Available online at www.sciencedirect.com
ScienceDirect
Pharmacology, Biochemistry and Behavior 87 (2007) 179–197
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia

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Received 4 October 2006; received in revised form 16 April 2007; accepted 19 April 2007
Available online 4 May 2007

Abstract

Ischemic stroke is a devastating disease with a complex pathophysiology. Animal modeling of ischemic stroke serves as an indispensable tool first to investigate mechanisms of ischemic cerebral injury, secondly to develop novel antiischemic regimens. Most of the stroke models are carried on rodents. Each model has its particular strengths and weaknesses. Mimicking all aspects of human stroke in one animal model is not possible since ischemic stroke is itself a very heterogeneous disorder. Experimental ischemic stroke models contribute to our understanding of the events occurring in ischemic and reperfused brain. Major approaches developed to treat acute ischemic stroke fall into two categories, thrombolysis and neuroprotection. Trials aimed to evaluate effectiveness of recombinant tissue-type plasminogen activator in longer time windows with finer selection of patients based on magnetic resonance imaging tools and trials of novel recanalization methods are ongoing. Despite the failure of most neuroprotective drugs during the last two decades, there are good chances to soon have effective neuroprotectives with the help of improved preclinical testing and clinical trial design. In this article, we focus on various rodent animal models, pathogenic mechanisms, and promising therapeutic approaches of ischemic stroke.

Keywords: Focal cerebral ischemia; Middle cerebral artery; Magnetic resonance imaging; Rat; Mouse; Experimental stroke model; Pathophysiology; Therapy

1. Introduction

Stroke is the second most common cause of death worldwide (Murray and Lopez, 1997) and 1/6 of all human beings will suffer at least one stroke in their lives (Seshadri et al., 2006). Furthermore, stroke is the leading cause of adult disability with approximately one third of patients who survive 6 months are dependent on others (Warlow, 1998). Because of its huge socioeconomic burden absorbing 6% of all health care budgets and with the fact that life expectancy increases globally one can assume that stroke is already, and will continue to be, the most challenging disease.

Ischemic stroke accounts for approximately 80% of all strokes and results from a thrombotic or embolic occlusion of a major cerebral artery (most often middle cerebral artery, MCA) or its branches. Clinical variability of stroke, mainly in terms of causes, duration, localization, and severity of ischemia and co-existing systemic diseases, raises the need for very large patient group sizes in clinical research to avoid confounding effects of the diversity. Experimental focal cerebral ischemia models have been developed to mimic human stroke and serve as an indispensable tool in the stroke research field. In an experimental stroke model, variables may be taken under strict control and researchers may address specific questions about either pathologic events occurring after ischemic stroke and how to develop novel stroke therapies.

The number and diversity of experimental focal ischemia models have increased over the recent decades and animal studies have provided most of our knowledge on pathophysiological mechanisms involved in focal cerebral ischemia. Consequences

Abbreviations: BBB, Blood-brain barrier; CBF, Cerebral blood flow; CVT, Cerebral venous thrombosis; DWI, Diffusion-weighted magnetic resonance imaging; MCA, Middle cerebral artery; MCAO, Middle cerebral artery occlusion; MRI, Magnetic resonance imaging; PWI, Perfusion-weighted magnetic resonance imaging; rt-PA, Recombinant tissue-type plasminogen activator; STAIR, Stroke Therapy Academic Industry Roundtable.

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doi:10.1016/j.pbb.2007.04.015

of blood flow reduction in a brain territory are complex that trigger a serial of multistep pathophysiologic events, the so-called ischemic cascade. Two major approaches have been developed to treat ischemic stroke: recanalization and neuroprotection. At present, alteplase, recombinant tissue-type plasminogen activator (rt-PA) is the only approved therapy for acute ischemic stroke (NINDS, 1995). Among more than 700 drugs which have been studied and found to be effective in animal stroke models, yet none has been proven efficacious on the basis of a positive phase III trial except a new free-radical tapering agent, NXY-059 (Kaste, 2005; Lees et al., 2006).

Large body of this review will deal with experimental ischemic stroke models. In the first part, we will briefly describe focal cerebral ischemia models while focusing on the two most relevant animal model to human stroke — embolic stroke model and intraluminal suture MCA occlusion (MCAO) model — we will also discuss strengths and weaknesses of each model and variability factors that may affect results of animal experiments. In the second part, the pathophysiology of ischemic stroke will be briefly discussed to point out its main mechanisms. Second part will also include therapeutic approaches to ischemic stroke and major compounds that have been developed to interfere with various steps of the ischemic cascade.

2. Experimental ischemic stroke models

2.1. Animal selection

Although initial scientific knowledge on stroke has come from higher species, now the majority of experiments are carried out in small animals such as the rat and the mouse. The use of small animals for stroke research studies presents clear advantages of lower cost and greater acceptability from ethical perspectives compared to larger animals. The rat is the most commonly used animal in stroke studies because of many reasons including its resemblance to humans in cerebrovascular anatomy and physiology (Macrae, 1992), its moderate size which allows monitoring easily the physiologic parameters and examining the brain specimens (Takizawa et al., 1991), the relative homogeneity within strains, and most of all, the ease of conducting reproducible studies (Brinker et al., 1999). Since mouse is the most appropriate animal to be applied genetic modifications, it is largely used in transgenic technology concerning studies in molecular pathophysiology of stroke (Fujimura et al., 1999; Chen et al., 2005). On the other hand, non-human primates have closer similarities with humans in terms of behavior and sensorimotor integration due to their gyrencephalic brains. It is recommended that once a positive result is achieved from a drug study in small animals, the study should be later replicated in higher species before proceeding to clinical trials (STAIR, 1999).

2.2. Model selection

Most animal stroke models were developed to induce cerebral ischemia within the MCA territory in order to be relevant to clinical situation (Del Zoppo et al., 1992). Animal stroke experiments may model either permanent or transient ischemia. Ischemic lesion size varies greatly according to the ischemia duration. To obtain reproducible infarct volumes, minimum 60 to 90 min of ischemia is required and for this reason, transient focal ischemia models are usually based on 90–120 min of ischemia. It is well known that lesion induced by more than 3 h of focal ischemia is not anymore reversible (Grotta, 1998). Permanent stroke models permit to study cerebral ischemia without the effect of reperfusion. When an occluded artery is recanalized by a model of transient cerebral ischemia, as it happens in most of human stroke, in addition, consequences of reperfusion in the ischemic territory (i.e. reperfusion injury) can be evaluated (Aronowski et al., 1997; Gursoy-Ozdemir et al., 2004).

Certainly there is not 'one' ideal ischemic stroke model since human stroke itself is a diverse condition. The first aspect to consider of the animal stroke model should be: how much does the model match with the clinical problem that will be taken under investigation? Among different animal models available for focal cerebral ischemia induction, those receiving following criteria may be 'more' ideal: (1) the ischemic processes and pathophysiologic responses should be relevant to human stroke, (2) the ischemic lesion size should be reproducible, (3) the technique used to perform the modeling should be relatively easy and minimally invasive, (4) physiologic variables can be monitored and maintained within normal range, (5) brain samples should be readily available for outcome measurements such as histopathological, biochemical, and molecular biological evaluation, and (6) the cost and effort should be reasonable (Li and Fisher, 2001).

Pathophysiological mechanisms and size of the ischemic lesion may vary according to the model. For instance, an embolus model using microembolic materials leads multiple small cerebral lesions (Mayzel-Oreg et al., 2004), but the lesion induced by direct surgical method that occludes proximal MCA involves most of the MCA territory (Tamura et al., 1981). Early photothrombotic models lack of penumbra, but there are detailed studies that expose large penumbra in the suture MCAO method in rats (Dietrich et al., 1987b; Carano et al., 2000).

Model selection is highly important in the field of drug developmental focal cerebral ischemia studies. First meeting of STAIR can be addressed as a guideline to design such studies (STAIR, 1999; Shuaib, 2006).

2.3. Major rodent models of focal cerebral ischemia

2.3.1. Models not requiring craniectomy

2.3.1.1. Embolic model. Embolic models of focal cerebral ischemia fall into two broad categories: thromboembolic models and non-clot embolic models.

Thromboembolic stroke models mimic human stroke more closely than other models of cerebral ischemia since most of the human strokes are caused by thromboembolism. Other advantages of thromboembolic models are their potential to test thrombolytic agents (Overgaard, 1994), to evaluate the ischemic lesion which underwent thrombolysis (Brinker et al.,

1999), and to study combination therapies, e.g., thrombolytic agents and neuroprotective drugs (Zhang et al., 2004).

Thromboembolic ischemia is induced most commonly by the injection of autologous thrombi into extracranial arteries to reach the more distal intracranial arteries. In early versions of thromboembolic models human blood clot (Papadopoulos et al., 1987) or a suspension of homologous small clot fragments (Kaneko et al., 1985) were used to produce embolism. The fact that, infarcts induced by these methods were variable and early spontaneous recanalization took place, various attempts have been made to produce autolysis resistant fibrin-rich emboli resembling human arterial thrombi (Overgaard et al., 1992; Takano et al., 1998). Busch et al. injected multiple fibrin-rich autologous clots into the external carotid artery one after another. A consistent reduction of cerebral blood flow (CBF) and histological damage in the MCA territory were observed, without any spontaneous recanalization 3 h after embolization (Busch et al., 1997).

Although a standardized thromboembolic animal model still lacks, recent experiences gathered from the thromboembolic focal ischemia models reveal clearly the importance of the formation and the composition of the emboli used to induce consistent CBF decline and reproducible lesions. When aimed to induce reproducible lesions, obstructing emboli should be located in the proximal segment of a large feeder artery (Busch et al., 1997).

Many compounds and artificial embolic materials, such as viscous silicone (Lauer et al., 2002), collagen (Purdy et al., 1989), polyvinylsiloxane (Yang et al., 2002), retractable silver ball (Molnar et al., 1988), and heterologous atheroemboli (Rapp et al., 2003) have been used to induce ischemia by injection into common carotid artery or internal carotid artery, most commonly in rats, but also in larger animals (Molnar et al., 1988) and primates (Watanabe et al., 1977). Among non-clot embolus models, microsphere-induced microembolization is the most extensively studied model in many aspects (Zivin et al., 1987; Fukuchi et al., 1999; Roos et al., 2003). The lesion development in the microsphere-induced stroke model is slow, increasing in size up to 24 h postinjection. Microsphere-induced embolic model may provide larger therapeutic window for drug testing in stroke. On the other hand, another unique characteristic of the microsphere model is the multifocal and heterogenous nature of the developing lesions (Mayzel-Oreg et al., 2004).

2.3.1.2. Intraluminal suture MCAO model. Among experimental ischemic stroke models, the intraluminal suture MCAO in rats and in mice is the most frequently used model. This model is less invasive and easy to perform both permanent and transient ischemia in a controlled manner. Intraluminal suture MCAO model involves inserting a monofilament into the internal carotid artery and advancing until it blocks blood flow to MCA (Fig. 1). This model provides reproducible MCA territory infarctions (involving both frontoparietal cortex and lateral caudoputamen) and allows reperfusion by retracting the suture. Depending on the shape, size, and insertion length of the thread, the MCA can be either occluded selectively or in conjunction with the posterior communicating artery, branches

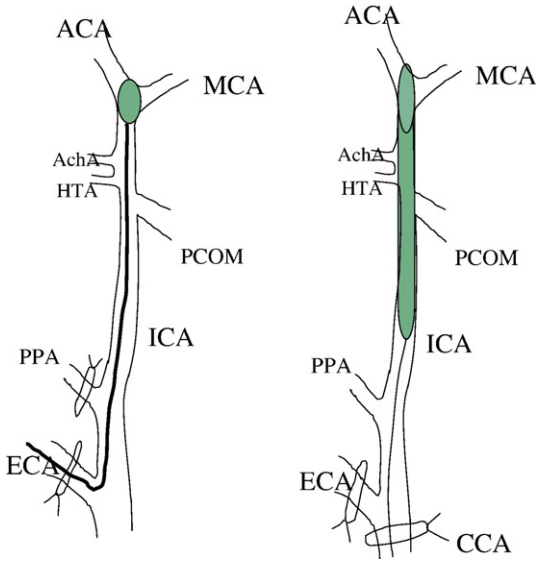

Fig. 1. Intraluminal suture middle cerebral artery occlusion technique. On the left, Longa's method, insertion of the uncoated suture into external carotid artery, where branches of internal carotid artery except MCA are kept patent; on the right Koizumi's method, insertion of the silicone-coated suture into common carotid artery, blocking MCA, AchA, and HTA. ACA, anterior carotid artery; AchA. anterior choroidal artery; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; HTA, hypothalamic artery; PPA, pterygopalatine artery; PCOM, posterior communicating artery.

of internal carotid artery and the common carotid artery (Fig. 1). The selective MCAO model, which provides higher ischemic lesion growth size within 24 h, may be advantageous for studies of neuroprotective strategies (Woitzik et al., 2006). Suture occlusion technique can be performed in a magnet bore, consequently this model allows evaluating very early events of focal cerebral ischemia. Li et al. have shown that suture MCAO induced in the magnet may be as successful as MCAO induced outside of the magnet, and in-bore reperfusion is applicable (Li et al., 1998).

Since Koizumi's first description of the intraluminal suture MCAO in rats (Koizumi et al., 1986), several modifications of the initial model, that frequently have employed different types of threads, have been published (Longa et al., 1989; Belayev et al., 1996a). Lesion reproducibility and size seem to be affected by many specific factors in this technique such as suture diameter (3.0 or 4.0 filament), coating of the suture (with silicone or poly-l-lysine), or insertion length of the thread. Size of the filament correlates well with the size of the infarct (Abraham et al., 2002). In contrast to uncoated suture silicone-coated suture was shown to cause larger and more consistent infarcts (Laing et al., 1993; Shimamura et al., 2006) with good reproducibility and reliability even among investigators of varying experience (Takano et al., 1997a). Lesion size grows the deeper the suture insertion is (Zarow et al., 1997; He et al., 1999). There are continuous efforts to optimize suture design and methodology of intraluminal suture MCAO technique (Schmid-Elsaesser et al., 1998; Gerriets et al., 2004b; Ma et al., 2006).

Intraluminal suture occlusion technique is increasingly applied in mice (Hata et al., 1998; Li et al., 2004, 2006),

transgenic and knockout mouse mutants (Huang et al., 1994; Tureyen et al., 2005) and also in other species such as rabbits (Kong et al., 2004).

Disadvantages of the suture occlusion method include: (1) vessel rupture and subsequent subarachnoid hemorrhage, (2) hyperthermia, and (3) inadequate MCAO. Silicone coating of the suture and laser Doppler-guided placement of the suture could reduce the incidence of subarachnoid hemorrhage (Schmid-Elsaesser et al., 1998). Spontaneous hyperthermia appears to be associated with hypothalamic injury and occurs in most, if not all, animals subjected to suture occlusion of MCA lasting 2 h or more (Li et al., 1999b).

2.3.1.3. Photothrombosis model. Photothrombosis induces a cortical infarct by the systemic injection of a photoactive dye (most often Rose Bengal) in combination with irradiation by a light beam at a specific wavelength (Watson et al., 1985). Generation of singlet oxygen leads to focal endothelial damage, platelet activation and aggregation in both pial and intraparenchymal vessels within the irradiated area (Dietrich et al., 1987b). The region of irradiation can be determined so as to induce ischemic lesion in any desired cortical area. Photothrombotic ischemic lesion lacks of penumbra because vasogenic edema and BBB breakdown in the lesion occur within minutes (Dietrich et al., 1987a). Thus, the model is undesirable for preclinical drug studies where the chief target is penumbra. Recent photothrombotic ring models with modified irradiation features, in terms of either beam intensity or duration, seem to be able to induce cortical ischemic lesions involving a penumbra-like lesion (Wester et al., 1995; Pevsner et al., 2001; Hilger et al., 2004). Although rats are frequently the most preferred species for the photothrombotic ischemic stroke model, a krypton laser-induced photothrombotic distal MCAO model in mice is also available (Sugimori et al., 2004).

Another disadvantage of the photothrombosis model is that the lesion induced by this method is end-arterial occlusive in nature and is resistant to therapies based on the enhancement of collateral perfusion. Although it has some limitations, photothrombotic ischemic stroke model may address specific questions, e.g., in the field of restorative drug studies and neuronal repair evaluation.

2.3.1.4. Endothelin-1 induced stroke. Endothelin-1 acts as a potent vasoconstrictor and can be applied directly onto the exposed MCA (Robinson et al., 1990) or adjacent to the MCA by stereotaxic intracerebral injection (Sharkey et al., 1993) or onto the cortical surface of the rat (Fuxe et al., 1997). While direct or stereotaxic applications provide significant decrease of CBF in the MCA territory (70–93%) that result in ischemic lesion pattern similar to that induced by direct surgical MCAO (Macrae et al., 1993; Sharkey et al., 1993), cortical applications provide sufficient reduction in blood supply of frontoparietal cortex and induce a semicircular infarct involving all layers of the neocortex (Fuxe et al., 1997). Less invasiveness and ability to induce ischemia in any desired region of the brain are the chief advantages of endothelin-1 application, but dose dependent action of endothelin-1 reduces the control on ischemia duration and intensity. Moreover, findings suggest that endothelin-1 application induces astrocytosis and facilitates axonal sprouting that may interfere with the production and interpretation of neural repair experiments (Carmichael, 2005).

2.3.2. Models requiring craniectomy

These methods include direct surgical occlusion of MCA and models combining MCA and other vessel occlusions. Surgical focal cerebral ischemia models are invasive, and more or less they require craniotomy which expose the brain to the atmosphere and affect intracranial pressure and blood-brain barrier (BBB) function.

Surgical MCA occlusion procedures have been performed in a variety of animals including non-human primates (Hudgins and Garcia, 1970), pigs (Imai et al., 2006), cats (MacDonald et al., 1972), dogs (Suzuki et al., 1980), and rabbits (Meyer et al., 1986). Since the first study by Robinson et al. (Robinson et al., 1975) describing ligation of the distal MCA in Sprague-Dawley rats, the rat is most widely used species to undergo stroke with a surgical technique. Among several approaches orbital route has been found less traumatic (O'Brien and Waltz, 1973). While electrocauterization of a portion of MCA results in permanent occlusion, the use of microclips and ligature snares allows reperfusion (Shigeno et al., 1985; Buchan et al., 1992).

Tamura's model (Tamura et al., 1981) has been applied widely because it allows access to the more proximal regions of the MCA compare to the previous techniques. Tamura's method produces infarction, similar to the infarction induced by intraluminal suture MCAO model, that involves both cortex and striatum in the MCA territory. Bederson et al. investigated occlusion of the MCA at various sites (Bederson et al., 1986). The necessity to extend the length of the portion of MCA to be occluded appears to isolate lenticulostriate and small cortical arteries from both proximal and distal collateral supplies, thus to produce reproducible focal infarction in rats.

Tandem vessel occlusion techniques involve electrocauterization of the distal MCA on the surface of the brain, and unilateral (Brint et al., 1988) or bilateral common carotid artery occlusion (Chen et al., 1986) (latter is so-called three vessel occlusion model). Although MCA is occluded distally and with a short length, the reduction in collateral blood flow caused by common carotid artery occlusion consolidates the ischemic damage (McAuley, 1995). Three vessel occlusion model produces large neocortical infarcts with a high mortality (Buchan et al., 1992). Release of the contralateral common carotid artery after one hour of occlusion reduces the mortality (Chen et al., 1986).

An "ideal" direct surgical MCAO method should have following features: (1) minimal injury to the brain (e.g., drilling or electrocoagulation may lead to thermal and mechanical damage), (2) minimal exposure of intracranial contents to the atmosphere (minimal craniotomy), (3) ease to perform (to avoid intersurgeon variability), (4) adaptability to more than one animal species, and (5) ability to ensure reperfusion (thus being compatible with preclinical drug development studies).

2.3.3. Posterior cerebral circulation stroke models

Animal modeling for vertebrobasilar stroke is challenging. Previously, there has not been a reproducible small animal

model of hindbrain ischemia. Moreover, most of the available models are surgically demanding and are performed in large animals (Kuwabara et al., 1988; Guo et al., 1995; Inui et al., 1996). Electrocoagulation of the basilar artery results in neurological deficits only in 20–30% of rats (Kameyama et al., 1985). Wojak et al. (1991) were the first to mimic basilar artery territory infarct in the rat. They used a surgical approach by which basilar artery was thermocoagulated at various locations. Even with two-point occlusions of the basilar artery, infarcts were small and variable and no neurological deficit was observed. Thromboembolic vertebrobasilar stroke was studied in a rabbit model (Pan and Wright, 1987) and in a rat model recently (Henninger et al., 2006). While rabbit model has introduced an angiographic technique to deliver blood clots into the vertebral artery, the rat model involves a thoracotomy to expose vertebral artery for clot injection. When combined with transfemoral digital subtraction angiography, which seems a reliable tool for evaluating vertebral artery occlusion (Plaschke et al., 2000), modeling thromboembolic vertebrobasilar artery stroke may be less invasive. Besides such a model may be suitable for mimicking human vertebrobasilar artery occlusion, the most feared form of ischemic stroke and testing efficacy of thrombolytic agents (Henninger et al., 2006).

2.3.4. Cerebral venous thrombosis models

Experimental data about cerebral venous thrombosis (CVT) are scarce. First report of CVT was induced in a rabbit model by tying both jugular veins in 1836, since then various attempts have been made to develop a reproducible and clinically relevant animal model of CVT (Cooper, 1836). Cats (Schaller et al., 2003), pigs (Fries et al., 1992), dogs (Sarwar et al., 1985), and rabbits (Alexander et al., 1990) have been used in the models of CVT (Schaller et al., 2003). The rat model, however, is more suitable for CVT because the anatomy of its venous system is more consistent (Schumacher, 1984).

Occlusion of the superior sagittal sinus alone, by injection of different agents (e.g., sclerosing agents, cyanoacrylate, ethanolamine) into the superior sagittal sinus or by heating or compression of the superior sagittal sinus, were insufficient to mimic neuropathological consequences of CVT. The combination of superior sagittal sinus ligation with the injection of thrombogenic cephalin suspension in rats reliably induces superior sagittal sinus thrombosis and causes parenchymal damage due to additional involvement of the cortical draining veins (Rother et al., 1996). This model was originally developed by Frerichs et al. in 1994 and may provide a better understanding of the pathophysiological events underlying CVT (Frerichs et al., 1994; Liebetrau et al., 2003; Vosko et al., 2003). A rat model of cortical vein occlusion by the photochemical thrombotic technique, so-called two-vein occlusion model, is available and serve as a tool to investigate venous cerebral infarction (Nakase et al., 1995; Kimura et al., 2005; Nakagawa et al., 2005).

2.4. Sources of variability

Although animal modeling has provided most of our knowledge about pathogenic mechanisms and potential treatment of ischemic stroke, the relevance of animal stroke models to human stroke has been increasingly debated, because many compounds showing neuroprotective effects in preclinical studies have failed to show efficacy in clinical studies (Li and Fisher, 2001). The objective of an experimental model is to achieve homogenous and reproducible lesions with minimum variability, in order to maximize reliability (Alonso de Lecinana et al., 2005). Severeness of ischemia and size of infarction are closely related to local CBF (Zhao et al., 1997). Methods for monitoring CBF (such as laser-Doppler flowmetry) substantially improve the quality and reliability of animal stroke models (Woitzik and Schilling, 2002). A number of different parameters, if not stringently controlled, can confound the validity of the model by exacerbating inter- and intramodel variability (Macrae, 1992). Main sources of variability in animal stroke modeling are briefly discussed below.

2.4.1. Animal related factors

Rats are ideal subjects for mimicking human stroke due to close similarities between the cerebrovascular anatomy (including the presence of a circle of Willis) and physiology of rat and man (Tamura et al., 1981; Macrae, 1992) and although microvascular anomalies do occur in rat brain they do not significantly affect the incidence or size of the cortical infarction induced by surgical MCAO method (Menzies et al., 1992). Most models of ischemic stroke are conducted in young animals with no underlying chronic diseases or any genetic predisposition to such diseases. When aimed to assess whether aged rats are at greater risk of neuronal damage from experimental stroke, some studies found no correlation between the infarct volume and age of the rat (Duverger and MacKenzie, 1988; Wang et al., 2003), whereas some others suggest susceptibility to bigger infarct volumes in older rats (Davis et al., 1995), or in younger rats (Shapira et al., 2002). Aging found to be associated with reduction in angiogenesis and poor neurological functional recovery after stroke in rats (Zhang et al., 2005).

Some studies have demonstrated that certain rat strains are more sensitive to MCAO and produce more extensive infarct volumes (Duverger and MacKenzie, 1988; Oliff et al., 1996) probably due to the differences in intracranial collateral anastomoses (Oliff et al., 1997). Some rat strains might not be suitable to use in any stroke models, for instance surgical difficulties accompanied by high complication rates due to their cerebrovascular anatomy make Fischer rats quite unsuitable for suture MCAO, although significant vendor differences may also exist (Dittmar et al., 2006). Surprisingly, in addition to final infarct size, dynamics of the penumbra have also been found different between two rat strains in a study using diffusion and perfusion imaging (Bardutzky et al., 2005). The spontaneously hypertensive rat and its stroke-prone cohort are species susceptible to develop larger and much less variable infarcts following MCAO (Coyle, 1986; Duverger and MacKenzie, 1988). Recent findings strongly suggest that spontaneously hypertensive stroke-prone rat has a genetic susceptibility to stroke independent of blood pressure (Nabika et al., 2004). This unique strain may provide a model to investigate genetic susceptibility to stroke.

Female rats sustain smaller infarcts after MCAO compared with male rats and gender difference in infarct size is lost when female animals are ovariectomized at an early age (Alkayed et al., 1998). When testing neuroprotective agents, if a positive result is received in male rats, the study should be repeated in female animals. But this requires precise timing with monitoring the estrous cycle, in order to control potential neuroprotective effects of cyclic female hormone levels (Merchenthaler et al., 2003).

2.4.2. Physiological parameters

Temperature, arterial blood pressure, arterial blood gases, and blood glucose are the essential parameters to monitor and control during an animal experiment. Both hyperthermia and hypothermia affect ischemic lesion size. While mild to moderate hypothermia confers marked neuroprotection, hyperthermia is associated with an extension of neuronal damage (Krieger and Yenari, 2004; McIlvoy, 2005). Hyperglycemia has been shown to worsen the ischemic damage in the majority of experiments (Nedergaard, 1987; Huang et al., 1996), some other researchers have reported decrease (Ginsberg et al., 1987) or no change (Prado et al., 1988) in infarct volumes with hyperglycemia. The use of fasted animals tends to minimize interanimal variability of plasma glucose levels (Ginsberg and Busto, 1989). Because ischemic brain looses capacity of autoregulation, brain tissue becomes more vulnerable to changes in arterial blood gases and blood pressure, these parameters should be continuously monitored and controlled in any cerebral ischemia experiment (Pulsinelli and Jacewicz, 1992).

2.4.3. Model related factors

Direct surgical MCAO models include confounding factors associated with their invasiveness: skull trauma, external blood vessel injury, changes in intracranial pressure (Wiebers et al., 1990). In addition, skill demanding surgical operations may lead to intermodel variability which stems from the operator. Endovascular models (i.e., embolic models and suture occlusion model) and phothotrombotic model are less invasive. Although pathophysiology of embolic models has close resemblance with clinical situation, if the blood clots lodge not into the MCA stem but into distal branches, ischemic lesions induced with these techniques become highly variable in terms of location and size. Intraluminal suture technique of MCAO induces relatively bigger and reproducible infarct volumes, however MCAO lasting more then 2 h has a disadvantage of inducing hyperthermia even if anesthesia is maintained for a long period (6 h) (Li et al., 1999b). This aspect is particularly important in case of neuroprotective drug studies and among permanent stroke models for instance macrosphere model may be more appropriate than permanent suture MCAO to test neuroprotective compounds (Gerriets et al., 2003). Ischemic characteristics may differ according to the model and some models may not produce ischemic regions amenable to therapeutic regimens that target penumbra. Large ischemic penumbra has been identified in the suture occlusion model (Memezawa et al., 1992; Carano et al., 2000) in rat, on the other hand, there is relatively little ischemic penumbra in the photothrombotic stroke model (Carmichael, 2005). However induction of photothrombosis with a ring filter may provide a region of penumbra (Hu et al., 2001).

2.4.4. Anesthesia

Experimental stroke models usually are carried on anesthetized animals. Many of commonly used anesthetics have been shown to cause a degree of neuroprotection (Kirsch et al., 1996). Thus selecting the anesthesia regimen to apply in animal models has a chief importance in the preclinical neuroprotective drug studies. Different anesthesia methods are available in rats, such as intraperitoneal injection, inhalation via face-mask in spontaneously breathing animal or inhalation via face-mask in intubated and mechanically ventilated animal. In one study using suture MCAO model in rats, inhalation anesthesia under mechanical ventilation has been found to be superior to other methods investigated, because it has provided better control of physiological parameters and lower mortality during 7 days postischemia (Zausinger et al., 2002).

2.5. Outcome measures

In animal ischemic stroke studies the most precise outcome measure is the infarct volume. Infarct volume can easily be measured post mortem with a number of histologic techniques. In vivo magnetic resonance imaging (MRI) is in use not for only measuring the final infarct size, but it is also used to investigate temporal evolution of ischemic lesion. Functional assessment is an essential measure of outcome since functional recovery is universally used as a primary endpoint in clinical trials.

2.5.1. Histopathology

Traditional histological staining techniques are hematoxylin-eosin and triphenyltetrazolium chloride staining methods that are performed after the animals' sacrifice at an appropriate time point following ischemic insult. Traditional histological staining techniques accurately and reliably distinguish infarcted tissue from normal tissue in both early and delayed periods of focal ischemia (Garcia et al., 1995). Digital camera-based imaging and computer-based image-analyzing systems enable to calculate infarct volumes by multiplying the area of the lesion with the slice thickness. Enlargement of the infarcted tissue by edema results in overestimation of infarct volume, that is why the calculation of corrected infarct volume is used to compensate for the effect of brain edema (Takano et al., 1997b; Tatlisumak et al., 1998b). Besides absolute infarct volumes, the percentage of the hemisphere involved with ischemia can be provided (Tatlisumak et al., 1998a). Edema leads a space-occupying effect and results in a strong horizontal displacement of midline structures on the magnetic resonance images. Gerriets et al. used T2-weighted images to display the shift of the midline structures and to calculate hemispheric volumes (Gerriets et al., 2004a). They observed that this method correlated well with the calculation of the hemispheric lesion volume with edema correction on triphenyltetrazolium chloride staining. In mice ischemic stroke studies, more extensive analysis of infarct topography with paraffin-embedding and close sectioning may be advantageous, because such material is readily amenable to

detailed analysis by sophisticated image-averaging methods (Belayev et al., 1999). Influence of fixation procedures on the quantification of ischemic lesion is a confounding factor in focal cerebral ischemia models. Relative measurements of infarct volume and edema, since they are independent of the investigated fixation procedures, may solve this problem.

2.5.2. Brain physiology

Cortical electroencephalographic activity can be easily and continuously monitorized in rats. Recording of peri-infarct depolarizations and seizure discharges in rat models of stroke allows evaluating neurophysiological aspects of ischemic brain injury including the contribution of pathologic spreading depressions to the expansion of infarction and therapeutic mechanisms of neuroprotective drugs (Tatlisumak et al., 1998c; Hartings et al., 2003b).

2.5.3. Magnetic resonance imaging

Magnetic resonance diffusion weighted (DWI) and perfusion-weighted imaging (PWI) are very sensitive to early ischemic changes. DWI is able to detect ischemic lesions in the hyperacute phase of ischemia even within minutes of injury (Tatlisumak and Li, 2003; Tatlisumak et al., 2004). In animal stroke models, DWI signal abnormalities correlate highly with the location and volume of pathologically confirmed cerebral infarction (Mack et al., 2003). The brighter the DWI lesion, the greater the neuronal damage (Rivers and Wardlaw, 2005). PWI demonstrates the region of hypoperfusion. Combination of DWI with PWI allows a more complete understanding of the underlying ischemic pathophysiology. Mismatch between these two techniques crudely reflects ischemic penumbra. The definition of PWI/DWI mismatch as penumbra is suggested to be modified after the recognition that the PWI boundary includes a region of benign oligemia and that a portion of the DWI core is potentially salvageable with rapid reperfusion (Maulaz et al., 2005). The ischemic changes demonstrated by DWI may be permanently or temporarily reversible in the case of transient ischemia depending on the duration of ischemia (Li et al., 1999a). The amount of cellular damage underlying DWI lesion appearance is unclear and more experimental data are needed (Rivers and Wardlaw, 2005). Use of MRI modalities in preclinical studies is an opportunity to overcome potential pretreatment bias, because lesion detection before treatment and in vivo lesion monitoring posttreatment are easily performed. Several more sophisticated MRI modalities (e.g., functional MRI, pharmacological MRI, diffusion tensor imaging, molecular imaging) are rapidly advancing and they seem promising as novel tools for investigating many aspects of stroke (Reese et al., 2002; Dijkhuizen and Nicolay, 2003; Barber et al., 2004; Numano et al., 2006).

2.5.4. Neurological outcome

Neurological deficits unfortunately are relatively difficult to assess in small laboratory animals and especially in rodents. In addition, small animals recover easier so neurological deficits may clear rapidly within hours or days after ischemia due to high degree of brain plasticity. Motor deficits are relatively objective end points of a rat stroke model and can be evaluated by a number of easy and quick methods (Bederson et al., 1986; Longa et al., 1989; Menzies et al., 1992).

2.5.5. Behavioral outcome

The ability of rat to reach and grasp food with a single paw, enables this strain to serve as a model for exploring neural networks of skilled limb movements (Gharbawie and Whishaw, 2006). Tests to examine the effects of focal stroke on refined sensorimotor function include: limb placing, beam walking, grid walking, rotarod, sticky label test, and staircase test (Hunter et al., 2000). Laboratory Animal Behavioral Observation, Registration and Analysis System (LABORAS™) is a validated automatic behavioral classification system, in assessing long-term functional outcome and one transient MCAO study in the rat showed that in addition to motor deficits, animals display changes in home cage behavior which, in contrast to motor function, are prolonged over time (Quinn et al., 2005). A number of cognitive tests are also available: among them Morris water maze is the prototype (D'Hooge and De Deyn, 2001). Combining appropriate behavioral tests to histological measurements becomes more critical with the growing interest in neurorestorative drug studies (Roof et al., 2001). One obstacle is that, data from neuroprotective drug studies in animals suggest a poor correlation between pathologic and functional improvement (Green, 2002). For instance, despite the lack of a clear pathological evidence of infarct size improvement, behavioral assessment might reveal effectiveness of a neuroprotective drug (Yamaguchi et al., 1995; Kawamata et al., 1996). Lack of correlation between different outcome measures indicates that both behavioral, neurological, and histological endpoints are necessary for effectively and comprehensively examining the putative protective effect of a drug in models of stroke.

3. Pathophysiology and therapy of acute ischemic stroke

3.1. Pathophysiology of acute ischemic stroke

Ischemia is defined as a reduction in CBF, sufficient to cause metabolic or functional deficits. Ischemia is typically caused by the occlusion of an artery supplying a specific territory of the brain. The characteristics of the brain injury depends on the severity and duration of the CBF reduction. In animal models, blood flow is most crucially reduced in the central region of the brain (infarct core), and in a graded fashion centrifugally from the core (ischemic penumbra) normally supplied by the occluded artery, due to residual perfusion from collateral blood vessels. The ischemic penumbra is nonfunctional, however, retains structural integrity. Evidence indicates that, if the blood flow is not restored within hours, the penumbral region becomes part of the core region (Green et al., 2003). The process of cellular injury and death are remarkably different in these two regions (Smith, 2004). There appear two major modes of cell death that participate in ischemic cell death: necrosis and apoptosis. The thinking that brain infarction is a classic example of necrosis has let the place to another approach: apoptosis and necrosis seem like the two poles of a continuum of cellular death ('aponecrosis') after ischemic stroke (MacManus and

Buchan, 2000; Unal-Cevik et al., 2004). While necrosis is more dominant in the core tissue, penumbral cells die by means of either mode, with apoptosis being more common for cells further away from the core (Smith, 2004). The concept of neurovascular unit emphasizes that also the milieu (extracellular matrix, glial and endothelial cells) influences the fate of the neurons (Lo et al., 2003).

Within minutes of vascular occlusion a complex sequence of pathophysiological spatial and temporal events (ischemic cascade) occurs following each other in a certain order which in fact is not strictly in order, because these events demonstrate overlapping features. Ischemic brain injury may last hours or even days. Major events occurring after CBF reduction are illustrated in Fig. 2, with the maximal effort to include and reflect most interactions within each step.

Leading pathogenic mechanisms of ischemic cascade include energy failure, elevation of intracellular Ca^{2+} level, excitotoxicity, spreading depression, generation of free radicals, BBB disruption, inflammation, and apoptosis.

3.1.1. Energy failure

Brain is almost exclusively dependent on the continuous steady flow of glucose and oxygen to undergo oxidative phosphorylation for energy production because it has no stores of energy and deprivation occurs in minutes only. The first consequence of CBF reduction is the depletion of substrates, particularly oxygen and glucose, that causes accumulation of lactate via anaerobic glycolysis. Acidosis may enhance free-radical formation, interfering with intracellular protein synthesis and worsen ischemic brain injury; yet the mechanisms of the deleterious effects of acidosis are still unknown (Siesjo et al., 1996; Huang and McNamara, 2004; Mergenthaler et al., 2004). Energy failure leads to perturbation of the Na^+/K^+-ATPase, and Ca^{2+}/H-ATPase pumps; in addition Na^+-Ca^{2+} transporter is reversed (Phan et al., 2002). Subsequent ion dyshomeostasis (elevation of intracellular Na^+, Ca^{2+}, Cl^- and elevation of extracellular K^+ levels) causes to cytotoxic edema and leads to events triggered by excess of intracellular Ca^{2+} (Fig. 2).

3.1.2. Excitotoxicity

With the energy depletion, membrane potential is lost and consequently neurons and glia depolarize (Dirnagl et al., 1999). After depolarization, excitotoxic amino acids, especially glutamate, are released into the extracellular compartment from presynaptic neurons in large amounts, already in the very early phase of ischemia. Besides direct neurotoxicity of glutamate on neurons, the activation of glutamate receptors (NMDA-, AMPA-, and metabotropic glutamate receptors) leads to a further increase of the intracellular Ca^{2+}, Na^+, and Cl^- levels (Fig. 2); thus increases the amount of the edema and toxicity via intracellular Ca^{2+} excess. Excitotoxic mechanisms can cause necrosis but can also initiate molecular events that lead to apoptosis (Dirnagl et al., 1999).

3.1.3. Spreading depression

Cellular injury within the penumbral region may also occur from recurrent waves of depolarization starting within the ischemic core and extending outwards to surrounding tissue. Spreading depression consumes energy and increases infarct volume. Several studies demonstrated the contribution of repetitive pathologic peri-infarct (spreading depression-like) depolarizations to the recruitment of penumbral tissue into the infarct core (Takano et al., 1996; Hartings et al., 2003a). There is some evidence that spreading depression-like depolarizations occur in human, but lack of reliable non-invasive detection techniques have hindered progress in human research (Dreier et al., 2006). The most likely source for these depolarizations is the elevated extracellular K^+ level and increase in the glutamate release at the boundaries between the ischemic core and the penumbra (Takano et al., 1996; Dijkhuizen et al., 1999). Direct current-magnetoencephalography technique seems promising to noninvasively evaluate spreading depressions in humans (Mackert, 2004). Currently near infrared spectroscopy and direct current-electroencephalography are under investigation for detecting human spreading depressions.

3.1.4. Elevation of intracellular Ca^{2+} level

Ca^{2+} enters the neurons via NMDA- and AMPA receptor-operated channels, voltage gated Ca^{2+} channels, store-operated channels and the reverse operation of the Na^+-Ca^{2+} exchanger. Additionally release from the organelles such as endoplasmic reticulum, mitochondria, synaptic vesicles and from Ca^{2+} binding proteins may further increase intracellular Ca^{2+} level. Calcium plays a unique role on the ischemic pathophysiology since it causes several damaging events by activation of a variety of Ca^{2+} dependent enzymes, including protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, calcium-dependent nitric oxide synthase, calpain and various proteases and endonucleases (Fig. 2). As a result of formation of cytotoxic products such as free radicals and leukotrienes, irreversible mitochondrial damage, and inflammation, both necrotic and programmed cell death are triggered by excess of intracellular Ca^{2+} (Fisher and Schaebitz, 2000; McIlvoy, 2005).

3.1.5. Generation of free radicals

As a consequence of focal cerebral ischemia and particularly when reperfusion occurs, oxygen radicals are produced during enzymatic conversions, such as the cyclooxygenase-dependent conversion of arachidonic acid to prostanoids and the degradation of hypoxanthine (Lo et al., 2003). Formation of mitochondrial permeability transition pore occurs under ischemic conditions and leads to a burst of free oxygen radicals and the release of pro-apoptotic molecules (Mergenthaler et al., 2004). Furthermore, free radicals are also generated during the inflammatory response after ischemia (Lo et al., 2003). Reactive oxygen species react irreversibly with several cellular constituents such as proteins, double bonds of phospholipids, and nuclear DNA.

Free radicals cause lipid peroxidation, membrane damage, dysregulation of cellular processes, and mutations of the genome. In fact, reactive oxygen species can damage virtually any cellular component. Cell damage causes aberrations in ion homeostasis, cell signaling, and gene expression.

Oxygen radicals serve as important signaling molecules that trigger inflammation and apoptosis (Dirnagl et al., 1999).

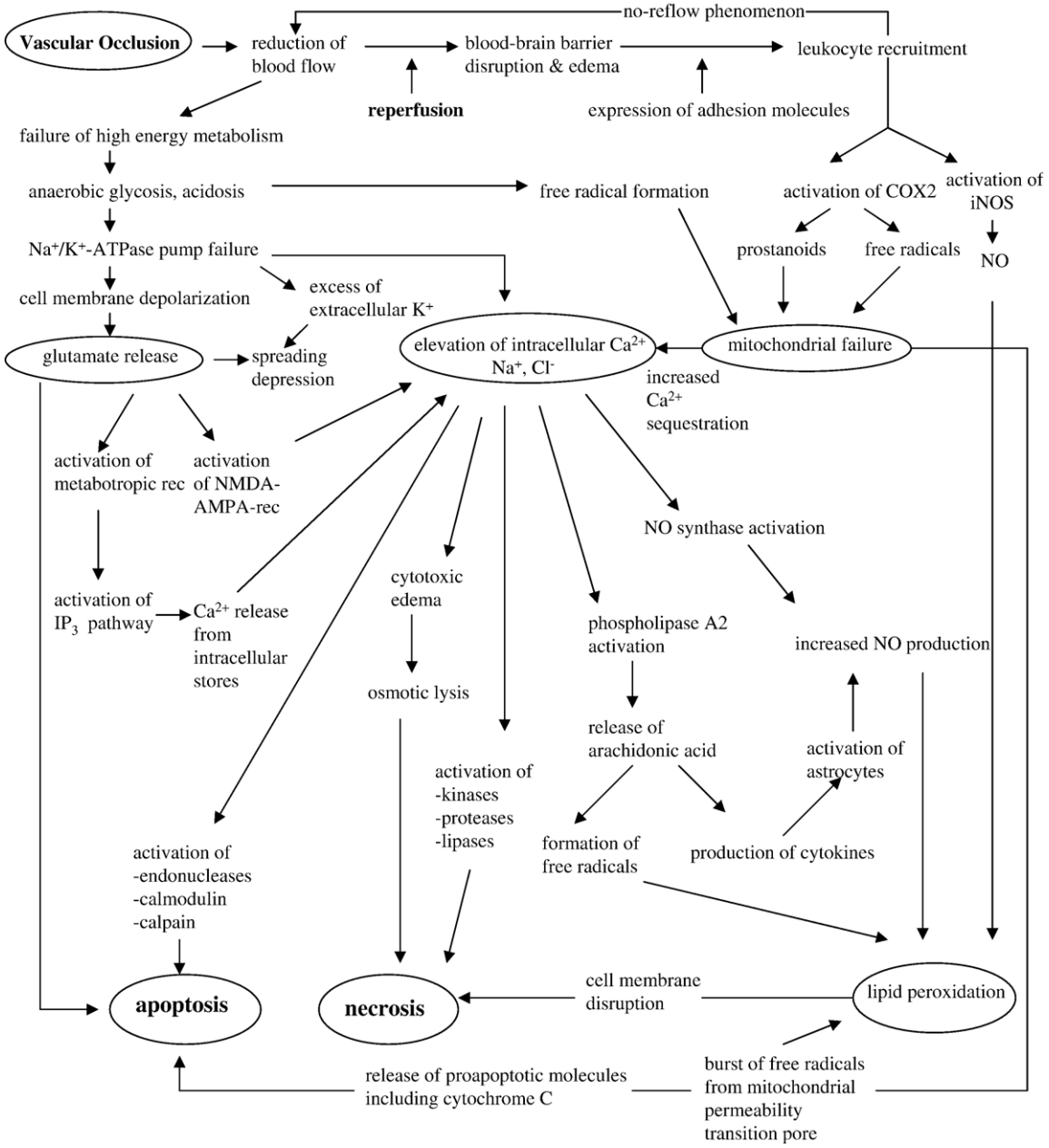

Fig. 2. Diagram depicting major events that occur after cerebral vessel occlusion and subsequent reperfusion. The figure is not fully comprehensive of all events. AMPA, α-amino-3-hydroxy-5-methylisoxasole-4-propionic acid; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IP_3, inositol 1,4,5-triphosphate; COX2, cyclooxygenase-2.

3.1.6. BBB disruption

Mechanical or hypoxic damage of vascular endothelium, toxic damage of inflammatory molecules and free radicals and especially destruction of the basal lamina by matrix metalloproteinases are potential causes of BBB disruption which leads to vasogenic edema, influx of toxic substances, inflammation, and presumably hemorrhagic complications after stroke. Brain edema aggravates the ischemic process by its volumetric effect causing to local compression of microcirculation, rise of intracranial pressure, and dislocation of parts of the brain. The degree of brain swelling is proportional to the size of the infarct and in the case of severe stroke (malignant MCA infarction) can give rise to fatal transtentorial herniation, responsible for 30–90% of acute stroke fatalities (Gartshore et al., 1997). Transient ischemia by the delayed restoration of blood flow seems to aggravate infarction mainly due to additional BBB damage which occurs earlier than in permanent ischemia (Yang et al., 1999). In experimental permanent brain ischemia breakdown of BBB has been reported to occur in a monophasic pattern. Although to date there is a consensus about biphasic opening of BBB after transient ischemic insult, careful examination of the literature on BBB changes after stroke reveals insufficiency of the methodology or inconsistency of the animal studies concerning this matter. To our opinion, BBB disruption continues for several

days, even weeks without reaching its normal functioning and configuration (Kuroiwa et al., 1985; Belayev et al., 1996b; Huang et al., 1999; Marsala et al., 2004). The role and pattern of BBB opening on ischemic injury needs further evaluation.

3.1.7. Inflammation

Ischemic injury triggers inflammatory cascades in the brain parenchyma that may further amplify tissue damage by many mechanisms. Within minutes of occlusion, there occurs upregulation of proinflammatory genes which produces mediators of inflammation such as platelet-activating factor, tumor necrosis factor α, and interleukin 1 β. After the expression of adhesion molecules (including intercellular adhesion molecule 1 and selectins) at the vascular endothelium, neutrophils transmigrate from the blood into the brain parenchyma, followed by macrophages and monocytes. Whereas microvascular obstruction by neutrophils (no-reflow phenomenon) can worsen the degree of ischemia, production of toxic mediators by activated inflammatory cells and injured neurons (cytokines, nitric oxide, superoxide and prostanoids) can amplify tissue damage. Neutrophils are the first inflammatory cells to arrive to the ischemic tissue as early as within hours after reperfusion (Emerich et al., 2002). Macrophages and monocytes arrive within few days. In addition, the inflammatory reaction might also be linked to apoptosis (Dirnagl et al., 1999) (Fig. 3). The pathogenic role of neutrophils and other leukocytes in cerebral ischemia is still a subject of debate (Emerich et al., 2002), microglia and macrophages may even be beneficial through their role in tissue remodeling (Danton and Dietrich, 2003).

3.1.8. Glial cell contribution

New data based on the use of radiation bone marrow-chimeric mice in transient focal cerebral ischemia suggest that resident microglial activation precedes macrophage infiltration and the vast majority of macrophages in the infracted area are derived from local microglia (Schilling et al., 2003). Although astrocytes protects neurons by multiple mechanisms, including regulation of ionic homeostasis, control of extracellular glutamate levels, and upregulating glycolytic capacity during ischemia (Dienel and Hertz, 2005), under certain conditions, activated astrocytes contribute to the ischemic cell death (Trendelenburg and Dirnagl, 2005). As recently was shown in a stroke model applying hyperbaric oxygen treatment (Gunther et al., 2005), pharmaceutical agents or therapies suppressing microglial activation but increasing astrocytic response, might improve postischemic outcome.

3.1.9. Changes in neurotransmitters and neuroactive substances

Postmortem (by immunohistochemical staining techniques (Allen et al., 1995)) and in vivo (by microdialysis (Matsumoto et al., 1993)) evaluation of neurochemical changes following stroke in animal models showed that while aspartate, glutamate, inosine, hypoxanthine, adenosine and γ-aminobutyrate increases in the acute ischemic period, glycine seems to increase with prolonged ischemia and some neuroactive substances increase in peri-infarct region (such as tyrosine hydroxylase, neuropeptide Y), and some (neuropeptide Y, leu-enkephalin, neurotensin, and dynorphin) in nuclei of amygdala which are not infarcted. Recently microdialysis method has been applied to patients with large MCA infarction, and similar results have been achieved (Dohmen et al., 2003).

3.1.10. Apoptosis

Triggered by a number of processes, including excitotoxicity, free-radical formation (damaging cellular lipids, proteins and nucleic acids), inflammation (activation of FAS-receptor, e.g., through binding of tumor necrosis factor α), mitochondrial and DNA damage, and cytochrome *c* release from mitochondria, apoptosis occurs after milder ischemic injury, particularly within the ischemic penumbra (Fisher and Schaebitz, 2000; Mergenthaler et al., 2004; Sugawara et al., 2004). Apoptosis is an energy-consuming process, so reperfusion could potentiate apoptosis by restoring cellular energy (Schaller and Graf, 2004). Several mediators facilitate cross-communication between apoptotic cell death pathways: the calpains, cathepsin B, nitric oxide, and poly-(ADP-ribose) polymerase (Lo et al., 2003). Following ischemia, caspase activation occurs in response to pro-apoptotic signals such as downregulation of Bcl-2 and upregulation of the Bax/Bid and Death receptor family. Caspase 3 activation may be a downstream event in the apoptotic cascade (Phan et al., 2002). Caspases 1, 3, 8, and 9 are involved in cerebral ischemia (Mergenthaler et al., 2004). Activated caspases are protein-cleaving enzymes and more than 30 proteins can be cleaved that lead to characteristic DNA-laddering and cleavage of structural proteins (such as laminin, actin, gelsolin), which are essential for the integrity of the nucleus and the cell. Apoptotic cell morphology differs greatly from necrotic cell morphology. Necrotic cells become edematous and loose their cellular architecture by cytoskeletal breakdown. Apoptotic cell is characterized by a number of morphological features: shrinkage of the cytoplasm, marked condensation of chromatin, membrane-blebbing, and fragmentation of the cell by separation of the protuberances to form multiple small membrane-bound bodies that contain intact organelles and/or dense clumps (apoptotic bodies) (Love, 2003; Mergenthaler et al., 2004). Apoptotic cells are rapidly removed by phagocytosis without eliciting an inflammatory reaction (Love, 2003). To date there are only few studies demonstrating apoptotic features in human stroke (Guglielmo et al., 1998; Love et al., 2000; Sairanen et al., 2006).

3.1.11. Genomics/proteomics

DNA microarray technology allows mapping the time course of gene response for differential expression of thousands of genes in the brain, that is triggered by ischemic insult (Schmidt-Kastner et al., 2002; Kim et al., 2004; Lu et al., 2004). Transcient focal cerebral ischemia induces a complex change in genomic profile, including expression of new genes, upregulation and downregulation of genes, occurring distinctly in a temporal manner. Detection of gene changes after ischemia is just the first step towards understanding different molecular pathways and proteomics and peptidomics studies provides supplemental insights (Schuhmann et al., 2005; Fonteh et al., 2006; Chen et al., 2006).

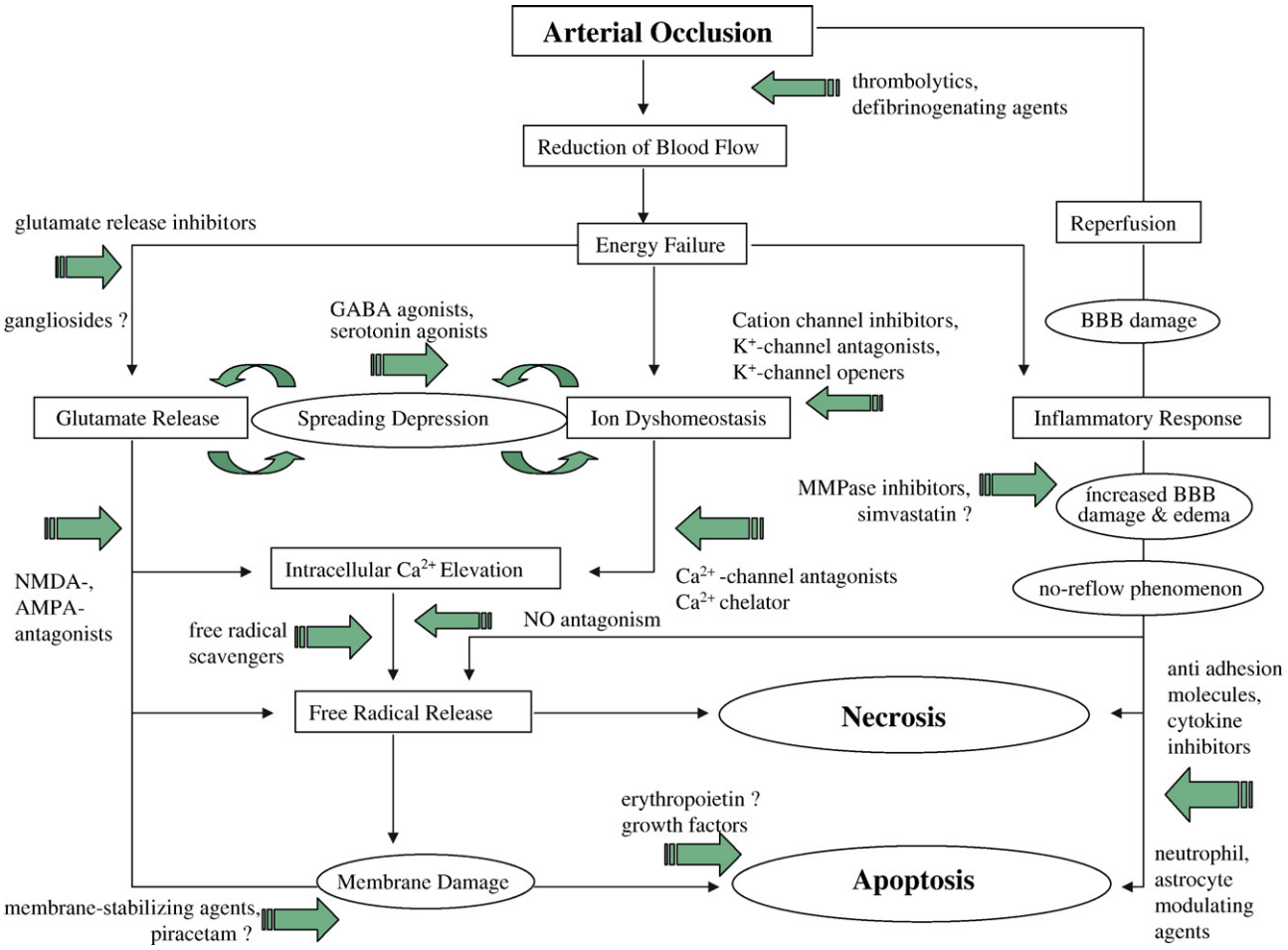

Fig. 3. Various compounds that interfere with ischemic cascade steps. NO, nitric oxide; GABA, gamma aminobutyric acid; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; MMPase, matrix metalloproteinase.

3.2. Acute ischemic stroke therapy

Major approaches developed to treat acute ischemic stroke fall into two categories: recanalization and neuroprotection. Whereas the goal of using thrombolytics or mechanical devices is the restoration of blood flow, neuroprotective regimen approach is aimed to protect the ischemic penumbra hence to prevent the ischemic core lesion growing.

3.2.1. Recanalization

To date rt-PA is the only pharmacological agent licensed to treat selected acute ischemic stroke patients when administered within 3 h after the onset of symptoms. However, only 1–8.5% of hospitalized patients receive treatment mainly due to the short time window for administration, need for a computed tomography scan, and exclusions due to other specific criteria (Millan and Davalos, 2006). Meta-analyses have provided evidence of an effect of intravenous thrombolysis beyond the 3 h time window, especially when improved selection criteria such as modern MRI protocols are applied (Schellinger et al., 2004). Ongoing DIFFUSE and EPITHET trials investigate whether specific MRI tools may predict a favourable clinical response to intravenous rt-PA therapy administered beyond 3 h after stroke onset. Data of ECASS III trial which was designed to look for safety and efficacy of rt-PA between 3 to 4 h after the onset of stroke are not yet available. Intra-arterial prourokinase application within 6 h of the onset of stroke resulted in better rates of recanalization then intravenous administration and improved clinical outcome but PROACT II trial data did not suffice for FDA approval (Furlan et al., 1999; Schellinger et al., 2004).

Endovascular revascularization has been an evolving treatment option due to positive results of MERCI trial using a mechanical embolectomy device for opening occluded vessels in stroke patients (Smith et al., 2005). Combination therapy of mechanical embolectomy and thrombolysis with t-PA is under clinical investigation (Multi MERCI trial) as an MRI guided trial of mechanical embolectomy (MR RESCUE). Optimism about potential efficacy of sonothrombolysis in the acute ischemic stroke therapy seems to have ended because of a phase II trial of low frequency transcranial ultrasound-mediated thrombolysis that was prematurely stopped due to increased rates of cerebral hemorrhages (Daffertshofer et al., 2005). Another technological advance of transcranial ultrasound uses targeted gaseous or fluid microbubbles to enhance thrombolysis and more data for the utility of this device will come from the continuing Interventional Management of Stroke study III (Sacco et al., 2007). Another alternative reperfusion strategy is

Table 1
Major recently completed and ongoing trials with neuroprotective drugs in acute ischemic stroke

Mechanism and compound	Trial*	Size**	Result
Glutamate antagonists			
Aptiganel (Cerestat)	Aptiganel acute stroke trial	628	No benefit
Gavestinel	GAIN international	1804	No benefit
	GAIN Americas	1367	No benefit
Dextrorphan	Phase II		No benefit
CGS 19755 (Selfotel)	ASSIST (abandoned)	567	No benefit, adverse effect
Eliprodil	Eliprodil Phase III (abandoned)		No results published
ACEA 1021	Phase II		Safe with low dose
YM872	2 phase II		Ongoing
ZK-200775	Phase II (abandoned)		Intolerable side-effects
Magnesium	FAST-MAG	1298	Ongoing
Glutamate release inhibition			
Sipatrigine	Phase II		Intolerable side-effects
Fosphenytoin	Fosphenytoin phase III	462	No benefit
Free-radical scavenging			
NXY-059 (Cerovive)	SAINT I	1722	Effective
Ebselen	Ebselen trial-phase III	394	Ongoing
Tirilazade mesylate	RANTTAS	556	No benefit
Edaravone	EAIS, phase II		Safe and effective
Calcium antagonism			
Nimodipine	VENUS	459	No benefit
	TRUST	1215	No benefit
Flunarizine	Flunarizine in stroke treatment	331	No benefit
Ca chelation			
DP-b99	Phase II		Ongoing
Potassium channel activation			
BMS-204352	Potassium channel opening trial	1978	No benefit
GABA agonism			
Clomethiazole	Clomethiazole acute stroke study	1360	No benefit
Diazepam	EGASIS	843	Initial results, no benefit
Serotonin agonism			
Bay×3702 (Repinotan)	RECT	660	Ongoing
SUN N4057 (Piclozotan)	Phase II		Ongoing
Opiate antagonism			
Nalmefene (Cervene, ReVex)	Cervene stroke study	368	No benefit
Naloxone	Phase II		Effective
Leukocyte adhesion inhibition			
Anti-ICAM-1 antibody (Enlimomab)	EAST (abandoned)	625	Adverse effect
HU23F2G	HALT	310	No benefit
Inhibition of cytokines			
IL-1 receptor antagonist	Phase II		Safe and effective
Membrane stabilization			
Citicoline	ICTUS	2600	Ongoing
Neutrophil modulation			
Neutrophil inhibitory factor	Phase II		No benefit
Unknown/multiple mechanisms			
Cerebrolysin	Phase II		Effective
Piracetam	PASS II		Ongoing
ONO-2506 (arundic acid)	RREACT	1320	Ongoing
Glycine	Phase II		Safe and effective
Lubeluzole	LUB-INT-13	1786	No benefit

Table 1 (*continued*)

Mechanism and compound	Trial*	Size**	Result
Unknown/multiple mechanisms			
Caffeine+ethanol	Phase I		Ongoing
Caffeinol+mild hypothermia	Phase I		Ongoing
GM-1 (ganglioside)	EST	792	No benefit
FGF (fibroblast growth factor)	Trafermin in acute ischemic stroke	302	Adverse effect
Erythropoietin	Phase II		Safe and effective
Simvastatin	Phase I		Safe and effective

*Trials are phase III otherwise it is noted.
**Number of patients included or planned.

desmoteplase, a salivatory plasminogen activator from the vampire bat that has been found safe and effective in both DIAS and DEDAS trials (Hacke et al., 2005; Furlan et al., 2006). Further dose safety and efficacy of desmoteplase is under investigation by the ongoing DIAS II trial researchers. Defibrinogenation is another strategy in acute stroke therapy. Ancrod, a purified fraction of venom from the Malaysian pit viper was found to be effective in the phase III STAT trial within 3 h of stroke onset (Sherman et al., 2000), but ineffective within 6 h in ESTAT trial (Hennerici et al., 2006) and ongoing two more phase III trials (ASP-I and ASP-II) have been designed to search for the safety and efficacy of a brief intravenous infusion of ancrod started within 6 h of stroke onset (Sherman et al., 2000). Potent antiplatelet agents (e.g., integrin IIbβ3 inhibitors) are available for vascular intervention, but their role in acute ischemic stroke has not been defined. Also uses of aspirin, clopidogrel or the combination aspirin/dipyridamole together with the plasminogen activator in acute ischemic stroke have not been studied yet but may confer benefit (Del Zoppo, 2004). Combination regimen of thrombolysis and glycoprotein IIb–IIIa inhibitor is a promising approach to increase recanalization rate according to case series (Lee et al., 2002). Phase III trial of abciximab (AbESTT-II), a monoclonal antibody directed against the platelet glycoprotein IIb–IIIa receptor, included acute ischemic stroke patients within 6 h after stroke onset, but it has been stopped prematurely due to a high rate of intracranial hemorrhage. The combination therapy of glycoprotein IIb–IIIa antagonist eptifibatide with rt-PA (CLEAR) might show the safety and efficacy of this approach. The use of streptokinase in acute stroke trials was abandoned because it had increased mortality and morbidity in several studies (MAST-1, 1995; Donnan et al., 1996). Another promising experimental approach to improve vessel recanalization by the use of a combination of systemic intravenous thrombolysis with local intra-arterial thrombolysis is to be tested in a phase III trial (Sacco et al., 2007).

Limitations of existing thrombolytic agents have prompted the development of new generation thrombolytic agents over the last decade: tenecteplase, reteplase, and staphylokinase. Tenecteplase which offers high-level fibrin-selectivity, a long half-life and increased thrombolytic potency on platelet-rich clots, has been tested for dose-escalating and further clinical trials are needed (Haley et al., 2005). A randomized phase-IIb trial comparing three different doses of tenecteplase with standard intravenous rt-PA, for patients presenting within 3 h is underway (Sacco et al., 2007). Reteplase also has a longer half-life and better penetration into a thrombus compared with rt-PA. Since reteplase seems to cause greater ADP-and thrombin-induced platelet aggregation and greater glycoprotein IIb/IIIa expression than rt-PA, to date safety of combinational therapy of reteplase with abciximab is being tested in different phase II trials (ROSIE, ROSIE-CT, Safety and Efficacy of Intra-arterial Reteplase and Intravenous Abciximab in Patients with Acute Ischemic Stroke Study) (Schumacher et al., 2005). Plasminogen activator derived from *Staphylococcus aureus* has a high specificity for fibrin and avoids systemic plasminemia but preclinical trials are still lacking (Toombs, 2001).

3.2.2. Neuroprotection

Experimental evidence supports the concept that establishing reperfusion alone is not enough to cease ischemic injury and each step of the ischemic cascade may be a genuine target for therapeutic intervention (Fig. 2). A large number of potentially neuroprotective agents directed at different harmful mechanisms in the ischemic cascade have been investigated in experimental animal stroke studies. Majority of the substances which were found to be neuroprotective in animals have failed in clinical trials. Potential reasons of this translation failure from laboratory to the clinic have been extensively discussed elsewhere and are beyond the scope of this review (Wiebers et al., 1990; Del Zoppo, 1995; Grotta, 1995; Ginsberg, 1996; De Keyser et al., 1999; Green et al., 2003; Fisher and Tatlisumak, 2005). Several suggestions by numerous investigators, to increase the quality of preclinical studies and for how to design clinical trials, are available (Hsu, 1993; STAIR, 1999; Alonso de Lecinana et al., 2005; Shuaib, 2006).

3.2.2.1. Pharmaceuticals. Various neuroprotective agents that may interfere with various steps of ischemic cascade are presented in Fig. 3. Ongoing neuroprotection clinical trials and major recently completed trials are listed in Table 1.

Key agents currently in development include citicoline (cell-membrane stabilizer), traxoprodil (NMDA antagonist), DP-B99 (metal ion chelator) and NXY-059 (a novel free-radical trapping agent) (Shuaib, 2006). Erythropoietin is thought to act as neuroprotectant by multiple mechanisms in experimental studies (Sadamoto et al., 1998). Erythropoietin is the first drug that was found effective in humans in acute ischemic stroke probably because the clinical trial was a "human rat trial" (Ehrenreich et al., 2002; Ehrenreich et al., 2004) and there is an urgent need for a

larger clinical trial. The new free-radical scavenger NXY-059 was tested in animal experiments according to STAIR criteria (Shuaib, 2006) and positive results could have been translated to humans successfully in a phase III study most likely due to appropriate clinical design based on laboratory findings (Lees et al., 2006), a larger phase III trial is ongoing (SAINT II, study size planned include 3200 patients).

It is appealing to develop combination therapies for ischemia that combine vascular and parenchymal effects, especially because preclinical data suggest that many drug combinations improve neuroprotection and extend the therapeutic window in ways not possible with a single agent (Lo et al., 2005).

3.2.2.2. Brain hypothermia. The transfer of hypothermia treatment from laboratory to the clinic undertaken in two pilot studies (COOLAID and cool aid) showed the feasibility and safety of induced hypothermia in acute stroke patients (Krieger et al., 2001; De Georgia et al., 2004). Possible neuroprotective effect of mild hypothermia induced by surface cooling technique (NOCSS, Nordic Cooling Stroke Study) in patients presented within 6 h after the onset of stroke is now under evaluation in a clinical trial. Besides invasive cooling technique via femoral-based catheter cooling system, that is currently used in Combined Cytoprotection TPA stroke Trial, new techniques providing selective brain hypothermia non- or minimal invasively are under development in rats (Taniguchi et al., 2005; Clark and Colbourne, 2007).

4. Summary

Ischemic stroke is a heterogeneous disorder with a complex pathophysiology. Experimental ischemic stroke models have contributed to our understanding of the mechanisms occurring during ischemic brain injury. Recognition of each step of the ischemic cascade has lead to the possibility of developing a new class of neuroprotective drug that interferes with a specific mechanism or multiple mechanisms of ischemic injury. Animal modeling has served for recognition of the pathophysiology and for testing novel antiischemic molecules, such as thrombolytic agents, neuroprotectives, and neurorestorative agents potentiating neuroplasticity. Although currently rt-PA is the only approved therapy of acute ischemic stroke within a 3 h time window, neuroprotection offers a viable strategy to treat acute ischemic stroke patients and may widen the time window of thrombolytic therapy. Novel imaging techniques such as DWI and PWI herald a better selection of stroke patients to apply thrombolysis and neuroprotectants. Despite the many failures of neuroprotective drug clinical studies during the last two decades, there are now good reasons to be optimistic. Novel antiischemic therapies will soon be available with the help of improved preclinical assessment and clinical trial design.

Acknowledgments

This work was supported in part by the University of Helsinki, the Helsinki University Central Hospital, the Sigrid Juselius Foundation, the Finnish Academy of Sciences, the Finnish Neurological Foundation, the Maire Taponen Foundation, and the Center for International Mobility. The authors declare no conflict of interest.

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