



Involvement of mitochondria on neuroprotective effect of sphingosine-1-phosphate in cell death in an in vitro model of brain ischemia

Alba Agudo-López, Begoña G. Miguel, Inmaculada Fernández, Ana M. Martínez*

Departamento de Bioquímica y Biología Molecular I, Facultad de Química, Universidad Complutense de Madrid, E-28040 Madrid, Spain

ARTICLE INFO

Article history:

Received 2 November 2009

Received in revised form

22 December 2009

Accepted 28 December 2009

Keywords:

Sphingosine-1-phosphate

Ischemia

Mitochondria

Neuroprotection

ABSTRACT

Sphingosine-1-phosphate (S1P) has been demonstrated to be an important regulator of cell death and survival. Although it has been suggested that the sphingolipid may act as a neuroprotector in the cell apoptosis induced by traumatic brain injury, the mechanisms involved in this action are unknown. In this study, the relationship between S1P and neuroprotective effect was studied in an in vitro model of ischemia, maintaining SH-SY5Y human neuroblastoma cells under oxygen–glucose deprivation (OGD). When cells were treated with 1 μ M S1P simultaneously with OGD and recovery, cell viability increases in a dose–response manner. S1P treatment reduces significantly both necrosis and apoptosis cell death. On the other hand, the treatment with specific PKC ϵ (V1-2), prevents S1P protective effect of OGD/recovery-induced necrosis. Moreover, S1P treatment provokes the translocation of PKC ϵ to the mitochondria. From these results, it is reasonable to assume that S1P protection from necrosis is mediated by PKC ϵ . We also studied the action of S1P on mitochondrial inner membrane potential and mitochondrial Ca^{2+} levels during ischemia. In this regard, we must point out that S1P treatment reduces the OGD-induced membrane depolarization and also reduces the increase of Ca^{2+} in mitochondria during OGD. Results also indicate that mitochondria from OGD treated cells have significantly less ability to resist swelling on Ca^{2+} loading than those obtained in presence of oxygen and glucose. Nevertheless, when S1P was added, this resistance increases considerably. These findings suggest that S1P may have a potential role as a neuroprotective agent in brain injury.

© 2009 Elsevier Ireland Ltd. All rights reserved.

Stroke is one of the main causes of death and disability in the western world. Mechanisms underlying neuronal cell death after brain ischemia are complex as they depend on multiple factors [8]. Delayed neuronal death includes the development of apoptotic and necrotic processes, and the specific contribution of these processes to brain damage depend on different factors [18].

The regulation of the cell death program in neurons is a subject of great interest. Among the known mediators of apoptosis, the sphingolipid metabolites such as ceramide and sphingosine-1-phosphate (S1P) have received much attention in the last decade as key regulators of cell death and survival [13,26]. S1P is formed by the phosphorylation of sphingosine by sphingosine kinase, and it has been implicated in many and diverse biological processes, such as cell growth, differentiation, cell survival, angiogenesis and cell migration [23,26]. Recently, it has been proposed that S1P may represent a novel neuroprotective target to counteract the pathophysiology of acute brain and spinal cord injury in regard to apoptotic cell death mechanisms, mitochondrial dysfunction, lipid hydrolysis, and oxidative damage mechanisms [25].

* Corresponding author. Fax: +34 913 944 159.

E-mail address: anamart@bbm1.ucm.es (A.M. Martínez).

It is noteworthy that during ischemia, some protein kinase C (PKC) isoforms are translocated to different cellular destinations which include the plasma membrane, golgi, nucleus, mitochondria and other cellular compartments. PKCs have been reported to interact with many target proteins upon activation [9]. PKC ϵ is expressed in neural tissue, and it has been implicated in protective roles against stroke and neural ischemia [2]. Besides, in the last years, mitochondria have been recognised as regulators of cell death [15]. In this sense, during hypoxia, PKC ϵ is known to translocate to mitochondria, where interacts with several targets. When mitochondria are exposed to high Ca^{2+} concentrations, especially when accompanied by oxidative stress and adenine nucleotide depletion, as happens after an ischemia/recovery insult, they undergo massive swelling and become uncoupled. This occurs as a result of the opening of the mitochondrial permeability transition pore (mPTP) with subsequent loss of ionic homeostasis, matrix swelling and outer membrane rupture. If the pore remains open, cells cannot maintain their ATP levels and this will lead to cell death by necrosis [11,12]. Prevention of mPTP opening, directly or indirectly by reducing oxidative stress or Ca^{2+} overload, provides a protective strategy against reperfusion injury [11]. It is widely considered that opening of the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) plays a crucial role in protection from ischemia. This

channel seems to affect mPTP opening. Recently, PKC ϵ has been identified as a component of the mitoK_{ATP} signalling cascade [16]. PKC ϵ is suspected to stabilize mitochondria activating mitoK_{ATP} and modifying mPTP opening [7].

Taking this into account, we aimed to elucidate the mechanism involved in neuroprotective role of S1P against ischemia-induced cell death.

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin and 1% glutamine in a humidified incubator in 95% air–5% CO₂ at 37 °C. The cell culture medium was replaced every 2 days.

The in vitro model of ischemia we used was achieved maintaining the cells under oxygen–glucose deprivation (OGD) for 3 h, followed by 16 h of recovery, as described previously [21]. Briefly, the standard culture medium was replaced with a glucose-free buffer (154 mM NaCl, 5.6 mM KCl, 5.0 mM HEPES, 3.6 mM NaHCO₃, 2.3 mM CaCl₂; pH 7.4), and bubbled with an anaerobic gas mixture (95% N₂, 5% CO₂) for at least 2 h before use. Cells were then placed under hypoxia in a hypoxic humidified incubator in 1% O₂–5% CO₂ at 37 °C of Thermo Electron Corporation (model 3141). After 3 h, buffer was changed by standard medium and placed in a humidified aerobic incubator at 37 °C for 16 h of recovery.

Cell viability was determined by MTT (3-[4,5-dimethylthiazole-2-yl]2,5-diphenyl tetrazolium bromide) assay as previously described [1]. Briefly, MTT to a final concentration of 0.5 mg/ml was added to each sample in a 96-well plate. The plates were incubated at 37 °C for 4 h, and the formazan granules generated by the live cells were dissolved in isopropyl alcohol acid (0.5% of HCl concentrate in isopropyl alcohol) and absorbance at 570 nm was monitored by using a Power-Wavex microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA).

The percentage of early apoptotic cells (intact cell membrane, affinity for annexin V-FITC and devoid of PI staining) and necrotic or late apoptotic cells (the cell membrane loses its integrity, the cell becomes PI staining) were analyzed by flow cytometry using an annexin V-FITC/PI kit (Biosource) following the manufacturer's instructions.

In order to measure mitochondrial membrane depolarization, cells were loaded for 15 min with 1 μ M TMRM (Anaspec) at 37 °C. S1P (1 μ M) was added just before OGD. Cells were then placed in a thermostated sealed chamber on a Leica confocal microscope in the buffer described above with or without glucose depending on the treatment. To induce OGD, the chamber was maintained under a N₂ atmosphere during the time of OGD. For recovery, chamber was submitted to a normal atmosphere and glucose (1 mg/ml) was added as in initial conditions. Images were then collected on a Leica confocal microscope and analyzed with LCS Lite software.

Immunoblot analysis were carried out in cytosolic and mitochondrial fractions as previously described [5].

For calcium measurements, cells were loaded at 37 °C for 15 min with 2.2 mM rhod-2 AM (Anaspec) to measure mitochondrial Ca²⁺. Cells were treated with 2 μ M thapsigargin for 20 min before the S1P addition to avoid interferences by endoplasmic reticulum Ca²⁺. Cells were then placed in a thermostated sealed chamber on a Leica confocal microscope as described above.

Opening of mPTP was monitored by analyzing mitochondrial swelling in isolated mitochondria. Cells were maintained under oxygen–glucose deprivation for 3 h and then mitochondria were isolated [19]. Mitochondrial swelling was assayed spectrophotometrically [24]. Briefly, isolated mitochondria were resuspended in swelling buffer (120 mM KCl, 10 mM Tris–HCl, 20 mM MOPS, and 5 mM KH₂PO₄, pH 7.4) to a final protein concentration of 0.25 mg/ml. Absorbance was measured spectrophotometrically at 520 nm every 15 s. After 2 min of stabilization 200 μ M CaCl₂ was added to induce mPTP opening.

Data are presented as means \pm S.D. in this article. Statistical comparisons were made using Student's *t*-test. Values of *p* < 0.05 was considered statistically significant.

Cells SH-SY5Y were subjected to 3 h of OGD and 16 h of recovery and then viability was assessed by MTT. A decrease in viability around 33% compared with control was observed. When cells were treated with S1P simultaneously with OGD and recovery, cell viability increased in a dose–response manner, obtaining a 100% viability with 1 μ M S1P, therefore, this concentration was used for all experiments (Fig. 1A). Identical results were obtained when S1P was added 3 h before OGD (data not shown), so in all the experiments, S1P was added at the same time as OGD. To test if this protection of S1P was mediated by receptor binding, pertussis toxin (PTX) (100 ng/ml) was added at the same time than S1P. PTX avoided the protective effect of the S1P, suggesting the involvement of G_{i/o} protein-coupled S1P receptors (Fig. 1A).

Neuronal cell death can occur by necrosis and/or apoptosis. These two types of cell death can be distinguished by flow cytometry assay. The results obtained with this technique, indicated that after 3 h of OGD and 16 h of recovery, cells died predominantly by necrosis, but also by apoptosis (20 \pm 1.3% for necrosis or late apoptosis and 8 \pm 1.5% for early apoptosis). S1P treatment reduced significantly both apoptosis and necrosis (Fig. 1B). These results are in agreement with other reported previously [10]. Bearing in mind that it has been described previously the involvement of PKC

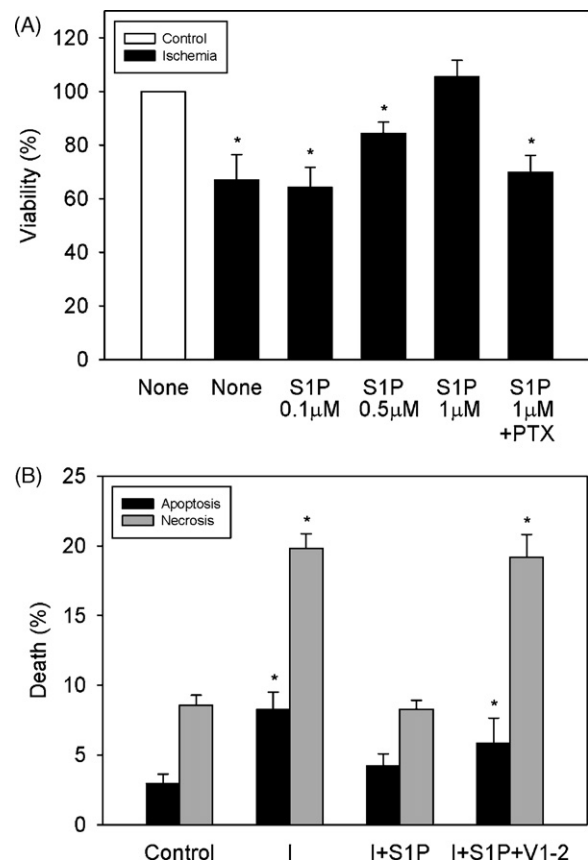


Fig. 1. Effect of exogenous S1P on the SH-SY5Y cells death subjected to OGD. (A) Viability was tested by MTT assay of control cells and cells submitted to 3 h OGD and 16 h of recovery with or without S1P (0.1, 0.5 and 1 μ M). PTX (100 ng/ml) was added when indicated. (B) Flow cytometry with annexin V and propidium iodide of control cells and cells submitted to 3 h OGD and 16 h of recovery (I) with or without 1 μ M S1P. Early apoptotic cells were positive to annexin V but not to propidium iodide and necrotic or late apoptotic cells were positive to propidium iodide. V1-2 (5 μ M) was added when indicated. Values are mean \pm S.D. of at least three independent experiments. *Statistically significant difference from control value (*p* < 0.05).

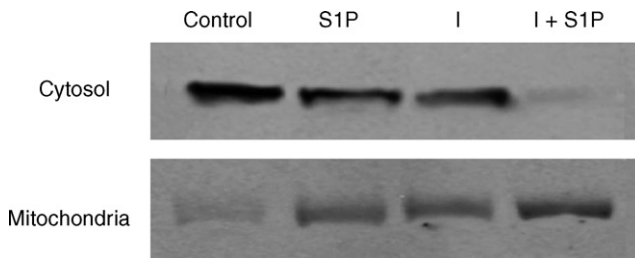


Fig. 2. PKC ϵ translocation from cytosol to mitochondria. Mitochondrial fractions of SH-SY5Y cells were submitted to 1 h OGD in the presence or absence of 1 μ M S1P. The immunoblots are representative of one of three experiments with similar results.

family, especially pKC ϵ isoform, in the mechanism of action of different protector agents of the ischemic/recovery insult [3,6,20], we tested whether this PKC isoform is involved in the protective mechanism of action of S1P. Our results indicated that the specific PKC ϵ translocation inhibitor V1-2, in a concentration of 5 μ M, prevents S1P protective effect of OGD/recovery-induced necrosis and apoptosis (Fig. 1B). From these results, one can speculate that S1P protection is mediated by PKC ϵ . On the other hand, preliminary results obtained by us, seem to involve the isoform of PKC ζ in the above mentioned apoptosis protection, but a more intense study would be necessary (data not shown).

In recent years, mitochondria have been recognized as regulators of cell death via both necrosis and apoptosis [17] and it is well known the implication of mitochondria in death induced by ischemia in different experimental models [15]. First, we studied how OGD/recovery affects the redistribution of pKC ϵ between mitochondria and cytosol. It is noteworthy that both OGD/recovery and S1P treatment alone lead to PKC ϵ translocation from cytosol to mitochondria. This effect was evident after 1 h OGD treatment (Fig. 2) and it was maintained for 4 h (data not shown). Moreover, when S1P was added during OGD, the effect seems to be additive (Fig. 2). The increase of PKC ϵ in mitochondria has been described as a mechanism of defence against the hypoxia/ischemia insult [4]. S1P seems to act, at least in part, through this mechanism.

Mitochondrial membrane potential is created by the electron transport chain, and changes are related to the cell death produced by ischemia, which make it an useful marker for neuronal death [15]. To investigate the changes in mitochondrial inner membrane potential, in the presence of OGD and with S1P treatment, we carried out experiments in an anaerobic chamber with influx of N_2 (atmosphere of approximately 1% of oxygen) placed on a confocal microscopy using TMRM as probe. Cells were subjected to 60 min of OGD and 25 min of recovery. OGD produced a strong membrane depolarization in a time-dependent manner maintained during recovery (Fig. 3). Our results are in agreement with others reported previously in similar experimental ischemia models [15]. When 1 μ M S1P was added during the time of OGD, depolarization produced by OGD was significantly reduced (nearly a 50%) and a peak of repolarization occurred during recovery. V1-2 prevented the S1P effect, indicating this fact that S1P action is mediated by PKC ϵ .

Mitochondrial membrane potential facilitates the mitochondrial permeability to ions, especially Ca^{2+} . Calcium enters then into the mitochondria by the force of negative charge in matrix. Mitochondria act as a Ca^{2+} buffer, regulating the cytoplasmic Ca^{2+} concentration by the Ca^{2+} uniporter. Under normal conditions, mitochondria store small amounts of Ca^{2+} but has a large capacity to accumulate and buffer Ca^{2+} under states of high cellular Ca^{2+} loading [22]. Mitochondrial Ca^{2+} overload has been suggested as a marker for injury in ischemia [14]. Abnormal cellular Ca^{2+} load can trigger mPTP opening. mPTP opening enables free passage into the mitochondria molecules smaller than 1.5 kDa, including pro-

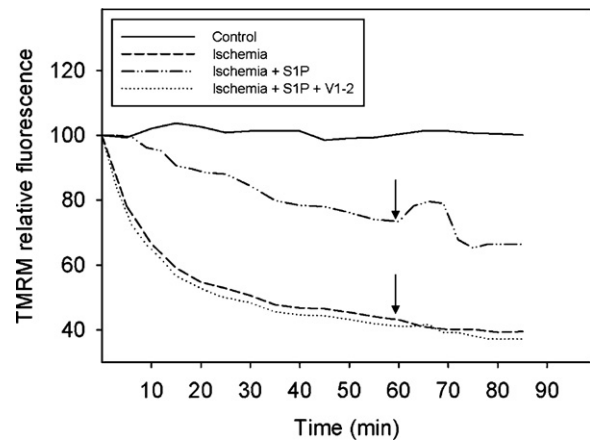


Fig. 3. Effects of S1P on mitochondrial inner membrane potential measured in mitochondria isolated from SH-SY5Y cells subjected to OGD. Cells were loaded with 1 μ M TMRM and placed in an anaerobic thermostated chamber on a confocal microscopy. Cells, except control, were subjected to 1 h of OGD and 25 min of recovery, with or without 1 μ M S1P. V1-2 (5 μ M) was added when indicated. Representative values from three independent experiments. Each experimental group had at least 60 cells analysed per experiment. Arrows indicate the recovery.

tons. The resulting uncoupling of oxidative phosphorylation leads to ATP depletion and cell death and it is now widely recognised that mPTP opening is a major cause of ischemia/recovery injury and an effective target for protection [12]. To investigate this in our system, we measured changes in Ca^{2+} levels with confocal microscopy using rhod-2 AM as probe. During OGD, mitochondrial calcium increased, as has been previously described [22]. S1P treatment during OGD produced a decrease in mitochondrial Ca^{2+} (Fig. 4). The decrease of mitochondrial Ca^{2+} overload during ischemia has been established to prevent or delay cell death [14]. This effect was partially prevented by V1-2 (Fig. 4), indicating this a possible mediation by PKC ϵ . PKC ϵ has been seen to activate mitoK_{ATP} channel opening. Numerous studies have demonstrated that opening of this channel exerts a protective effect against ischemia/recovery injury, which is thought to be due to regulation of Ca^{2+} levels among others factors, which is related with the inhibition of mPTP, principal mediator of necrotic death [4,12,17]. The mitoK_{ATP} inhibitor 5-hydroxidecanoate (5-HD) also prevented the S1P effect on mito-

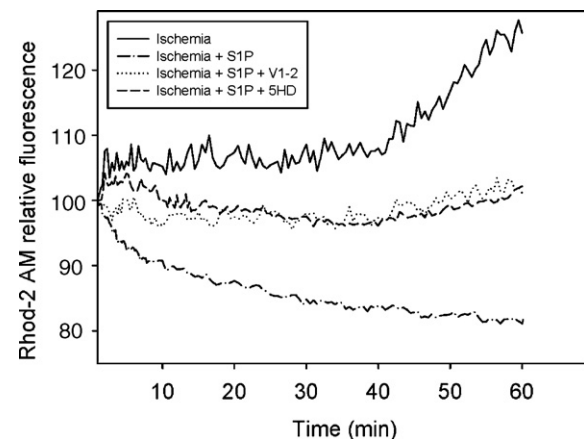


Fig. 4. Effects of S1P on mitochondrial calcium from SH-SY5Y cells subjected to OGD. Cells were pretreated with 2 μ M thapsigargin, loaded with 2.2 mM rhod-2 AM and placed in an anaerobic thermostated chamber in a confocal microscopy. Cells were subjected to 1 h of OGD, in the presence or absence of 1 μ M S1P. V1-2 (5 μ M) and 5-HD (0.3 mM) were added when indicated. Representative values from three independent experiments. Each experimental group had at least 60 cells analysed per experiment.

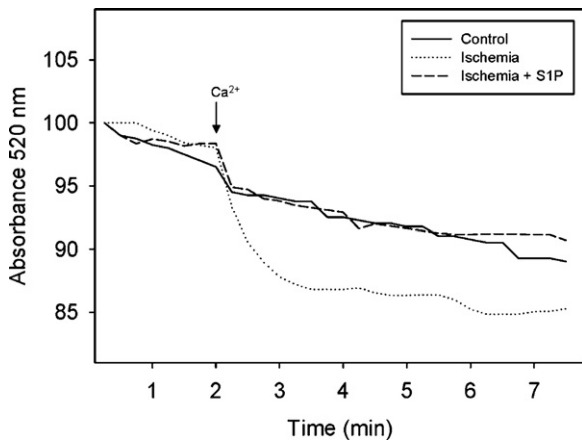


Fig. 5. Effects of S1P on mitochondrial swelling from SH-SY5Y cells subjected to OGD. Mitochondria were isolated from control cells or cells subjected to 3 h of OGD with or without 1 μ M S1P and swelling was assessed as a decrease in light absorbance at 520 nm. Representative rates of mitochondria swelling are shown. Arrow indicates the addition of 250 μ M CaCl_2 to isolated mitochondria suspension. Representative values from three independent experiments.

chondrial Ca^{2+} , so it seems that this S1P action is mediated by $\text{mitoK}_{\text{ATP}}$ too.

When the mPTP is open, in the presence of Ca^{2+} loading, solutes enter the inner matrix, causing the mitochondria to swell. This is observed as decrease in the optical density at 540 nm, indicating swelling of mitochondria in presence of Ca^{2+} . The ability of mitochondria to resist swelling has been related with protection [19]. To study this point, we have isolated mitochondria from cells subjected to ischemia in presence or absence of S1P. The same Ca^{2+} concentration produced a minor mitochondrial swelling when, together with OGD, S1P was added (Fig. 5). This fact indicates a higher resistance to mPTP opening due to S1P protecting effect.

These results demonstrate a protective effect of S1P in OGD/recovery injury. This protection involves mitochondrial membrane potential stabilization, a reduced Ca^{2+} loading during OGD and a less sensitivity to mPTP opening and is mediated by PKC ϵ and $\text{mitoK}_{\text{ATP}}$.

The findings of the present study demonstrate for the first time, the key role played by the mitochondria and the involvement of PKC ϵ in the mechanism of protection of S1P against brain injury.

Acknowledgements

We are extremely grateful to Dr. R.E. Catalán for helpful discussions and for reading of the manuscript. A. Agudo-López was supported by a fellowship from Ministerio de Educacion (Spain).

References

- [1] M.C. Alley, D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, M.R. Boyd, Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay, *Cancer Res.* 48 (1988) 589–601.
- [2] M.E. Barnett, D.K. Madgwick, D.J. Takemoto, Protein kinase C as a stress sensor, *Cell Signal.* 19 (2007) 1820–1829.

- [3] G.R. Budas, E.N. Churchill, D. Mochly-Rosen, Cardioprotective mechanisms of PKC isozyme-selective activators and inhibitors in the treatment of ischemia-reperfusion injury, *Pharmacol. Res.* 55 (2007) 523–536.
- [4] G.R. Budas, D. Mochly-Rosen, Mitochondrial protein kinase C ϵ (PKC ϵ): emerging role in cardiac protection from ischaemic damage, *Biochem. Soc. Trans.* 35 (2007) 1052–1054.
- [5] M.C. Calcerrada, B.G. Miguel, L. Martín, R.E. Catalán, A.M. Martínez, Involvement of phosphatidylinositol 3-kinase in nuclear translocation of protein kinase C ζ induced by C2-ceramide in rat hepatocytes, *FEBS Lett.* 514 (2002) 361–365.
- [6] A.D.T. Costa, K.D. Garlid, Intramitochondrial signaling: interactions among $\text{mitoK}_{\text{ATP}}$, PKC ϵ , ROS, and MPT, *Am. J. Physiol. Heart Circ. Physiol.* 295 (2008) H874–H882.
- [7] A.D.T. Costa, R. Jakob, C.L. Costa, K. Andrukiv, I.C. West, K.D. Garlid, The mechanism by which the mitochondrial ATP-sensitive K^+ channel opening and H_2O_2 inhibit the mitochondrial permeability transition, *J. Biol. Chem.* 281 (2006) 20801–20808.
- [8] U. Dirnagl, C. Iadecola, M.A. Moskowitz, Pathobiology of ischaemic stroke: an integrated view, *Trends Neurosci.* 22 (1999) 391–397.
- [9] R.D. Edmondson, T.M. Vondriska, K.J. Biederman, J. Zhang, R.C. Jones, Y. Zheng, D.L. Allen, J.X. Xiu, E.M. Cardwell, M.R. Pisano, P. Ping, Protein kinase C ϵ signaling complexes include metabolism- and transcription/translation-related proteins: complimentary separation techniques with LC/MS/MS, *Mol. Cell. Proteomics* 1 (2002) 421–433.
- [10] E. Fordel, L. Thijs, W. Martinet, D. Schrijvers, L. Moens, S. Dewilde, Anoxia or oxygen and glucose deprivation in SH-SY5Y cells: a step closer to the unraveling of neuroglobin and cytoglobin functions, *Gene* 398 (2007) 114–122.
- [11] A.P. Halestrap, Calcium, mitochondria and reperfusion injury: a pore way to die, *Biochem. Soc. Trans.* 34 (2006) 232–237.
- [12] A.P. Halestrap, What is the mitochondrial permeability transition pore? *J. Mol. Cell. Cardiol.* 46 (2009) 821–831.
- [13] Y.A. Hannun, L.M. Obeid, The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind, *J. Biol. Chem.* 277 (2002) 25847–25850.
- [14] D. Hausenloy, A. Wynne, M. Duchon, D. Yellon, Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection, *Circulation* 109 (2004) 1714–1717.
- [15] T. Iijima, Mitochondrial membrane potential and ischemic neuronal death, *Neurosci. Res.* 55 (2006) 234–243.
- [16] M. Jaburek, A.D.T. Costa, J.R. Burton, C.L. Costa, K.D. Garlid, Mitochondrial PKC ϵ and mitochondrial ATP-sensitive K^+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes, *Circ. Res.* 99 (2006) 878–883.
- [17] S. Javadov, M. Karmazyn, Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection, *Cell Physiol. Biochem.* 20 (2007) 1–22.
- [18] Y. Kametsu, S. Osuga, A.M. Hakim, Apoptosis occurs in the penumbra zone during short-duration focal ischemia in the rat, *J. Cereb. Blood Flow Metab.* 23 (2003) 416–422.
- [19] I. Khaliulin, H. Schwab, P. Wang, E. Houminer, L. Grinberg, H. Katzeff, J.B. Borman, S.R. Powell, Preconditioning improves postischemic mitochondrial function and diminishes oxidation of mitochondrial proteins, *Free Radic. Biol. Med.* 37 (2004) 1–9.
- [20] M.Y. Kim, M.J. Kim, I.S. Yoon, J.H. Ahn, S.H. Lee, E.J. Baik, C.H. Moon, Y.S. Jung, Diazoxide acts more as a PKC ϵ activator, and indirectly activates the mitochondrial K_{ATP} channel conferring cardioprotection against hypoxic injury, *Br. J. Pharmacol.* 149 (2006) 1059–1070.
- [21] G. Miglio, F. Varsaldi, E. Francioli, A. Battaglia, P.L. Canonico, G. Lombardi, Cabergoline protects SH-SY5Y neuronal cells in an in vitro model of ischemia, *Eur. J. Pharmacol.* 489 (2004) 157–165.
- [22] H. Miyata, E.G. Lakatta, M.D. Stern, H.S. Silverman, Relation of mitochondrial and cytosolic free calcium to cardiac myocyte recovery after exposure to anoxia, *Circ. Res.* 71 (1992) 605–613.
- [23] S.G. Payne, S. Milstien, S. Spiegel, Sphingosine-1-phosphate: dual messenger functions, *FEBS Lett.* 531 (2002) 54–57.
- [24] M. Ruiz-Meana, D. Garcia-Dorado, E. Miro-Casas, A. Abellan, J. Soler-Soler, Mitochondrial Ca^{2+} uptake during simulated ischemia does not affect permeability transition pore opening upon simulated reperfusion, *Cardiovasc. Res.* 71 (2006) 715–724.
- [25] I.N. Singh, E.D. Hall, Multifaceted roles of sphingosine-1-phosphate: how does this bioactive sphingolipid fit with acute neurological injury? *J. Neurosci. Res.* 86 (2008) 1419–1433.
- [26] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, *Nat. Rev. Mol. Cell. Biol.* 4 (2003) 397–407.