

Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage

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Abstract

Cerebral hypoxia/ischemia leads to mitochondrial dysfunction due to lack of oxygen leaving the glycolytic metabolism as a main pathway for ATP production. Inhibition of mitochondrial respiration thus triggers generation of lactate and hydrogen ions (H^+), and furthermore dramatically reduces ATP generation leading to dysregulation of cellular ion metabolism with subsequent intracellular calcium accumulation. Upon reperfusion, when mitochondrial dysfunction is (at least partially) reversed by restoring cerebral oxygen supply, bioenergetic metabolism recovers and brain cells are able to re-institute their normal ionic homeostatic mechanisms. However, the initial restoration of normal mitochondrial function may be only transient and followed by a secondary, delayed perturbation of mitochondrial respiratory performance seen as a decrease in cellular ATP levels and known as “secondary energy failure”. There have been several mechanisms considered responsible for delayed post-ischemic mitochondrial failure, the mitochondrial permeability transition (MPT) being one that is considered important. Although the amount of calcium available during early reperfusion *in vivo* is limited, relative to the amount needed to trigger the MPT *in vitro*; the additional intracellular conditions (of acidosis, high phosphate, and low adenine nucleotide levels) prevailing during reperfusion, favor MPT pore opening *in vivo*. Furthermore, the cellular redistribution and/or changes in the intracellular levels of pro-apoptotic proteins can alter mitochondrial function and initiate apoptotic cell death. Thus, mitochondria seem play an important role in orchestrating cell death mechanisms following hypoxia/ischemia. However, it is still not clear which are the key mechanisms that cause mitochondrial dysfunction and lead ultimately to cell death, and which have more secondary nature to brain damage acting as aggravating factors.

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1. Introduction

All cellular functions are either directly or indirectly dependent on cellular energy supplies. In order to preserve normal cellular function, mammalian cells require a constant supply of oxygen and substrates such as glucose to maintain adequate energy production. The brain in particular has low levels of storage forms of carbohydrates (in the form of glycogen), and is highly dependent on oxidative metabolism, since it represents only about 2% of total body weight and yet accounts for about 20% of total oxygen consumption (for review, see [1]). When the cellular oxygen supply is reduced to critical levels, as occurs in severe hypoxia or ischemia, damage to brain cells can occur. In this review, we will discuss the factors influencing hypoxia/ischemia-induced changes

in cerebral energy metabolism, and in particular the mechanisms altering mitochondrial function and their role in brain cell death. Before we consider detailed recent mechanistic information, and in view of its central role in the processes described above, we would like briefly review some basic information concerning cerebral energy metabolism under both physiological and pathophysiological conditions.

2. Major pathways of glucose metabolism yielding to ATP generation

Chemical free energy stored in the brain is mainly in the form of high-energy phosphate compounds, particularly adenosine-5'-triphosphate (ATP), which is made most notably under normoxic conditions, by the combined biochemical pathways of glycolysis and oxidative phosphorylation. In glycolysis, one molecule of glucose is anaerobically

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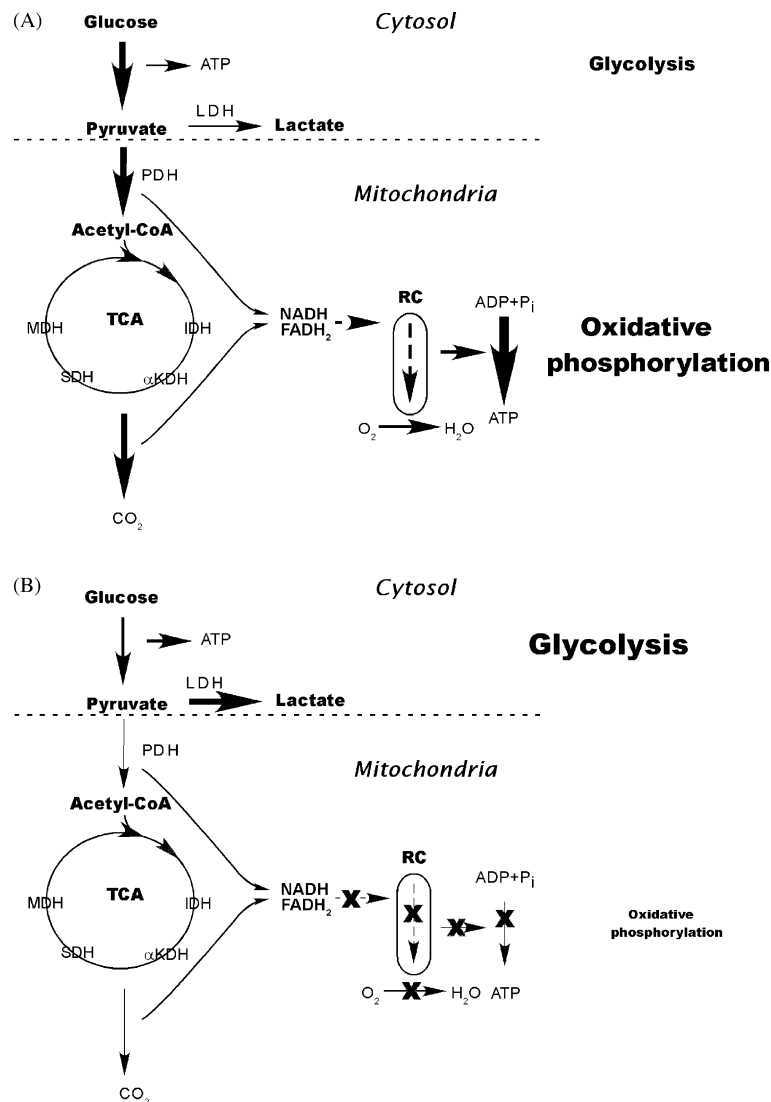


Fig. 1. Schematic diagram of basic pathways of glucose metabolism under normal physiological (A) and hypoxic/ischemic conditions (B). Glycolytic degradation of glucose generates pyruvate, which is under anaerobic conditions further converted to lactate by lactate dehydrogenase (LDH). During this process 2 mol of ATP are produced from one mol of glucose. In the presence of oxygen glucose is oxidized completely to CO₂ and H₂O. During this process pyruvate is transported into mitochondria where it is further processed to acetyl-CoA by pyruvate dehydrogenase (PDH) and NADH is produced. Acetyl-CoA then enters the tricarboxylic acid cycle (TCA). Isocitrate dehydrogenase (IDH), α -ketoglutarate dehydrogenase (α -KDH), malate dehydrogenase (MDH) within the TCA also generate NADH, and succinate dehydrogenase (SDH) generates FADH₂. Both NADH and FADH₂ serve as electron donors to respiratory complexes (RC) in mitochondria. Energy released during electron transport (dashed arrow) within the respiratory chain is utilized to generate ATP (oxidative phosphorylation). The electron flow in RC can continue only in the presence of oxygen since oxygen serves as final electron acceptor in this process. Under conditions of hypoxia/ischemia (B) the tissue oxygen tension is dramatically reduced causing arrest of mitochondrial respiration and ATP production by oxidative phosphorylation. This will stimulate glycolysis with increased lactate production. The thickness of the arrows represents the volume of metabolite fluxes.

converted to two molecules of pyruvate (Fig. 1). Under anoxic conditions pyruvate is further converted to lactate by lactate dehydrogenase (LDH).

Oxidative phosphorylation requires oxygen although more energy is released as glucose is oxidized completely to CO₂ and H₂O. During this process 36 mol of ATP are produced per one mole of glucose. Oxidative phosphorylation takes place in mitochondria where acetyl-CoA (derived from the oxidation of pyruvate) enters the tricarboxylic acid cycle (TCA) (Fig. 1).

Reducing equivalents in the form of NADH (produced by pyruvate dehydrogenase, isocitrate dehydrogenase, α -ketoglutarate dehydrogenase and malate dehydrogenase) and FADH₂ generated by succinate dehydrogenase serve as electron donors to respiratory complexes I and II, respectively, in mitochondria (Fig. 2). The ensuing process of electron transport within the respiratory chain to molecular oxygen proceeds with a very large decrease in free energy, much of which is used to generate an electrochemical gradient of hydrogen ions (H⁺) across the inner mitochondrial

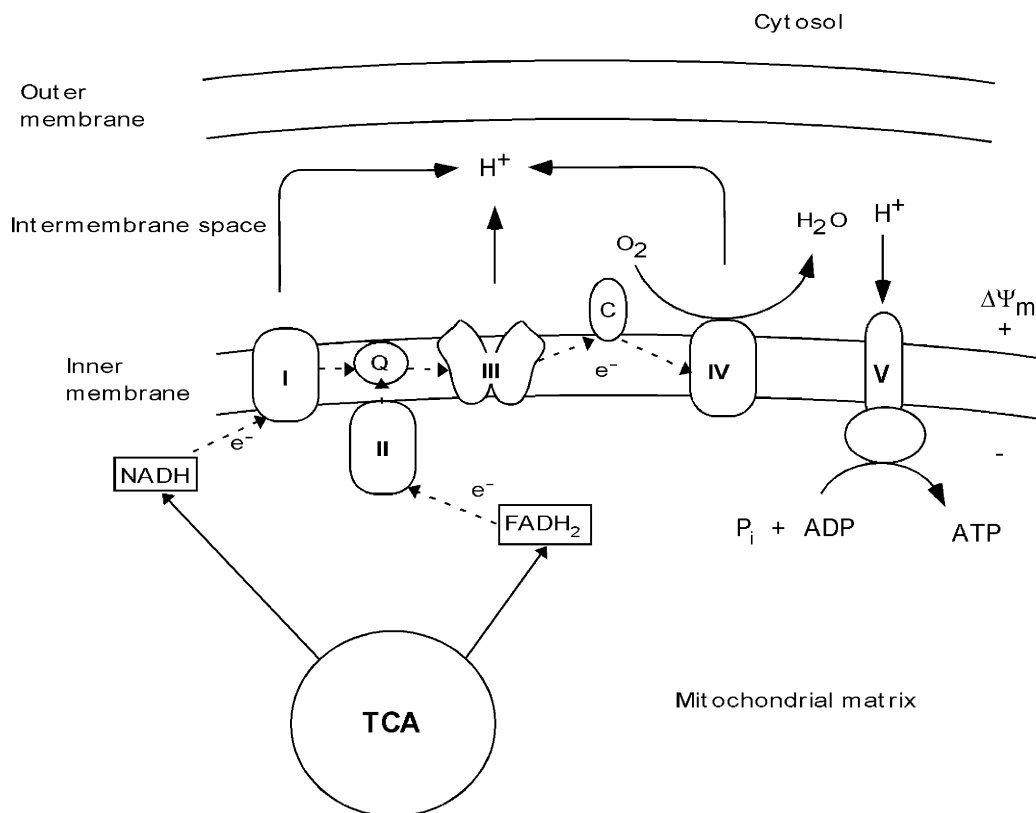


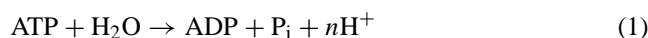
Fig. 2. Schematic diagram of mitochondrial respiration. NADH and FADH₂ produced within the TCA cycle donate electrons to complex I and complex II, respectively. Decline in free energy during the electron flow within the respiratory chain complexes generates an electrochemical gradient of hydrogen ions (H⁺) across the inner mitochondrial membrane. The energy from backflow of H⁺ ions through complex V is used to generate ATP from ADP and inorganic phosphate (P_i). Abbreviations: Q, ubiquinone; C, cytochrome c. Dashed arrows represent electron flow.

membrane and an electric potential, the inside of the mitochondria being negative. The energy stored in the electrochemical gradient of H⁺ is utilized for phosphorylation of ADP to yield ATP, in the process of oxidative phosphorylation. Under normal conditions 95% of carbohydrates that enter the brain are utilized ultimately by mitochondria, and more than 95% of ATP is generated by mitochondrial oxidative phosphorylation (for review, see [2]).

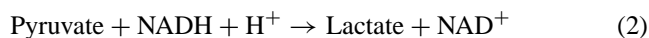
3. Changes in bioenergetic and calcium metabolisms induced by hypoxia/ischemia

Hypoxia/ischemia interferes with the oxidation of pyruvate (and other substrates) in mitochondria. Thus, decreases in tissue PO₂ leads to mitochondrial dysfunction. As a result ATP production by mitochondria is severely reduced or arrested and most of the ATP is generated only by the glycolytic pathway with the end product of lactate plus H⁺.

However, it must be emphasized, that H⁺ are produced by a variety of metabolic reactions, including glycolytic ones. A prime example is the ATPase reaction, which proceeds with the production of H⁺ thus:



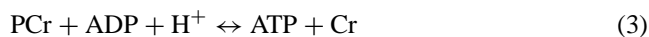
where n varies with pH and with the Mg²⁺ concentration. It has been demonstrated that H⁺ is not produced with lactate by lactate dehydrogenase reaction since this reaction:



consumes H⁺. However, if 1 mol of glucose is metabolized to 2 mol of lactate, and if the two ATPs, formed in this process, are again hydrolyzed to ADP and P_i, 2 mol of H⁺ will always be released.

Apart from glycolysis, during the first minutes after oxidative phosphorylation is discontinued the brain has two other mechanisms that help maintain cellular ATP levels. The first is the reaction catalyzed by the enzyme creatine kinase.

Creatine kinase catalyzes a reversible transfer of phosphate between phosphocreatine (PCr) and ATP thus:



The creatine kinase equilibrium favors ATP formation, thus any ADP formed will be converted back to ATP by translocation of phosphate from PCr to ADP. Therefore, during cerebral ischemia, only after PCr levels are decreased the ADP and AMP concentrations rise significantly due to ATP hydrolysis [3–6]. For example, in the adult rat, after 30 s of complete ischemia, brain PCr levels decrease to about 30%

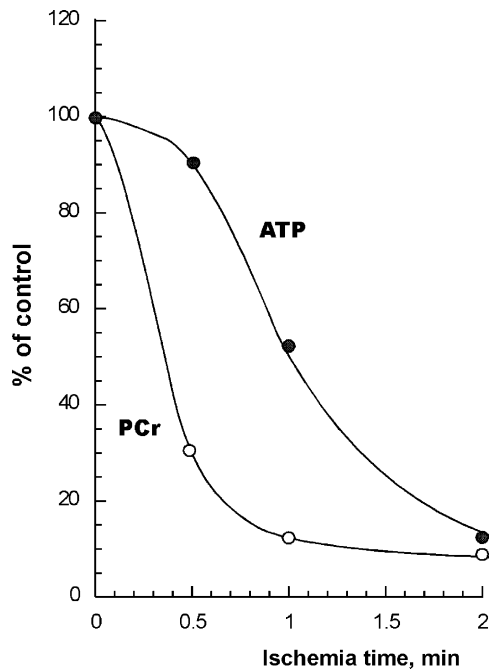


Fig. 3. Changes in PCr and ATP levels as % of control during the first 2 min of ischemia. Data were taken from [6].

of their pre-ischemic values while ATP levels are reduced only by 10% ([5,6]; see Fig. 3).

The other mechanism that helps to uphold brain ATP levels is reaction catalyzed by the enzyme adenylate kinase thus:



This process is readily reversible and is clearly observed under conditions of rapid ATP breakdown, as for example in ischemia, where AMP concentrations rise to higher levels than ADP. Thus during the first minute of energy failure due to hypoxia/ischemia there is a rapid decrease in PCr and ATP levels with rises in ADP, AMP, inorganic phosphate (P_i) and lactate concentrations and accompanying acidosis.

The glycolytic production of ATP under these conditions is regulated by several parameters. The main regulatory step in glycolysis is activation/inhibition of the enzyme phosphofructokinase (PFK), which catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate [7,8]. The enzyme is inhibited by ATP, PCr and citrate, and activated by ADP, P_i , AMP, and cAMP. Under normal physiological conditions this enzyme is inhibited by low pH [9]. However, a small decrease in cellular energy state releases PFK inhibition by hydrogen ions. After the onset of ischemia PFK activity increases greatly, due to a small decrease in cytosolic ATP levels and the subsequent increase ADP, AMP, and P_i concentrations [9].

3.1. Metabolic stages and ions homeostasis

In the brain, the majority of ATP is utilized to maintain the ion gradients across plasma membranes [2]. While the intracellular levels of K^+ (K_i^+) are about 30-fold higher than extracellular (K_e^+), the intracellular concentrations of Na^+ , Cl^- (Na_i^+ , Cl_i^-) are about seven-fold, and Ca^{2+} (Ca_i^{2+}) 10,000-fold lower than in the extracellular fluids (for review, see [10]). Therefore, an arrest of ATP production by mitochondria will compromise the mechanisms maintaining these gradients and lead to massive downhill ionic fluxes (for reviews, see [10–13]), and ion homeostasis is lost, even though carbohydrate substrates may still be available. This is because glycolysis only yields 2 mol of ATP per mole of glucose metabolized as opposed to 36 mol of ATP generated when both glycolysis and oxidative phosphorylation are fully functional (see above). Measurements of metabolites made from tissue rapidly frozen during cerebral ischemia have shown that high energy phosphate turnover during the first 10 s of ischemia is not significantly different from that during normoxia, but that this is followed by a period with lesser energy flux [14,15]. Thus, energy failure due to ischemia ultimately leads to dissipation of cellular ionic gradients [12,16,17].

The interval between the onset of ischemia and complete cellular depolarization is called the anoxic depolarization (AD) time. As mentioned above ischemia activates glycolysis, with production of lactate and H^+ , causing a gradual decrease in both extra-cellular and intracellular pH (pH_i and pH_e , respectively) [18–21]. The amount of lactate produced corresponds to the pre-ischemic tissue stores of glucose and glycogen, the former varying with plasma glucose concentration [22]. The pH_e starts to decrease immediately after onset of ischemia, and in normoglycemic animals the initial shift (about 0.4 pH units) is succeeded by an alkaline transient, which coincides with the AD (for review, see [23]). Following the AD the pH_e declines further to approximately pH 6.6 (see [24]). However, in hyperglycemic animals the ischemia-induced pH changes are even greater [19]. Apart from causing more marked changes in pH_e hyperglycemia also prolongs the AD time [17,25,26]. This is because the high tissue glucose concentrations can provide a substrate for glycolysis and allow production of ATP for longer periods to fuel membrane pumps in the absence of blood flow. Furthermore, acidosis generally inhibits ion fluxes through cellular membrane channels [27] thereby reducing the energy requirement for maintaining ion gradients across the plasma membrane. As expected, by reducing plasma glucose levels to about 2 mM the AD time is shortened [28]. Interestingly, excessive hypercapnia induced in normoglycemic animals before the ischemic insult also prolongs the AD time [17,29]. This would suggest that intra-ischemic acidosis has a major role in altering the time between the onset of ischemia and AD since the plasma glucose levels were not elevated in hypercapnic subjects. However, animals with superimposed hypercapnia (when the pH_e is already reduced

to 6.5) that are also subjected to ischemia undergo a further decrease in pH_e during ischemia to about pH 6.3 [29]. Therefore the aggravated acidosis at the onset of the ischemic insult is probably dramatically reducing the energy demand for ion transports in hypercapnic animals and leads to prolonged AD time when compared to normoglycemic normocapnic ones.

3.2. Calcium fluxes during anoxic depolarization

During anoxic depolarization cells take up more than 90% of the calcium from extracellular fluids [17,30]. The pre-ischemic level of Ca_e^{2+} is 1.2 mM and is reduced after anoxic depolarization to approximately 0.1 mM. In addition, the extracellular fluid space decreases to approximately 50% of control. Very likely, calcium influx into cells occurs by multiple pathways with the NMDA receptor-gated channels playing a dominant role. The second pathway for calcium entry into the cells is the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger since blockers of voltage-sensitive calcium channels seem to have little effect on ischemic calcium fluxes [31]. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger is an electrogenic pump working with

a reported stoichiometry of 3Na^+ that are moved inwards for each calcium transported outwards (for review, see [32]). The source of energy for the Na^+ -coupled Ca^{2+} movement is the electrochemical Na^+ gradient. At the time of anoxic depolarization the sodium gradient collapses and the electrochemical gradient of the calcium ions reverses the pump causing massive loading of cells with calcium [33]. The contribution of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to ischemic tissue injury was observed particularly in white matter where MK-801 (a noncompetitive NMDA receptor antagonist), did not prevent glutamate-induced damage [34,35].

3.3. Effect of pH on calcium fluxes related to AD

Apart from the effect on AD time, tissue glucose levels also affect the calcium fluxes during ischemia. When ischemia is induced at increased plasma glucose concentrations, the rate of fall in Ca_e^{2+} is reduced. In hyperglycemic subjects cellular Ca^{2+} uptake occurs in two phases, the initial phase represents a decrease in Ca_e^{2+} from normal 1.2 to ~ 0.4 mM followed by secondary phase which is characterized by a very gradual influx of calcium (see Fig. 4).

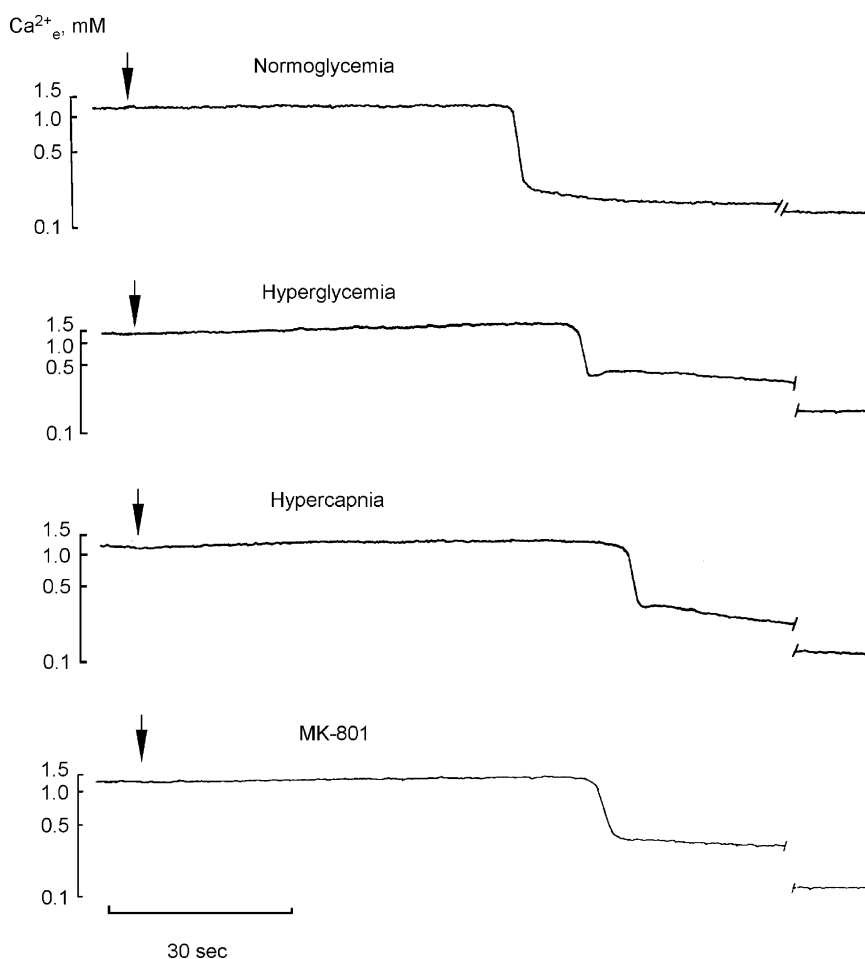


Fig. 4. Changes in extracellular calcium concentration (Ca_e^{2+}) in the rat cortex in complete ischemia during control (normoglycemic and normocapnic) conditions, in conditions of exaggerated acidosis (hyperglycemia and hypercapnia), and in animals given MK-801. Data were taken from [17].

The initial rate of Ca_e^{2+} decrease is directly proportional to tissue glucose concentrations. Since a comparable delay in calcium influx is observed when tissue pH during ischemia is additionally reduced, not by pre-ischemic hyperglycemia, but by excessive hypercapnia, and since a similar delay is seen when normoglycemic, normocapnic animals are given the NMDA antagonist MK-801, it seems clear that the delay in calcium uptake in hyperglycemic animals is due to the additional fall in pH_e , also that the effect is largely extended on the NMDA receptor-gated ion channels [17].

Pre-ischemic hyperglycemia, or extreme hypercapnia aggravates ischemic brain damage, involving pan-necrotic lesions in which glial cells and vascular epithelium are also affected (for review, see [36]). Since aggravated acidosis reduces the rate of calcium uptake by NMDA-gated channels it is very likely that most of the calcium will enter the cells via the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. This can lead to significant increase in the amount of calcium accumulated by glia after AD since neurons take up calcium at reduced rates. Therefore, the adverse effect of excessive intra-ischemic acidosis on glial survival can be, at least in part, due to more extensive calcium accumulation by these cells. However, MK-801 has a similar effect on ischemic calcium fluxes to aggravated acidosis (Fig. 4, see also [10,30]), yet the results show that MK-801 does not ameliorate nor aggravate damage due to global ischemia [37,38]. Most probably, the combination of increased calcium accumulation by glia and exaggerated acidosis leads to massive damage of these cells. Although exaggerated acidosis aggravates ischemic brain damage, in hyperglycemic subjects the adverse effect of glucose per se cannot be excluded [39,40].

4. Bioenergetic and calcium metabolism during recovery

If ischemia is followed by adequate reperfusion following a lag period of 1–2 min, the ion gradients at the plasma membrane are gradually normalized suggesting a recovery of cellular energy metabolism.

At the end of 10–15 min of global ischemia high-energy phosphate levels are markedly reduced and, as could be expected, tissue glucose and glycogen pools are depleted, and lactate is increased (see [41]).

There are also marked changes in citric acid cycle metabolites. Thus, the tissue is depleted of α -ketoglutarate and oxaloacetate, there is significant decrease in citrate and malate, a moderate fall in fumarate and a substantial increase in succinate. For example after 5 min of complete ischemia succinate concentrations rise to 270% of normal [42]. Ischemia further leads to highly significant increase in GABA, alanine and ammonia [43].

Fifteen minutes after blood flow is restored there is a marked decrease in glutamate, aspartate and ammonia, increase in glutamine, asparagine and in GABA [42]. The ma-

ior part of the ammonia is probably detoxified by the amidation of glutamate in astrocytes by the ATP-requiring enzyme glutamine synthetase, a conclusion that is supported by the elevated levels of glutamine, and the decreased concentration of glutamate.

The high-energy phosphate levels recover appreciably already after 5 min of reperfusion [41]. In the rat 1 min after cerebral circulation is restored cellular ion gradient starts to be re-instituted, at which time the brain ATP concentration is 0.9 mM, and further increases to 1.7 mM following 5 min of reperfusion. Similarly, PCr levels rise from 0.2 mM at the end of ischemia to 3.6 mM at the end of 5 min reperfusion period [41]. At 15 min of recovery there is a further increase in PCr and ATP levels the latter reaching about 80% of normal values [44]. Interestingly, during this period, lactate levels are still more than 200% of normal [41,44]. Thus, the lactate/pyruvate ratio is significantly elevated at 15 min of recovery. This suggests that the pyruvate utilization by mitochondria is inhibited during early reperfusion. Indeed, pyruvate dehydrogenase complex (PDH) inactivation after transient global ischemia has been reported by several groups [45–49]. Cardell and coworkers [44] reported an inhibition of the PDH complex activity at 15 min after the start of reperfusion. The inactivation of PDH is most probably confined to the E1 subunit of the PDH complex since the activity of the other two subunits (E2 and E3) were unchanged following the ischemic insult [49]. Furthermore, data showing that acetyl-L-carnitine (ALCAR) administration can significantly improve the recovery of the bioenergetic state of post-ischemic tissue also suggest that PDH activity is inhibited, since ALCAR most probably bypasses the PDH and serves as an exogenous donor of oxidizable acetyl groups that enter aerobic metabolism at a point just distal to the impaired PDH reaction [50,51].

Taking all the above data into consideration, it is not clear what serves as an oxidizable substrate for mitochondria during the first minutes of reperfusion. Since the PDH complex is inactivated the utilization of pyruvate by mitochondria for oxidative phosphorylation is impaired. One possibility is that the accumulated succinate during the ischemic insult (see above) serves as temporary source of substrate for complex II in mitochondria. This is because the levels of other intermediates of the TCA cycle are subnormal (see above). However, succinate supported respiration leads to higher rates of mitochondrial free radical production due to back-leak of electrons from complex II to complex I [52–54] and can cause oxidative damage to mitochondrial proteins which is observed after transient ischemia [47,55–57].

4.1. Lactate as an energy source for post-ischemic tissue

It is known that lactate can serve as an energy source since it can be converted in the brain to pyruvate by the lactate dehydrogenase isoenzyme LDH5 [58,59], which, in the brain is found characteristically in neurons [60]. This led to the conclusion that high levels of lactate after ischemia

are beneficial for the recovery of brain energy metabolism [61]. Lactate could support mitochondrial respiration after the ischemic insult by being converted to pyruvate, however, pyruvate utilization by mitochondria requires activity of the PDH complex. Since PDH can be inactivated during early reperfusion and the lactate/pyruvate ratio remains elevated during the first 15 min of recovery it is difficult to envisage how lactate can significantly support the cellular bioenergetic recovery following ischemic insult (see also [62,63]).

5. Mitochondrial dysfunction and calcium metabolism following transient ischemia

Following ischemia and reperfusion, brain energy levels/bioenergetic metabolism may appear to recover. However, after a period of several hours or days there may be a secondary decrease in tissue bioenergetic potential. This phenomenon has been termed “secondary energy failure” and is accompanied by delayed mitochondrial dysfunction and tissue calcium accumulation leading to cell death (for reviews, see [13,64,65]). Thus, transient cerebral ischemia is followed by a gradual rise in Ca_i^{2+} [21], by delayed calcium sequestration in mitochondria [66,67], and by delayed mitochondrial respiratory dysfunction [68]. There have been attempts to explain the mitochondrial respiratory dysfunction by the peroxidation of lipid components of mitochondrial membranes or by activation of phospholipase A2 that secondarily affects the membrane-embedded respiratory complexes [69,70]. Another hypothesis is that direct oxidation of proteins in these complexes by free radicals inhibits mitochondrial respiration [45,47,56], and in particular causes a blood flow dependant and reversible inhibition of complexes I, II, III and V activities and a delayed “secondary” irreversible decrease in complex IV activity [47,57]. Data showing a translocation of cytochrome *c* (cyt *c*) from mitochondria to the cytosol during the reperfusion period also suggests damage to mitochondrial membranes or a pathological alteration in membrane permeability [71–73]. Thus, it has become increasingly clear that during reperfusion following cerebral ischemia, a secondary (delayed) mitochondrial failure occurs that compromises cellular energy metabolism.

One of the mechanisms that could cause mitochondrial damage following an ischemic insult is the opening of mitochondrial permeability transition (MPT) pore (for reviews, see [13,65,74,75]; for heart ischemia, see [76]).

5.1. Mitochondrial permeability transition (MPT) and its regulation during ischemia/recovery

Studies on isolated mitochondria have revealed that when mitochondria accumulate a large amount of calcium, and/or when they are exposed to oxidative stress, a large conductance pore in the mitochondrial inner membrane is opened. The opening of this MPT pore leads to collapse of the mitochondrial membrane potential and dissipation of proton

and ion gradients [77–79], resulting in the uncoupling of oxidative phosphorylation and the cessation of mitochondrial ATP synthesis. The MPT pore is a mega-channel regulated by multiple effectors [79–82]. In addition to high intramitochondrial calcium concentration and exposure of mitochondria to oxidizing agents, high levels of inorganic phosphate (P_i), depletion of mitochondrial glutathione and alkaline pH also favor MPT pore opening. Alternatively, adenine nucleotides (particularly ATP and ADP), magnesium ions, and low pH will decrease the probability of the MPT pore opening.

The MPT is inhibited by CsA, most likely due to its binding to cyclophilin D (cyc D), a matrix peptidyl-prolyl *cis*, *trans*-isomerase, which is the endogenous MPT modulator.

5.2. Detection of MPT in vivo

Since MPT pore formation and the regulation of its opening is complex, and since many effectors could potentially affect the pore synergistically in vivo, one cannot readily predict when and under what conditions the MPT is induced in intact tissue. Moreover, recent studies have suggested that the MPT activity of brain mitochondria is regulated differently than that isolated from liver or heart [83–85]. Although MPT pore opening is readily studied in vitro, methods that allow accurate, quantifiable, time-dependant detection and study of the MPT in vivo have yet to be established.

An early study showing mitochondrial membrane damage in post-ischemic brain, was reported by Ouyang et al. [86]. These authors detected a leak of the mitochondrial isoform of aspartate aminotransferase from mitochondria to cytosol in non-synaptic brain mitochondria isolated from rats during first hour of reperfusion. Additional, indirect evidence that the MPT contributes to the events leading to ischemic cell death is the observation that the MPT inhibitor CsA can dramatically ameliorate brain damage due to ischemic insult when it is allowed to pass the blood–brain barrier [87,88]. The protective effect of CsA was later shown also in brain damage induced by hypoglycemic coma [89], focal ischemia [90,91] and trauma [92]. Furthermore, it has been shown that there is a significant decrease in heart mitochondrial NAD^+ content after an ischemic insult [93]. In addition, an uptake of glutathione by brain mitochondria following cerebral ischemia was reported [94,95]. These results were interpreted as indirect evidence of the MPT because the robust increase in permeability of the mitochondrial inner membrane allowed diffusion of NAD^+ and glutathione between the cytosol and the mitochondrial matrix, and since the observed post-ischemic changes were reversed by CsA pretreatment.

Another attempt to detect MPT in vivo was reported by Griffith and Halestrap [96]. Radioactive deoxyglucose (DOG) was used as an intracellular marker molecule whose entry into the mitochondria can only occur when the MPT pore is opened. Rat hearts were perfused with medium containing DOG prior to ischemia, and the presence of DOG in mitochondria isolated from post-ischemic heart tissue was

examined. An increased entrapment of DOG in mitochondria suggested that a transient opening of the MPT pore during reperfusion had occurred.

5.3. Early reperfusion and the MPT

During the first minutes of reperfusion, mitochondria are exposed to high concentrations of calcium and P_i and to lower than normal pH. Since previous studies have demonstrated that low pH inhibits the MPT, it was assumed that brain lactic acidosis during the early reperfusion period protects the mitochondria from the MPT. However, it has been demonstrated that acidic pH actually stimulates calcium-induced MPT in non-synaptic brain mitochondria, when tested under energized conditions [97]. Thus, during the early reperfusion period mitochondria are exposed to several conditions that should favor opening of the MPT pore, including high cytosolic calcium and phosphate levels and low pH. When sufficient O_2 and substrate are delivered to the reperfused tissue, resumption of respiration reestablishes the mitochondrial membrane potential that drives calcium uptake by mitochondria. Therefore, calcium overload will predictably lead to activation of the MPT *in vivo*.

5.4. Calcium-induced MPT *in vitro* and mitochondrial calcium accumulation *in vivo*

As discussed above, ischemia leads to translocation of calcium ions from extracellular fluids into cells. The ion movements across cell membranes *in vivo* are, at the time of AD, restricted to intra- and extracellular fluids. This is because the blood–brain barrier (BBB) tightly controls ion exchange between blood and extracellular fluids and is intact for many hours, even following a transient ischemic insult [98]. Thus, although any efflux or influx of ions markedly influences the extracellular and intracellular calcium concentrations, the total tissue concentration is unaltered by a transient ischemic episode for hours or days [99,100].

Extracellular fluids occupy about 20% of tissue volume *in vivo* and have a Ca_c^{2+} of about 1.2 mM. In complete or dense ischemia, virtually all of this extracellular calcium enters brain cells. This means that the Ca_i^{2+} should increase to about 240 μ M. If calcium preferentially enters neurons (via NMDA-gated channels) the increase may be at least twice as high. However, the Ca_i^{2+} measured experimentally rises during ischemia from about 0.1 to 30–60 μ M [30]. The reason for this relatively small (compared to the theoretical maximum) increase is that the calcium entering the cell is partly bound or sequestered within the cytosol. However, the uptake of cytosolic calcium by mitochondria at the time of AD is, at least partly, compromised since mitochondria are probably depolarized due to lack of oxygen. This conclusion is supported by data showing only a moderate rise of mitochondrial calcium content at the end of ischemic period [67] (see Fig. 5). However, during the first minutes of reperfusion when mitochondria are re-energized and regain their

membrane potential, calcium accumulation by mitochondria is much more pronounced [67] (Fig. 5). The mitochondrial calcium content increases from a pre-ischemic level of 3 to ~15 nmol $Ca\ mg^{-1}$ protein at 10 min of reperfusion.

This increase in mitochondrial calcium content is much smaller than one can observe *in vitro*. For example if brain mitochondria are incubated in potassium based buffer containing NADH-linked substrates and phosphate the calcium uptake capacity is about 180 nmol $Ca\ mg^{-1}$ protein [85]. In the presence of physiological levels of ATP, the mitochondrial capacity to retain calcium increases to over 1000 nmol $Ca\ mg^{-1}$ protein [84,101,102]. The presence of high levels of ADP also increases brain mitochondrial calcium uptake capacity, however to less extent, about 800 nmol $Ca\ mg^{-1}$ protein [101,103].

The moderate rise of total mitochondrial calcium content following ischemia *in vivo* suggests that the available pools of calcium are limited (see above). Furthermore, the calcium uptake capacity of mitochondria is reduced by acidic conditions or high phosphate concentrations [97,103], which are conditions both present during early reperfusion.

Since the highest levels of calcium accumulation capacity were determined in the presence of physiological concentrations of ATP, magnesium, and phosphate and at pH 7.0, much of the reported data cannot be readily interpolated to ischemic or post-ischemic tissue when the ATP levels do not fully recover, the pH is acidic and the phosphate concentration is elevated. Furthermore, the direct comparison of *in vitro* and *in vivo* data requires that the amount of calcium taken up by cells during ischemia is expressed per mass of mitochondria within the calcium accumulating cells.

As mentioned above at the time of AD intracellular calcium concentration theoretically can rise up to 240 μ M or even to 480 μ M if only neurons preferentially accumulate the calcium from extracellular fluids. Yet, if all this calcium were accumulated only by mitochondria the mitochondrial calcium load would be between 3 and 16 nmol $Ca\ mg^{-1}$ of mitochondrial proteins. This is because mitochondria represents 3–8% of cell volume in brain [104] and since a mitochondrial matrix volume of 1 μ l corresponds to about 1 mg of mitochondrial proteins [103]. This is a relatively small amount of calcium, which will under physiological conditions induce the MPT pore opening only in a small fraction of the whole mitochondrial population that is particularly sensitive to calcium-induced damage [85,101]. The MPT triggering probability however will be higher during conditions of immediate reperfusion following transient ischemia because of intracellular acidosis, high phosphate concentration and low levels of ATP. However, the significance of possible damage to a sub-population of mitochondria during the immediate recirculation for cell survival remains to be determined.

The situation is different at the time of delayed mitochondrial failure when there is a progressive secondary accumulation of calcium in cells [21,99] and mitochondria [66,67] (see Fig. 5). The increase in tissue calcium content

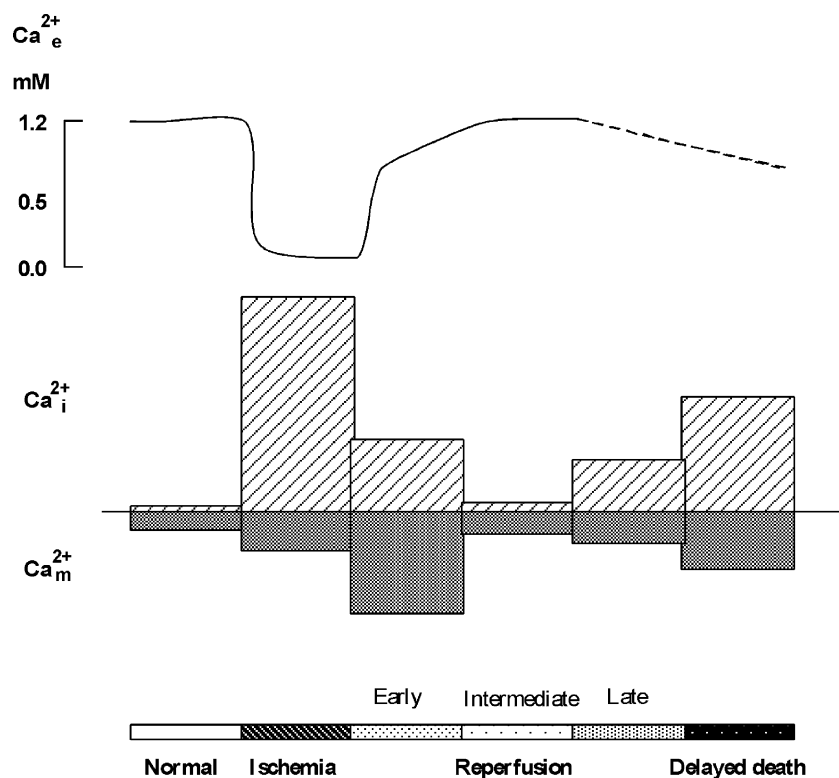


Fig. 5. Schematic illustration of changes in extracellular calcium (Ca_e^{2+}), intracellular calcium (Ca_i^{2+}), and in mitochondrial calcium (Ca_m^{2+}) during global ischemia and reperfusion. Data were taken from [74].

after prolonged recovery suggest that even slow delivery of calcium from blood to post-ischemic cells during several hours or days can lead to massive calcium overload of post-ischemic tissues. This can lead to large amounts of cellular/mitochondrial calcium accumulation, which ultimately will cause mitochondrial dysfunction and bioenergetic failure compromising cellular function and survival. The mechanisms of this delayed post-ischemic calcium perturbation are not fully understood. A similar delayed calcium dysregulation (DCD) was observed *in vitro* in neurons during prolonged glutamate exposure [105], for review see [106,107]. Although the pathways of calcium entry have yet not been identified mitochondrial calcium metabolism seems to play a significant role in the mechanisms leading to DCD [107].

6. Apoptotic proteins and mitochondrial dysfunction during post-ischemic period

The finding that cytochrome *c* (cyt *c*) is released from mitochondria together with apoptosis inducing factor (AIF) and procaspase-9 and that caspase-3, an executioner of apoptotic cell death, is activated following ischemia suggested that apoptotic mechanisms are triggered by ischemic insult [108–112]. Although the mechanisms causing cyt *c* release from mitochondria are not well understood there are at least two possibilities discussed in the literature. One is

the mechanical damage of outer mitochondrial membrane due to MPT induced swelling. This usually leads to permanent damage to both inner and outer mitochondrial membrane and the affected mitochondria are becoming dysfunctional. The other mechanism of cyt *c* release is based on the observations that translocation of the pro-apoptotic protein Bax into mitochondrial membranes can result in changes of the outer membrane permeability and cyt *c* release (for review, see [113]). The interactions of anti-apoptotic proteins alike Bcl-2/Bcl-x_L at the mitochondrial membrane interfere with this process. Interestingly there are reports showing that Bax can initiate the release of cyt *c* also through an interaction with the MPT pore [114–117] or in the absence of MPT (thus in the absence of mitochondrial swelling) leaving the intramitochondrial structures intact (see [113]). Thus, the complex interaction of anti- and pro-apoptotic proteins at the mitochondrial membrane can regulate also the MPT [118,119] therefore suggesting that the MPT can be an integral mechanism of apoptosis [120].

Studies *in vitro* show that moderate excitotoxic insults may activate mechanisms of apoptosis, whereas dense insults are prone to cause necrosis [121]. This is probably related to the observation that caspase-dependent cell death utilizes energy [122]. Therefore cells with most of the mitochondria being irreversibly damaged will become necrotic and cells with partially compromised mitochondria will be prone to undergo apoptosis. Typical injury manifesting both moderate and dense insult is focal ischemia. Focal ischemia

can be permanent or transient lasting 30–90 min and is characterized by two ischemic regions [24]. The infarct core (or ischemic focus) which represents a brain tissue with dense ischemia where most of the cells are subjected to conditions that lead to energy depletion, complete loss of ion homeostasis with calcium accumulation [123,124] and necrosis. The ischemic core is surrounded by penumbral regions where the ischemia is less severe and the cells within these tissues can be rescued by appropriate treatment [24]. Interestingly, cells in the penumbra exhibited morphological changes similar to apoptosis [108], which is consistent with their ability to maintain ATP levels high enough to allow apoptosis to proceed. Another example of dense ischemia though with shorter duration (10–15 min) is global ischemia. Although there are reports suggesting involvement of apoptotic mechanisms in cell death following global ischemia, interestingly this type of cell injury has the morphological characteristics of necrosis [125–127] and the classical apoptotic DNA fragmentation has not been consistently observed in these tissues [128,129]. Therefore, it seems that after global ischemia, what appears to be necrosis (as defined pathologically) could be the end result of some type of apoptotic program. Thus, this type of ischemia shows a unique form of cell death within the brain where both apoptotic and necrotic features can be recognized.

7. Conclusion

Most of the biochemical pathways identified following cerebral ischemia and reperfusion involve mitochondria either as primary targets and/or regulators of cell death mechanisms. The extent of mitochondrial dysfunction caused by ischemia primarily affects the cellular energy metabolism, yet also probably determines which particular molecular mechanism is going to play a significant role in the ensuing cell death processes and which will become an epiphenomenon of the death process itself. There are however, many fundamental unresolved issues concerning the involvement of mitochondrial energy metabolism in the mechanisms of ischemic brain damage.

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References

- [1] I.A. Silver, M. Erecinska, Oxygen and ion concentrations in normoxic and hypoxic brain cells, in: A.G. Hudetz, D.F. Brulye (Eds.), *Oxygen Transport to Tissue XX*, New York, Plenum Press, 1998, pp. 7–15.
- [2] M. Erecinska, I.A. Silver, ATP and brain function, *J. Cereb. Blood Flow Metab.* 9 (1989) 2–19.
- [3] B. Ljunggren, R.A. Ratcheson, B.K. Siesjo, Cerebral metabolic state following complete compression ischemia, *Brain Res.* 73 (1974) 291–307.
- [4] T.E. Duffy, S.R. Nelson, O.H. Lowry, Cerebral carbohydrate metabolism during acute hypoxia and recovery, *J. Neurochem.* 19 (2003) 959–977.
- [5] J. Folbergrova, H. Minamisawa, A. Ekholm, B.K. Siesjo, Phosphorylase alpha and labile metabolites during anoxia: correlation to membrane fluxes of K^+ and Ca^{2+} , *J. Neurochem.* 55 (1990) 1690–1696.
- [6] A. Ekholm, B. Asplund, B.K. Siesjo, Perturbation of cellular energy state in complete ischemia: relationship to dissipative ion fluxes, *Exp. Brain Res.* 90 (1992) 47–53.
- [7] J.V. Passanneau, O.H. Lowry, The role of phosphofructokinase in metabolic regulation, *Adv. Enzym. Regul.* 2 (1964) 265–274.
- [8] B.K. Siesjo, *Brain Energy Metabolism*, John Wiley & Sons, New York, 1978.
- [9] M. Erecinska, J. Deas, I.A. Silver, The effect of pH on glycolysis and phosphofructokinase activity in cultured cells and synaptosomes, *J. Neurochem.* 65 (1995) 2765–2772.
- [10] M. Erecinska, I.A. Silver, Ions and energy in mammalian brain, *Prog. Neurobiol.* 43 (1994) 37–71.
- [11] C. Nicholson, Dynamics of the brain cell microenvironment, *Neurosci. Res. Program Bull.* 18 (1980) 175–322.
- [12] A.J. Hansen, Effect of anoxia on ion distribution in the brain, *Physiol. Rev.* 65 (1985) 101–148.
- [13] T. Kristián, B.K. Siesjo, Calcium in ischemic cell death, *Stroke* 29 (1998) 705–718.
- [14] O.H. Lowry, J.V. Passanneau, D.W. Schulz, Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain, *J. Biol. Chem.* 239 (2003) 18–30.
- [15] B. Nilsson, K. Norber, C.H. Nordstrom, B.K. Siesjo, Rate of energy utilization in the cerebral cortex of rats, *Acta Physiol. Scand.* 93 (1975) 569–571.
- [16] R.J. Harris, L. Symon, Extracellular pH, potassium, and calcium activities in progressive ischaemia of rat cortex, *J. Cereb. Blood Flow Metab.* 4 (1984) 178–186.
- [17] T. Kristián, K. Katsura, G. Gido, B.K. Siesjo, The influence of pH on cellular calcium influx during ischemia, *Brain Res.* 641 (1994) 295–302.
- [18] W.A. Mutch, A.J. Hansen, Extracellular pH changes during spreading depression and cerebral ischemia: mechanisms of brain pH regulation, *J. Cereb. Blood Flow Metab.* 4 (1984) 17–27.
- [19] M.L. Smith, R. von Hanwehr, B.K. Siesjo, Changes in extra- and intracellular pH in the brain during and following ischemia in hyperglycemic and in moderately hypoglycemic rats, *J. Cereb. Blood Flow Metab.* 6 (1986) 574–583.
- [20] K. Katsura, A. Asplund, A. Ekholm, B.K. Siesjo, Extra- and intracellular pH in the brain during ischaemia, related to tissue lactate content in normo- and hypercapnic rats, *Eur. J. Neurosci.* 4 (1992) 166–176.
- [21] I.A. Silver, M. Erecinska, Ion homeostasis in rat brain in vivo: intra- and extracellular $[Ca^{2+}]$ and $[H^+]$ in the hippocampus during recovery from short-term, transient ischemia, *J. Cereb. Blood Flow Metab.* 12 (1992) 759–772.
- [22] B. Ljunggren, H. Schutz, B.K. Siesjo, Changes in energy state and acid–base parameters of the rat brain during complete compression ischemia, *Brain Res.* 73 (1974) 277–289.
- [23] T. Kristián, B.K. Siesjo, Changes in ionic fluxes during cerebral ischaemia, *Int. Rev. Neurobiol.* 40 (1997) 27–45.
- [24] B.K. Siesjo, Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment, *J. Neurosurg.* 77 (1992) 337–354.

- [25] A. Ekholm, T. Kristián, B.K. Siesjo, Influence of hyperglycemia and of hypercapnia on cellular calcium transients during reversible brain ischemia, *Exp. Brain Res.* 104 (1995) 462–466.
- [26] M. Erecinska, I.A. Silver, Calcium handling by hippocampal neurons under physiologic and pathologic conditions, *Adv. Neurol.* 71 (1996) 119–136.
- [27] W. Moody, Effects of intracellular H^+ on the electrical properties of excitable cells, *Annu. Rev. Neurosci.* 7 (1984) 257–278.
- [28] P.A. Li, T. Kristián, K. Katsura, M. Shamloo, B.K. Siesjo, The influence of insulin-induced hypoglycemia on the calcium transients accompanying reversible forebrain ischemia in the rat, *Exp. Brain Res.* 105 (1995) 363–369.
- [29] K. Katsura, T. Kristián, M.L. Smith, B.K. Siesjo, Acidosis induced by hypercapnia exaggerates ischemic brain damage, *J. Cereb. Blood Flow Metab.* 14 (1994) 243–250.
- [30] I.A. Silver, M. Erecinska, Intracellular and extracellular changes of $[Ca^{2+}]$ in hypoxia and ischemia in rat brain in vivo, *J. Gen. Physiol.* 95 (1990) 837–866.
- [31] Y. Xie, E. Zacharias, P. Hoff, F. Tegtmeier, Ion channel involvement in anoxic depolarization induced by cardiac arrest in rat brain, *J. Cereb. Blood Flow Metab.* 15 (1995) 587–594.
- [32] M.P. Blaustein, W.J. Lederer, Sodium/calcium exchange: its physiological implications, *Physiol. Rev.* 79 (1999) 763–854.
- [33] P.K. Stys, R.M. LoPachin, Mechanisms of calcium and sodium fluxes in anoxic myelinated central nervous system axons, *Neuroscience* 82 (1998) 21–32.
- [34] S. Li, Q. Jiang, P.K. Stys, Important role of reverse Na^+ – Ca^{2+} exchange in spinal cord white matter injury at physiological temperature, *J. Neurophysiol.* 84 (2000) 1116–1119.
- [35] S. Li, P.K. Stys, Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter, *J. Neurosci.* 20 (2000) 1190–1198.
- [36] B.K. Siesjo, K. Katsura, T. Kristián, Acidosis-related damage, *Adv. Neurol.* 71 (1996) 209–233.
- [37] N.H. Diemer, F.F. Johansen, M.B. Jorgensen, *N*-Methyl-D-aspartate and non-*N*-methyl-D-aspartate antagonists in global cerebral ischemia, *Stroke* 21 (1990) III39–III42.
- [38] A. Buchan, H. Li, W.A. Pulsinelli, The *N*-methyl-D-aspartate antagonist, MK-801, fails to protect against neuronal damage caused by transient, severe forebrain ischemia in adult rats, *J. Neurosci.* 11 (1991) 1049–1056.
- [39] K.H. Moley, M.M. Chi, C.M. Knudson, S.J. Korsmeyer, M.M. Mueckler, Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways, *Nat. Med.* 4 (1998) 1421–1424.
- [40] A.L. Keim, M.M. Chi, K.H. Moley, Hyperglycemia-induced apoptotic cell death in the mouse blastocyst is dependent on expression of p53, *Mol. Reprod. Dev.* 60 (2001) 214–224.
- [41] A. Ekholm, K. Katsura, T. Kristián, M. Liu, J. Folbergrova, B.K. Siesjo, Coupling of cellular energy state and ion homeostasis during recovery following brain ischemia, *Brain Res.* 604 (1993) 185–191.
- [42] J. Folbergrova, B. Ljunggren, K. Norberg, B.K. Siesjo, Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex, *Brain Res.* 80 (1974) 265–279.
- [43] R.A. Lovell, S.J. Elliott, K.A. Elliott, The gamma-aminobutyric acid and factor i content of brain, *J. Neurochem.* 10 (1963) 479–488.
- [44] M. Cardell, T. Koide, T. Wieloch, Pyruvate dehydrogenase activity in the rat cerebral cortex following cerebral ischemia, *J. Cereb. Blood Flow Metab.* 9 (1989) 350–357.
- [45] K.R. Wagner, M. Kleinholz, R.E. Myers, Delayed decreases in specific brain mitochondrial electron transfer complex activities and cytochrome concentrations following anoxia/ischemia, *J. Neurol. Sci.* 100 (1990) 142–151.
- [46] Y.E. Bogaert, R.E. Rosenthal, G. Fiskum, Postischemic inhibition of cerebral cortex pyruvate dehydrogenase, *Free Radic. Biol. Med.* 16 (1994) 811–820.
- [47] A. Almeida, K.L. Allen, T.E. Bates, J.B. Clark, Effect of reperfusion following cerebral ischaemia on the activity of the mitochondrial respiratory chain in the gerbil brain, *J. Neurochem.* 65 (1995) 1698–1703.
- [48] E. Zaidan, N.R. Sims, Selective reductions in the activity of the pyruvate dehydrogenase complex in mitochondria isolated from brain subregions following forebrain ischemia in rats, *J. Cereb. Blood Flow Metab.* 13 (1993) 98–104.
- [49] E. Zaidan, K.F. Sheu, N.R. Sims, The pyruvate dehydrogenase complex is partially inactivated during early recirculation following short-term forebrain ischemia in rats, *J. Neurochem.* 70 (1998) 233–241.
- [50] M. Miljkovic-Lolic, G. Fiskum, R.E. Rosenthal, Neuroprotective effects of acetyl-L-carnitine after stroke in rats, *Ann. Emerg. Med.* 29 (1997) 758–765.
- [51] R.E. Rosenthal, R. Williams, Y.E. Bogaert, P.R. Getson, G. Fiskum, Prevention of postischemic canine neurological injury through potentiation of brain energy metabolism by acetyl-L-carnitine, *Stroke* 23 (1992) 1312–1317.
- [52] J.F. Turrens, Superoxide production by the mitochondrial respiratory chain, *Biosci. Rep.* 17 (1997) 3–8.
- [53] T.E. Bates, S.J. Heales, S.E. Davies, P. Boakye, J.B. Clark, Effects of 1-methyl-4-phenylpyridinium on isolated rat brain mitochondria: evidence for a primary involvement of energy depletion, *J. Neurochem.* 63 (1994) 640–648.
- [54] A.A. Starkov, B.M. Polster, G. Fiskum, Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax, *J. Neurochem.* 83 (2002) 220–228.
- [55] K.R. Wagner, M. Kleinholz, R.E. Myers, Delayed onset of neurologic deterioration following anoxia/ischemia coincides with appearance of impaired brain mitochondrial respiration and decreased cytochrome oxidase activity, *J. Cereb. Blood Flow Metab.* 10 (1990) 417–423.
- [56] Y. Liu, R.E. Rosenthal, P. Starke-Reed, G. Fiskum, Inhibition of postcardiac arrest brain protein oxidation by acetyl-L-carnitine, *Free Radic. Biol. Med.* 15 (1993) 667–670.
- [57] K.L. Allen, A. Almeida, T.E. Bates, J.B. Clark, Changes of respiratory chain activity in mitochondrial and synaptosomal fractions isolated from the gerbil brain after graded ischaemia, *J. Neurochem.* 64 (1995) 2222–2229.
- [58] P.G. Bittar, Y. Charnay, L. Pellerin, C. Bouras, P.J. Magistretti, Selective distribution of lactate dehydrogenase isoenzymes in neurons and astrocytes of human brain, *J. Cereb. Blood Flow Metab.* 16 (1996) 1079–1089.
- [59] D. Smith, A. Pernet, W.A. Hallett, E. Bingham, P.K. Marsden, S.A. Amiel, Lactate: a preferred fuel for human brain metabolism in vivo, *J. Cereb. Blood Flow Metab.* 23 (2003) 658–664.
- [60] M. McKenna, J. O'Brien, K. Kla, I.B. Hopkins, Differential distribution of LDH isozymes in cortical synaptic terminals and primary cultures of cortical neurons, *J. Neurochem.* 74 (2000) S87.
- [61] A. Schurr, R.S. Payne, M.T. Tseng, J.J. Miller, B.M. Rigor, The glucose paradox in cerebral ischemia. New insights, *Ann. N.Y. Acad. Sci.* 893 (1999) 386–390.
- [62] P.A. Li, Q.P. He, K. Csizsar, B.K. Siesjo, Does long-term glucose infusion reduce brain damage after transient cerebral ischemia? *Brain Res.* 912 (2001) 203–205.
- [63] M.C. McKenna, J.T. Tildon, J.H. Stevenson, I.B. Hopkins, X. Huang, R. Couto, Lactate transport by cortical synaptosomes from adult rat brain: characterization of kinetics and inhibitor specificity, *Dev. Neurosci.* 20 (1998) 300–309.
- [64] B.K. Siesjo, E. Elmer, S. Janelidze, et al., Role and mechanisms of secondary mitochondrial failure, *Acta Neurochir. Suppl. (Wien)* 73 (1999) 7–13.
- [65] B.K. Siesjo, T. Kristián, F. Shibazaki, H. Uchino, The role of mitochondrial dysfunction in reperfusion damage in the brain, in: S. Klumpp, J. Kriegstein (Eds.), *Pharmacology of Cerebral Ischemia*, Springer-Verlag, New York, 2000, pp. 163–175.

- [66] E. Dux, G. Mies, K.A. Hossmann, L. Siklos, Calcium in the mitochondria following brief ischemia of gerbil brain, *Neurosci. Lett.* 78 (1987) 295–300.
- [67] E. Zaidan, N.R. Sims, The calcium content of mitochondria from brain subregions following short-term forebrain ischemia and recirculation in the rat, *J. Neurochem.* 63 (1994) 1812–1819.
- [68] N.R. Sims, W.A. Pulsinelli, Altered mitochondrial respiration in selectively vulnerable brain subregions following transient forebrain ischemia in the rat, *J. Neurochem.* 49 (1987) 1367–1374.
- [69] I. Nakahara, H. Kikuchi, W. Taki, et al., Changes in major phospholipids of mitochondria during postischemic reperfusion in rat brain, *J. Neurosurg.* 76 (1992) 244–250.
- [70] D.D. Gilboe, D. Kintner, J.H. Fitzpatrick, et al., Recovery of postischemic brain metabolism and function following treatment with a free radical scavenger and platelet-activating factor antagonists, *J. Neurochem.* 56 (1991) 311–319.
- [71] M. Fujimura, Y. Morita-Fujimura, K. Murakami, M. Kawase, P.H. Chan, Cytosolic redistribution of cytochrome *c* after transient focal cerebral ischemia in rats, *J. Cereb. Blood Flow Metab.* 18 (1998) 1239–1247.
- [72] M.A. Perez-Pinzo, G.P. Xu, J. Born, et al., Cytochrome *c* is released from mitochondria into the cytosol after cerebral anoxia or ischemia, *J. Cereb. Blood Flow Metab.* 19 (1999) 39–43.
- [73] T. Sugawara, M. Fujimura, Y. Morita-Fujimura, M. Kawase, P.H. Chan, Mitochondrial release of cytochrome *c* corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia, *J. Neurosci.* 19 (1999) RC39.
- [74] T. Kristián, B.K. Siesjo, Calcium-related damage in ischemia, *Life Sci.* 59 (1996) 357–367.
- [75] H. Friberg, T. Wieloch, Mitochondrial permeability transition in acute neurodegeneration, *Biochimie* 84 (2002) 241–250.
- [76] F. Di Lisa, M. Canton, R. Menabo, G. Dodoni, P. Bernardi, Mitochondria and reperfusion injury. The role of permeability transition, *Basic Res. Cardiol.* 98 (2003) 235–241.
- [77] P. Bernardi, K.M. Broekemeier, D.R. Pfeiffer, Recent progress on regulation of the mitochondrial permeability transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane, *J. Bioenerg. Biomembr.* 26 (1994) 509–517.
- [78] A.P. Halestrap, C.P. Connern, E.J. Griffiths, P.M. Kerr, Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury, *Mol. Cell Biochem.* 174 (1997) 167–172.
- [79] M. Zoratti, I. Szabo, The mitochondrial permeability transition, *Biochim. Biophys. Acta* 1241 (1995) 139–176.
- [80] T.E. Gunter, D.R. Pfeiffer, Mechanisms by which mitochondria transport calcium, *Am J. Physiol.* 258 (1990) C755–C786.
- [81] P. Bernardi, V. Petronilli, The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal, *J. Bioenerg. Biomembr.* 28 (1996) 131–138.
- [82] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, *Physiol. Rev.* 79 (1999) 1127–1155.
- [83] B.S. Kristal, J.M. Dubinsky, Mitochondrial permeability transition in the central nervous system: induction by calcium cycling-dependent and -independent pathways, *J. Neurochem.* 69 (1997) 524–538.
- [84] A. Andreyev, B. Fahy, G. Fiskum, Cytochrome *c* release from brain mitochondria is independent of the mitochondrial permeability transition, *FEBS Lett.* 439 (1998) 373–376.
- [85] T. Kristián, J. Gertsch, T.E. Bates, B.K. Siesjo, Characteristics of the calcium-triggered mitochondrial permeability transition in non-synaptic brain mitochondria: effect of cyclosporin A and ubiquinone O, *J. Neurochem.* 74 (2000) 1999–2009.
- [86] Y.B. Ouyang, S. Kuroda, T. Kristián, B.K. Siesjo, Release of mitochondrial aspartate aminotransferase (mAST) following transient focal ischemia suggests the opening of a mitochondrial permeability transition pore, *Neurosci. Res. Commun.* 20 (1997) 167–173.
- [87] H. Uchino, E. Elmer, K. Uchino, O. Lindvall, B.K. Siesjo, Cyclosporin A dramatically ameliorates CA1 hippocampal damage following transient forebrain ischaemia in the rat, *Acta Physiol. Scand.* 155 (1995) 469–471.
- [88] H. Uchino, E. Elmer, K. Uchino, et al., Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat, *Brain Res.* 812 (1998) 216–226.
- [89] H. Friberg, M. Ferrand-Drake, F. Bengtsson, A.P. Halestrap, T. Wieloch, A. Cyclosporin, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death, *J. Neurosci.* 18 (1998) 5151–5159.
- [90] T. Yoshimoto, B.K. Siesjo, Posttreatment with the immunosuppressant cyclosporin A in transient focal ischemia, *Brain Res.* 839 (1999) 283–291.
- [91] S. Matsumoto, 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) 736–741.
- [92] P.G. Sullivan, M. Thompson, S.W. Scheff, Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury, *Exp. Neurol.* 161 (2000) 631–637.
- [93] F. Di Lisa, R. Menabo, M. Canton, M. Barile, P. Bernardi, Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD⁺ and is a causative event in the death of myocytes in postischemic reperfusion of the heart, *J. Biol. Chem.* 276 (2001) 2571–2575.
- [94] E. Zaidan, N.R. Sims, Alterations in the glutathione content of mitochondria following short-term forebrain ischemia in rats, *Neurosci. Lett.* 218 (1996) 75–78.
- [95] E. Zaidan, M. Nilsson, N.R. Sims, Cyclosporin A-sensitive changes in mitochondrial glutathione are an early response to intrastriatal NMDA or forebrain ischemia in rats, *J. Neurochem.* 73 (1999) 2214–2217.
- [96] E.J. Griffiths, A.P. Halestrap, Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion, *Biochem. J.* 307 (Pt 1) (1995) 93–98.
- [97] T. Kristián, P. Bernardi, B.K. Siesjo, Acidosis promotes the permeability transition in energized mitochondria: implications for reperfusion injury, *J. Neurotrauma* 18 (2001) 1059–1074.
- [98] S. Ohta, G. Gido, B.K. Siesjo, Influence of ischemia on blood–brain and blood–CSF calcium transport, *J. Cereb. Blood Flow Metab.* 12 (1992) 525–528.
- [99] J.K. Deshpande, B.K. Siesjo, T. Wieloch, Calcium accumulation and neuronal damage in the rat hippocampus following cerebral ischemia, *J. Cereb. Blood Flow Metab.* 7 (1987) 89–95.
- [100] D.S. Warner, M.L. Smith, B.K. Siesjo, Ischemia in normo- and hyperglycemic rats: effects on brain water and electrolytes, *Stroke* 18 (1987) 464–471.
- [101] T. Kristián, T.M. Weatherby, T.E. Bates, G. Fiskum, Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria, *J. Neurochem.* 83 (2002) 1297–1308.
- [102] C. Chinopoulos, A.A. Starkov, G. Fiskum, Cyclosporin A-insensitive permeability transition in brain mitochondria: inhibition by 2-aminoethoxydiphenyl borate, *J. Biol. Chem.* 278 (2003) 27382–27389.
- [103] S. Chalmers, D.G. Nicholls, The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria, *J. Biol. Chem.* 278 (2003) 19062–19070.
- [104] J.J. Pysh, T. Khan, Variations in mitochondrial structure and content of neurons and neuroglia in rat brain: an electron microscopic study, *Brain Res.* 36 (1972) 1–18.
- [105] M. Tymianski, M.P. Charlton, P.L. Carlen, C.H. Tator, Secondary Ca²⁺ overload indicates early neuronal injury which precedes staining with viability indicators, *Brain Res.* 607 (1993) 319–323.
- [106] M. Tymianski, C.H. Tator, Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and

- ischemic central nervous system injury, *Neurosurgery* 38 (1996) 1176–1195.
- [107] D.G. Nicholls, S. Vesce, L. Kirk, S. Chalmers, Interactions between mitochondrial bioenergetics and cytoplasmic calcium in cultured cerebellar granule cells, *Cell Calcium* 34 (2003) 407–424.
- [108] P. Lipton, Ischemic cell death in brain neurons, *Physiol. Rev.* 79 (1999) 1431–1568.
- [109] U. Dirnagl, C. Iadecola, M.A. Moskowitz, Pathobiology of ischaemic stroke: an integrated view, *Trends Neurosci.* 22 (1999) 391–397.
- [110] G. Fiskum, Mitochondrial participation in ischemic and traumatic neural cell death, *J. Neurotrauma* 17 (2000) 843–855.
- [111] A.K. Liou, R.S. Clark, D.C. Henshall, X.M. Yin, J. Chen, To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways, *Prog. Neurobiol.* 69 (2003) 103–142.
- [112] E.H. Lo, T. Dalkara, M.A. Moskowitz, Mechanisms, challenges and opportunities in stroke, *Nat. Rev. Neurosci.* 4 (2003) 399–415.
- [113] Y. Tsujimoto, S. Shimizu, VDAC regulation by the Bcl-2 family of proteins, *Cell Death Differ.* 7 (2000) 1174–1181.
- [114] I. Marzo, C. Brenner, N. Zamzami, et al., The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins, *J. Exp. Med.* 187 (1998) 1261–1271.
- [115] J.G. Pastorino, S.T. Chen, M. Tafani, J.W. Snyder, J.L. Farber, The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition, *J. Biol. Chem.* 273 (1998) 7770–7775.
- [116] J.G. Pastorino, M. Tafani, R.J. Rothman, A. Marcineviciute, J.B. Hoek, J.L. Farber, Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore, *J. Biol. Chem.* 274 (1999) 31734–31739.
- [117] C. Brenner, H. Cadiou, H.L. Vieira, et al., Bcl-2 and Bax regulate the channel activity of the mitochondrial adenine nucleotide translocator (in process citation), *Oncogene* 19 (2000) 329–336.
- [118] X. Deng, L. Xiao, W. Lang, F. Gao, P. Ruvolo, W.S. May Jr., Novel role for JNK as a stress-activated Bcl2 kinase, *J. Biol. Chem.* 276 (2001) 23681–23688.
- [119] J. Zhong, J. Troppmair, U.R. Rapp, Independent control of cell survival by Raf-1 and Bcl-2 at the mitochondria, *Oncogene* 20 (2001) 4807–4816.
- [120] G. Kroemer, J.C. Reed, Mitochondrial control of cell death, *Nat. Med.* 6 (2000) 513–519.
- [121] P. Nicotera, S.A. Lipton, Excitotoxins in neuronal apoptosis and necrosis, *J. Cereb. Blood Flow Metab.* 19 (1999) 583–591.
- [122] P. Nicotera, Caspase requirement for neuronal apoptosis and neurodegeneration, *IUBMB Life* 49 (2000) 421–425.
- [123] G. Gido, T. Kristián, B.K. Siesjo, Extracellular potassium in a neocortical core area after transient focal ischemia, *Stroke* 28 (1997) 206–210.
- [124] T. Kristián, G. Gido, S. Kuroda, A. Schutz, B.K. Siesjo, Calcium metabolism of focal and penumbral tissues in rats subjected to transient middle cerebral artery occlusion, *Exp. Brain Res.* 120 (1998) 503–509.
- [125] J. Deshpande, K. Bergstedt, T. Linden, H. Kalimo, T. Wieloch, Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death, *Exp. Brain Res.* 88 (1992) 91–105.
- [126] F. Colbourne, G.R. Sutherland, R.N. Auer, Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia, *J. Neurosci.* 19 (1999) 4200–4210.
- [127] K.A. Hossmann, U. Oeschies, W. Schwandt, H. Krep, Electron microscopic investigation of rat brain after brief cardiac arrest, *Acta Neuropathol. (Berl.)* 101 (2001) 101–113.
- [128] J.P. MacManus, H. Fliss, E. Preston, I. Rasquinha, U. Tuor, Cerebral ischemia produces ladder DNA fragments distinct from cardiac ischemia and archetypal apoptosis, *J. Cereb. Blood Flow Metab.* 19 (1999) 502–510.
- [129] J.P. MacManus, A.M. Buchan, Apoptosis after experimental stroke: fact or fashion? *J. Neurotrauma* 17 (2000) 899–914.