hrs of reoxygenation with and without the silencing against NCX1 and NCX3. Cell viability was measured by propidium iodide and fluorescein diacetate assay at the end of reoxygenation. *P<0.05 Vs CTL and IPC; **P<0.05 Vs OGD/REOXY; #p<0.05 Vs cx not silenciated.

DISCUSSION

The results of the present study demonstrated that the two isoforms of the Na^+/Ca^{2+} exchanger, NCX1 and NCX3, which are involved in several pathophysiological aspects of cerebral ischemia, can be included among the members of the growing family of the mediators involved in the ischemic brain tolerance due to their ability to regulate neuronal calcium homeostasis. In particular, we proved evidence that neuroprotection observed in preconditioned neurons exposed to OGD/reoxygenation was correlated to the increase in NCX1 and NCX3 protein expression. Indeed, the treatment with siRNA against NCX1 and NCX3 was able to prevent this effect. These data are in accordance with results recently observed *in vivo* in an animal model of ischemic preconditioning (Pignataro et al., 2011). Consistently with these results we found that the upregulation of NCX1 and NCX3 protein expression in neurons exposed to IPC was dependent on PI3K/Akt activation, since the treatment with LY294002 was able to abolish this increase. Interestingly, in our experimental model, we demonstrated that NO plays a key role in the triggering PI3K/Akt pathway as the increase in the phosphorylated form of Akt observed within 30 minutes after IPC was completely abolished by the treatment with L-NAME. More importantly, the treatment with L-NAME was able to inhibit NCX3 but did not affect NCX1 protein expression. We recently demonstrated that in neurons NCX3, apart

its localization on the plasmamembrane, is also expressed on the outer mitochondrial membrane where it contributed to the extrusion of calcium from mitochondria (Scorziello et al., submitted paper). It is well known that mitochondria, in addition to the generation of cellular energy, play an important role in regulating cellular calcium homeostasis (Babcock et al., 1997; Budd and Nicholls, 1996; Werth and Thayer, 1994; Jouaville et al., 1995) in concert with the sarco-endoplasmic reticulum Ca^{2+}-ATPase, the plasma membrane Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger (Saris and Carafoli, 2005). On the other hand, the maintenance of mitochondrial calcium homeostasis is an important requirement ensuring mitochondrial function. In fact, Ca^{2+}-sensitive dehydrogenases can regulate oxidative phosphorylation and ATP synthesis during times of high cellular demand (McCormack and Denton, 1980). Therefore it is possible to hypothesize that the increased expression of NCX3, within 48hrs from the sub-lethal insult, might exert neuroprotective effects regulating calcium handling and improving mitochondrial oxidative capacity. This finding is in line with the results previously obtained in an *in vitro* model of IPC and demonstrating that the increase in nNOS expression and $NO^{\bullet}$ production through the activation of Ras/ERK1/2 pathway stimulated mitochondrial MnSOD. These effects were associated with a reduction in free radical production, cytocrome c release from mitochondria to cytosol, and in turn to an improvement of neuronal survival (Scorziello et al., 2007). On the basis of the tight correlation among

mitochondrial calcium content, oxidative metabolism and neuronal survival, the results of the present study add new insight to the molecular mechanisms involved in the ischemic brain preconditioning. Indeed, the over-expression of NCX3 might help mitochondria to preserve their energetic capacity making them less vulnerable to the subsequent lethal insult represented by OGD/reoxygenation. This hypothesis was strongly supported by the results obtained measuring the activity of NCX during IPC. In fact, we demonstrated that IPC induced an increase of NCX activity in the reverse mode of operation that was still observed in preconditioned neurons exposed to OGD/Reoxygenation. This is an early event since it occurred within 30 minutes after IPC stimulus and is due to the contribution of NCX1 and NCX3 isoforms and was promoted by NO because was abolished by the treatment with L-NAME. Intriguingly, this effect was associated with an increase in ER calcium content. Consistently with data previously published (Sirabella et al., 2009), the experiments of the present study suggested that NCX1, working in the reverse mode of operation, play a key role in the regulation of ER calcium refilling in the early phase of IPC. Indeed, the treatment with siNCX1, but not siNCX3, was able to prevent this phenomenon. The novel aspect of the study is the demonstration that NO promoted NCX1-induced ER refilling that was hampered by L-NAME pretreatment. This finding is in accordance with data recently published by Secondo et al., and demonstrating that NO was able to stimulate NCX1 to work in the reverse mode of operation, whereas NO did

not affect NCX3 activity (Secondo et al. 2011). The possibility that NCX1 activation in the early phase of IPC could affect mitochondrial calcium content promoting mitochondrial calcium uptake could not be excluded. However, in the late phase of IPC mitochondrial calcium handling is mainly regulated by NCX3 that is able to promote mitochondrial calcium extrusion. This data is supported by the finding that NCX3 expression increased 48 hrs after the IPC insult. We have previously demonstrated that NCX3 is distributed also at mitochondrial level, therefore, it is possible to speculate that during IPC the increased expression of NCX3 on mitochondria might contribute to the efflux of calcium from mitochondria thus protecting neurons by the subsequent lethal OGD/reoxygenation. The finding that the treatment with CGP and siNCX3 counteracted the effect of IPC on mitochondrial calcium content leading to the lack of IPC-neuroprotection further supports this hypothesis.

Collectively, we proposed a model (Figure 9) in which a functional interplay between NCX1 and NCX3 occurs during IPC. This phenomenon is tightly dependent on NO and Akt activation and, by contributing to the modulation of intracellular ionic homeostasis, could represent the mechanism responsible for neuroprotection induced by ischemic preconditioning.

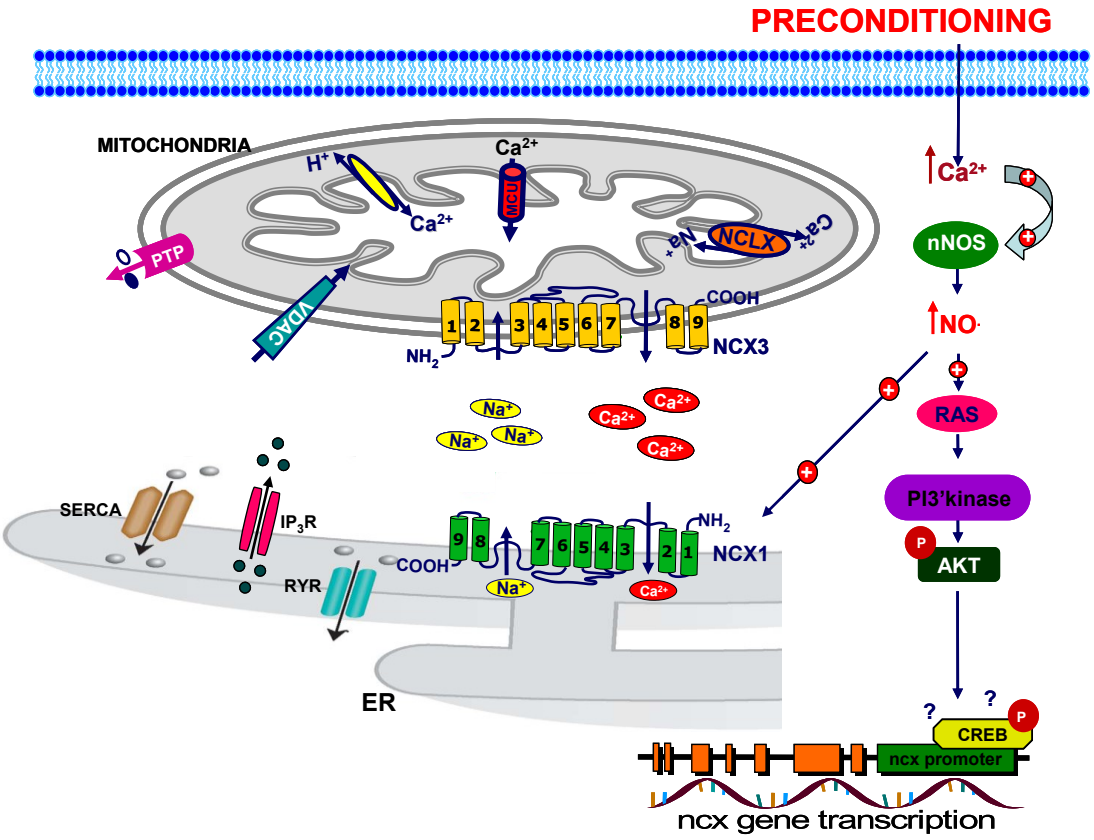


Figure 9: Proposed model

REFERENCES

Abe H, Nowak Jr TS (1996*a*) The stress response and its role in cellular defense mechanisms after ischemia. *Adv Neurol* 71:451-466.

Abe H, Nowak Jr TS (1996*b*) Gene expression and induced ischemic tolerance following brief insults. *Acta Neurobiol Exp (Warsz)* 56:3-8.

Abe H, Nowak Jr TS (2000) Postischemic temperature as a modulator of the stress response in brain: dissociation of heat shock protein 72 induction from ischemic tolerance after bilateral carotid artery occlusion in the gerbil. *Neurosci Lett* 295:54-58.

Adhami F, Liao G, Morozov YM, Schloemer A, Schmithorst VJ, Lorenz JN, (2006). Cerebral ischemia-hypoxia induces intravascular coagulation and autophagy. Am J Pathol; 169: 566–83.

Alderton WK, Cooper CE & Knowles RG (2001). Nitric oxide synthases: structure, function and inhibition. Biochem J 357, 593–615.

Algeciras-Schimnich A., Barnhart, B. C. & Peter, M. E (2002). Apoptosis-independent functions of killer caspases. Curr. Opin. Cell Biol. 14, 721–726.

Alsbo CW, Wrang ML, Johansen FF, Diemer NH (2000) Quantitative PCR analysis of AMPA receptor composition in two paradigms of global ischemia. *Neuroreport* 11:311-315.

Amin V, Cumming DV, Latchman DS (1996) Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischemic stress but with different efficiencies. *Neurosci Lett* 206:45-48.

Amoroso S, De Maio M, Russo GM, Catalano A, Bassi A, Montagnani S, Di Renzo GF and Annunziato L (1997) Pharmacological evidence that the activation of the Na^{+}-Ca^{2+} exchanger protects C6 glioma cells during chemical hypoxia. *Brit J Pharmacol* 121: 303-309.

Amoroso S, Gioielli A, Cataldi M, Di Renzo G, Annunziato L (1999) In the neuronal cell line SH-SY5Y, oxidative stress induced free radical

overproduction causes cell death without any partecipation of intracellular Ca^{++} increase *Biochem.Biophys.Acta* 1425:151-160.

Amoroso S, Tortiglione A, Secondo A, Catalano A, Montagnani S, Di Renzo G and Annunziato L (2000) Sodium nitroprusside prevents chemical hypoxia-induced cell death through iron ions stimulating the activity of the Na^{+}-Ca^{2+} exchanger in C6 glioma cells. *J Neurochem* 744:1505-1513.

Arthur PG, Lim SC, Meloni BP, Munns SE, Chan A, Knuckey NW (2004). The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. Brain Res; 1017: 146–54.

Asai A, Tanahashi N, Qiu JH, Saito N, Chi S, Kawahara N, Tanaka K, Kirino T (2002) Selective proteasomal dysfunction in the hippocampal CA1 region after transient forebrain ischemia. *J Cereb Blood Flow Metab* 22:705-710.

Asano S, Matsuda T, Takuma K, Kim HS, Sato T, Nishikawa T and Baba A (1995) Nitroprusside and cyclic GMP stimulate Na^{+}/Ca^{2+} exchange activity in neuronal preparations and cultured rat astrocytes. *J Neurochem* 64: 2437-2441.

Atochin DN, Clark J, Demchenko IT, Moskowitz MA, Huang PL (2003). Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. Stroke; 34: 1299–303.

Atochin DN, Clark J, Demchenko IT,Moskowitz MA&Huang PL (2003). Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. Stroke 34, 1299–1303.

Badaut J, Hirt L, Price M, de Castro Ribeiro M, Magistretti PJ, Regli L. Hypoxia/hypoglycaemia preconditioning prevents the loss of functional electrical activity in organotypic slice cultures. Brain Res 2005;1051:117–122.

Baines C.P., R.A. Kaiser, N.H. Purcell, N.S. Blair, H. Osinska, M.A. Hambleton, E.W. Brunskill, M.R. Sayen, R.A. Gottlieb, G.W. Dorn, J. Robbins, J.D. Molkentin, Loss of cyclophilin D reveals a critical role for

mitochondrial permeability transition in cell death, Nature 434 (2005) 658e662.

Baker PF, McNaughton PA.(1976) Kinetics and energetics of calcium efflux from intact squid giant axons. *J Physiol* 259: 103-144.

Balduini W, Carloni S, Buonocore G. Autophagy in hypoxia-ischemia induced brain injury: Evidence and speculations. Autophagy 2009; 5: 221–3.

Barac YD, Zeevi-Levin N, Yaniv G, Reiter I, Milman F, Shilkrut M, Coleman R, Abassi Z, and Binah O (2005) The 1,4,5-inositol trisphosphate pathway is a key component in Fas-mediated hypertrophy in neonatal rat ventricular myocytes. Cardiovasc Res 68:75–86.

Baranova O, Miranda LF, Pichiule P, Dragatsis I, Johnson RS, Chavez JC. Neuron-specific inactivation of the hypoxia inducible factor 1 alpha increases brain injury in a mouse model of transient focal cerebral ischemia. J Neurosci. 2007;27:6320-6332

Barone FC, White RF, Spera PA, Ellison J, Currie RW, Wang X, Feuerstein GZ (1998) Ischemic preconditioning and brain tolerance: temporal histological and functional outcomes, protein syntesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 29: 1937-1950.

Basso E., L. Fante, J. Fowlkes, V. Petronilli, M.A. Forte, P. Bernardi, Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D, J. Biol. Chem. 280 (2005) 18558e18561.

Beckman JS, Beckman TW, Chen J,Marshall PA & Freeman BA (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 87, 1620–1624.

Belayev L, Ginsberg MD, Alonso OF, Singer JT, Zhao W, Busto R. Bilateral ischemic tolerance of rat hippocampus induced by prior unilateral transient focal ischemia: relationship to c-fos mRNA expression. Neuroreport 1996;8:55–59.

Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR (2000) Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol 48:285–296

Bergeron M, Yu AY, Solway KE, Semenza GL, Sharp FR (1999b) Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischemia in rat brain. Eur J Neurosci 11:4159–4170

Bergeron M., A.Y. Yu, K.E. Solway, G.L. Semenza, F.R. Sharp (1999), Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal cerebral ischaemia in rat brain, *Eur. J. Neurosci*. 11 4159–4170.

Bergeron M., J.M. Gidday, A.Y. Yu, G.L. Semenza, D.M. Ferriero, F.R. Sharp (2000) Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain, *Ann. Neurol*. 48, 285–296.

Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 79:1127-1155.

Bernardi P, Petronilli V, Di Lisa F, and Forte M (2001) A mitochondrial perspective on cell death *Trends Biochem Sci.* 26:112-117.

Bernardi P., Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (1999) 1127e1155.

Bernardi P., S. Vassanelli, P. Veronese, R. Colonna, I. Szabo, M. Zoratti, Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations, J. Biol. Chem. 267 (1992) 2934e2939.

Bernaudin M, Tang Y, Reilly M, Petit E, Sharp FR. Brain genomic response following hypoxia and re-oxygenation in the neonatal rat. Identifi cation of genes that might contribute to hypoxia-induced ischemic tolerance. J Biol Chem 2002; 277: 39728–38.

Beyer Jr W. F., Wang Y. and Fridovich I. (1986) Phosphate inhibition of the copper- and zinc-containing superoxide dismutase: a reexamination. *Biochemistry* 25, 6084–6088.

Bickler PE, Zhan X, Fahlman CS. Isoflurane preconditions hippocampal neurons against oxygenglucose deprivation: role of intracellular Ca2+ and mitogen-activated protein kinase signaling. Anesthesiology 2005;103:532–539.

Blair LAC, Bence-Hanulec KK, Mehta S, Franke T, Kaplan D, and Marshall J (1999) Akt-dependent potentiation of L channels by insulin-like growth factor-1 is required for neuronal survival. J Neurosci 19:1940–1951.

Blaustein MP (1977) Effects of internal and external cations and ATP on sodium-calcium exchange and calcium-calcium exchange in squid axons. *Biophys J* 20: 79-111.

Blaustein MP and Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79: 763-854.

Blondeau N, Widmann C, Lazdunski M, Heurteaux C (2001) Activation of the nuclear factor-κB is a key event in brain tolerance. *J Neurosci* 21:4668-4677.

Blondeau N, Widmann C, Lazdunski M, Heurteaux C (2002) Polyunsaturated fatty acids induce ischemic and epileptic tolerance. *Neuroscience* 109, 231–241.

Bolanos J. P., Garcia-Nogales P. and Almeida A. (2004) Provoking neuroprotection by peroxynitrite. *Curr. Pharm. Des*. 10, 867–877.

Boscia F, Gala R, Pignataro G, de Bartolomeis A, Cicale M, Ambesi-Impiombato A, Di Renzo G, Annunziato L. Permanent focal brain ischemia induces isoform-dependent changes in the pattern of na+/ca2+ exchanger gene expression in the ischemic core, periinfarct area, and intact brain regions. J Cereb Blood Flow Metab. 2006;26:502-517

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. *Anal. Biochem*. 72, 248–254.

Brambrink AM, Schneider A, Noga H, Astheimer A, Gotz B, Korner I, Heimann A, Welschof M, Kempski O, (2000) Tolerance-Inducing dose of 3-nitropropionic acid modulates bcl-2 and bax balance in the rat brain: a potential mechanism of chemical preconditioning. *J Cereb Blood Flow Metab* 20, 1425–1436.

Brdiczka D., G. Beutner, A. Ruck, M. Dolder, T. Wallimann, The molecular structure of mitochondrial contact sites. Their role in regulation of

energy metabolism and permeability transition, Biofactors 8 (1998) 235e242.

Bright R, Mochly-Rosen D. The role of protein kinase C in cerebral ischemic and reperfusion injury. Stroke 2005; 36: 2781–90.

Brown IR, Rush S, Ivy GO (1989) Induction of a heat shock gene at the site of tissue injury in the rat brain. *Neuron* 2:1559-1564.

Bruer U, Weih MK, Isaev NK, Meisel A, Ruscher K, Bergk A, Trendelenburg G, Wiegand F, Victorov IV, Dirnagl U. Induction of tolerance in rat cortical neurons: hypoxic preconditioning. FEBS Lett 1997;414:117–121.

Bruno V., G. Battaglia, A. Copani, et al., Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs, J. Cereb. Blood Flow Metab. 21 (2001) 1013–1033.

Budihardjo I, Oliver H, Lutter M, Luo X &Wang X (1999). Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 15, 269–290.

Calabrese V, Cornelius C, Rizzarelli E, Owen JB, Dinkova-Kostova AT & Butterfield DA (2009). Nitric oxide in cell survival: a janus molecule. Antioxid Redox Signal 11, 2717–2739.

Cambier J., Masson M, Dehen H (2001) Neurologia.

Canitano A, Papa M, Boscia F, Castaldo P, Sellitti S, Taglialatela M and Annunziato L. (2002) Brain distribution of the Na^+/Ca^{2+} exchanger-encoding genes NCX1, NCX2, and NCX3 and their related proteins in the central nervous system. *Ann N Y Acad Sci*. 976:394-404.

Carafoli E (1985) The homeostasis of calcium in heart cells. *Mol Cell Cardiol* 17: 203-212.

Carloni S, Buonocore G, Balduini W. Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. Neurobiol Dis 2008; 32: 329–39.

Caroni P, Carafoli E. (1983) The regulation of the Na^+ -Ca^{2+} exchanger of heart sarcolemma. *Eur J Biochem*. 132:451-460.

Castro J., I. Ruminot, O.H. Porras, C.M. Flores, T. Hermosilla, E. Verdugo, F. Venegas, S. Hartel, L. Michea, L.F. Barros, ATP steal between cation pumps: a mechanism linking Naþ influx to the onset of necrotic Ca2þ overload, Cell Death Differ. 13 (2006) 1675e1685.

Centeno JM, Orti M, Salom JB, Sick TJ, Pérez-Pinzón MA (1999) Nitric oxide is involved in anoxic preconditioning neuroprotection in rat hippocampal slices. *Brain Res* 836:62-69.

Chan PH (2001) Reactive oxygen radicals in signaling and damage in ischemic brain *J Cereb Blood Flow Metab* 21:2-14.

Chen H, Luo J, Kintner DB, Shull GE, Sun D (2005) Na_-dependent chloride transporter (NKCC1)-null mice exhibit less gray and white matter damage after focal cerebral ischemia. *J Cereb Blood Flow Metab* 25, 54–66.

Chen J, Graham SH, Zhu RL, Simon RP (1996) Stress proteins and tolerance to focal cerebral ischemia. *J Cereb Blood Flow Metab* 16:566-577.

Chen J., Simon R. (1997*a*) Ischemic tolerance in the brain *Neurology* 48 306-311.

Chen JJ, Lin F, Qin ZH. The roles of the proteasome pathway in signal transduction and neurodegenerative diseases. Neurosci Bull 2008; 24: 183–94.

Chen LJ, Su XW, Qiu PX, Huang YJ, Yan GM. Thermal preconditioning protected cerebellar granule neurons of rats by modulating HSP70 expression. Acta Pharmacol Sin 2004; 25: 458–61.

Chen RW and Chuang DM (1999) Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. *J Biol Chem* 274: 6039-6042.

Chen X., D.B. Kintner, J. Luo, A. Baba, T. Matsuda, D. Sun, Endoplasmic reticulum Ca2+ dysregulation and endoplasmic reticulum stress following in vitro neuronal ischemia: role of Na+–K+–Cl− cotransporter, J. Neurochem. 106 (2008) 1563–1576.

Chen ZL, and Strickland S (1997*b*) Neuronal death in hippocampus is promoted by plasmin catalyzed degradation of laminin *Cell* 91, 917-925.

Cho S, Park EM, Zhou P, Frys K, Ross ME & Iadecola C (2005). Obligatory role of inducible nitric oxide synthase in ischemic preconditioning. J Cereb Blood Flow Metab 25, 493–501.

Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 11: 465-469.

Chopp M, Chen H, Ho K-L, Dereski MO, Brown E, Hetzel FW, Welch KMA (1989) Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. *Neurology* 39:1396-1398.

Chopp M, Li Y (1996) Apoptosis in focal cerebral ischemia Acta Neurochir. Suppl. 66:21-26.

Ciechanover A, Orian A, Schwartz AL (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22:442-451.

Colasanti M & Persichini T (2000). Nitric oxide: an inhibitor of NF-κB/Rel system in glial cells. Brain Res Bull 52, 155–161.

Corbett D, Crooks P (1997) Ischemic preconditioning: a long term survival study using behavioral and histological endpoints. *Brain Res* 760:129-136.

Costa ADT & Garlid KD (2008). Intramitochondrial signaling: interactions among mitoKATP, PKCε, ROS, andMPT. Am J Physiol Heart Circ Physiol 295, H874–H882.

Cregan SP, Arbour NA, Maclaurin JG, Callaghan SM, Fortin A, Cheung EC, Guberman DS, Park DS, Slack RS (2004). p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. *J Neurosci* 24, 10003–10012.

Crompton M., A. Costi, Kinetic evidence for a heart mitochondrial pore activated by Ca2þ, inorganic phosphate and oxidative stress. A potential mechanism for mitochondrial dysfunction during cellular Ca2þ overload, Eur. J. Biochem. 178 (1988) 489e501.

Csordas G., C. Renken, P. Varnai, L. Walter, D. Weaver, K.F. Buttle, T. Balla, C.A. Mannella, G. Hajnoczky, Structural and functional features and significance of the physical linkage between ER and mitochondria, J. Cell Biol. 174 (2006) 915e921.

Csordas G., P. Varnai, T. Golenar, S. Roy, G. Purkins, T.G. Schneider, T. Balla, G. Hajnoczky, Imaging interorganelle contacts and local calcium dynamics at the ERemitochondrial interface, Mol. Cell 39 (2010) 121e132.

Cunningham KW and Fink GR (1996) Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in Saccharomyces cerevisiae. *Mol Cell Biol* 16: 2226-2237.

Curtis J, Finkbeiner S (1999) Sending signals from the synapse to the nucleus: possible roles for CaMK, Ras/ERK, and SAPK pathways in regulation of synaptic plasticity and neuronal growth *J Neurosci Res* 58:88-95.

Danielisova V., Nemethova M., Gottlieb M. and Burda J. (2005) Changes of endogenous antioxidant enzymes during ischemic tolerance acquisition. *Neurochem. Res*. 30, 559–565.

Dave K. R., Saul I., Busto R., Ginsberg M. D., Sick T. J. and Perez-Pinzon M. A. (2001) Ischemic preconditioning preserves mitochondrial function after global cerebral ischemia in rat hippocampus. *J. Cereb. Blood Flow Metab*. 21, 1401–1410.

Dave KR, DeFazio RA, Raval AP, Torraco A, Saul I, Barriento A, et al. Ischemic preconditioning targets the respiration of synaptic mitochondria via protein kinase Cε. J Neurosci 2008; 28: 4172–82.

Dawson V. L. and Dawson T. M. (2000) Neuronal ischemic preconditioning. *Trends Pharmacol. Sci*. 21, 423–424.

De Cesare D, Fimia GM, Sassone-Corsi P (1999) Signaling route sto CREM and CREB: plasticità in trascriptional activation *Tends in Biochemical sciences* 24:281-285.

De Marchi U., E. Basso, I. Szabo, M. Zoratti, Electrophysiological characterization of the Cyclophilin D-deleted mitochondrial permeability transition pore, Mol. Membr. Biol. 23 (2006) 521e530.

De Marchi U., S. Campello, I. Szabo, F. Tombola, J.C. Martinou, M. Zoratti, Bax does not directly participate in the Ca(2þ)-induced permeability transition of isolated mitochondria, J. Biol. Chem. 279 (2004) 37415e37422.

De Souza Wyse AT, Streck EL, Worm P, Wajner A, Ritter F & Netto CA (2000) Preconditioning prevents the inhibition of Na+,K+-ATPase activity after brain ischemia. Neurochem Res 25, 971–975.

DeGracia DJ, Hu BR. Irreversible translation arrest in the reperfused brain. J Cereb Blood Flow Metab 2007; 27: 875–93.

Delgado-Esteban M., Martin-Zanca D., Andres-Martin L., Almeida A. and Bolanos J. P. (2007) Inhibition of PTEN by peroxynitrite activates the phosphoinositide-3-kinase/Akt neuroprotective signalling pathway. *J. Neurochem*. 102, 194–205.

Denton R.M., Regulation of mitochondrial dehydrogenases by calcium ions, Biochim. Biophys. Acta 1787 (2009) 1309e1316.

Dhalla NS, Elmoselhi AB, Hata T, Makino N. Status of myocardial antioxidants in ischemia-reperfusion injury. Cardiovasc Res 2000; 47; 446–56.

Di Capua N, Sperling O, Zoref-Shani E. Protein kinase C-epsilon is involved in the adenosine-activated signal transduction pathway conferring protection against ischemia-reperfusion injury in primary rat neuronal cultures. J Neurochem 2003; 84: 409–12.

Di Lisa F., A. Carpi, V. Giorgio, P. Bernardi, The mitochondrial permeability transition pore and cyclophilin D in cardioprotection, Biochim. Biophys. Acta (2011).

Di Lisa F., R. Menabo, M. Canton, M. Barile, P. Bernardi, Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NADþ and is a causative event in the death of myocytes in postischemic reperfusion of the heart, J. Biol. Chem. 276 (2001) 2571e2575.

Di Polo R and Beaugé LA (1998) Differential up-regulation of Na^{+}-Ca^{2+} exchange by phosphoarginine and ATP in dialysed squid axons. *J Physiol* 507: 737-747.

Di Polo R, Berberian G, Beauge L (2004) Phosphoarginine regulation of the squid nerve Na^+/Ca^{2+} exchanger: metabolic pathway and exchanger-ligand interactions different from those seen with ATP. *J Physiol* 15: 387-401.

Di Polo R. (1979) Calcium influx in intenally dialyzed squid giant axons. *J. Gen. Physiol.* 73: 91-113.

Digicaylioglu M. and Lipton S. A. (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature* 412, 641–647.

Dimmeler S., Fleming I., Fisslthaler B., Hermann C., Busse R., Zeiher A.M. (1999), Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation, *Nature* 399, 601–605.

Dirnagl U & Meisel A (2008) Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? Neuropharmacology 55, 334–344.

Dirnagl U, Becker K, Meisel A, Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. Lancet Neurol. 2009 Apr;8(4):398-412.

Dirnagl U., Iadecola C., Moskowitz M.A. (1999) Pathobiology of ischaemic stroke: an integrated view *Trends Neurosci.* 22: 391-397

Doering AE and Lederer WJ (1993) The mechanism by which cytoplasmic protons inhibit the sodium-calcium exchanger in guinea-pig heart cells. *J Physiol* 466: 481-499.

Doering AE and Lederer WJ (1994) The action of Na^+ as a cofactor in the inhibition by cytoplasmic protons of the cardiac Na^+-Ca^{2+} exchanger in the guinea-pig. *J Physiol* 480: 9-20.

Dosenko VE, Nagibin VS, Tumanovska LV, Moibenko AA. Protective effect of autophagy in anoxia-reoxygenation of isolated cardiomyocyte? Autophagy 2006; 2: 305–6.

Dowden J, Corbett D. Ischemic preconditioning in 18- to 20-month-old gerbils: long-term survival with functional outcome measures. Stroke 1999;30:1240–1246. [PubMed: 10356107]

Duchen M.R., Mitochondria in health and disease: perspectives on a new mitochondrial biology, Mol. Aspects Med. 25 (2004) 365e451.

Dugan LL and Choi DW (1994) Excitotoxicity, free radicals, and cell membrane changes. Suppl: S17-21 *Ann Neurol* 35, S17-21.

Ebert BL, Gleadle JM, O'Rourke JF, Bartlett SM, Poulton J, Ratcliffe PJ (1996) Isoenzyme-specific regulation of genes involved in energy metabolism by hypoxia: similarities with the regulation of erythropoietin. Biochem J 313:809–814

Endres M, Meisel A, Biniszkiewicz D, et al. DNA methyltransferase contributes to delayed ischemic brain injury. J Neurosci 2000; 20: 3175–81.

Espinosa-Tanguma R, DeSantiago J, Rasgado-Flores H (1993) Alpha-chymotrypsin deregulation of the sodium-calcium exchanger in barnacle muscle cells. *Am J Physiol* 265: C1118-C1127.

Esterbauer H. and Cheeseman K. H. (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. *Methods Enzymol*. 186, 407–421.

Facchinetti F, Dawson VL, Dawson TM (1998) Free radicals as mediators of neuronal injury *Cell.and Mol.Biol.* 18,667-682.

Farooqui AA, Haun SE, Harrocks LA (1994) Ischemia and hypoxia *Basic Neurochemistry. New York: Raven press.* pp 867-883.

Feuerstein GZ, Wang YX, Barone FC (1996) Inflammation related gene expression and stroke: implications for new therapeutic agents *Pharmacology of cerebral ischemia* pp 405-419.

Fiskum G, Murphy AN, Beal MF (1999) Mithocondria in neurodegeneration: acute ischemia and chronic neurodegenerative disease *J Cereb Blood Flow Metab* 19:351-369.

Formisano L., Saggese M., Secondo A., Sirabella R., Vito P., Valsecchi V., Molinaro P., Di Renzo G., Annunziato L. (2008) The two isoform of the Na^+/Ca^{2+} Exchanger, NCX1 and NCX3, costitute novel additional target for the prosurvival action of Akt/Protein kinase B pathway. *Molec Pharmacol* 73, 727-737.

Frieden M., R. Malli, M. Samardzija, N. Demaurex, W.F. Graier, Subplasmalemmal endoplasmic reticulum controls K(Ca) channel activity upon stimulation with a moderate histamine concentration in a human umbilical vein endothelial cell line, J. Physiol. 540 (2002) 73e84.

Frieden M., S. Arnaudeau, C. Castelbou, N. Demaurex, Subplasmalemmal mitochondria modulate the activity of plasma membrane Ca2þ-ATPases, J. Biol. Chem. 280 (2005) 43198e43208.

Fujimura M., Morita-Fujimura Y., Kawase M., Copin J. C., Calagui B., Epstein C. J. and Chan P. H. (1999) Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J. Neurosci*. 19, 3414–3422.

Fujioka Y, Hiroe K and Matsuoka S (2000) Regulation kinetics of Na^{+}-Ca^{2+} exchange current in guinea-pig ventricular myocytes. *J Physiol* 529: 611-623.

Fukunaga K and Kawano T (2003) Akt is a molecular target for signal transduction therapy in brain ischemic insult. J Pharmacol Sci 92:317–327.

Furuichi T., Liu W., Shi H., Miyake M. and Liu K. J. (2005) Generationof hydrogen peroxide during brief oxygen-glucose deprivation induces preconditioning neuronal protection in primary cultured neurons. *J. Neurosci. Res*. 79, 816–824.

Furukawa K (1997) The actin-severing protein gelsolin modulates calcium channel and NMDA receptor activities and vulnerability to excitotoxicity in hippocampal neurons *J. Neurosci*. 17, 8178-8186.

Furukawa K and Mattson MP (1995) Cytochalasins protect hippocampal neurons against amyloid beta-peptide toxicity: evidence that actin depolymerization suppresses Ca^{2+} influx. *J Neurochem* 65: 1061-1068.

Gallo EF & Iadecola C (2011). Neuronal nitric oxide contributes to neuroplasticity-associated protein expression through cGMP, protein kinase G, and extracellular signal-regulated kinase. J Neurosci 31, 6947–6955.

Garcia-Nogales P., Almeida A. and Bolanos J. P. (2003) Peroxynitrite protects neurons against nitric oxide-mediated apoptosis. A key role for glucose-6-phosphate dehydrogenase activity in neuroprotection. *J. Biol. Chem*. 278, 864–874.

Gaspar T, Kis B, Snipes JA, Lenzser G, Mayanagi K, Bari F, Busija DW. Transient glucose and amino acid deprivation induces delayed preconditioning in cultured rat cortical neurons. J Neurochem 2006;98:555–565.

Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, et al. Global analysis of protein expression in yeast. Nature 2003; 425: 737–41.

Ghosh S, Strum JC, Bell RM (1997) Lipid biochemistry: functions of glycerolipids and sphingolipids in cellular signaling *FASEB J* 11:45-50.

Giacomello M., I. Drago, M. Bortolozzi, M. Scorzeto, A. Gianelle, P. Pizzo, T. Pozzan, Ca2þ hot spots on the mitochondrial surface are generated by Ca2þ mobilization from stores, but not by activation of store-operated Ca2þ channels, Mol. Cell 38 (2010) 280e290.

Gidday J. M., Fitzgibbons J. C., Shah A. R. and Park T. S. (1994) Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neurosci. Lett*. 168, 221–224.

Gidday JM (2006) Cerebral preconditioning and ischaemic tolerance *Nature* 7:437-448.

Gidday JM, Fitzgibbons JC, Shah AR, Park TS (1994) Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci Lett 168:221-224.

Gidday JM, Shah AR, Maceren RG, Wang Q, Pelligrino DA, Holtzman DM, Shah AR (1999) Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J Cereb Blood Flow Metab* 19:331-340.

Ginis I, Schweizer U, Brenner M, Liu J and al. (1999) TNF-α preteatment prevents subsequent activation of cultured brain cells with TNF-α and hypoxia via ceramide *Am.J Physiol.Cell.Physiol.* 276:C1171-C1183.

Ginis I., Jaiswal R., Klimanis D., Liu J., Greenspon J. and Hallenbeck J. M. (2002) TNF-alpha-induced tolerance to ischemic injury involves differential control of NF-kappaB transactivation: the role of NF-kappaB association with p300 adaptor. *J. Cereb. Blood Flow Metab*. 22, 142–152.

Glazier SS, O'Rourke DM, Graham DI, Welsh FA. Induction of ischemic tolerance following brief focal ischemia in rat brain. J Cereb Blood Flow Metab 1994;14:545–553.

Goldberg M. P. and Choi D. W. (1990) Intracellular free calcium increases in cultured cortical neurons deprived of oxygen and glucose. *Stroke* 21, III75–III77.

Goldberg MP, Choi DW. Combined oxygen and glucose deprivation in cortical cell culture: calciumdependent and calcium-independent mechanisms of neuronal injury. J Neurosci 1993;13:3510–3524.

Gonzalez-Zulueta, M. et al. Requirement for nitric oxide activation of p21ras/extracellular regulated kinase in neuronal ischemic preconditioning. Proc. Natl Acad. Sci. USA 97, 436–441 (2000).

Gouriou Y, Demaurex N, Bijlenga P, De Marchi U, Mitochondrial calcium handling during ischemia-induced cell death in neurons. Biochimie. 2011 Aug 10

Gozal E, Simakajornboon N, Dausman JD et al. (1999) Hypoxia induces selective SAPK/JNK-2-AP-1 pathway activation in the nucleus tractus solitarii of the conscious rat *J Neuroch* 73:665-674.

Grabb MC, Choi DW (1999) Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. *J Neurosci* 19:1657-1662.

Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S. and Tannenbaum S. R. (1982) Analysis of nitrate, nitrite, and [15 N]nitrate in biological fluids. *Anal. Biochem*. 126, 131–138.

Griffiths E.J., A.P. Halestrap, Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion, Biochem. J. 307 (Pt 1) (1995) 93e98.

Gross SS &Wolin MS (1995). Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57, 737–769.

Gu GJ, Li YP, Peng ZY, Xu JJ, Kang ZM, Xu WG, et al. Mechanism of ischemic tolerance induced by hyperbaric oxygen preconditioning involves upregulation of hypoxia-inducible factor-1and erythropoietin in rats. J Appl Physiol 2008; 104: 1185–91.

Gunter KK, Zuscik MJ, Gunter TE (1991) The Na+-independent Ca^{2+} efflux mechanism of liver mitochondria is not a passive $Ca^{2+}/2H^{+}$ exchanger. J Biol Chem 266:21640–21648.

Halestrap A.P., A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection, Biochem. Soc. Trans. 38 (2010) 841e860.

Halestrap A.P., What is the mitochondrial permeability transition pore? J. Mol. Cell Cardiol. 46 (2009) 821e831.

Halestrap AP, Clarke SJ & Khaliulin I (2007) The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 1767, 1007–1031.

Han BH, Holtzman DM (2000) BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway *J Neurosci* 20:5775-5781.

Hanada M, Feng J, and Hemmings BA (2004) Structure, regulation and function of PKB/AKT—a major therapeutic target. Biochim Biophys Acta 1697:3–16.

Hang TM and Hilgemann DW (2004) Multiple transport modes of the cardiac Na^{+}/Ca^{2+} exchanger. *Nature* 427: 544-548.

Hansen M. B., Nielsen S. E. and Berg K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* 119, 203–210.

Hashiguchi A, Yano S, Morioka M, Hamada J, Ushio Y, Takeuchi Y & Fukunaga K (2004). Up-regulation of endothelial nitric oxide synthase via phosphatidylinositol 3-kinase pathway contributes to ischemic tolerance in the CA1 subfield of gerbil hippocampus. J Cereb Blood Flow Metab 24, 271–279.

Hashiguchi, A. et al. Up-regulation of endothelial nitric oxide synthase via phosphatidylinositol 3-kinase pathway contributes to ischemic tolerance in the CA1 subfield of gerbil hippocampus. J. Cereb. Blood Flow Metab. 24, 271–279 (2004).

Hassen GW, Tian D, Ding D, Bergold PJ. A new model of ischemic preconditioning using young adult hippocampal slice cultures. Brain Res Brain Res Protoc 2004;13:135–143.

Haworth R.A., D.R. Hunter, The Ca2þ-induced membrane transition in mitochondria. II. Nature of the Ca2þ trigger site, Arch. Biochem. Biophys. 195 (1979) 460e467.

Hebert DN, Molinari M. In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiol Rev 2007; 8: 1377–408.

Hendrick JP, Hartl FU (1993) Molecular chaperon function of hsp *Annu.Rev Biochem*. 62:349-384.

Hernández-SanMiguel E, Vay L, Santo-Domingo J, Lobatón CD, Moreno A, Montero M, Alvarez J (2006) The mitochondrial Na^+/Ca^{2+} exchanger plays a key role in the control of cytosolic Ca2+ oscillations. Cell Calcium 40:53–61.

Heurteaux C, Bertania V, Lazdunski M, Widmann C (1993) K channel openers prevent global ischemia induced expression of c-fos and c-jun, hsp, and amyloid β-protein precursor genes and neuronal death in rat hippocampus *Proc Natl Acad Sci U S A* 90:9431-9435.

Heurteaux C, Lauritzen I, Widmann C, Lazdunski M (1995) Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. *Proc Natl Acad Sci U S A* 92:4666-4670.

Hilgemann DW (1990) Regulation and deregulation of cardiac Na^+/Ca^{2+} exchange in giant excised sarcolemmal membrane patches. *Nature* 344: 242-245.

Hilgemann DW, Matsuoka S, Nagel GA and Collins A (1992) Steady-state and dynamic properties of cardiac sodium-calcium exchange. Sodium-dependent inactivation. *J Gen Physiol* 100: 905-932.

Hirai F., Motoori S., Kakinuma S. et al. (2004) Mitochondrial signal lacking manganese superoxide dismutase failed to prevent cell death by reoxygenation following hypoxia in a human pancreatic cancer cell line, KP4. *Antioxid. Redox Signal*. 6, 523–535.

Hiraide T, Katsura K, Muramatsu H, Asano G, Katayama Y (2001) Adenosine receptor antagonist cancelled the ischemic tolerance phenomen in gerbil.*Brai Res* 910:94-98.

Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. Surg Neurol 2006; 66: 232–45.

Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxiainducible factor 1 is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 95:7987–7992

Huang P. L. (2004) Nitric oxide and cerebral ischemic preconditioning. *Cell Calcium* 36, 323–329.

Hui L, Pei DS, Zhang QG, Guan QH, and Zhang GY (2005) The neuroprotection of insulin on ischemic brain injury in rat hippocampus through negative regulation of JNK signalling pathway by PI3K/AKT activation. Brain Res 1052:1–9.

Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.*20: 132-139.

Iadecola C, Kahles T, Gallo EF, Anrather J, Neurovascular protection by ischaemic tolerance: role of nitric oxide. J Physiol. 2011 Sep 1;589

Ide T, Takada K, Qiu JH, Saito N, Kawahara N, Asai A, Kirino T (1999) Ubiquitin stress response in postischemic hippocampal neurons under non tolerant and tolerant conditions. *J Cereb Blood Flow Metab* 19:750-756.

Impey S, Obrietan K, Storm DR (1999) Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity *Neuron* 23:11-14.

Iwamoto T, Pan Y, Nakamura TY, Wakabayashi S, Shigekawa M (1998a) Protein kinase C-dependent regulation of Na^+/Ca^{2+} exchanger isoforms NCX1 and NCX3 does not require their direct phosphorylation *Biochemistry*. 37: 17230-17238.

Jäättelä M (1999) Heat shock proteins as cellular lifeguards. Ann Med 31:261-271.

Janoff A (1964) Alterations in lysosomes during shock; effects of preconditioning (tolerance) and protective drugs. *Int. Anesthesiol. Clin.* 2:251-259.

Jiang B.-H., Rue E., Wang G.L., Roe R., Semenza G.L. (1996) Dimerization, DNA binding and transactivational properties of hypoxia-inducible factor-1, *J. Biol. Chem*. 271 17771-17778.

Jiang B-H, Rue E, Wang GL, Roe R, Semenza GL (1996a) Dimerization, DNA binding and transactivational properties of hypoxiainducible factor-1. J Biol Chem 271:17771–17778

Jin K.L., Mao X.O., Nagayama T., Goldsmith P.C., Greenberg D.A. (2000), Induction of vascular endothelial growth factor and hypoxia-inducible factor-1a by global ischemia in rat brain, *Neuroscience* 99 577–585.

Jin KL, Mao XO, Nagayama T, Goldsmith PC, Greenberg DA (2000) Induction of vascular endothelial growth factor and hypoxiainducible factor-1 by global ischemia in rat brain. Neuroscience 99:577–585

Jin ZQ, Zhou HZ, Cecchini G, Gray MO, Karliner JS. MnSOD in mouse heart: acute responses to ischemic preconditioning and ischemiareperfusion injury. Am J Physiol Heart Circ Physiol 2005; 288: 2986–94.

Jones N. M. and Bergeron M. (2001) Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. *J. Cereb. BloodFlow Metab*. 21, 1105–1114.

Jones, N. M. & Bergeron, M. Hypoxia-induced ischemic tolerance in neonatal rat brain involves enhanced ERK1/2 signaling. J. Neurochem. 89, 157–167 (2004).

Jope RS, Zhang L & Song L (2000). Peroxynitrite modulates the activation of p38 and extracellular regulated kinases in PC12 cells. Arch Biochem and Biophys 376, 365–370.

Jouaville L.S., P. Pinton, C. Bastianutto, G.A. Rutter, R. Rizzuto, Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming, Proc. Natl. Acad. Sci. U S A 96 (1999) 13807e13812.

Jousset H., M. Frieden, N. Demaurex, STIM1 knockdown reveals that storeoperated Ca2þ channels located close to SERCA pumps silently refill the endoplasmic reticulum, J. Biol. Chem. (2007).

Kakizawa H., Matsui F. and Tokita Y., et al. (2006) Neuroprotective effect of nipradilol, an NO donor, on hypoxic-ischemic brain injury of neonatal rats. *Early Hum. Dev*.doi:10.1016/j.earlhumandev. 2006.10.003.

Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L (1999) Regulation of the hypoxia-inducible transcription factor 1 by the ubiquitin proteasome pathway. J Biol Chem 274:6519–6625

Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeurle PA (1994) Constitutive NF-κB Activity in neurons *Mol Cell biol* 14:3981-3992.

Kapinya KJ, Prass K, Dirnagl U. Isoflurane induced prolonged protection against cerebral ischemia in mice: a redox sensitive mechanism? Neuroreport 2002; 13: 1431–5.

Kariko K, Weissmann D, Welsh FA. Inhibition of toll-like receptor and cytokine signaling – a unifying theme in ischemic tolerance. J Cereb Blood Flow Metab 2004; 24: 1288–304.

Kato H, Kogure K, Araki T, Liu XH, Kato K, Itoyama Y (1995) Immunohistochemical localization of superoxide dismutase in the hippocampus following ischemia in a gerbil model of ischemic tolerance. *J Cereb Blood Flow Metab* 15:60-70.

Kato H, Liu Y, Araki T, Kogure K (1991) Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: cumulative damage and protective effects. *Brain Res* 553:238-242.

Kato H, Liu Y, Araki T, Kogure K (1992) MK-801, but not anisomycin, inhibits the induction of tolerance to ischemia in the gerbil hippocampus. *Neurosci Lett* 139:118-121.

Kato H, Liu Y, Araki T, Kogure K. MK-801, but not anisomycin, inhibits the induction of tolerance to ischemia in the gerbil hippocampus. Neurosci Lett 1992;139:118–121.

Kato H, Liu Y, Araki T, Kogure K. Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: cumulative damage and protective effects. Brain Res 1991; 553: 238–42.

Kato H., Kogure K., Araki T., Liu X. H., Kato K. and Itoyama Y. (1995) Immunohistochemical localization of superoxide dismutase in the hippocampus following ischemia in a gerbil model of ischemic tolerance. *J. Cereb. Blood Flow Metab*. 15, 60–70.

Kato K, Shimazaki K, Kamiya T et al. (2005) Differential effects of sublethal ischemia and chemical preconditioning with 3-nitropropionic acid on protein expression in gerbil hippocampus. Life Sci 77, 2867–2878.

Katsura K, Kristian and Siesjo BK (1994) Energy metabolism, ion homeostasis, and cell damage in the brain *Biochem Soc Trans* 22:991-996.

Kawahara N, Croll SD, Wiegand SJ, Klatzo I (1997) Cortical spreading depression induces long-term alterations of BDNF levels in cortex and hippocampus distinct from lesion effects: implications for ischemic tolerance*. Neurosci Res* 29:37-47.

Kawahara N, Ide T, Saito N, Kawai K, Kirino T (1998) Propentofylline potentiates induced ischemic tolerance in gerbil hippocampal neurons via adenosine receptor. *J Cereb Blood Flow Metab* 18:472-475.

Kawahara N, Ruetzler CA, Klatzo I (1995) Protective effect of spreading depression against neuronal damage following cardiac arrest cerebral ischemia. *Neurol Res* 17:9-16.

Kawano T, Kunz A, Abe T, Girouard H, Anrather J, Zhou P & Iadecola C (2007). iNOS-derived NO and nox2-derived superoxide confer tolerance to excitotoxic brain injury through peroxynitrite. J Cereb Blood Flow Metab 27, 1453–1462.

Keller J. N., Kindy M. S., Holtsberg F. W. et al. (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J. Neurosci*. 18, 687–697.

Khaspekov L, Shamloo M, Victorov I, Wieloch T. Sublethal in vitro glucose-oxygen deprivation protects cultured hippocampal neurons against a subsequent severe insult. Neuroreport 1998;9:1273– 1276.

Khaspekov L., H. Friberg, A. Halestrap, I. Viktorov, T. Wieloch, Cyclosporin A and its non immunosuppressive analogue N-Me-Val-4-cyclosporin A mitigate neurons, Eur. J. Neurosci. 11 (1999) 3194e3198.

Kim J.S., Y. Jin, J.J. Lemasters, Reactive oxygen species, but not Ca2þ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H2024eH2034.

Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnelle DR, Stamler JS & Lipton SA (1999). Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO. Neuron 24, 461–469.

Kim YK, Kim SJ, Yatani A, Huang Y, Castelli G, Vatner DE, Liu J, Zhang Q, Diaz G, Zieba R, et al. (2003) Mechanism of enhanced cardiac function in mice with hypertrophy induced by overexpressed Akt. J Biol Chem 278:47622–47628.

Kinga S and Tymianski M, (2010) Calcium, ischemia and excitotoxicity. Cell calcium 47(2):122-9

Kinouchi H, Epstein CJ, Mizui T, Carlson E, Chen SF, Chan PK (1991) Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. *Proc Natl Acad Sci U S A* 88:11158-11162.

Kintner DB, Su G, Lenart B, Ballard AJ, Meyer JW, Ng LL, Shull GE, Sun D (2004). Increased tolerance to oxygen and glucose deprivation in astrocytes from Na_/H_ exchanger isoform 1 null mice. *Am J Physiol Cell Physiol* 287, C12–C21.

Kirichok Y., G. Krapivinsky, D.E. Clapham, The mitochondrial calcium uniporter is a highly selective ion channel, Nature 427 (2004) 360e364.

Kirino T, Tsujita Y, Tamura A (1991) Induced tolerance to ischemia in gerbil hippocampal neurons *J Cereb Blood Flow Metab* 11:299-307.

Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982;239:57–69.

Kis B, Rajapakse NC, Snipes JA, Nagy K, Horiguchi T, Busija DW. Diazoxide induces delayed preconditioning in cultured rat cortical neurons. J Neurochem 2003;87:969–980

Kitagawa K, Matsumoto M, Matsushita K, Mandai K, Mabuchi T, Yanagihara T, Kamada T (1996) Ischemic tolerance in moderately symptomatic gerbils after unilateral carotid occlusion. *Brain Res* 716:39-46.

Kitagawa K, Matsumoto M, Ohtsuki T, Kuwabara K, Mabuchi T, Yagita Y, Hori M, Yanagihara T. Extended neuronal protection induced after sublethal ischemia adjacent to the area with delayed neuronal death. Neuroscience 2000;96:141–146.

Kitagawa K, Matsumoto M, Ohtsuki T, Kuwabara K, Mabuchi T, Yagita Y, Hori M, Yanagihara T (2000) Extended neuronal protection induced after sublethal ischemia adjacent to the area with delayed neuronal death. *Neuroscience* 96:141-146.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T (1990) ”Ischemic tolerance” phenomen found in the brain. Brain Res 528:21-24.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K. 'Ischemic tolerance' phenomenon found in the brain. Brain Res 1990;528:21–24.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T (1990) ”Ischemic tolerance” phenomen found in the brain. *Brain Res* 528:21-24.

Kitagawa K., Matsumoto M., Kuwabara K. et al. (1991) ‘Ischemic tolerance’ phenomenon detected in various brain regions. *Brain Res*. 561, 203–211.

Kluck RM, Bossywetzel E, Green DR, Newmeyer DD (1997) the release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis *Science* 275, 1132-1136.

Knepper B. R. and Kurylo D. D. (1998) Effects of nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester on spatial and cued leaning. *Neuroscience* 83, 837–841.

Kobayashi S, Harris VA, Welsh FA (1995) Spreading depression induces tolerance of cortical neurons to ischemia in rat brain. *J Cereb Blood Flow Metab* 15:721-727.

Kofuji P, WJ Lederer, and Schulze DH (1994) Mutually exclusive and cassette exons underlie alternatively spliced isoforms of the Na^{+}/Ca^{2+}exchanger. *J Biol Chem* 269: 5145-5149.

Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, et al. Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 2008; 172: 454–69.

Kokoszka J.E., K.G. Waymire, S.E. Levy, J.E. Sligh, J. Cai, D.P. Jones, G.R. MacGregor, D.C. Wallace, The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore, Nature 427 (2004) 461e465.

Kolch W., Meaningful relationships: the regulation of the Ras/Raf/ MEK/ERK pathway by protein interactions, Biochem. J. 351 (2000) 289–305.

Kolesnick R, Gold DW (1994) The sphingomyelin pathway in TNF and IL-1 signaling *Cell* 77:325-328.

Krauskopf A., O. Eriksson, W.J. Craigen, M.A. Forte, P. Bernardi, Properties of the permeability transition in VDAC1(_/_) mitochondria, Biochim. Biophys. Acta 1757 (2006) 590e595.

Kristian T., B.K. Siesjo, Calciumin ischemic cell death, Stroke 29 (1998) 705e718.

Kroemer G (1998) The mitochondrion as an integrator/coordinator of cell death pathways. *Cell Death Differ* 5(6),547.

Kroemer G and Reed JC (2000) Mitochondrial control of cell death *Nature Med*. 6, 513-519.

Kunz A, Park L, Abe T, Gallo EF, Anrather J, Zhou P & Iadecola C (2007). Neurovascular protection by ischemic tolerance: Role of nitric oxide and reactive oxygen species. J Neurosci 27, 7083–7093.

Lander H.M., J.S. Ogiste, S.F. Pearce, R. Levi, A. Novogrodsky, Nitric oxide-stimulated guanine nucleotide exchange on p21ras, J. Biol. Chem. 270 (1995) 7017–7020.

Lange-Asschenfeldt C., Raval A. P., Dave K. R., Mochly-Rosen D., Sick T. J. and Perez-Pinzon M. A. (2004) Epsilon protein kinase C mediated ischemic tolerance requires activation of the extracellular regulated kinase pathway in the organotypic hippocampal slice.

Lee SH, Kwon HM, Kim YJ, Lee KM, Kim M, Yoon BW. Effects of HSP70.1 gene knockout on the mitochondrial apoptotic pathway after focal cerebral ischemia. Stroke 2004; 35: 2195–9.

Lee SL, AS Yu and Lytton J (1994) Tissue-specific expression of Na^+-Ca^{2+} exchanger isoforms. *J Biol Chem* 269: 14849-14852.

Lefer DJ, Scalia R, Campbell B, Nossuli T, Hayward R, Salamon M, Grayson J & Lefer AM (1997). Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia-reperfusion injury in rats. J Clin Invest 99, 684–691.

Lei SZ, Pan ZH, Aggarwal SK, Chen HSV, Hartman J, Sucher NJ & Lipton SA (1992). Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. Neuron 8, 1087–1099.

Leist M and Nicotera P (1998) Apoptosis, excitotoxicity, and neuropathology *Exp. Cell Res.* 239, 183-201.

Levitsky DO, Nicoll DA, and Philipson KD (1994) Identification of the high affinity Ca^{2+}-binding domain of the cardiac Na^+-Ca^{2+} exchanger. *J Biol Chem* 269: 22847-22852.

Li MH, Cha YN & Surh YJ (2006). Peroxynitrite induces HO-1 expression via PI3K/Akt-dependent activation of NF-E2-related factor 2 in PC12 cells. Free Radic Biol and Med 41, 1079–1091.

Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM and Burke EP (1994) Cloning of the NCX2 isoform of the plasma membrane Na^+-Ca^{2+} exchanger. *J Biol Chem* 269: 17434-17439.

Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM and Philipson KD (1991) Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+/Ca^{2+} exchanger. *J Biol Chem* 266: 1014-1020.

Liaudet L, Vassalli G & Pacher P (2009). Role of peroxynitrite in the redox regulation of cell signal transduction pathways. Front Biosci 14, 4809–4814.

Lin B.,Globus MY, Dietrich WD et al. (1992) Differing neurochemical and morphological sequelae of global ischemia: comparison of singe and multiple insult paradigms *J Neurochem* 59:2213-2223.

Linck B, Qiu Z, He Z, Tong Q, Hilgemann DW and Philipson KD (1998) Functional comparison of the three isoforms of the Na^+/Ca^{2+} exchanger (NCX1, NCX2, NCX3). *Am J Physiol* 274: C415-C423.

Lipton P (1999) Ischemic cell death in brain neuron *Physiol Rev* 79: 1431-1568.

Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HSV, Sucher NJ, Loscalzo J, Singel DJ & Stamler JS (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature 364, 626–632.

Liu C, Chen S, Kamme F, Hu BR. Ischemic preconditioning prevents protein aggregation after transient cerebral ischemia. Neuroscience 2005; 134: 69–80.

Liu D., Lu C., Wan R., Auyeung W. W. and Mattson M. P. (2002) Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release. *J. Cereb. Blood Flow Metab*. 22, 431–443.

Liu J, Ginis I, Spatz M, Hallenbeck JM. Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-alpha and ceramide. Am J Physiol Cell Physiol 2000; 278: C144–53.

Liu S, Beckman JS & Ku DD (1994). Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. J Pharm and Exp Therap 268, 1114–1121.

Liu X, Sheng R, Qin Z, (2009) The neuroprotective mechanism of brain ischemic preconditioning. Acta Pharmacol Sin. 30(8):1071-80

Liu y, Kato H, Nakata N, Kogure K (1992) Protection of rat hippocampus against ischemic neuronal damage by pre-treatment with sublethal ischemia. *Brain Res* 586:121-124.

Liu Y, Kato H, Nakata N, Kogure K (1993) Temporal profile of heat shock protein 70 synthesis in ischemic tolerance induced by preconditioning ischemia in rat hippocampus. *Neuroscience* 56:921-927.

Liu Y, Xiong L, Chen S, Wang Q. Isoflurane tolerance against focal cerebral ischemia is attenuated by adenosine A1 receptor antagonists. Can J Anaesth 2006; 53: 194–201.

Lobner D, Choi DW (1996) Preincubation with protein synthesis inhibitors protects cortical neurons against oxygen-glucose deprivation-induced death. *Neuroscience* 72:335-341.

Lonze BE & Ginty DD (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron 35, 605–623.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem*.193, 265–275.

Lu A, Tang Y, Ran R, Clark JF, Aronow BJ, Sharp FR (2003) Genomics of the periinfarction cortex after focal cerebral ischemia *J Cereb Blood Flow Metab* 23:786-810.

Luo J, Chen H, Kintner DB, Shull GE, Sun D (2005) Decreased neuronal death in Na_/H_ exchanger isoform 1-null mice after in vitro and in vivo ischemia. *J Neurosci* 25, 11256–11268.

Lytton J, Li XF, Dong H and Kraev A (2002) K^+-dependent Na^+/Ca^{2+} exchangers in the brain. *Ann N Y Acad Sci* 976:382-393.

MacManus JP, Linnik MD (1997) Gene expression induced by cerebral ischemia: an apoptotic perspective. *J Cereb Blood Flow Metab* 17:815-832.

Maehara K, Hasegawa T, Xiao H et al.(1999) Cooperative interaction of NF-κB and C/EBP binding sites is necessary for Mn-SOD gene transcription mediated by lipopolysaccharide and interferon-γ *FEBS Lett* 449:115-199.

Majda BT, Meloni BP, Rixon N, Knuckey NW (2001) Suppression subtraction hybridization and Northern analysis reveal upregulation of heat shock, trkB, sodium calcium exchanger genes following global cerebral ischemia in the rat. *Brain Res* 93, 173–179.

Makar TK et al. (1994) Vitamin e, ascorbate, glutathione metabolism in cultures of chick asrtocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain *J Neurochem*. 62:45-53.

Manabe T, Aiba A, Yamada A et al (2000) Regulation of long term potentiation by H-Ras through NMDA receptor phosphorylation *J Neurosci* 273:29394-29399.

Marini AM, rabin SJ, Lipsky RH, Mocchetti I (1998) Activity-dependent release of BDNF underlies the neuroprotective effects of NMDA *J Biol Chem* 273:29394-29399.

Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, Risau W (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol 156:965–976

Martin RL, Lloyd HG, Cowan AI. The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neurosci 1994;17:251–257.

Marzo I., C. Brenner, N. Zamzami, J.M. Jurgensmeier, S.A. Susin, H.L. Vieira, M.C. Prevost, Z. Xie, S. Matsuyama, J.C. Reed, G. Kroemer, Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis, Science 281 (1998) 2027e2031.

Massa S. M., Swanson R. A. and Sharp F. R. (1996) The stress gene response in brain. Cerebrovasc. *Brain Metab. Rev*. 8, 95–158.

Matsumoto S., H. Friberg, M. Ferrand-Drake, T. Wieloch, Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion, J. Cereb. Blood Flow Metab. 19 (1999) 736e741.

Matsuoka S, Nicoll DA, He Z and Philipson KD (1997) Regulation of cardiac Na^{+}-Ca^{2+} exchanger by the endogenous XIP region. *J Gen Physiol* 109: 273-286.

Matsushima K, Hakim AM (1995) Transient forebrain ischemia protects against subsequent focal cerebral ischemia without changing cerebral perfusion. *Stroke* 26:1047-1052.

Matsushima K, Hogan MJ, Hakim AM (1996) Cortical spreading depression protects against subsequent focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* 16:221-226.

Mayanagi K, G´asp´ar T, Katakam PVG, Kis B & Busija DW (2006). The mitochondrial KATP channel opener BMS-191095 reduces neuronal damage after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 27, 348–355.

McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, et al. Caspase 3 activation is essential for neuroprotection in preconditioning. Proc Natl Acad Sci USA 2003; 100: 715–20.

Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 2007; 54: 34–66.

Meldrum D. R. (1997) Mechanisms of cardiac preconditioning: ten years after the discovery of ischemic preconditioning. *J. Surg. Res*. 73, 1–13.

Melino G, Bernassola F, Knight RA, Corasaniti MT, Nistic G & Finazzi-Agr A (1997). S-nitrosylation regulates apoptosis. Nature 388, 432–433.

Meller R., Cameron J. A., Torrey D. J. et al. (2006) Rapid degradation of Bim by the ubiquitin-proteasome pathway mediates short-term ischemic tolerance in cultured neurons. *J. Biol. Chem*. 281, 7429–7436.

Meller R., Minami M., Cameron J. A., Impey S., Chen D., Lan J. Q., Henshall D. C. and Simon R. P. (2005) CREB-mediated Bcl-2 protein expression after ischemic preconditioning. *J. Cereb. Blood Flow Metab*. 25, 234–246.

Meloni B.P., Majda B.T., Knuckey N.W (2002) Evaluation of preconditioning treatments to protect near-pure cortical neuronal cultures from in vitro ischemia induced acute and delayed neuronal death *Brain Res.* 928:69-75.

Merrill AG, van Echten G, Wang E, Sandhoff K (1993) Fumosin B1 inhibits sphingosine*N*-acyltansferase and de novo sphingolipid biosynthesis in cultured neurons in situ *J Biol Chem* 268:27299-27306.

Minet E, Arnould T, Michel G, Roland I, Mottet D, Raes M, et al. ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett 2000; 46: 53–8.

Miyashita K, Abe H, Nakajima T, Ishikawa A, Nishiura M, Sawada T, Naritomi H. Induction of ischaemic tolerance in gerbil hippocampus by pretreatment with focal ischaemia. Neuroreport 1994;6:46–48.

Molinaro P, Viggiano D, Nisticò R, Sirabella R, Secondo A, Boscia F, Pannaccione A, Scorziello A, Mehdawy B, Sokolow S, Herchuelz A, Di Renzo GF, Annunziato L. Na+ -Ca2+ exchanger (NCX3) knock-out mice display an impairment in hippocampal long-term potentiation and spatial learning and memory. J Neurosci. 2011 May 18;31(20):7312-21

Moncayo J, de Freitas GR, Bogousslavsky J, Altieri M, van Melle G (2000) Do transient ischemic attacks have a neuroprotective effect. *Neurology* 54:2089-2094.

Moro MA, Darley-Usmar VM, Goodwin DA, Read NG, Zamora-Pino R, Feelisch M, RadomskiMW & Moncada S (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. Proc Natl Acad Sci U S A 91, 6702–6706.

Mrsi'c-Pelıc'J, eupan G, Maysinger D, PelcI'c G, Vitezi'c D, Simoni'c A (2002) The influence of MK-801 on the hippocampal free arachidonic acid level and Na_,K_-ATPase activity in global cerebral ischemia-exposed rats. *Prog Neuropsychopharmacol Biol Psychiatry* 26, 1319–1326.

Munoz A, Nakazaki M, Goodman JC, Barrios R, Onetti CG, Bryan J, Aguilar-Bryan L. (2003) Ischemic preconditioning in the hippocampus of a knockout mouse lacking SUR1-based KATP channels. *Stroke* 34, 164–170.

Murakami K, Kondo T, Kawase M (1998) Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with Mn-SOD deficiency *J.Neurosci.* 18:205-213.

Murphy AN, Fiskum G, Beal MF (1999) Mitochondria in neurodegeneration: Bioenergetic function in cell life and death *J Cereb Blood Flow Metab* 19:231-245.

Murry C. E., Jennings R. B. and Reimer K. A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74, 1124–1136.

Nakagawa T., S. Shimizu, T. Watanabe, O. Yamaguchi, K. Otsu, H. Yamagata, H. Inohara, T. Kubo, Y. Tsujimoto, Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death, Nature 434 (2005) 652e658.

Nakajima T, Iwabuchi S, Miyazaki H, Okuma Y, Inanami O, Kuwabara M. Relationship between the activation of cyclic AMP responsive element binding protein and ischemic tolerance in the penumbra region of rat cerebral cortex. Neurosci Lett 2002; 331: 13–6.

Nakajima T, Iwabuchi S, Miyazaki H, Okuma Y, Kuwabara M, Nomura Y, et al. Preconditioning prevents ischemia-induced neuronal death through persistent Akt activation in the penumbra region of the rat brain. J Vet Med Sci 2004; 66: 521–7.

Nakamura, M., Nakakimura, K., Matsumoto, M. & Sakabe, T. Rapid tolerance to focal cerebral ischemia in rats is attenuated by adenosine A1 receptor antagonist. J. Cereb. Blood Flow Metab. 22, 161–170 (2002).

Nakasaki Y, Iwamoto T, Hanada H, Imagawa T and Shigekawa M (1993) Cloning of the rat aortic smooth muscle Na^+/Ca^{2+} exchanger and tissue-specific expression of isoforms. *J Biochem* 114: 528-534.

Nakata N, Kato H, Kogure K (1993) Inhibition of ischemic tolerance in the gerbil hippocampus by quercetin and anti-heat shock protein-70 antibody. *Neuroreport* 4:695-698.

Nakatsuka H, Ohta S, Tanaka J, Toku K, Kumon Y, Maeda N, et al. Cytochrome c release from mitochondria to the cytosol was suppressed in the ischemia-tolerance-induced hippocampal CA1 region after 5-min forebrain ischemia in gerbils. Neurosci Lett 2000; 278: 53–6.

Nandagopal K, Dawson TM, Dawson VL (2001) Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *J Pharmacol Exp Ther* 297:474-478.

Narita M., S. Shimizu, T. Ito, T. Chittenden, R.J. Lutz, H. Matsuda, Y. Tsujimoto, Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria, Proc. Natl. Acad. Sci. U S A 95 (1998) 14681e14686.

Nawashiro H, Tasaki K , Ruetzler CA , Hallenbeck JM. TNF-alpha pretreatment induces protective effects against focal cerebral ischemia in mice. J Cereb Blood Flow Metab 1997; 17: 483–90.

Nedergaard M, Hansen AJ (1988) Spreading depression is not associated with neuronal injury in the normal brain. *Brain Res* 449:395-398.

Nicholls D.G., S.L. Budd, Mitochondria and neuronal survival, Physiol. Rev. 80 (2000) 315e360.

Nicoll DA, Longoni Sand Philipson KD (1990) Molecular cloning and functional expression of the cardiac sarcolemmal Na^{+}-Ca^{2+} exchanger. *Science* 250: 562-565.

Nishi S, Taki W, Uemura Y, Higashi T, Kikuchi H, Kudoh H, Satoh M, Nagata K (1993) Ischemic tolerance due to the induction of HSP70 in a rat ischemic recirculation model. *Brain Res* 615:281-288.

Nogawa S et al. (1998) Interaction between inducible NOS and cox-2 ater cerebral ischemia. *Proc Natl. Acad Sci. USA* 95:10966-10971.

Noshita N., Sugawara T., Fujimura M., Morita-Fujimura Y. and Chan P. H. (2001) Manganese superoxide dismutase affects cytochrome c release

and caspase-9 activation after transient focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab*. 21, 557–567.

Nowicky AV, and Duchen MR (1998) Changes in $[Ca^{+2}]_i$ and membrane currents during impaired mitochondrial metabolism in dissociated rat hippocampal neurons *J. Phisiol* 507.1:131-145.

Nozaki K, Nishimura M, Hashimoto N. Mitogen-activated protein kinases and cerebral ischemia. Mol Neurobiol 2001; 23: 1–19.

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

Obrenovitch T. P. (2008) Molecular Physiology of Preconditioning-Induced Brain Tolerance to Ischemia. *Physiol Rev* 88, 211–247

Ohta S, Furuta S, Matsubara I, Kohno K, Kumon Y & Sakaki S (1996) Calcium movement in ischemia-tolerant hippocampal CA1 neurons after transient forebrain ischemia in gerbils. J Cereb Blood Flow Metab 16, 915–922.

Ohtsuki T, Matsumoto M, Kitagawa K, Taguchi A, Maeda Y, Hata R, Ogawa S, Ueda H, Handa N, Kamada T (1993) Induced resistance and susceptibility to cerebral ischemia in gerbil hippocampal neurons by prolonged but mild hypoperfusion. *Brain Res* 614:279-284.

Omata N, Murata T, Takamatsu S, Maruoka N, Wada Y, Yonekura Y, Fujibayashi Y. (2002) Hypoxic tolerance induction in rat brain slices following hypoxic preconditioning due to expression of neuroprotective proteins as revealed by dynamic changes in glucose

Orban PC, Chapman PF, Brambilla R (1999) Is the Ras-MAPK signalling pathway necessary for long-term memory formation? *Trends in Neurosci* 22:38-44.

Orio M, Kunz A, Kawano T, Anrather J, Zhou P & Iadecola C (2007). Lipopolysaccharide induces early tolerance to excitotoxicity via nitric oxide and cGMP. Stroke 38, 2812–2817.

Pacher P, Beckman JS & Liaudet L (2007). Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87, 315–424.

Pacher P., A.P. Thomas, G. Hajnoczky, Ca2+ marks: miniature calcium signals in single mitochondria driven by ryanodine receptors, Proc. Natl. Acad. Sci. U S A 99 (2002) 2380e2385.

Palty R, Silverman WF, Hershfinkel M, Caporale T, Sensi SL, Parnis J, Nolte C, Fishman D, Shoshan-Barmatz V, Herrmann S, Khananshvili D, Sekler I (2010) NCLX is an essential component of mitochondrial Na+/Ca^{2+} exchange. Proc Natl Acad Sci U S A 107:436-441.

Paolillo M., Feliciello A., Porcellini A. et al. (1999) The type and the localization of cAMP-dependent protein kinase regulate transmission of cAMP signals to the nucleus in cortical and cerebellar granule cells. *J. Biol. Chem*. 274, 6546–6552.

Papa M, Canitano A, Boscia F, Castaldo P, Sellitti S, Porzig H, Taglialatela M and Annunziato L (2003) Differential expression of the Na^{+}-Ca^{2+} exchanger transcripts and proteins in rat brain regions. *J Comp Neurol*. 461: 31-48.

Park HK, Chu K, Jung KH, Lee ST, Bahn JJ, Kim M, et al. Autophagy is involved in the ischemic preconditioning. Neurosci Lett 2009; 451:16–9

Paschen W., J. Doutheil, Disturbances of the functioning of endoplasmic reticulum: a key mechanism underlying neuronal cell injury? J. Cereb. Blood Flow Metab. 19 (1999) 1–18.

Patel A., van de Poll M. C., Greve J. W. et al. (2004) Early stress protein gene expression in a human model of ischemic preconditioning. *Transplantation* 78, 1479–1487.

Pedersen SF, O'Donnell ME, Anderson SE, Cala PM (2006). Physiology and pathophysiology of Na+/H+ exchange and Na+-K+-2Cl+ cotransport in the heart, brain, blood. *Am J Physiol Regul Integr Comp Physiol* 291, R1–R25.

Perez-Pinzon M. A. (2004) Neuroprotective effects of ischemic preconditioning in brain mitochondria following cerebral ischemia. *J. Bioenerg. Biomembr*. 36, 323–327.

Perez-Pinzon M. A., Dave K. R. and Raval A. P. (2005) Role of reactive oxygen species and protein kinase C in ischemic tolerance in the brain. *Antioxid. Redox Signal*. 7, 1150–1157.

Pérez-Pinzón MA, Born JG. Rapid preconditioning neuroprotection following anoxia in hippocampal slices: role of K+ ATP channel and protein kinase C. Neuroscience 1999; 89: 453–9.

Pérez-Pinzón MA, Mumford PL, Rosenthal M, Sick TJ (1996) Anoxic preconditioning in hippocampal slices: role of adenosine. *Neuroscience* 75:687-694.

Pesse B, Levrand S, Feihl F,Waeber B, Gavillet B, Pacher P & Liaudet L (2005). Peroxynitrite activates ERK via Raf-1 and MEK, independently from EGF receptor and p21Ras in H9C2 cardiomyocytes. J Mol Cell Cardiol 38, 765–775.

Peters O, Back T, Lindauer U, Busch C et al. (1998) Increased formation of ROS after permanent and reversible middle cerebral artery occlusion in the rat *J Cereb Blood Flow Metab* 18:196-205.

Petronilli V., C. Cola, P. Bernardi, Modulation of the mitochondrial cyclosporine A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca2þ, J. Biol. Chem. 268 (1993) 1011e1016.

Pfeiffer DR, Gunter TE, Eliseev R, Broekemeier KM, Gunter KK (2001) Release of Ca^{2+} from mitochondria via the saturable mechanisms and the permeability transition. IUBMB Life. 52:205-212.

Philipson KD and Nicoll DA (2000) Sodium-calcium exchange: a molecular perspective. *Ann Rev Physiol* 62:111-133.

Pignataro G, Gala R, Cuomo O, Tortiglione A, Giaccio L, Castaldo P, Sirabella R, Matrone C, Canitano A, Amoroso S, Di Renzo G, Annunziato L. Two sodium/calcium exchanger gene products, ncx1 and ncx3, play a major role in the development of permanent focal cerebral ischemia. Stroke. 2004;35:2566-2570

Pignataro G, Tortiglione A, Scorziello A, Giaccio L, Secondo A, Severino B, Santagada V, Caliendo G, Amoroso S, Di Renzo GF and Annunziato L (2004) Evidence for a protective role played by the Na^{+}/Ca^{2+} exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. *Neuropharmacology* 46:439-448.

Pignataro G, Boscia F, Esposito E, Sirabella R, Cuomo O, Vinciguerra A, Di Renzo G, Annunziato L. NCX1 and NCX3: Two new effectors of delayed preconditioning in brain ischemia. Neurobiol Dis. 2011 Oct 17. [Epub ahead of print].

Pisani A., P. Bonsi, D. Contenzone, P. Giacomini, P. Calabresi, Involvement of intracellular calcium stores during oxygen/glucose deprivation in striatal large aspiny interneurons, J. Cereb. Blood Flow Metab. 20 (2000) 839–846.

Plamondon, H., Blondeau, N., Heurteaux, C. & Lazdunski, M. Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death: involvement of adenosine A1 receptors and KATP channels. J. Cereb. Blood Flow Metab. 19, 1296–1308 (1999).

Poburko D., J. Santo-Domingo, N. Demaurex, Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations, J. Biol. Chem. 286 (2011) 11672e11684.

Pozos TC, Sekler I and Cyert MS.(1996) The product of HUM1, a novel yeast gene, is required for vacuolar Ca^{2+}/H^{+} exchange and is related to mammalian Na^{+}/Ca^{2+} exchangers. *Mol Cell Biol* 16: 3730-3741.

Puisieux, F. et al. Brain ischemic preconditioning is abolished by antioxidant drugs but does not up-regulate superoxide dismutase and glutathione peroxidase. Brain Res. 1027, 30–37 (2004).

Puzzo D, Staniszewski A, Deng SX, Privitera L, Leznik E, Liu S, Zhang H, Feng Y, Palmeri A, Landry DW& Arancio O (2009). Phosphodiesterase 5 inhibition improves synaptic function, memory, and amyloid-β load in an Alzheimer's disease mouse model. J Neurosci 29, 8075–8086.

Qiu Z, Nicoll DA and Philipson KD (2001) Helix packing important regions of the cardiac Na^{+}/Ca^{2+} exchanger. *J Biol Chem* 276: 194-199.

Quednau BD, Nicoll DA and Philipson KD (1997) Tissue specificity and alternative splicing of the Na^{+}/Ca^{2+} exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am J Physiol* 272: C1250-C1261.

Raghavendra Rao VL, Bowen KK, Dhodda VK, Song G, Franklin JL, Gavva NR, Dempsey RJ (2002) Gene expression analysis of spontaneously

hypertensive rat cerebral cortex following transient focal cerebral ischemia. *J Neurochem.* 83:1072-86.

Rami A, Langhagen A, Steiger S. Focal cerebral ischemia induces upregulation of Beclin 1 and autophagy-like cell death. Neurobiol Dis 2008; 29: 132–41.

Ran R, Xu H, Lu A, Bernaudin M. Hypoxia preconditioning in the brain. Dev Neurosci 2005; 27: 87–92.

Rasola A., P. Bernardi, The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis, Apoptosis 12 (2007) 815e833.

Rauca C, Zerbe R, Jantze H, Krug M. The important of free hydroxyl radicals to hypoxia preconditioning. Brain Res 2000; 868: 147–9.

Raval AP, Dave KR, DeFazio RA, Perez-Pinzon MA. εPKC phosphorylates the mitochondrial K+ ATP channel during induction of ischemic preconditioning in the rat hippocampus. Brain Res 2007; 1184: 345–53.

Raval AP, Dave KR, Mochly-Rosen D, Sick TJ, Pérez-Pinzón MA. Epsilon PKC is required for the induction of tolerance by ischemic and NMDA-mediated preconditioning in the organotypic hippocampal slice. JNeurosci 2003; 23: 384–91.

Ravati A, Ahlemeyer B, Becker A, Klumpp S, Krieglstein J. Preconditioning-induced neuroprotection is mediated by reactive oxygen species and activation of the transcription factor nuclear factorkappaB. J Neurochem 2001; 78: 909–19.

Reed JC (1997) Cytochrome-c:can't live with it- can't live without it *Cell* 91:559-562.

Reeves JP and Hale CC (1984) The stoichiometry of the cardiac sodium-calcium exchange system. *J Biol Chem* 259: 7733-7739.

Rejdak R., Rejdak K., Sieklucka-Dziuba M., Stelmasiak Z. and Grieb P. (2001) Brain tolerance and preconditioning. *Pol. J. Pharmacol*. 53, 73–79.

Reshef A, Sperling O, Zoref Shani E (1998) Opening of ATP-sensitive potassium channels by cromakalim confers tolerance against chemical ischemia in rat neuronal cultures *Neurosc.Lett*. 250:111-114.

Riccio A, Alvania RS, Lonze BE, Ramanan N, Kim T, Huang Y, Dawson TM, Snyder SH & Ginty DD (2006). A nitric oxide signaling pathway controls CREB-mediated gene expression in neurons. Mol Cell 21, 283–294.

Rizzuto R., M. Brini, M. Murgia, T. Pozzan, Microdomains with high Ca2þ close to IP3-sensitive channels that are sensed by neighboring mitochondria, Science 262 (1993) 744e747.

Rizzuto R., T. Pozzan, Microdomains of intracellular Ca2+: molecular determinants and functional consequences, Physiol. Rev. 86 (2006) 369–408.

Ruscher K, Isaev N, Trendelenburg G, Weih M, Iurato L, Meisel A, Dirnagl U (1998) Induction of hypoxia inducible factor 1 by oxygen glucose deprivation is attenuated by hypoxic preconditioning in rat cultured neurons. Neurosci Lett 254:117–120

Rybnikova E, Sitnik N, Gluschenko T, Tjulkova E, Samoilov MO. (2006) The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobarichypoxia in rats. *Brain Res* 1089, 195–202.

Rybnikova E. A., Khozhai L. I., Tyul'kova E. I., Glushchenko T. S., Sitnik N. A., Pelto-Huikko M., Otellin V. A. and Samoilov M. O. (2005) Expression of early gene proteins, structural changes in brain neurons in hypobaric hypoxia, and the correcting effects of preconditioning. *Neurosci. Behav. Physiol*. 35, 383–388.

Saba RI, Ruysschaert JM, Hercuelz A and Goormaghtigh E (1999) Fourier transform infrared spectroscopy study of the secondary and tertiary structure of the reconstituted Na^{+}-Ca^{2+} exchanger 70 kDa polypeptide. *J Biol Chem* 274: 15510-15518.

Sakurai M, Hayashi T, Abe K, Aoki M, Sadahiro M, Tabayashi K, et al. Enhancement of heat shock protein expression after transient ischemia in the preconditioned spinal cord of rabbits. J Vasc Surg 1998; 27: 720–5.

Sakurai M, Hayashi T, Abe K, Aoki M, Sadahiro M, Tabayashi K, et al. Enhancement of heat shock protein expression after transient ischemia in the preconditioned spinal cord of rabbits. J Vasc Surg 1998; 27: 720–5.

Santacruz-Toloza L, Ottolia M, Nicoll DA and Philipson KD (2000) Functional analysis of a disulfide bond in the cardiac Na^+/Ca^{2+} exchanger. *J Biol Chem* 275: 182-188.

Santillo M., Mondola P., Seru R. et al. (2001) Opposing functions of Kiand Ha-Ras genes in the regulation of redox signals. *Curr. Biol*. 11, 614–619.

Saris NE, Carafoli E (2005) A historical review of cellular calcium handling, with emphasis on mitochondria. Biochemistry 70:187-194.

Schaller B. and Graf R. (2002) Cerebral ischemic preconditioning. An experimental phenomenon or a clinical important entity of stroke prevention?. *J. Neurol*. 249, 1503–1511.

Schinzel A.C., O. Takeuchi, Z. Huang, J.K. Fisher, Z. Zhou, J. Rubens, C. Hetz, N.N. Danial, M.A. Moskowitz, S.J. Korsmeyer, Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia, Proc. Natl. Acad. Sci. U S A 102 (2005) 12005e12010.

Schinzel A.C., O. Takeuchi, Z. Huang, J.K. Fisher, Z. Zhou, J. Rubens, C. Hetz, N.N. Danial, M.A. Moskowitz, S.J. Korsmeyer, Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia, Proc. Natl. Acad. Sci. U S A 102 (2005) 12005e12010.

Schnetkamp PP, Basu DK, Szerencsei RT. (1989) Na^+/Ca^{2+} exchange in bovine rod outer segments requires and transports K^+. *Am J Physiol* 257: C153-C157.

Schultz JEJ, Qian YZ, Gross GJ, Kukreja RC. The ischemia selective K-ATP channel antagonist, 5-hydroxydecanoate, blocks ischemic preconditioning in the rat heart. J Mol Cell Cardiol 1997; 29: 1055–60.

Schulze DH, Muqhal M, Lederer WJ, Ruknudin AM (2003) Sodium/calcium exchanger (NCX1) macromolecular complex. *J Biol Chem* 278: 28849-28855.

Scorziello A, Pellegrini C, Forte L, Tortiglione A, Gioielli A, Iossa S, Amoroso S, Tufano R, Di Renzo GF, Annunziato L (2001) Differential vulnerability of cortical and cerebellar neurons in primari culture to oxygen glucose deprivation followed by reoxygenation. *J Neurosci Res* 63:20-26.

Scorziello A., Pellegrini C., Secondo A. et al. (2004) Neuronal NOS activation during oxygen and glucose deprivation triggers cerebellar granule cell death in the later reoxygenation phase. *J. Neurosci. Res*. 76, 812–821.

Secondo A., Sirabella R., Formisano L., D'Alessio A., Castaldo P., Amoroso S., Ingleton P., Di Renzo G. and Annunziato L. (2003) Involvement of PI3'-K, mitogen-activated protein kinase and protein kinase B in the up-regulation of the expression of nNOSalpha and nNOSbeta splicing variants induced by PRL-receptor activation in GH3 cells. *J. Neurochem.* 84, 1367–1377.

Secondo A, Molinaro P, Pannaccione A, Esposito A, Cantile M, Lippiello P, Sirabella R, Iwamoto T, Di Renzo G, Annunziato L. Nitric oxide stimulates NCX1 and NCX2 but inhibits NCX3 isoform by three distinct molecular determinants. Mol Pharmacol. 2011 Mar;79(3):558-68.

Semenza G.L., Jiang B.-H., Leung S.W., Passantino R., Concordet J.-P., Maire P., Giallongo A. (1996), Hypoxia response elements in aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor-1, *J. Biol. Chem*. 271 32529–32537.

Semenza G.L., Roth P.H., Fang H.-M., Wang G.L. (1994), Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor-1, *J. Biol. Chem*. 269 23757–23763.

Semenza GL (2000) HIF-1: mediator of physiological and pathological responses to hypoxia. J Appl Physiol 88:1474–1480

Semenza GL, Jiang B-H, Leung SW, Passantino R, Concordet J-P, Maire P, Giallongo A (1996) Hypoxia response elements in aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential

binding sites for hypoxia-inducible factor-1. J Biol Chem 271:32529–32537

Semenza GL, Roth PH, Fang H-M, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxiainducible factor-1. J Biol Chem 269:23757–23763

Shamloo M, Kamme F, Wieloch T (2000) Subcellular distribution and autophosphorylation of calcium/calmodulin-dependent protein kinase II-alpha in rat hippocampus in a model of ischemic tolerance. *Neuroscience* 96:665-674.

Shamloo M, Rytter A, Wieloch T (1999*a*) Activation of the extracellular signal-regulated protein kinase cascade in the hippocampal CA1 region in a rat model of global cerebral ischemic preconditioning. *Neuroscience* 93:81-88.

Shamloo M, Wieloch T (1999*b*) Changes in protein tyrosine phosphorylation in the rat brain after cerebral ischemia in a model of ischemic tolerance. *J Cereb Blood Flow Metab* 19:173-183.

Shaul O, Hilgemann DW, de-Almeida-Engler J, Van Montagu M, Inz D and Galili G (1999) Cloning and characterization of a novel $Mg(^{2+})/H(^{+})$ exchanger. *EMBO J* 18: 3973-3980.

Shiga Y., H. Onodera, Y. Matsuo, K. Kogure, Cyclosporin A protects against ischemiaereperfusion injury in the brain, Brain Res. 595 (1992) 145e148.

Shimazaki K, Ishida A, Kawai N (1994) Increase in bcl-2 oncoprotein and the tolerance to ischemia-induced neuronal death in the gerbil hippocampus. *Neurosci Res* 20:95-99.

Shimazaki K, Nakamura T, Nakamura K et al. (1998) Reduced calcium elevation in hippocampal CA1 neurons of ischemia-tolerant gerbils. Neuroreport 9, 1875–1878.

Shimizu S, Nagayama T, Jin KL, Zhu L, Loeffer JE, Watkins SC, et al. Bcl-2 antisense treatment prevents induction of tolerance to focal ischemia in the rat brain. J Cereb Blood Flow Metab 2001; 21: 233– 43.

Shintani T, Klionsky DJ. Autophagy in health and disease: a doubleedged sword. Science 2004; 306: 990–5.

Shiva S, Sack MN, Greer JJ et al. (2007) Nitrite augments tolerance to ischemia／reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med 204, 2089–2102.

Siegelbaum SA (1999) Creb can get you depressed *Neuron* 23:414-415.

Simon RP, Niiro M, Gwinn R (1993) Prior ischemic stress protects against experimental stroke*. Neurosci Lett* 163:135-137.

Sirabella R., A. Secondo, A. Pannaccione, et al., Anoxia-induced NF-kappa B dependent upregulation of NCX1 contributes to Ca2+ refilling into endoplasmic reticulum in cortical neurons, Stroke 40 (2009) 922–929.

Snider B.J., Lobner D., Yamada K.A., Choi D.W. (1998) Conditioning heat stress reduces excitotoxic and apoptotic components of oxygen–glucose deprivation-induced neuronal death in vitro *J. Neurochem.* 70:120-129.

Sommer C, Gass P, Kiessling M (1995) Selective c-jun expression in CA1 neurons of the gerbil hippocampus durin and after acquisition of an ischemia-tolerant state. *Brain Pathol* 5:135-144.

Song G, Ouyang G, and Bao S (2005) The Activation of AKT/PKB signalling pathway and cell survival. J Cell Mol Med 9:59–71.

Stagliano NE, Pérez-Pinzón MA, Moskowitz MA, Huang PL (1999) Focal ischemic preconditioning induces rapid tolerance to middle cerebral artery occlusion in mice*. J Cereb Blood Flow Metab* 19:757-761.

Starkov A.A., C. Chinopoulos, G. Fiskum, Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury, Cell Calcium 36 (2004) 257e264.

Stenzel-Poore MP, Stevens SL, King JS, Simon RP. Preconditioning reprograms the response to ischemic injury and primes the emergence of unique endogenous neuroprotective phenotypes: a speculative synthesis. Stroke 2007; 38: 680–85.

Stenzel-Poore MP, Stevens SL, Xiong Z, et al. Eff ect of ischaemic preconditioning on genomic response to cerebral ischaemia: similarity to neuroprotective strategies in hibernation and hypoxia-tolerant states. Lancet 2003; 362: 1028–37.

Stojanovski D, Johnston AJ, Streimann I, Hoogenraad NJ, and Ryan MT (2003) Import of nuclear-encoded proteins into mitochondria. Exp Physiol 88:57-64.

Sugino T, Nozaki K, Takagi Y, Hashimoto N (1999) 3-Nitropropionic acid induces ischemic tolerance in gerbil hippocampus *in vivo. Neurosci Lett* 259:9-12.

Sun S, Ganchi PA, Ballard DW, Greene WC (1993) NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregolatory pathway *Science* 259:1912-1915.

Szabadkai G & Duchen MR (2008) Mitochondria: the hub of cellular Ca^{2+} signaling. Physiology 23:84-94.

Tanaka H, Calderone A, Jover T, Grooms SY, Yokota H, Zukin RS, Bennett MV (2002) Ischemic preconditioning acts upstream of GluR2 down-regulation to afford neuroprotection in the hippocampal CA1. *Proc Natl Acad Sci U S A* 99:2362-2367.

Tang Y, Pacary E, Freret T, et al. Eff ect of hypoxic preconditioning on brain genomic response before and following ischemia in the adult mouse: identifi cation of potential neuroprotective candidates for stroke. Neurobiol Dis 2006; 21: 18–28.

Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM (1997) Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. *Brain Res* 748:267-270.

Tauskela JS, Comas T, Hewitt K, Monette R, Paris J, Hogan M, Morley P. Cross-tolerance to otherwise lethal N-methyl-D-aspartate and oxygen-glucose deprivation in preconditioned cortical cultures. Neuroscience 2001;107:571–584.

Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 2000; 279: L1005–28.

Tomasevic G, Shamloo M, Israeli D, Wieloch T (1999) Activation of p53 and its target genes p21(WAF1/Cip1) and PAG608/Wig-1 in ischemic preconditioning. *Brain Res Mol Brain Res* 70:304-313.

Toyoda T, Kassell NF, Lee KS (1997) Induction of ischemic tolerance and antioxidant activity by brief focal ischemia. *Neuroreport* 8:847-851.

Tremblay R., Chakravarthy B., Hewitt K., Tauskela J., Morley P., Atkinson T., Durkin J.P (2000) Transient NMDA receptor inactivation provides long-term protection to cultured cortical neurons from a variety of death signals *J. Neurosci.* 20:7183-7192.

Tsan MF, Gao B. Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 2004; 286: 739–44.

Tsan MF, Gao B. Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 2004; 286: 739–44.

Valsecchi V, Pignataro G, Del Prete A, Sirabella R, Matrone C, Boscia F, Scorziello A, Sisalli MJ, Esposito E, Zambrano N, Di Renzo G, Annunziato L., (2010) NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic brain preconditioning. Stroke 42(3):754-63

Vanhoutte P, Barnier JV, Guibert B, Pages C et al. (1999) Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brai slices *Mol and Cell Biol* 19:136-146.

Vasington F.D., P. Gazzotti, R. Tiozzo, E. Carafoli, The effect of ruthenium red on Ca 2þ transport and respiration in rat liver mitochondria, Biochim. Biophys. Acta 256 (1972) 43e54.

Vellimana AK, Milner E, Azad TD, Harries MD, Zhou ML, Gidday JM, Han BH & Zipfel GJ (2011). Endothelial nitric oxide synthase mediates endogenous protection against subarachnoid hemorrhage-induced cerebral vasospasm. Stroke 42, 776–782.

Vlasov, T. D., Korzhevskii, D. E. & Polyakova, E. A. Ischemic preconditioning of the rat brain as a method of endothelial protection from ischemic/repercussion injury. Neurosci. Behav. Physiol. 35, 567–572 (2005).

Voellmy R. Transcriptional regulation of the metazoan stress protein response. Prog Nucleic Acid Res Mol Biol 2004; 78: 143–85.

Voellmy R. Transcriptional regulation of the metazoan stress protein response. Prog Nucleic Acid Res Mol Biol 2004; 78: 143–85.

Wada K, Miyazawa T, Nomura N, Tsuzuki N, Nawashiro H, Shima K, et al. Preferential conditions for and possible mechanisms of induction of ischemic tolerance by repeated hyperbaric oxygenation in gerbil hippocampus. Neurosurgery 2001; 49: 160–7.

Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor-1. J Biol Chem 270:1230–1237

Wang J, Bright R, Mochly-Rosen D, Giffard RG. Cell-specific role for epsilon- and betaI-protein kinase C isozymes in protecting cortical neurons and astrocytes from ischemia-like injury. Neuropharmacology 2004; 47: 136–45.

Wang X, Li X, Currie RW, Willette RN, Barone FC, Feuerstein GZ (2000) Application of real-time polymerase chain reaction to quantitate induced expression of interleukin-1beta mRNA in ischemic brain tolerance. *J Neurosci Res* 59:238-246.

Weih M, Kallenberg K, Bergk A, Dirnagl U, Harms L, Wernecke KD, Einhaupl KM (1999 a) Attenuated stroke severity after prodromal TIA: a role for ischemic tolerance in the brain. Stroke 30:1851-1854.

Weih M, Kallenberg K, Bergk A, Dirnagl U, Harms L, Wernecke KD, Einhaupl KM (1999 *a*) Attenuated stroke severity after prodromal TIA: a role for ischemic tolerance in the brain. *Stroke* 30:1851-1854.

Weih M., Bergk A., Isaev N.K., Ruscher K., Megow D., Riepe M., Meisel A., Victorov IV, Dirnagl U (1999 *b*) Induction of ischemic tolerance in rat cortical neurons by 3-nitropropionic acid: chemical preconditioning *Neurosci. Lett.*, 272:207-210.

Wen YD, Sheng R, Zhang LS, Han R, Zhang X, Zhang XD, et al. Neuronal injury in rat model of permanent focal cerebral ischemia is associated with activation of autophagic and lysosomal pathways. Autophagy 2008; 4: 762–9.

White JE, Lin HY, Davis FB, Davis PJ, Tsan MF (2000) Differential induction of TNF-α and Mn-SOD by endotoxin in human monocytes: role of protein tyrosine kinase, mitogen activated protein kinase, and NF-κB *J Cell Physiol* 182:381-389.

Wick A, Wick W, Waltenberger J, Weller M, Dichgans J, Schulz JB, et al. Neuroprotection by hypoxic preconditioning requires sequential activation of vascular endothelial growth factor receptor and Akt. J Neurosci 2002; 22: 6401–7.

Wiegand F, Liao W, Busch C, Castell S, Knapp F, Lindauer U, Megow D, Meisel A, Redetzky A, Ruscher K, Trendelenburg G, Victorov I, Riepe M, Diener HC, Dirnagl U (1999) Respiratory chain inhibition induces tolerance to focal cerebral ischemia. *J Cereb Blood Flow Metab* 19:1229-1237.

Wiener CM, Booth G, Semenza GL (1996) In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Comm 225:485–488

Wingrove, J. A. & O'Farrell, P. H. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in Drosophila. Cell 98, 105–114 (1999).

Wrang ML, Moller F, Alsbo CW, Diemer NH (2001) Changes in gene expression following induction of ischemic tolerance in rat brain: detection and verification. *J Neurosci Res* 65:54-58.

Wu JC. Liang ZQ. Qin ZH. Quality control system of the endoplasmic reticulum and related diseases. Acta Biochem Biophys Sin 2006; 38: 219–26.

Wu JC. Liang ZQ. Qin ZH. Quality control system of the endoplasmic reticulum and related diseases. Acta Biochem Biophys Sin 2006; 38: 219–26.

Xu Y (1999) An intronic NF-κB element is ettentiel for induction of the human Mn-SOD gene by TNF-αand IL-1β *DNA Cell Biol* 18:709-722.

Yang J, Liu X, Bhalla K (1997) Prevention of apoptosis by Bcl-2: relese of cytochrome *c* from mitochondria blocked *Science* 275:1129-1132.

Yano S, Morioka M, Fukunaga K, Kawano T, Hara T, Kai Y, Hamada J, Miyamoto E, Ushio Y (2001) Activation of Akt/protein kinase B contributes to induction of ischemic tolerance in the CA1 subfield of gerbil hippocampus. *J Cereb Blood Flow Metab* 21:351-360.

Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK. The neuroprotective potential of heat shock protein 70 (HSP70). Mol Med Today 1999; 5: 525–31.

Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK. The neuroprotective potential of heat shock protein 70 (HSP70). Mol Med Today 1999; 5: 525–31.

Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, Ron D. Compartment- specific perturbation of protein handling activates genes encoding mitochondrial chaperones. J Cell Sci 2004; 117: 4055–66.

Yoshida, M., Nakakimura, K., Cui, Y. J., Matsumoto, M. & Sakabe, T. Adenosine A1 receptor antagonist and mitochondrial ATP-sensitive potassium channel blocker attenuate the tolerance to focal cerebral ischemia in rats. J. Cereb. Blood Flow Metab. 24, 771–779 (2004).

Yu L and Colvin RA (1997) Regional differencies in expression of transcripts for Na^{+}/Ca^{2+} exchanger isoforms in rat brain. *Mol Brain Res* 50: 285-292.

Yun Hy, Gonzale-Zulueta M, Dawson T, Dawson V (1998) Nitric oxide mediates NMDA receptor-induced activation of p21 Ras. *Proc Natl Acad Sci Usa* 95:5773-5778.

Yun HY, Gonzalez-Zulueta M, Dawson VL & Dawson TM (1998). Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. Proc Natl Acad Sci U S A 95, 5773–5778.

Zaman K, Ryu H, Hall D, O'Donovan K, Lin K-I, Miller MP, Marquis JC, Baraban JM, Semenza GL, Ratan RR (1999) Protection from oxidative stress-induced apoptosis in cortical neuronal cultures by iron chelators is associated with enhanced DNA binding of hypoxia-inducible factor-1 and ATF-1/CREB and increased expression of glycolytic enzymes, p21waf1/cip1, and erythropoietin. J Neurosci 19:9821–9830

Zhan R. Z., Fujihara H., Baba H., Yamakura T. and Shimoji K. (2002) Ischemic preconditioning is capable of inducing mitochondrial tolerance in the rat brain. *Anesthesiology* 97, 896–901.

Zhang R, Chopp M, Zaloga C, et al (1995) The temporal profiles of ICAM-1 protein and mRNA expression after transient MCA occlusion in the rat *J Cereb Blood Flow Metab* 682:182-188.

Zhang R, Phillips ML, Chopp M, et al (1996) E-selectin in focal cerebral ischemia and reperfusion in the rat *J Cereb Blood Flow Metab* 16:1126-1136.

Zhang Y, Wu YX, Hao YB, Dun Y, Yang SP. Role of endogenous opioid peptidesin protection of ischemic preconditioning in rat small intestine. Life Sci 2001; 68: 1013–9.

Zhang, X. et al. Preconditioning with prolonged oxygen exposure induces ischemic tolerance in the brain via oxygen free radical formation. Can. J. Anaesth. 51, 258–263 (2004).

Zhao H, Sapolsky RM & Steinberg GK (2006) Phosphoinositide-3-kinase／akt survival signal pathways are implicated in neuronal survival after stroke. Mol Neurobiol 34, 249–270.

Zhao P, Huang Y, Zuo Z. Opioid preconditioning induces opioid receptor-dependent delayed neuroprotection against ischemia in rats. Neuropathol Exp Neurol 2006; 65: 945–52.

Zheng S, Zuo Z. Isoflurane preconditioning induces neuroprotection against ischemia via activation of p38 mitogen-activated protein kinases. Mol Pharmacol 2004; 65: 1172–80.

Zhou AM, Li WB, Li QJ, Liu HQ, Feng RF, Zhao HG. A short cerebral ischemic preconditioning up-regulates adenosine receptors in the hippocampal CA1 region of rats. Neurosci Res 2004; 48: 397–404.

Zhou P, Qian L & Iadecola C (2005). Nitric oxide inhibits caspase activation and apoptotic morphology but does not rescue neuronal death. J Cereb Blood Flow Metab 25, 348–357.

Zimmermann C, Ginis I, Furuya K, Klimanis D, Ruetzler C, Spatz M, Hallenbeck JM (2001) Lipopolysaccharide-induced ischemic tolerance is

associated with increased levels of ceramide in brain and in plasma. *Brain Res* 895:59-65.

Zoratti M., I. Szabo, U. De Marchi, Mitochondrial permeability transitions: how many doors to the house? Biochim. Biophys. Acta 1706 (2005) 40e52.

Zoratti M., U. De Marchi, L. Biasutto, I. Szabo, Electrophysiology clarifies the mega riddles of the mitochondrial permeability transition pore, FEBS Lett. 584 (2010) 1997e2004.

Zweier JL, Talukder MAH. The role of oxidants and free radicals in reperfusion injury. Cardiovas Res 2006; 70: 181–90.