MECHANISMS, CHALLENGES AND OPPORTUNITIES IN STROKE

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Over the past two decades, research has heavily emphasized basic mechanisms that irreversibly damage brain cells after stroke. Much attention has focused on what makes neurons die easily and what strategies render neurons resistant to ischaemic injury. In the past few years, clinical experience with clot-lysing drugs has confirmed expectations that early reperfusion improves clinical outcome. With recent research emphasizing ways to reduce tissue damage by both vascular and cell-based mechanisms, the spotlight is now shifting towards the study of how blood vessels and brain cells communicate with each other. This new research focus addresses an important need in stroke research, and provides challenges and opportunities that can be used to therapeutic advantage.

NEUROLOGICAL DISEASES

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Stroke, a brain attack, is the third leading cause of death in the Western world. Worldwide, about 5.5 million people died from stroke in 1999 — approximately 10% of all deaths. There are more than 3.5 million survivors in the United States alone, and the disease remains a major cause of disability. In practice, 'stroke' refers to an umbrella of conditions caused by the occlusion or haemorrhage of blood vessels supplying the brain. Most often, blood flow is compromised within the territory of an occluded blood vessel. Less commonly, stroke results from the absence of blood flow to the entire brain due to cardiac arrest. In all instances, stroke ultimately involves death and dysfunction of brain cells, and neurological deficits that reflect the location and size of the compromised brain area.

In July 2001, the National Institutes of Neurological Disorders and Stroke convened the Stroke Program Review Group[1] to advise on directions for basic and clinical stroke research for the next decade. This meeting emphasized the relevance of dynamic interactions between endothelial cells, vascular smooth muscle, astroglia and microglia, neurons and associated tissue matrix proteins — the neurovascular unit — and its importance to disease pathophysiology. The neurovascular unit places stroke in the context of an integrative tissue response in which all cellular and matrix elements, not just neurons or blood vessels, are players in the evolution of tissue injury. The concept might also apply to other brain disorders in which there is a significant vascular component, such as VASCULAR DEMENTIA, migraine, trauma, MULTIPLE SCLEROSIS and, possibly, the ageing brain.

This review will focus on emerging concepts in stroke involving relevant components of the neurovascular unit, as well as highlighting those that are promising targets for stroke therapy. It will begin by briefly discussing risk factors. It will then highlight major mechanisms of cellular injury, and focus on mechanisms and tissue processes that we see as crucial to potential positive developments in stroke therapy.

Risk factors and prevention

Ischaemic strokes share certain common features with myocardial infarction, such as overlapping risk factors (for example, diabetes and elevated homocysteine blood levels), similar initiating events, and the need for acute treatment to salvage dying tissues. Elevated arterial blood pressure is a particularly notable risk factor for stroke, and modest decreases (< 5 mm Hg) significantly reduce the frequency of stroke events, fatality rates and functional impairment in high-risk subjects, even when blood pressure readings at baseline are only marginally elevated[2]. Angiotensin-converting enzyme (ACE)

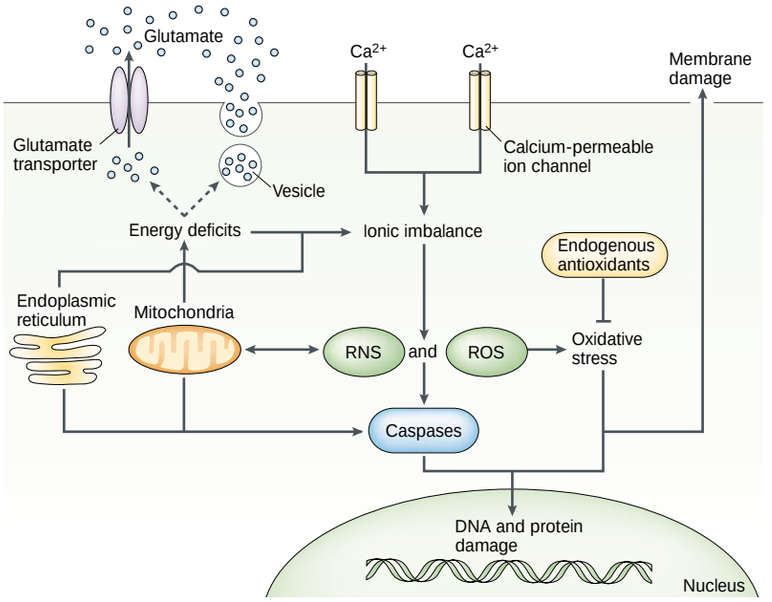


Figure 1 | **Major pathways implicated in ischaemic cell death: excitotoxicity, ionic imbalance, oxidative and nitrosative stresses and apoptotic-like mechanisms.** There is extensive interaction and overlap between multiple mediators of cell injury and cell death. After ischaemic onset, loss of energy substrates leads to mitochondrial dysfunction and generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Additionally, energy deficits lead to ionic imbalance, excitotoxic glutamate efflux and build-up of intracellular calcium. Downstream pathways ultimately include free radical damage to membrane lipids, cellular proteins and DNA, as well as calcium-activated proteases and caspase cascades that dismantle a wide range of homeostatic, reparative and cytoskeletal proteins.

VASCULAR DEMENTIA
A state of diminished cognition that results from repeated cerebral strokes, with a step-like deterioration in intellectual functions.

MULTIPLE SCLEROSIS
A neurodegenerative disorder characterized by demyelination of central nervous system tracts. Symptoms depend on the site of demyelination and include sensory loss, weakness in leg muscles, speech difficulties, loss of coordination and dizziness.

ATHEROSCLEROSIS
A condition in which lipids accumulate on the inner walls of arteries and eventually obstruct blood flow.

POLYMORPHISM
The simultaneous existence in the same population of two or more genotypes in frequencies that cannot be explained by recurrent mutations.

REPERFUSION
The restoration of blood flow to an ischaemic region. Reperfusion might cause additional tissue damage after a stroke.

inhibitors and diuretics lower the risk of stroke, as does antiplatelet therapy, in addition to other antihypertensive therapies.

ATHEROSCLEROSIS is another important risk factor. Large clinical trials substantiate claims that lipid-lowering drugs — 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors (statins) — that normalize blood vessel wall function are promising drugs for stroke prophylaxis[3]. For example, statins reduce stroke risk for both first, and secondary events by at least 25–30%, and prevention can be successful even when blood lipid values are only minimally elevated when treatment is started. It has been estimated that the benefits of combining prophylactic strategies is more than additive, so that risk reduction might approach 70%, at least for heart attack[4]. Similar risk reductions are anticipated for stroke, although no estimates have been published to date. Despite these encouraging gains in preventive therapy, in the future there will be an even greater need for acute stroke treatments, as shifting demographics and an ageing population demand better acute therapy, as well as better outcomes after stroke.

Genetic background is also acknowledged as an important stroke risk factor. In addition to a higher concordance rate among monozygotic versus dizygotic siblings, a maternal or a paternal family history confers a higher risk of stroke. Except for several important, albeit infrequent, autosomal disorders[5], most pedigrees do not follow simple Mendelian inheritance, and the evidence points to a complex trait with combined effects of several genes interacting with environmental cues. POLYMORPHISMS in candidate genes such as *ACE*, endothelial nitric oxide synthase (*eNOS*), *APOE* and β-fibrinogen (FGB) reportedly increase the risk of stroke, but these findings await confirmation. Most investigators agree that genomics and proteomics are the most promising recent developments impacting the future of stroke prevention, diagnosis, treatment and outcome.

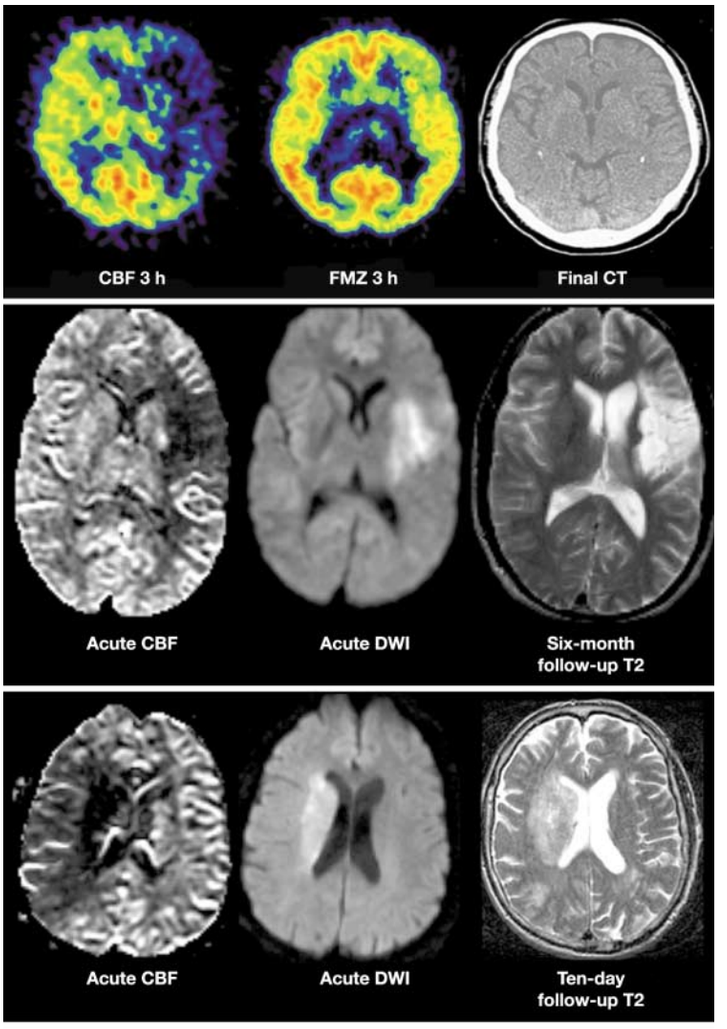
Cerebral pathophysiology

Temporal and spatial events after stroke. Ischaemic stroke is characterized by complex spatial and temporal events evolving over hours or even days. Within the centre or core of the ischaemic territory, blood flow deficits, low ATP levels and energy stores, ionic disruption and metabolic failure are severe, and cell death progresses in minutes. However, the peripheral zones within the flow-compromised territory — the ischaemic penumbra (BOX 1) — suffer milder insults due to residual perfusion from collateral blood vessels. During the early stages of occlusion, the penumbra might comprise as much as a third to half the lesion volume, and actively metabolizes glucose. In this perinfarct margin of metabolically and ionically challenged, metastable tissues, cells die more slowly as the penumbra collapses and the lesion expands over time[6]. In the penumbra, active cell death mechanisms are recruited, and targeting these mechanisms provides promising therapeutic approaches. Within the core territory, salvage of rapidly dying brain cells might not be feasible without early REPERFUSION. In fact, once tissues are damaged beyond a critical point, cell death seems inevitable, despite restoration of both blood flow and ATP levels.

Active cell death mechanisms. There are at least three fundamental mechanisms leading to cell death during ischaemic brain injury: excitotoxicity and ionic imbalance, oxidative/nitrosative stress, and apoptotic-like cell death (FIG. 1). These mechanisms demonstrate overlapping and redundant features. They mediate injury within neurons, glia and vascular elements, and at the subcellular level, they impact the function of mitochondria, nuclei, cell membranes, endoplasmic reticula and lysosomes. Cell bodies and their processes and synaptic endings are all at risk, and cell death might proceed by mechanisms promoting rupture, lysis, phagocytosis or involution and shrinkage.

Just as cell death occurs in well-defined subsets of neurons in chronic neurodegenerative diseases, so does selective death develop in well-defined subsets of cells in the brain after transient global cerebral ischaemia[7]. In general, neurons and oligodendrocytes seem to be more vulnerable to cell death than astroglial or endothelial cells, and among neurons, specific populations seem to be especially susceptible. CA1 hippocampal pyramidal neurons, cortical projection neurons in layer 3, subsets of neurons in dorsolateral striatum and Purkinje cells of the cerebellum are particularly susceptible. We presume that 'susceptible or vulnerable' brain cells reflect a phenotype and genotype less well endowed to survive ischaemic cell stresses based on the mechanisms that are described later.

Box 1 | Imaging the penumbra



In focal strokes, the 'core' territory refers to the region with the most severe reduction in blood flow and within which brain cells rapidly die. Adjacent to the core is the 'penumbra', a peripheral zone of moderate to mild ischaemia where residual blood flow might transiently sustain tissue viability. Imaging studies have validated the concept that tissue viability is heterogeneous distal to an occluded brain blood vessel. In animal models, the ischaemic penumbra can be visualized by autoradiographic techniques that compare regions of reduced blood flow with regions of actively metabolizing tissue (2-deoxyglucose), or larger regions of suppressed protein synthesis with core areas in which there is complete loss of ATP. In humans, positron-emission tomography (PET) and magnetic resonance imaging (MRI) can be used to visualize the ischaemic penumbra. PET can detect oxygen-utilizing tissue (oxygen extraction fraction) within regions of low blood flow, as well as locate ^{11}C-flumazenil recognition sites on viable neurons within underperfused brain areas. With MRI, there is often a volume mismatch between tissue showing reduced water molecule diffusion (a signature for cell swelling and ischaemic tissue) and a larger area of compromised tissue perfusion early after stroke onset. The difference reflects potentially salvageable tissue. Imaging methods such as these can optimize the selection of candidates for thrombolytic therapy or for adjunctive therapy many hours after stroke onset. Importantly, imaging might also provide quantitative surrogate endpoints for clinical trials. The upper panel of the figure shows PET scans taken within 3 hours of stroke onset (left and middle images) in a 69 year-old female showing significant cerebral blood flow (CBF) deficits consistent with focal ischaemia. However, within the area of compromised blood flow neurons remain viable at this time based on the distribution of ^{11}C-flumazenil (FMZ) binding to benzodiazepine receptors within the ischaemic territory. Prompt treatment with tissue plasminogen activator (tPA) lysed the clot, reperfused the brain and rescued all tissues. Follow-up computerized axial tomography scans at 3 weeks showed no evidence of infarction. (Images courtesy of W. D. Heiss, Cologne, Germany.) The middle panel of the figure shows MRI scans obtained within 4 hours of onset of hemiplegia and aphasia (left and middle images) in a 33 year-old male. CBF images showed a clear area of reduced perfusion in the middle cerebral artery territory, involving basal ganglia and cortex. A more restricted zone of reduced diffusion was detected on diffusion-weighted imaging (DWI) indicative of cell swelling and early ischaemic injury in the basal ganglia. This patient's clot was not lysed. At 6 months, infarction in both basal ganglia (core) and cortex (penumbra) was present on the T2 weighted image (right image). (Images courtesy of O. Wu and G. Sorensen, Boston, USA.) The lower panel shows MRI scans obtained between 4 and 5 hours after stroke onset, immediately before tPA treatment (left and middle images) in a 78 year-old male. Ischaemia was present within large areas of the middle cerebral artery territory including the cortex and deeper nuclei, although only the areas of the basal ganglia were affected on DWI. A follow-up T2 weighted MRI scan (right image) at 10 days showed that cortical penumbral tissue was rescued after tPA treatment, and that only core territory in the basal ganglia proceeded to infarction. (Images courtesy of O. Wu and G. Sorensen, Boston, USA.)

Excitotoxicity and ionic imbalance. After stroke onset, the loss of energy stores results in ionic imbalance, neurotransmitter release and inhibition of reuptake (for example, of glutamate, the major excitatory transmitter in the mammalian brain). Subsequently, binding of glutamate to ionotropic NMDA (*N*-methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors promotes excessive calcium influx, which triggers an array of downstream phospholipases and proteases that degrade membranes and proteins that are essential for cellular integrity. Mitochondria have also been implicated in toxicity, because of oxygen radical generation and the release of death-inducing factors (discussed later). In addition, ionotropic glutamate receptors (GluRs) promote an excessive influx of sodium with concomitant cell swelling and OEDEMA. Over the past decade, a large number of papers have been published relating excitotoxicity to ischaemic cell death[8], and readers are referred to these articles for more detailed information.

In experimental models of stroke, extracellular glutamate levels increase in the MICRODIALYSATE[9], and blockade of GluRs can reduce infarction[10]. NMDA receptor antagonists prevent the expansion of infarction in part by blocking spontaneous and spreading depolarizations of neurons and glia (cortical SPREADING DEPRESSION)[11]. More recently, activation of GluRs of the metabotropic subfamily (mGluRs) have been implicated in ischaemic cell death. Depending on the subtype, mGluRs can trigger either pro-survival or pro-death signals in ischaemic neurons[12].

Upregulation or downregulation of specific GluR subunits contributes to stroke pathophysiology in different ways[13]. For example, after global cerebral ischaemia, there is a relative reduction of calcium-impermeable GluR2 subunits in the AMPA-type receptors, which makes these receptors more permeable to deleterious calcium influx[14]. Antisense knockdown of calcium-impermeable GluR2 subunits significantly increased hippocampal injury in a rat model of transient global cerebral ischaemia, confirming the importance of these regulatory subunits in mediating neuronal vulnerability[15]. Variations in NMDA receptor subunit composition also affect tissue outcome. Knockout mice deficient in the NR2A subunit showed decreased cortical infarction after focal cerebral ischaemia[16]. Medium spiny striatal neurons, which are selectively vulnerable to ischaemia and excitotoxicity, preferentially express NR2B subunits[17]. Understanding how the expression of specific GluR subunits modifies cell survival should stimulate the discovery of drugs that selectively target specific subunits for stroke therapy. As noted above, perturbations in ionic homeostasis also have a crucial role in cerebral ischaemia. For example, L, P/Q and N-type calcium channel functions mediate excessive calcium influx, and calcium channel antagonists reduce ischaemic brain injury in preclinical studies (for reviews, see REFS 18–20).

Besides calcium, imbalances in other ions are important after ischaemia. Large amounts of zinc are stored in vesicles of excitatory neurons and are co-released upon depolarization[21]. *In vitro*, excessive zinc is neurotoxic[22], and loss of zinc from presynaptic terminals correlates with zinc translocation into cell bodies and subsequent neuronal death after focal cerebral ischaemia[23]. Recently, imbalances in potassium have also been implicated in ischaemic cell death. Neurons express a class of calcium-sensitive high-conductance potassium channels, and compounds that selectively modulate these channels protect the brain against stroke in animal models[24].

Oxidative and nitrosative stress. The reactive oxygen radical is a key mediator of tissue damage after reperfusion in many organs including heart, kidney and brain. Mitochondria are strongly implicated, and this might be due to excessive superoxide production during electron transport and inhibition of mitochondrial electron transport mechanisms by free radicals, leading to even more oxygen radical generation[25,26]. High calcium, sodium and ADP levels in ischaemic cells stimulate excessive mitochondrial oxygen radical production, as does the addition of NMDA to cultured cells[27]. Oxygen radicals are also produced during enzymatic conversions, such as the cyclooxygenase-dependent conversion of arachidonic acid to prostanoids and the degradation of hypoxanthine, especially upon reperfusion. Furthermore, free radicals are also generated during the inflammatory response after ischaemia (see later discussion). Not surprisingly, then, oxidative stress, excitotoxicity, energy failure and ionic imbalances are inextricably linked, and contribute to ischaemic cell death.

Oxygen radical production might be especially harmful to injured brain because levels of endogenous antioxidant enzymes (including superoxide dismutase (SOD), catalase and glutathione) and antioxidant vitamins (for example, α-tocopherol and ascorbic acid) are normally not high enough to match excess radical formation[28]. After ischaemia and particularly reperfusion, production of reactive oxygen species, including superoxide and hydroxyl radicals, overwhelms endogenous scavenging mechanisms and directly damages lipids, proteins, nucleic acids and carbohydrates. Importantly, oxygen radicals and oxidative stress facilitate MITOCHONDRIAL TRANSITION PORE (MTP) formation. MTP dissipates the proton motive force that is required for oxidative phosphorylation and ATP generation, and, as a result, mitochondria release their constituents — including apoptosis-related proteins — within the inner and outer mitochondrial membranes[29] (see later discussion). Upon reperfusion and renewed tissue oxygenation, dysfunctional mitochondria might generate oxidative stress and MTP formation[30].

Oxidative and nitrosative stresses are modulated by enzyme systems such as SOD and the NOS family (FIG. 2). Mice with enhanced expression of SOD show reduced injury after cerebral ischaemia, whereas those with a deficiency show increased injury, proving that excessive oxygen radical production is fundamental to ischaemic brain injury[31–34]. In the case of NOS, its activation during ischaemia might lead to nitric oxide combining with superoxide to generate the strong oxidant peroxynitrite[35]. Mice deficient in expression of the neuronal NOS isoform[36] or the inducible isoform[37] (in white blood cells,

OEDEMA
The presence of abnormally large amounts of fluid in the intercellular tissue spaces.

MICRODIALYSATE
A product of microdialysis — a technique to monitor the composition of the extracellular space in living tissue. A physiological solution is slowly pumped through a microdialysis probe. With time, this solution equilibrates with the extracellular fluid, making it possible to measure the concentration of the molecules of interest in the microdialysate.

SPREADING DEPRESSION
A slowly moving depression of electrical activity in the cerebral cortex. It consists of a wave of depolarization that can last for up to 2 minutes and travels at a speed between 3 and 12 mm min^{-1}. Wave passage is accompanied by increased blood flow and is followed by a prolonged period of vasodilation. Spreading depression seems to be related to migraine, and has been observed to accompany cerebral ischaemia.

MITOCHONDRIAL TRANSITION PORE
Regulated mitochondrial megachannel, the formation of which presumably requires the apposition of proteins of the inner and outer mitochondrial membranes. Opening of this pore can lead to the collapse of the mitochondrial transmembrane potential, uncoupling of the respiratory chain, production of superoxide ions, outflow of calcium and release of soluble intermembrane proteins.

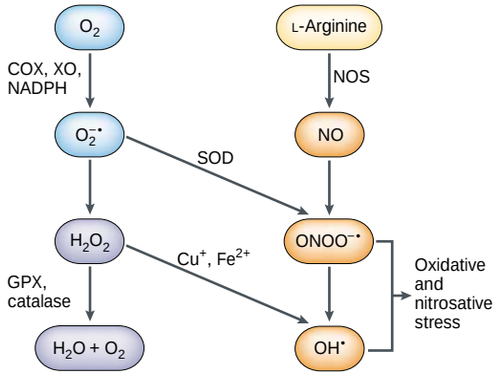


Figure 2 | **Interactions between pathways that generate oxygen and nitrogen radicals.** Combination of superoxide ($O_2^{-\bullet}$) and nitric oxide (NO) generates the potent radical peroxynitrite anion ($ONOO^{-\bullet}$). Metal (Cu^+ and Fe^{2+}) catalysed pathways can also produce the hydroxyl radical ($OH^\bullet$) from hydrogen peroxide (H_2O_2). COX, cyclooxygenase; GPX, glutathione peroxidase; NOS, nitric oxide synthase; SOD, superoxide dismutase; XO, xanthine oxidase.

primarily) show less tissue damage, compared with their wild-type counterparts, after cerebral ischaemia. Similarly, the generation of nitric oxide and oxidative stress is linked to DNA damage and activation of poly-(ADP-ribose) polymerase (PARP1), a nuclear enzyme that facilitates DNA repair and regulates transcription[38]. PARP1 catalyses the transformation of β-nicotinamide adenine dinucleotide (NAD^+) into nicotinamide and poly(ADP-ribose). In response to DNA strand breaks, PARP1 activity becomes excessive and depletes the cell of NAD^+ and possibly ATP. Ischaemic cell death by necrotic and apoptotic mechanisms is suppressed by inhibiting PARP1 activity or by deleting the *parp1* gene[39,40], indicating the potential of this enzyme as a therapeutic target.

Apoptotic-like pathways. Loss of membrane integrity and organelle failure are the most prominent mechanisms of cell death in ischaemia. However, research clearly implicates mechanisms that follow apoptotic-like pathways and cascades, particularly within the penumbra. Both caspase-dependent and caspase-independent mechanisms have been described. Caspases, a family of cysteine aspartases, are constitutively expressed in adult and especially newborn brain cells, particularly neurons. They are cleaved and activated in a sequential manner — triggered by stimuli either extrinsic or intrinsic to cells[41]. Mild ischaemic injury preferentially induces cell death by an apoptotic-like process rather than by necrosis, although 'aponecrosis' more aptly describes the pathology. Cell type, cell age and brain location render cells more or less resistant to apoptosis or necrosis. Importantly, caspase-dependent cell death utilizes energy in the form of ATP. Because ATP levels decrease rapidly after severe ischaemia, necrotic cell death usually predominates[42]. Although ischaemia and ATP depletion typically cause acute cell swelling, ionic imbalances can also trigger cell shrinkage and apoptotic-like cell death under certain conditions[43]. Moreover, potassium efflux through the NMDA channel induces cell body shrinkage, increases caspase activity and augments apoptosis in cultured neurons when extracellular concentrations of sodium and calcium are reduced[44]. So, there are mechanistic links between glutamate-mediated excitotoxicity and apoptotic programs, which might provide multiple targets for combination stroke therapy.

Apoptogenic triggers[45] include excessive oxygen radical formation[46], death-receptor ligation[47], DNA damage[48] and, possibly, lysosomal protease activation[49,50]. Cross-talk between cell death pathways leading to apoptosis or necrosis contributes to a complex phenotype that is difficult to determine on the basis of morphological grounds alone. Several mediators that facilitate cross-communication between cell death pathways[51] include the calpains[14], cathepsin B[49,50], nitric oxide[51–54] and PARP[55].

The normal human brain expresses caspases 1, 3, 8 and 9, apoptotic protease-activating factor 1, death receptors, the transcription factor p53, DNA fragmentation factor DFF45, plus several members of the Bcl2 family of proteins, all of which are implicated in apoptosis. Presumably, caspases are cleaved and activated in human brain by mechanisms similar to those documented in experimental models including acute ischaemia, trauma and neurodegenerative diseases[56]. When activated, executioner caspases (caspases 3 and 7) target and degrade numerous substrate proteins in several cell compartments, leading to cell demise. Caspase 3 is the most abundant cysteine protease in brain. It is cleaved acutely in neurons and is present in the ischaemic core, as well as the penumbra, in the early stages of reperfusion[57]. A second wave of caspase cleavage usually follows within hours or days, and probably participates in delayed ischaemic cell death. Cytosolic Bid, a pro-apoptotic Bcl2 family member lying upstream from mitochondrial activation, facilitates cytochrome *c* release and promotes APOPTOSOME formation[58]. Shortly after, cleaved products of numerous caspase-3 substrate proteins are formed (for example, gelsolin, actin, PARP1 and inhibitor of caspase-activated deoxyribonuclease (ICAD)), and there is evidence of internucleosomal endonuclease activity and DNA fragmentation.

Cell death can be suppressed by administering caspase inhibitors during and after vessel occlusion[59,60]. In fact, the therapeutic window seems to be temporally related to the onset of caspase activation, and caspase inhibitors attenuate ischaemic brain injury and neurological function when administered up to the point of protease activation[61]. Gene deletions of Bid or caspase 3 (REF. 62) render mice more resistant to ischaemic injury than their wild-type counterparts, and cultured neurons from these mutant mice survive better when exposed to oxygen/glucose deprivation. Strategies to silence caspases or suppress apoptosis-related gene products using antisense oligonucleotides or viral vector-mediated gene transfer substantiate these observations[63]. However, caspase inhibitors do not reduce infarct size in all brain ischaemia models. This might relate to the intensity and duration of ischaemia, robustness of caspase expression and cleavage, upregulation of caspase-independent or redundant cell death pathways and/or shortcomings of the administered agent.

APOPTOSOME
A multiprotein complex that consists of several (probably seven) molecules of APAF1 bound to cytochrome *c* and caspase 9. The apoptosome represents a holoenzyme complex, which maintains caspase 9 in an active conformation.

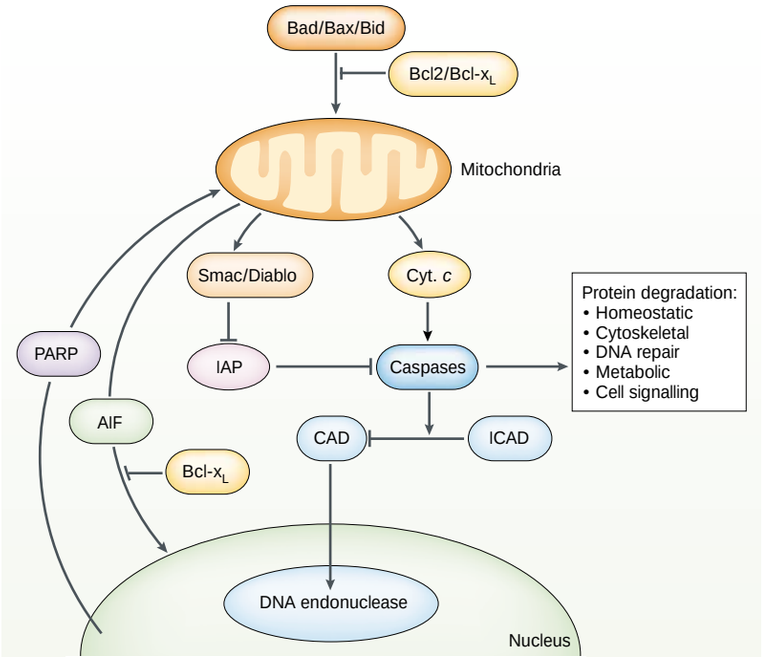


Figure 3 | **Cell death pathways that are relevant to an apoptotic-like mechanism in cerebral ischaemia.** Release of cytochrome *c* from the mitochondria is modulated by pro- as well as anti-apoptotic Bcl2 family members. Cytochrome *c* release activates downstream caspases through apoptosome formation (not shown) and caspase activation can be modulated by secondary mitochondria-derived activator of caspase (Smac/Diablo) indirectly through suppressing protein inhibitors of apoptosis (IAP). Effector caspases (caspases 3 and 7) target several substrates, which dismantle the cell by cleaving homeostatic, cytoskeletal, repair, metabolic and cell signalling proteins. Caspases also activate caspase-activated deoxyribonuclease (CAD) by cleavage of an inhibitor protein (ICAD). Caspase-independent cell death might also be important. One mechanism proposes that poly-(ADP ribose) polymerase (PARP) activation promotes the release of apoptosis-inducing factor (AIF), which translocates to the nucleus, binds to DNA and promotes cell death through a mechanism that awaits clarification.

The tumour necrosis factor (TNF) superfamily of death receptors regulates upstream caspase processes in brain and spinal cord ischaemia, as well as in central nervous system (CNS) trauma. In spinal cord ischaemia and brain trauma, Fas assembles as part of a death-inducing signalling complex along with FADD (Fas-associated protein with death domain) and procaspase 8, and recruitment of this complex temporally corresponds to cell death based on co-localization of cleaved caspases in TUNEL (terminal deoxynucleotidyl transferase labelling)-positive cells[64]. Hybrid mice that are deficient in both Fas and TNF expression are strongly resistant to ischaemic injury compared with the wild-type strain[48]. Also, significant brain protection is achieved when neutralizing antibodies against both FasL and TNF are injected into wild-type mice. The probable targets for protection by this mechanism include those cells and tissues that express both Fas and TNF receptors, such as inflammatory or immune cells, astrocytes and neurons, and those cells that reside within the microvasculature.

Caspase-independent apoptosis has recently been recognized as an important component of cell death pathways. It develops in cultured neurons following activation of PARP1 induced by NMDA receptor activation, and in fibroblasts following exposure to oxidizing agents. Apoptosis-inducing factor (AIF), a 67-kDa flavoprotein that was first reported in 1999 by Susin *et al.*[65], is implicated as a key signalling molecule in this cascade. Yu *et al.*[55] found that PARP1 activation promotes the release of AIF from mitochondria. AIF then relocates to the nucleus, binds DNA, promotes chromatin condensation and ANNEXIN STAINING, and kills cells by a complex series of incompletely understood events. Cell death by AIF seems to be resistant to treatment with pan-caspase inhibitors but can be suppressed by neutralizing AIF before its nuclear translocation. If AIF contributes to cell death in *in vivo* models of injury, it deserves careful scrutiny as a new therapeutic target for stroke.

Whereas caspases cause cell demise, upregulation or overexpression of Bcl2 or Bcl-x$_L$ suppresses cell death through several mechanisms, including stabilization of the MTP, suppression of cytochrome *c* or AIF release, and silencing of pro-apoptotic Bcl2 family members. The Bcl2 family of proteins is homologous to the *ced-9* gene in *Caenorhabditis elegans* that suppresses pro-apoptotic pathways. Mice overexpressing Bcl2, or wild-type animals administered a Bcl-x$_L$ fusion protein containing the human immunodeficiency virus (HIV)/transactivating activator of transcription (TAT) protein-transduction domain (PTD), are more resistant to ischaemia[10], as are cells and tissues exposed to a super anti-apoptotic artificial protein fused to the same PTD[66].

The mitochondria and the nucleus occupy centre stage in the arena of cell death and apoptosis[51,67,68] (FIG. 3). The mitochondria, with their complement of pro-apoptotic proteins (for example, cytochrome *c*, secondary mitochondria-derived activator of caspase (Smac/Diablo) and endonuclease), transition pore formation and role in oxidative phosphorylation, are uniquely positioned to detect and amplify cell death signalling processes. Mitochondria also possess membrane recognition elements for pro-apoptotic signalling molecules, such as Bid, Bad and Bax, that reside upstream of the cascade. Until recently, the nucleus was viewed primarily as a target of pro-death cytosolic proteins. Emerging data indicate that the nucleus also releases signalling molecules that recruit subcellular organelles in the cell death process, such as during caspase-independent apoptosis. Treatment of stroke patients by manipulating apoptotic pathways remains a daunting task but might one day be achievable using a non-peptide, brain- and cell-penetrant drug, probably combined with a second neuroprotectant targeting mechanism that aims to block necrosis and enhance reperfusion.

Targeting the neurovascular unit

Although much progress has been made in dissecting the molecular pathways of excitotoxicity, oxidative stress and apoptosis in ischaemic cell death, clinically effective stroke treatments remain elusive. Focusing on a single intracellular pathway or cell type might not suffice, and ultimately, strategies must look beyond the single cell for a more integrative answer to brain damage after ischaemic injury. So, the neurovascular unit

TUNEL METHOD
This method enables the visualization of cells undergoing apoptosis by labelling the broken ends of the double-stranded DNA with biotin-conjugated dUTP, using the enzyme terminal deoxynucleotidyl transferase.

ANNEXIN STAINING
Annexin V is a calcium- and phospholipid-binding family of proteins with vascular anticoagulant activity. As apoptotic cells express phosphatidylserine in their membranes, the affinity of annexin for this phospholipid makes this protein useful for labelling cells undergoing programmed cell death.

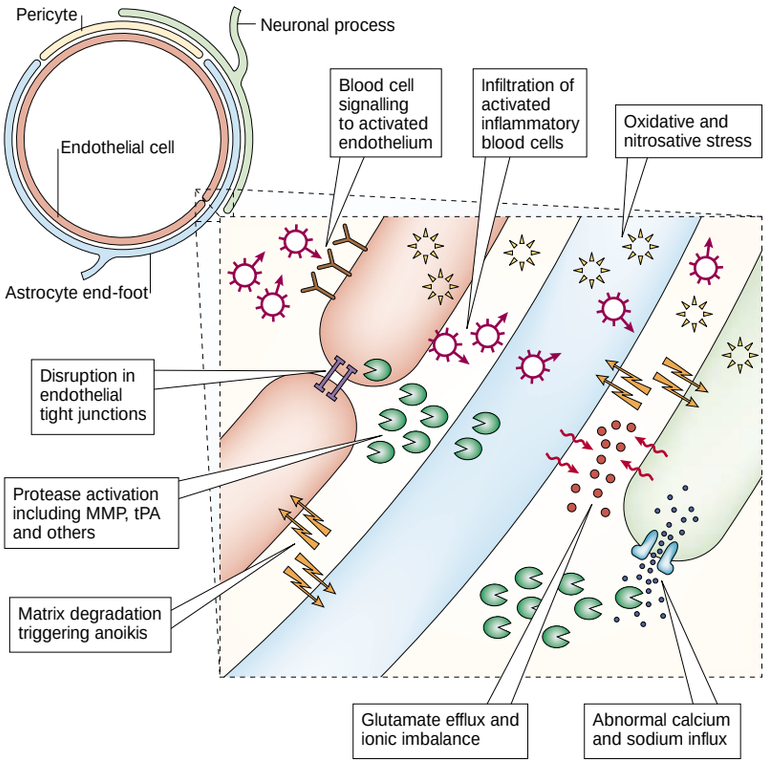


Figure 4 | **Schematic view of the neurovascular unit or module, and some of its components.** Circulating blood elements, endothelial cells, astrocytes, extracellular matrix, basal lamina, adjacent neurons and pericytes comprise the neurovascular unit. After ischaemia, perturbations in neurovascular functional integrity initiate several cascades of injury. Upstream signals such as oxidative stress, together with neutrophil and/or platelet interactions with activated endothelium, upregulate matrix metalloproteinases (MMPs), plasminogen activators and other proteases, which degrade matrix and lead to blood–brain barrier leakage. Inflammatory infiltrates through the damaged blood–brain barrier amplify brain tissue injury. Additionally, disruption of cell-matrix homeostasis might also trigger anoikis-like cell death in both vascular and parenchymal compartments. Overlaps with excitotoxicity have also been documented through tissue plasminogen activator (tPA)-mediated interactions with the NMDA (*N*-methyl-D-aspartate) receptor, which augment ionic imbalance and cell death.

provides a conceptual model comprised of cerebral endothelial cells, astrocytes and neurons, along with an extracellular matrix that maintains the integrity of brain tissue (FIG. 4). This modular concept emphasizes the dynamics of vascular, cellular and matrix signalling in the brain in both the grey and white matter[1] (BOX 2). For example, efficacy of the blood–brain barrier is crucially dependent on endothelial–astrocyte–matrix interactions[69]. Perturbation of the neurovascular matrix — which includes basement membrane components such as type IV collagen, heparan sulphate proteoglycan, laminin and fibronectin — disrupts the cell–matrix and cell–cell signalling that maintains neurovascular homeostasis. Although many proteases, including cathepsins and heparanases, might contribute to extracellular matrix proteolysis, we focus on the roles of plasminogen activator (PA) and matrix metalloproteinase (MMP) in stroke for three main reasons. First, these are the two major protease systems that modulate matrix in the brain. Second, plasminogen activators (for example, tissue plasminogen activator or tPA) are used for reperfusion therapy in stroke patients. Third, emerging data show important linkages between tPA, MMPs, oedema, and haemorrhage after stroke.

Proteolysis and the neurovascular matrix. MMPs are a family of over 20 zinc endopeptidases that have been divided into five classes including gelatinases (MMP2 and 9), collagenases (MMP1, 8 and 13), stromelysins (MMP3, 10 and 11), membrane-type MMPs (MMP14–17) and others (for example, MMP7 and 12) (REF. 70). Many MMPs can be produced by all cell types of the neurovascular unit[71]. Together with the PA system, MMPs play a central part in brain development, as they modulate the extracellular matrix to allow neurite outgrowth and cell migration[72]. In the adult brain, MMPs are generally downregulated, although some data indicate that active microregions of proteolysis persist and facilitate neuronal plasticity for learning and memory[73]. MMPs are tightly regulated at transcriptional and translational levels. Furthermore, they are secreted as ZYMOGENS that require cleavage for enzymatic activation (FIG. 5). Finally, endogenous inhibitors, such as tissue inhibitor of metalloproteinases, are also expressed in the brain.

MMP levels are increased in experimental models of ischaemia[74–76], haemorrhage[77] and trauma[78]. Similarly, MMP levels are increased in the brain and plasma of stroke patients[79–80]. Upstream triggers of MMP include the mitogen-activated protein kinase pathways[81] and oxidative stress[82]. Nitric oxide can directly activate MMP9 through *S*-nitrosylation at the catalytic site[83], thereby linking MMP signalling with another well-recognized pathway in stroke. Excessive MMP activity is deleterious — direct injection of MMP7, 8 or 9 into brain causes cell death and inflammation[84]. In experimental stroke models, treatment with MMP inhibitors reduces infarction and oedema[85,86]. MMP9-knockout mice are protected against cerebral ischaemia[85] and trauma[78], whereas MMP2-knockout mice are not resistant to focal ischaemia, indicating that MMP9 might have a more dominant role[87].

The PA axis comprises the other major proteolytic system in mammalian brain. Urokinase PA and tPA have crucial roles in modulating the matrix during neural development[88]. In adult brain, PAs are synthesized by neurons, astrocytes and microglia, and PA levels change in response to injury[89]. Endogenous inhibitors (for example, plasminogen activator inhibitor-1 and neuroserpin) are also expressed in brain. Unlike MMPs, however, the role of PAs in stroke is controversial. Primary neuronal cultures genetically deficient in tPA are resistant to oxygen/glucose deprivation[90], and tPA knockout mice are protected against excitotoxic injury[91]. In a mouse model of focal ischaemia, treatment with neuroserpin reduces infarction[92]. By contrast, responses are variable in tPA knockouts — they are protected against focal cerebral ischaemia in some studies[93] but not in others[94]. In part, these inconsistencies might reflect the impact of differences in genetic background, plus complexities arising from the beneficial clot-lysing effects of tPA versus its neurotoxic properties in brain parenchyma[95].

ZYMOGEN
Any inactive enzyme precursor that, following secretion, is chemically altered to the active form of the enzyme.

Proteolysis of the neurovascular matrix leads to disruption of the blood–brain barrier after reperfusion[96]. The gelatinases MMP2 and MMP9 are implicated because they degrade collagen IV, a major component of the basal lamina. Tight-junction proteins and MMP substrates such as zona occludens-1 (ZO-1) might also be targeted. ZO-1 degradation and the development of oedema are reduced in MMP9 knockout mice after transient focal stroke[85]. Patients with high plasma MMP9 levels after stroke are susceptible to haemorrhagic transformation[97]. In a non-human primate model of focal ischaemia, areas of haemorrhage co-localize with areas of increased MMP9 (REF. 76). Most importantly, MMP9 levels are amplified by tPA after embolic focal ischaemia[98,99], and MMP inhibitors reduce tPA-induced haemorrhagic transformation in experimental models[99,100]. Taken together, these data indicate a possible mechanism by which administered tPA and MMP9 mediate injury to the neurovascular unit after stroke. Therefore, modulating tPA and MMP9 activity might provide a new approach to ameliorate the complications of oedema and reperfusion injury.

Besides blood–brain barrier leakage, proteolysis of the neurovascular matrix might also promote ANOIKIS. In a primate model of focal cerebral ischaemia, areas in which vascular matrix antigens are lost correlate with regions of neuronal injury[101]. Extracellular disruption of neuron–lamina interactions promotes hippocampal cell death after excitotoxic lesions *in vivo*[102]. Active MMP9 disrupts neuron–matrix integrin pathways[83] that might lead to neuronal anoikis by suppressing cell survival Akt pathways[103]. Integrin–laminin matrix interactions might also be necessary for oligodendrocyte and astrocyte homeostasis[104]. In cultured cerebral endothelial cells that are exposed to hypoxia, pharmacological inhibition of MMPs reduces caspase activation and prevents cell death[105]. Besides anoikis, direct pathways that promote apoptosis might be implicated because some MMPs cleave and activate pro-death TNF and soluble Fas ligand[106].

Inflammation and the neurovascular unit. Inflammation in the blood-vessel wall and brain parenchyma contributes to stroke risk, and to tissue damage after ischaemia. Stroke risk has been linked to serologic markers of inflammation, such as C-reactive protein and soluble intercellular adhesion molecule (sICAM)[107,108]. Stroke onset is often triggered by several processes involving endothelial activation, pro-inflammatory and prothrombotic interactions between vessel wall and circulating blood elements, and ultimately THROMBOGENESIS[109]. Within minutes, several pro-inflammatory cascades are initiated. These events are promoted, in part, by the binding of cell adhesion molecules (from the selectin and immunoglobulin gene families that are expressed in endothelial cells) to glycoprotein receptors that are expressed on the neutrophil surface[110]. In support of this, ischaemic infarction is reduced in *Icam1* knockout mice[111] and exacerbated in mice that overexpress P-selectin[112]. Consistent with these findings, P-selectin is expressed on vascular endothelium within 90 minutes of cerebral ischaemia, ICAM1 is expressed within 4 hours, and E-selectin is expressed within 24 hours[113]. The inhibition of both selectin adhesion molecules, and the activation of COMPLEMENT, reduces brain injury and suppresses neutrophil and platelet accumulation after focal ischaemia in mice[114]. In fact, neutrophil and complement

Box 2 | White matter ischaemia

White matter is also susceptible to stroke. Because of notable differences in the responses of grey and white matter to ischaemic insult, careful delineation of targets and pathways is vital.

The main cell types comprising the neurovascular module within white matter are the endothelial cell, perinodal astrocyte, axon, oligodendrocyte and myelin. Laminin, fibronectin and chondroitin proteoglycans are matrix proteins that envelop these cells. White matter blood flow is lower than in grey matter, and white matter ischaemia is typically severe, with rapid cell swelling and tissue oedema because there is little collateral blood supply in deep white matter. Minor white matter strokes often cause extensive neurological deficits by interrupting the passage of large axonal bundles, such as those within the internal capsule.

Excitotoxicity in white matter differs slightly from that in grey matter. Loss of energy stores leads to depolarization and transmitter accumulation, as reported for the rat optic nerve model of oxygen/glucose deprivation[202]. But unlike grey matter, there are no synapses in white matter, so vesicular release does not occur. Instead, there is reversal of sodium-dependent carrier-mediated glutamate efflux from axons and oligodendrocytes in anoxic dorsal column tracts of the spinal cord[203]. Ultimately, activation of sodium, potassium and calcium channels leads to ionic imbalance and loss of normal axonal function. These energetic and ionic perturbations in axons trigger pathways of oxidative stress and downstream executioners of cell death, reminiscent of neuronal responses. Axons contain abundant mitochondria — a source of reactive oxygen species. Free radical scavenging significantly reduces white matter injury in a rat model of stroke[204]. However, small and large axons might not respond in the same way[205]. Small axons reportedly recover from short periods of oxygen and glucose deprivation, whereas large axons show only transient recovery followed by secondary deterioration after reperfusion. The mechanisms that underlie these different responses warrant further investigation.

White matter ischaemia might activate proteases, such as calpains, that degrade neurofilament substrates[206]. Additionally, phosphorylation of axonal microtubule proteins, such as tau, might also participate in axonal injury[207]. Together, these changes not only weaken the structural integrity of axons but might also impair axonal mechanisms for anterograde and retrograde transport.

Oligodendrocytes are abundant in white matter and possess AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) glutamate receptors (GluRs) comprising homomeric or heteromeric entities formed by GluR3 and GluR4 subunits. *In vitro*, AMPA receptor blockers protect oligodendrocytes from hypoxic and excitotoxic injury[208]. In fact, oligodendrocytes may be especially vulnerable because their AMPA receptors lack calcium-impermeable GluR2 subunits[209]. Death signals, such as tumour necrosis factor and Fas ligand, are expressed by damaged oligodendrocytes, and caspase-mediated apoptotic-like pathways are also recruited[210].

The response of myelin-synthesizing oligodendrocytes is crucial to white matter function during ischaemia. Even if outright cell death does not occur, metabolic dysfunction in oligodendrocytes might still impair the normal replenishment of myelin and synthesis of myelin-associated proteins. The importance of myelin–axon interactions has been demonstrated by the development of axonal degeneration in knockout mice lacking myelin components such as myelin-associated glycoprotein[211] and proteolipid protein[212].

Finally, direct attack by matrix metalloproteinases (MMPs) on myelin components such as myelin-basic protein affect injury[213], and degradation of myelin-basic protein is reduced in MMP9 knockout mice during ischaemia[85]. Clinically, chronic white matter lesions have been associated with upregulation of MMPs in autopsied samples from patients with vascular dementia[214]. These data indicate that proteolytic pathways operating in grey matter might also be recruited in white matter, indicating that there are common cascades that might be therapeutically targeted in ischaemia.

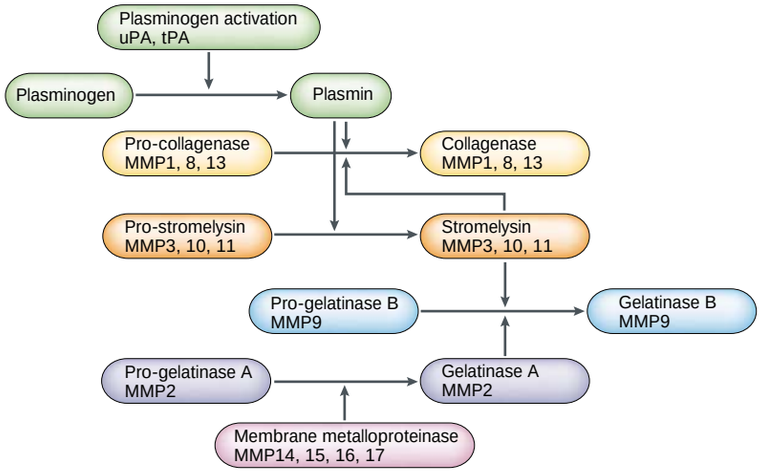


Figure 5 | **Summary of a protease cascade involving members of the matrix metalloproteinase (MMP) family of endopeptidases.** Because MMPs are generated as zymogens, cleavage by activator proteases is required to produce the active enzyme. Cascades involving upstream and downstream MMPs form a complex network in which several regulatory points are present, not unlike what has been described for caspases or the blood-clotting cascades. Linkage between the MMP system and the plasminogen system is an important aspect of stroke pathophysiology. tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator. Modified, with permission, from REF. 70 © (1999) Elsevier Science.

activation made patient outcomes significantly worse in a clinical trial using humanized mouse antibodies directed against ICAM (Enlimomab)[115,116]. Therefore, the complexities of interactions between several pathways will have to be carefully considered for optimal translation to the clinic.

After the onset of blood vessel occlusion, ischaemic injury triggers inflammatory cascades in the parenchyma that further amplify tissue damage[117,118]. As reactive microglia, macrophages and leukocytes are recruited into the ischaemic brain, inflammatory mediators are generated by these cells or by neurons and astrocytes. Among these, inducible NOS (iNOS), cyclooxygenase 2 (COX2), interleukin 1 (IL1), and monocyte chemoattractant protein 1 (MCP1) have crucial roles, as evidenced by the reduction of ischaemic injury in mutant mice with targeted disruption of these genes[37,118–122]. Within minutes of occlusion, there is transient upregulation of immediate early genes encoding transcription factors (for example, *Fos* and *Jun*). This is followed by a wave of expression of heat shock genes (for example, *Hsp70*, *Hsp72*) within 1–2 hours that decreases within 1–2 days. Around 12–24 hours after stroke, a third wave follows in which chemokines and cytokines are expressed (for example, IL1, IL6, IL8, TNFα, MCP1 and so on). Whether or not these three waves are causally related is not known. Nevertheless, therapies that seek to target these pathways need to be carefully timed to match the complex temporal evolution of tissue injury.

Inflammatory cascades stimulate both detrimental and potentially beneficial pathways after ischaemia. For example, administering TNFα-binding proteins reduces brain injury after focal ischaemia in rats[123], whereas ischaemic injury increases in TNF-receptor (TNFR) knockout mice[124]. In part, these contrasting results might reflect signal transduction cascades activated by TNFR1 and TNFR2; TNFR1 augmenting cell death and TNFR2 mediating neuroprotection[125]. Similarly, the vascular endothelial growth factor peptide exacerbates oedema in the acute phase of cerebral ischaemia but promotes vascular remodelling during stroke recovery[126]. Ultimately, the net effect of these mediators depends on the stage of tissue injury or the predominance of a single signalling cascade among several divergent pathways.

Emerging data indicate a multiplicity of reciprocal interactions between blood vessels, neurovascular matrix proteolysis, the transmigration of inflammatory cells, and neuronal injury[127]. For example, every cell in the neurovascular matrix expresses components of the complement system, and this system has been implicated in the initiation and regulation of the inflammatory response[128]. Activators of the complement pathway are expressed on ischaemic cortical neurons, and transient exposure to sublethal excitotoxic stress amplifies cell death owing to the complement membrane-attack complex[129]. It therefore seems prudent for future studies to consider the combined consequences of vascular activation, blood–brain barrier disruption and neuronal injury in the context of inflammatory signalling within the neurovascular unit (FIG. 4).

Selective treatments for multiple targets

Combination therapy. Most ischaemic strokes are caused by thromboembolic occlusions of major arteries that supply the brain. Agents that lyse these clots reperfuse ischaemic brain and form the basis of thrombolytic therapy. Indeed, thrombolysis using recombinant tPA is currently the only therapy for acute stroke approved by the US Food and Drug Administration. Because the risk of haemorrhage increases with time, treatment is currently limited to the 3-hour period immediately following vascular occlusion[130]. However, clot lysis might be therapeutically useful at later times because large numbers of necrotic neurons do not appear until 6 hours after ischaemia, at least in rat brain[131]. In fact, recently released results from the PROACT (prolyse in acute cerebral THROMBOEMBOLISM) II study, in which recombinant pro-urokinase was administered intra-arterially to patients with middle cerebral artery (MCA) occlusion, indicate that some tissue is salvageable at 6 hours[132]. Supporting this view, a recent positron-emission tomography (PET) study reports that a portion of ischaemic human brain might remain viable for up to 12 hours[133,134]. Furthermore, many ischaemic brains still show a perfusion–diffusion mismatch on magnetic resonance imaging (MRI) between 3 and 6 hours[135], indicating the potential benefits of thrombolysis for this pre-selected population. However, the use of thrombolysis must be weighed against the risk of intracerebral haemorrhage and brain oedema after 3 hours.

Most preclinical observations indicate that treatment is suboptimal without combining neuroprotective therapy with clot-lysing drugs. This combination reduces reperfusion injury and inhibits downstream targets in cell death cascades. Synergistic or additive effects were reported when thrombolysis was used in conjunction

ANOIKIS
Induction of programmed cell death by detachment of cells from the extracellular matrix.

THROMBOGENESIS
The formation of a thrombus. A thrombus is an aggregation of blood factors — primarily platelets and fibrin — with entrapment of cellular elements, which frequently causes vascular obstruction at its site of formation.

COMPLEMENT
A set of plasma proteins that attack extracellular pathogens. The pathogen becomes coated with complement proteins that facilitate pathogen removal by phagocytes. Complement components are also involved in inflammation and tissue destruction.

THROMBOEMBOLISM
The obstruction of a blood vessel with thrombotic material carried by the blood stream from the site of origin to plug another vessel.

Table 1 | Neuroprotection after preconditioning

Changes in protein expression	Reference
Increased expression	
Heat shock proteins	215
Bcl2	216
Sodium calcium exchanger	217
DNA repair protein Ku 70	218
Hypoxia-inducible factor-1α	220
Kinases in MAPK and ERK pathway	161
Erythropoietin	221
Decreased expression or inhibition	
NMDA receptor NR2A and NR2B subunits	222
JNK	223
Cytokines	219

ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate.

with neuroprotectants such as oxygen radical scavengers[136], AMPA[137] and NMDA receptor antagonists[138], MMP inhibitors[99], citicoline[139], topiramate[140], anti-leukocytic adhesion antibodies[141] and anti-thrombotics[142]. Combination therapies might decrease dosages for each agent, thereby reducing the occurrence of adverse events. Two recent clinical trials reported the feasibility and safety of treatment with intravenous tPA followed by neuroprotectants, clomethiazole[143] or lubeluzole[144]. Combining hypothermia with drugs[145] or with forced brain perfusion might provide additive benefits, as might devices that enhance tPA-induced thrombolysis by ultrasound. Ultimately, any method that sustains tissue viability should benefit stroke outcome. For example, breathing normobaric oxygen (100% oxygen) transiently reverses diffusion MRI deficits in a rat model of stroke[146]. The precise mechanism remains to be fully elucidated but might include improved collateral blood supply and increased tissue oxygen in penumbral zones.

Considering that several pathways leading to cell death are activated in cerebral ischaemia, effective neuroprotection might require the combination or addition of drugs in series that target distinct pathways during the evolution of ischaemic injury. Although seemingly independent treatments might not always yield additive results[147], the basic idea of simultaneously targeting several pathways is a rational approach. For example, iNOS and caspase inhibitors or anti-inflammatory strategies might prove more useful in some ischaemic situations when administered over days, as compared with treatments to reduce the impact of early events (for example, excitotoxicity or oxidative stress). In animal models, various neuroprotective combinations have been used with some success, including co-administration of an NMDA receptor antagonist with GABA (γ-aminobutyric acid) receptor agonists[148], free radical scavengers[149], citicholine[150], the protein synthesis inhibitor cycloheximide[151], caspase inhibitors[152] or growth factors such as basic fibroblast growth factor (bFGF)[153]. Synergy is also observed between two different antioxidants[154], and also between citicoline and bFGF[155]. Combination treatments with caspase inhibitors extend the therapeutic window and reduce doses of bFGF or NMDA antagonists required for effective therapy[152,156].

Most of the agents listed earlier have been tested in monotherapy clinical trials without success, and have been abandoned as therapies for acute stroke indications. This issue therefore needs revisiting. There are many reasons why clinical stroke trials have been unable to detect improved outcomes, and these complex issues have been explored by others[157,158]. In our view, rational therapy based on inhibiting multiple cell death mechanisms might ultimately prove as useful for stroke as it has for cancer chemotherapy.

Lessons from preconditioning. Tolerance to ischaemic/hypoxic challenge develops following brief but intense episode(s) of a non-damaging brain insult. This brief challenge protects against subsequent, prolonged and detrimental ischaemic/hypoxic episodes by upregulating powerful endogenous pathways that increase resistance to injury. Tolerance develops in heart, liver, small intestine, skeletal muscle, kidney and lung in response to preconditioning stimuli[159–161]. In brain, these stimuli include several potentially noxious transient events such as cortical spreading depression, potassium depolarization, inhibition of oxidative phosphorylation, exposure to excitotoxins and cytokines, and brief episodes of brain ischaemia.

Two temporally distinct types of tolerance are induced by preconditioning stimuli: acute — observed within minutes — and delayed — developing after hours[161]. Signalling cascades for both types of tolerance might share common mediators. Delayed tolerance is sustained for days to weeks and requires new gene expression and protein synthesis. Acute protective effects are short-lasting and are mediated by post-translational protein modifications.

Cardiologists use acute preconditioning before invasive procedures such as ANGIOPLASTY and coronary artery bypass grafting; tolerance might also protect the heart during preinfarction angina[162]. However, the advantages of preconditioning for the human brain have not yet been established, although one recent report suggests that transient ischaemic attacks confer a more favourable prognosis on subsequent stroke[163].

Preconditioning might offer insights into the molecular mechanisms responsible for endogenous neuroprotection, and so provide new strategies for making brain cells more resistant to ischaemic injury. TABLE 1 summarizes major mediators of preconditioning. Calcium influx triggered during ischaemic preconditioning might directly or indirectly activate a cascade that regulates gene expression leading to upregulation of neuroprotective molecules and downregulation of cell death mediators[161]. Ischaemic tolerance that is induced by brief exposure of cultured neurons to oxygen/glucose deprivation is dependent on NMDA receptor activation, calcium influx and new protein synthesis. Nitric oxide promotes tolerance by Ras-dependent signalling

ANGIOPLASTY
The surgical repair of a blood vessel. A balloon angioplasty is a non-invasive procedure during which a balloon-tipped catheter is introduced into a diseased blood vessel. As the balloon is inflated, the vessel opens further allowing improved blood flow.

Table 2 | Hypothermic protection

Mechanisms	Reference
Decreases:	
Cerebral metabolic rate	224
Intracellular acidosis	225
Prevents inhibition of protein kinase C	226
Release of excitatory amino acids	227
Formation of free radicals	228
Tissue lactate levels	229
Mitochondrial release of cytochrome *c*	230
Apoptotic cell death	231
Leukotriene B4	232
Microglial activation	233
iNOS-generated NO and peroxynitrite	234
Inflammatory response	235
BBB disruption and brain oedema	236
Peri-infarct depolarizations	237
Increases:	
Post-traumatic hypoperfusion	229
Modulates:	
Enzyme activity	226

BBB, blood–brain barrier; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

pathways through phosphatidylinositol 3-kinase (PI3K)/Akt and Raf/extracellular signal-regulated kinase (Erk). The PI3K/Akt pathway is involved in anti-apoptotic signalling in cerebellar granule cells and in peripheral sympathetic and sensory neurons, but it is not essential for ischaemic preconditioning in forebrain neurons[164]. On the other hand, Ras inhibition during preconditioning prevents tolerance. In fact, preconditioning might protect by transcriptional activation of neuroprotective proteins of the NMDA/nitric oxide/p21Ras/Erk pathway. Therefore, long-term changes leading to neuroprotection elicited by preconditioning can develop from Erk-induced phosphorylation of transcriptional elements such as Elk1 and cyclic-AMP-responsive element (CRE)-binding protein (CREB), which regulate the expression of neuroprotective genes[165].

Hypothermia and multiple targets. It is known that prolonged submersion in icy water (for example, for 30 minutes) protects the hypoxic brain and promotes, in some cases, a surprisingly favourable outcome, particularly in children. Taking advantage of this observation, induced hypothermia was first used to treat head trauma in the early 1940s. Several non-randomized trials followed, but were hindered by serious complications owing to low core temperatures (24–33°C), thereby making this treatment sometimes lethal (for example, ARRHYTHMIAS, COAGULOPATHY and infection)[166]. In the past decade, however, it was recognized that a small decrease in core temperature (from normothermia (~37°C) to 33–36°C) was sufficient to reduce neuronal death after experimental ischaemia[167]. This led to renewed interest in mild hypothermia, which is safer and technically easier to achieve than moderate hypothermia (28–32°C). From experimental animal studies, we now know that minor reductions in brain temperature (< 2°C) protect the brain after trauma and neonatal asphyxia[166]. Moreover, the neuroprotective effects of certain drugs (for example, GluR antagonists such as MK-801, or adenosine, diazepam and pentobarbital) partially correspond to lowering of brain temperature[168].

Despite robust protection in most of the experimental studies, there are still unsolved practical issues concerning the duration of treatment and the time limits for initiating treatment after stroke. The consensus from preclinical data indicates that the opportunity to treat does not extend beyond minutes after reversible MCA occlusion when hypothermia is maintained for a short duration (a few hours)[169]. In a global model of hippocampal ischaemia, hypothermia is beneficial if begun 30 minutes before, but not 10 minutes after, stroke onset[170]. However, if cooling is prolonged (12–48 hours), protection against injury is substantial following focal, as well as global, cerebral ischaemia[171,172]. In two successful clinical trials, investigators used an empirical approach and cooled patients after cardiac arrest[173,174] for either 12 or 24 hours, despite a relatively delayed interval (105 minutes) from ischaemic onset to initiation of cooling. Brain cooling can be achieved more rapidly (and spontaneously) when blood flow to the entire brain ceases following cardiac arrest, and thermoregulation might be abnormal owing to hypothalamic dysfunction. If only a segment of brain is ischaemic or traumatized, non-injured brain remains a metabolically active heat source. This could explain the lack of success so far in patients with traumatic brain injury[175]. Unfortunately, recommendations based on studies in rodent brains weighing only a few grams might have limited value in this context.

In a study from Australia, 49% of patients that were treated with hypothermia survived with a good neurological outcome as compared with 26% in the normothermia group (n = 43) (REF. 174). In a multi-centre European study, 55% of those rendered hypothermic (n = 136) showed a favourable outcome as compared with 39% of controls (n = 137) (REF. 173). Adverse event rates did not differ significantly between groups in either study. These encouraging findings corroborate more than a decade of preclinical studies, convincingly showing that neuroprotection by hypothermia decreases brain injury in several animal models.

Based on these results, additional controlled trials are now underway to test the therapeutic impact of hypothermia in focal ischaemia and embolic stroke, when combined with thrombolysis. Preliminary data justify enthusiasm. Schwab *et al.*[176] reported that mild hypothermia (33°C maintained for 48–72 hours) significantly reduced morbidity and improved long-term neurological outcome in small numbers of patients (n = 25) after acute, large, complete MCA infarction (that is, focal ischaemia). However, pneumonia developed during hypothermia in 40% of patients, a figure that was improved on by Kammersgaard *et al.*[177].

ARRHYTHMIA
Any variation from the normal rhythm of the heartbeat.

COAGULOPATHY
A defect in the mechanism of blood clotting.

Recently, investigators showed that intra-arterial thrombolysis in conjunction with mild hypothermia was safe in an open clinical trial (Cooling for Acute Ischemic Brain Damage; COOL AID) and now a multi-centre, randomized trial is being initiated in patients with embolic stroke[178].

The mechanisms of cerebroprotection by mild hypothermia remain unclear and controversial. Initially, protection was attributed to alterations in brain metabolism[179]. The most simple hypothesis posits that lower temperature slows metabolic demand. However, many events in ischaemia are modulated by temperature. Generally, most biological processes have a Q_{10} of approximately 2.5, which means that a 1°C reduction in temperature reduces the rate of cellular respiration, oxygen demand and carbon dioxide production by approximately 10% (REF. 180). Reduced temperature also slows the rate of pathological processes such as lipid peroxidation, or possibly even the activity of certain cysteine or serine proteases. Of course, detoxification and repair processes are also slowed, so the net outcome might be complex. It seems more likely that hypothermia increases resistance against several deleterious events, some of which are listed (TABLE 2). Therefore, these results emphasize the therapeutic value of inhibiting one or more death pathways and of targeting multiple injury mechanisms. Of course, it might be difficult to distinguish the relative importance of any given mechanism that improves tissue outcome in a given study, but nevertheless, the listed mechanisms positively impact tissue and cell outcome in ischaemia studies *in vivo* and *in vitro*.

Whereas hypothermia is protective, elevated core temperatures worsen the outcome from brain ischaemia in experimental animals and according to preliminary evidence from humans[181]. In patients, temperatures greater than 37.5°C are predictive of a poorer outcome than lower core temperatures[182]. In a recent prospective study of 390 consecutive cases admitted to hospital, initial body temperature correlated with stroke severity, infarct size, mortality and poor outcome[183]. For each 1°C elevation in body temperature, the relative risk of a poorer outcome increased 2.2-fold. In experimental animals, intra-ischaemic brain temperatures of 39°C enhanced and accelerated severe damage after global ischaemia, and increased infarction volume significantly after MCA occlusion[181]. Therefore, reducing elevated temperatures is imperative in stroke patients.

The microcirculation. Armed with the new knowledge that patients and experimental animals show improved outcome after early restoration of blood flow, many strategies have emerged that preserve cerebral blood flow and render the microcirculation more resistant to acute ischaemic injury (TABLE 3). Among the most promising are methods to enhance nitric oxide synthesis by the vascular endothelium; both increasing vascular endothelial NOS expression or increasing NOS enzymatic activity seem to be effective in experimental models.

Nitric oxide relaxes vascular smooth muscle and increases cerebral blood flow. It also has beneficial effects through limiting aggregation of platelets and adhesivity of white blood cells, both of which impede microvascular flow during stroke. In experimental studies, infusing L-arginine — the eNOS substrate — or nitric oxide donors increases vascular nitric oxide production and improves blood flow — an effect not observed in mice with an *eNOS* gene deletion[184,185]. As a consequence of improved flow in the ischaemic penumbra, electrical activity is restored[186]. Statins (HMG-CoA reductase inhibitors) that are given prophylactically can also increase vascular nitric oxide production, but do so through a mechanism that augments eNOS protein levels[187]. After chronic administration, absolute brain blood-flow increases and blood flow that was compromised distal to an occluded blood vessel improves, probably by enhanced collateral blood flow[184]. Statins, which were originally introduced as inhibitors of cholesterol biosynthesis, also reduce cerebral infarct size in experimental animals — the effect being dependent, in part, on eNOS protein levels and independent of cholesterol lowering[188]. Isoprenoid intermediates, which lie downstream from HMG-CoA and mevalonic acid, are implicated in the statin effect, along with the signalling molecule Rho[189]. Both eNOS expression and the effects of statins relate to disruption of Rho-mediated endothelial changes *in vivo* and *in vitro*. Other pleiotropic statin effects, such as suppression of pro-thrombotic activity (upregulating endogenous tPA and inhibiting plasminogen inhibitor 1), or of protein-C serum levels and inflammation in the ATHEROMATOUS PLAQUE, might all contribute to stroke mitigation. Indeed, meta-analysis of several clinical statin trials indicates a decreased stroke risk and reduced mortality among patients treated with statins[190].

Whereas statins increase eNOS protein levels over several hours to days, high-dose corticosteroids acutely upregulate eNOS activity by transcriptionally independent mechanisms[191]. Glucocorticoid receptors, PI3K and protein kinase Akt are all implicated in these mechanisms and promote eNOS phosphorylation and thereby activation. In experimental models of ischaemia-reperfusion, acute high-dose steroids reduce stroke injury volume, and decrease the size of infarcted myocardium — effects not seen in eNOS-deficient mice[192]. In wild-type animals, glucocorticoid effects on eNOS activity are blocked by both glucocorticoid receptor antagonists and eNOS inhibitors. Glucocorticoids improve short-term survival after acute myocardial infarction[193]. However, glucocorticoids increase the risk of infection and aggravate diabetes, and they might accelerate apoptotic cell death in injured brain through transcriptional events. So, targeting one or more downstream proteins in the Akt pathway to by-pass transcriptional events might obviate the need to engage the glucocorticoid receptor to upregulate eNOS activity. Importantly, NOS is also activated by oestrogen administration[194], which might partly explain oestrogen's powerful neuroprotective effects in experimental models[195].

Infusing albumin also mitigates the effects of blood flow stasis on the brain macro- and microcirculation

ATHEROMATOUS PLAQUE
A well-demarcated yellow area or swelling on the inner surface of an artery, produced by the deposition of lipids.

Table 3 | Therapeutic targets for microcirculation

Target	Desired effect
TXA2	Decreased platelet activation and thrombosis
PA1	Decreased thrombosis
MMP	Decreased plaque instability
NO	Improved endothelial function
ROS	Improved endothelial function
Adhesion molecules	Decreased endothelial inflammation
ET-1	Decreased vasoconstriction

ET, endothelin; MMP, matrix metalloproteinase; NO, nitric oxide; PA1, plasminogen activator 1; ROS, reactive oxygen species; TXA, thromboxane. Adapted, with permission, from REF. 238 © (2001) American Heart Association.

during acute stroke[196]. Within hours of stroke onset, capillary diameter shrinks owing to endothelial swelling and microvilli formation. Flow is obstructed and vascular resistance increases owing to mechanical plugging by erythrocytes, platelets and leukocytes. In this context, albumin infusion enhances red blood cell perfusion, and suppresses thrombosis and leukocyte adhesion in the brain microcirculation, particularly during the early reperfusion phase following experimental focal ischaemia. Albumin reduces infarct size in experimental animals, improves neurological score and lessens cerebral oedema[197]. These effects might reflect a combination of therapeutic properties including albumin's antioxidant effects, binding of free fatty acids and anti-apoptotic effects on the endothelium, in addition to mechanical unplugging effects within the microcirculation. Albumin also significantly lowers the HAEMATOCRIT and consequently improves microcirculatory flow, viscosity of plasma and cell deformability, as well as oxygen transport capacity. Clinical trials to test the effects of albumin are now being organized.

Numerous clinical trials targeting the microcirculation are in various stages of completion for acute stroke and for stroke prophylaxis. Most trials focus on both the macro- and microcirculation because platelets, thrombin, fibrin and the endothelium are important in clot formation and inflammation in large and small blood vessels. Clinical trials are testing the effectiveness of inhibiting thrombin formation, lysing fibrin (chemically or mechanically) or inhibiting platelet aggregation (blocking GPIIb/IIIa binding sites) in acute stroke. Cyclooxygenase and phosphodiesterase inhibitors, ticlopidine and clopidogrel (ADP antagonists with a complex mechanism of action) are already being used as antiplatelet treatments with benefit in primary or secondary stroke prevention, and the overlap with drugs that are useful for the prevention/treatment of myocardial infarction is notable[198,199].

Although not exclusively modulating the microcirculation and its response to ischaemia, immunological tolerance to vascular or CNS antigens was recently reported to improve outcome after experimental ischaemia[200]. The size and number of reported, spontaneously occurring ischaemic and haemorrhagic events was reduced in stroke-prone spontaneously hypertensive rats by inducing immune tolerance to E-selectin[201]. Protection was also achieved when myelin-basic protein was used as a tolerance-inducing antigen. Although the precise mechanisms underlying these therapeutic actions await clarification, repeated nasal instillation of E-selectin, but not ovalbumin, suppressed both the delayed hypersensitivity and cytokine response to E-selectin after challenge with a proinflammatory stimulus, lipopolysaccharide. Manipulation of the immune system through mucosal tolerance might provide a new tool for stroke prophylaxis in humans.

HAEMATOCRIT
The relative volume of blood occupied by red blood cells.

Conclusion

A large body of research has disclosed the intricate molecular cascades that contribute to brain cell death after stroke. Complex and overlapping pathways involving excitotoxicity, ionic imbalance, oxidative and nitrosative stress, and apoptotic-like mechanisms have been well documented in experimental models *in vitro* and *in vivo*. However, the successful translation of experimental results into clinical practice remains elusive. Clinicians and basic scientists remain frustrated by the repeated lack of success when agents intended for neuroprotection fail to improve outcome in clinical stroke trials. Wherein lies the problem? Is it the drugs, the models or the trial design? Fault probably lies with all three. Clinical trial design often does not replicate the narrow therapeutic time-windows observed in animal models. For example, previous trials involving GluR antagonists were overly optimistic because conditions triggering excitotoxic pathways do not persist beyond the first 1–2 hours after stroke onset, in contrast to their longer-lasting downstream effects. Furthermore, although neuroprotection can be robustly quantified in experimental models, the same is not true in clinical stroke. Clinical examination is not sufficiently sensitive to reliably estimate brain tissue loss or salvage. Imaging technology sensitive enough to quantitatively evaluate an evolving stroke in its infancy. In our view, objective assessment of lesion volumes, although ultimately not a substitute for patient outcome, is at the present time an essential endpoint for clinical trials. Animal models should also be modified to more closely match the clinical scenarios of the typical stroke patient: elderly, with concomitant vascular disease as well as hypertension or diabetes. Finally, there might be inherent limitations to current pharmacological approaches that typically target a single intracellular mechanism within a single cell type.

Although many challenges lie ahead, an attitude of cautious optimism seems justified at this time. Several international trials for thrombolysis firmly establish the idea that timely reperfusion can salvage brain. The efficacy of hypothermia so far confirms that multiple molecular cascades are indeed operational in human brain and that neuroprotection is an achievable goal. Ultimately, combination therapies that target the entire neurovascular unit, promote cell survival mechanisms and extend the therapeutic time-window for reperfusion therapy will provide the required opportunities to meet the challenges for stroke.

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