Biochemical pathways and their clinical applications in acute ischemic stroke

Raf Brouns\textsuperscript{a} MD PhD, Ann De Smedt\textsuperscript{a} MD, Robbert-Jan Van Hooff\textsuperscript{a} MD, Maarten Moens\textsuperscript{b} MD, Jacques De Keyser\textsuperscript{a,c} MD PhD.

\textbf{a.} Department of Neurology, Universitair Ziekenhuis Brussel, Center for Neurosciences (C4N), Vrije Universiteit Brussel (VUB), Brussel, Belgium
\textbf{b.} Department of Neurosurgery, Universitair Ziekenhuis Brussel, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Brussel, Belgium
\textbf{c.} Department of Neurology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

*Corresponding author: Prof. dr. Raf Brouns, Department of Neurology, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, 1090, Brussel, Belgium.
E-mail: Raf.Brouns@uzbrussel.be

Received: 26.05.2012 Accepted: 23.08.2012 Published: 24.09.2012

\textbf{Abstract}

Tissue damage following acute focal cerebral ischemia results from multiple pathophysiological mechanisms called the ischemic cascade. This is a complex series of neurochemical processes involving cellular bioenergetic failure, excitotoxicity, oxidative stress, blood–brain barrier dysfunction, microvascular injury, hemostatic activation, post-ischemic inflammation and finally cell death of neurons, glial and endothelial cells. These key biochemical pathways are targets for the development of improved stroke treatments and novel diagnostic tools.

\textbf{Keywords}: stroke, ischemic cascade, reperfusion, neuroprotection, biomarkers
INTRODUCTION

Stroke is a rapidly developing loss of brain function due to a disturbed cerebral blood supply. The functional loss can be caused by ischemia (lack of perfusion due to thrombosis or embolism) or by hemorrhage. In Western societies, about 80% of the strokes are secondary to focal cerebral ischemia. The remaining 20% are caused by hemorrhages (1). Ischemic stroke is the second most common cause of death and the leading cause of acquired disability in adults (2). World-wide about 22 million people suffer from a stroke each year (3) and in western countries, stroke causes 10-12% of all deaths (4). From a socio-economical point of view, the burden of stroke cannot be underestimated and will continue to increase greatly during the next decades. The total cost of stroke in the United States of America from 2005 to 2050 is projected to be more than two trillion dollar (5).

Decades of intensive research and development have tremendously improved our understanding of the vascular, cellular, and molecular mechanisms leading to brain tissue injury after acute ischemic stroke. Nonetheless, the current routine therapeutic arsenal for acute stroke is limited to intravenous administration of recombinant tissue plasminogen activator, which has proven to be safe and effective (6). Utilization rates of thrombolytics, however, remain low (7). As a result, there still is a need for improved diagnostics and therapeutics for acute stroke patients. To achieve these aims, a better insight in the basic disease mechanisms and the development of new diagnostic tools and prognostic markers are essential.

NEUROBIOLOGICAL PROCESSES IN ACUTE ISCHEMIC STROKE

THE ISCHEMIC CASCADE

The ischemic cascade refers to a series of neurochemical processes that are unleashed by transient or permanent focal cerebral ischemia (Figures 1 and 2) (8). The key processes are cellular bioenergetic failure due to focal cerebral hypoperfusion, followed by excitotoxicity, oxidative stress, blood-brain barrier dysfunction, microvascular injury, hemostatic activation, post-ischemic inflammation and finally cell death of neurons, glia and endothelial cells. This cascade usually goes on for hours but can last for several days, even after restoration of blood circulation (9). Although reperfusion of ischemic brain tissue is critical for restoring normal function, it can paradoxically result in secondary damage, called reperfusion injury.
Figure 1. Diagrammatic representation of the main pathophysiological mechanisms leading to cerebral damage in acute ischemic stroke

THE ISCHEMIC CORE AND THE PENUMBRA

The amount of permanent damage depends on the degree and the duration of ischemia. The ischemic core is a region with severely impaired blood flow, which is swiftly and irreversibly injured after onset of critical cerebral hypoperfusion (10). Cells in the core are killed rapidly by lipolysis, proteolysis, disaggregation of microtubules, total bio-energetic failure and breakdown of ion homeostasis. The ischemic penumbra is an area of constrained blood flow with partially preserved energy metabolism surrounding the lethally damaged core. It contains functionally impaired but structurally intact tissue and it is the battle field where the ischemic cascade is triggered, resulting in ongoing cellular injury and infarct progression (Figure 2) (11). The penumbra can be rescued by improving the blood flow and/or interfering with the ischemic cascade. Precisely this is the target for acute stroke therapy.
Figure 2. Graph representing the temporal profile of the main pathophysiological mechanisms underlying acute focal cerebral ischemia and their impact on the final ischemic damage. In the absence of early reperfusion, cells in the ischemic ischemic penumbra ( ) subside due to ongoing ischemic injury, resulting in expansion of the infarcted core ( ).

CELLULAR BIO-ENERGETIC FAILURE

Focal hypoperfusion restricts the delivery of essential substrates and causes the brain cells’ normal process for adenosine triphosphate production for energy to fail. This quickly leads to dysfunction of energy-dependent ion transport pumps, depolarization of neurons and glia and release of excitatory amino acids (12). Elevated extracellular potassium levels additionally stimulate ion transport proteins resulting in osmotic cell swelling, predominantly of astrocytes, with decreased glial reuptake of excitatory amino acids as a consequence (13). Failure of other energy-dependent processes, like presynaptic reuptake of excitatory amino acids, further increases the accumulation of excitatory amino acids in the extracellular space (14). Limited oxygen availability also results in anaerobic glycolysis and accumulation of lactate, which may be used as a marker of anaerobic metabolism in stroke and may be involved in secondary brain damage (15, 16).

EXCITOTOXICITY

Excitotoxicity refers to the secondary damage caused by pathological
activation and calcium uptake by neurons due to abnormal release of excitatory neurotransmitters from dying cells (17). Bio-energetic failure gives rise to accumulation of excitatory neurotransmitters, especially glutamate, in the extracellular space (18). This results in overstimulation of glutamate receptors and neuronal depolarization, causing more calcium influx and more glutamate release leading to local amplification of the initial ischemic insult (19). The intracellular calcium increase initiates the generation of free radicals and the activation of calcium-dependent degradative enzymes leading to acute cell death through necrosis. Excitotoxic mechanisms can also initiate molecular events that induce apoptosis and they can trigger the expression of genes that initiate post-ischemic inflammation (20).

OXIDATIVE STRESS

Oxidative stress occurs when the production of free radicals overpowers the endogenous scavenging capacity of cellular antioxidant defenses (21). Reactive oxygen and nitrogen molecules can be generated through several pathways and are important mediators of tissue injury in acute ischemic stroke (22, 23). Free radicals exhibit a spectrum of cellular effects including inactivation of enzymes, release of calcium from intracellular stores, mitochondrial dysfunction, protein denaturation, lipid peroxidation, damage to the cytoskeleton and DNA (24, 25). Severe oxidative stress causes cell death through necrosis, while more moderate oxidation can trigger apoptosis (26). Besides cerebral cellular oxidative injury, these substances may also aggravate brain damage through dysregulation of the cerebral vasomotor reactivity, blood-brain barrier dysfunction and platelet aggregation (27, 28).

BLOOD-BRAIN BARRIER DYSFUNCTION

Several mechanisms contribute to ischemic damage of the blood-brain barrier, that appears to be biphasic, particularly after reperfusion (Figure 2) (29). Oxidative stress is an early stimulus for blood-brain barrier injury and may trigger release of proteases, among which matrix metalloproteinases, by neurons, glia and endothelial cells. These enzymes induce blood-brain barrier damage through digestion of the endothelial basal lamina (30). Within 24 to 72 h after the infarction, a second phase of blood-brain barrier injury takes place. This phase is more complicated and results in greater tissue damage through leukocyte infiltration and marked matrix metalloprotein-9 release from neutrophils transmigrated to the ischemic brain (31, 32).

Disruption of the blood-brain barrier allows leakage of blood components into the brain parenchyma. Extravasation of high molecular weight molecules is followed by water due to osmosis and leads to vasogenic edema, which may cause secondary damage through intracranial hypertension. Additionally, extravasation of red blood cells leads to hemorrhagic transformation of the infarcted area. Finally, the leaky
blood-brain barrier facilitates transmigration of inflammatory cells, promoting the post-ischemic inflammatory response (33).

**ISCHEMIA-INDUCED MICROVASCULAR INJURY**

Microvasculature injury contributes to cerebral tissue damage by increased endothelial cell permeability, matrix degradation, loss of cerebrovascular autoregulation, leukocyte-endothelial cell adhesion and the “no-reflow” phenomenon. The pathophysiology of impaired cerebrovascular autoregulation in acute ischemic stroke is still controversial, but ischemia-induced endothelial damage may play a role, as this may reduce the release of nitric oxide and prostacyclin and may induce endothelin-1 production (34-36). Additional decrease in nitric oxide bioavailability may be caused by inhibition of nitric oxide synthetase by asymmetrical dimethylarginine (37). These processes lead to vasoconstriction which may further impair blood flow in the area of the cerebral infarction, thereby enhancing the ischemic injury. Additionally, dysfunctional autoregulation leaves the vulnerable ischemic penumbra unprotected against potentially harmful blood pressure changes (38, 39).

In response to focal ischemia, the endothelial cells present of leukocyte adhesion receptors on their surface (40). This not only is an essential step in the post-ischemic inflammatory response, but also contributes to the “no-reflow” phenomenon, which is the obstruction of the downstream microvascular bed after reperfusion of the occluded supply arteries. It is attributed to extrinsic compression from edema, endothelial swelling and intravascular obstruction due to local activation of leukocytes, platelets and coagulation (41, 42).

**HEMOSTATIC ACTIVATION**

In acute ischemic stroke, the endogenous fibrinolysis is usually outweighed by ongoing activation of the coagulation cascade and platelet activation, which is reflected in elevated levels of hemostatic indicators among which D-dimer (43, 44).

Endothelial cell injury results in exposure of tissue factor to blood, leading to activation of the coagulation cascade and formation of a fibrin network trapping platelets, clotting factors and erythrocytes to form a clot (45). Thrombin, plasmin and the thrombin/thrombomodulin complex are participants in this process that will activate procarboxypeptidase U (also denoted thrombin-activatable fibrinolysis inhibitor). Activation of this pro-enzyme produces carboxypeptidase U, which attenuates the endogenous fibrinolysis (46). Marked decrease in procarboxypeptidase U activity occurs in the first 72 hours after ischemic stroke (47) and in patients with poor response to thrombolytic therapy, probably reflecting stronger activation of the procarboxypeptidase U / carboxypeptidase U pathway and thrombus propagation (48).

Platelets are activated under conditions of ischemia and high shear
stress (49). Activated platelets accumulate in microvessels within 2 hours of vascular occlusion (50) and release a variety of biochemical mediators, catalyse interactions between coagulation factors and contribute to the “no-reflow phenomenon” by adhering to both leukocytes and microvascular endothelial cells (51). Additionally, platelets can cause temporary vasospasm by releasing thromboxane A2 and free radicals and they may exacerbate the inflammatory cascade by releasing chemotactic mediators for leukocyte migration (52).

POST-ISCHEMIC INFLAMMATION

A robust inflammatory reaction follows focal cerebral ischemia and the ongoing amplification of processes in both the cellular and humoral immune system aggravates cell damage as well as microvascular stasis and disruption of the blood-brain barrier (53, 54).

Both resident brain cells, especially astrocytes and microglia, and blood-borne leukocytes contribute to the post-ischemic inflammatory response by production of pro-inflammatory and anti-inflammatory mediators such as cytokines, chemokines and several enzymes (55, 56). The arachidonic acid cascade results in production of prostaglandins and leukotrienes, which are potent chemoattractants that are implicated in blood-brain barrier dysfunction, edema and neuronal death (57). Upregulation of nitric oxide synthases occurs in circulating leukocytes, microglia and astrocytes and causes damage through several mechanisms (58). Inflammatory cells also generate reactive oxygen species and produce matrix metalloproteinases inducing more damage to the ischemic brain (59).

ISCHEMIA-INDUCED CELL DEATH

Ischemic injury produces necrosis, a fulminant form of cell death associated with failure of the plasma membrane and cytotoxic edema of both the cell and internal organelles (60). A cell that dies through necrosis releases more glutamate and toxins into the environment, affecting surrounding neurons. Apoptosis is the consequence of a genetically regulated program that allows cells to die with minimal inflammation or release of genetic material (61). This process may be promoted by several pathways involving non-specific cellular injury, caspase activation and mitochondrial proteins such as apoptosis-inducing factor and Bcl-2/adenovirus E1B – interacting protein (62-65).

The local degree of ischemia, the cell maturity, the concentration of intracellular free calcium and the cellular micro-environment determine which process will dominate (66). Acute, permanent vascular occlusion is mostly followed by necrosis, whereas milder injury, particularly within the ischemic penumbra, often results in apoptosis.

CEREBRAL REPERFUSION INJURY

Prompt restoration of the blood supply can limit the infarct size and can
improve clinical outcome in patients with ischemic stroke (67) but reperfusion may also paradoxically exacerbate the brain damage (68). Cerebral reperfusion injury can be defined as a deterioration of ischemic but salvageable brain tissue after reperfusion and has a multifactorial etiology (69). Leukocytes appear to play a critical role through damaging of the endothelium, obstruction of the microcirculation, disruption the blood-brain barrier and infiltration in the brain tissue where they propagate inflammation (70). Platelets play a synergistic role with leukocytes in reperfusion injury via the “no-reflow phenomenon” and the release a variety of biochemical mediators that may lead to vasospasm and exacerbation of oxidative stress and the inflammatory cascade (71). Furthermore, experimental studies have shown that complement activation is an important component of reperfusion injury through the formation of several inflammatory mediators and the membrane attack complex (72). Finally, microvascular injury and post-ischemic hyperperfusion may cause vasogenic brain edema and hemorrhage (73).

CLINICAL APPLICATIONS OF BIOCHEMICAL PROCESSES IN ACUTE ISCHEMIC STROKE

THERAPEUTIC APPLICATIONS

The two cornerstones for the management of acute ischemic stroke are neuroprotection and early recanalisation. The actual therapeutic arsenal for acute ischemic stroke is highly limited and to date, no new treatment has been proven to be efficacious and safe in randomized clinical trials (74). Animal experiments have demonstrated that the focally ischemic brain can be protected pharmacologically, reducing infarction and improving functional outcome. Regrettably, so far every attempt to translate this preclinical success into clinically effective therapies has failed (75). Despite this apparent translational roadblock, at least 39 clinical phase II and III trials are currently recruiting patients for the evaluation of new therapeutics for acute ischemic stroke (Table 1) (76).

Most of them are based on key biochemical and molecular mechanisms underlying acute focal cerebral ischemia, including improvement of focal blood flow, optimising aerobic glycolysis, interventions in deleterious processes like excitotoxicity, oxidative stress, microvascular injury or inflammation, and finally prevention of cerebral cell death or support of brain regeneration. In order to optimize research in acute stroke therapy, recommendations for standardization and quality control in drug development have been advanced by the Stroke Therapy Academic Industry Roundtable (77) and initiatives to improve preclinical research through international consortia become increasingly more important (78).

DIAGNOSTIC APPLICATIONS

Biomarkers are substances that can be measured reliably and that reflect the presence, the severity or the evolution of a disease. Biochemical parameters have the
Table 1. Overview of currently active phase II and III clinical trials for acute ischemic stroke therapy, classified according to the underlying pathophysiological mechanism

<table>
<thead>
<tr>
<th>Main therapeutic mechanism</th>
<th>Therapeutic class</th>
<th>Treatment in evaluation</th>
<th>Clinical trial registry number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improving focal blood flow</td>
<td>Thrombolytics and anticoagulants</td>
<td>Intra-arterial thrombolysis versus or in combination with systemic thrombolysis using rt-PA</td>
<td>NCT00540527 NCT00640367 NCT01455935</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combined use of Eptifibatide and rt-PA</td>
<td>NCT00401310</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combined use of THR-18 and rt-PA</td>
<td>NCT01253512</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Newer generation thrombolytic agents (Desmoteplase, Tenecteplase)</td>
<td>NCT00790920 NCT00856661 NCT01104467 NCT01472926</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra-arterial application of plasmin</td>
<td>NCT01014975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combined use of Argatroban and rt-PA</td>
<td>NCT01464788</td>
</tr>
<tr>
<td></td>
<td>Devices</td>
<td>Combined use of transcranial ultrasound and rt-PA</td>
<td>NCT00401310</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intra-arterial embolectomy</td>
<td>NCT01492725 NCT01584609</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transcranial laser therapy with or without rt-PA</td>
<td>NCT01120301 NCT01220739</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Augmentation of cerebral blood flow by sphenopalatine ganglion stimulator</td>
<td>NCT00826059</td>
</tr>
</tbody>
</table>

potential to improve the accuracy of acute stroke diagnosis, to help distinguish between ischemic and hemorrhagic stroke and to support the selection patients for specific therapeutic options.

The diagnostic and prognostic value of numerous biochemical substances has been evaluated in animal models and in stroke patients. A non-limitative list of biochemical markers assessed in acute ischemic stroke patients is presented in Table 2. Most of these biomarkers are believed to reflect a relevant pathophysiological process in acute focal cerebral ischemia and therefore contribute to a better understanding of the underlying disease mechanisms. Ideally, biomarkers for this indication should be based on a blood test that can easily be obtained at the patient’s bedside with limited cost.

Recent research suggests that the plasma concentration of glial fibrillary acidic protein in acute stroke patients has the potential to differentiate between intracerebral hemorrhage and cerebral ischemia (79), but until now, no biomarker
Table 1. Non-limitative overview of biochemical markers evaluated in acute ischemic stroke patients, classified by its underlying pathophysiological mechanism

<table>
<thead>
<tr>
<th>Main pathophysiological mechanism</th>
<th>Etiology biomarker</th>
<th>Description biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cell damage</td>
<td>Aspecific release upon cell death</td>
<td>DNA fragments</td>
</tr>
<tr>
<td></td>
<td>Neuronal</td>
<td>Neuron-specific enolase, tau protein</td>
</tr>
<tr>
<td></td>
<td>Gliarial</td>
<td>Protein S100β, myelin basic protein, glial fibrillary acidic protein, secretagogin</td>
</tr>
<tr>
<td>Bioenergetic failure</td>
<td>Anaerobic glycolysis</td>
<td>Lactate</td>
</tr>
<tr>
<td>Excitotoxicity</td>
<td>Amino acids</td>
<td>Glutamate, N-acetyl-aspartate, cystein</td>
</tr>
<tr>
<td></td>
<td>Glutamate receptor</td>
<td>Autoantibodies to NMDA receptors</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>(Anti-) oxidants</td>
<td>malondialdehyde, uric acid, glutathione peroxidase, superoxide dismutase, catalase, nitric oxide, ascorbic acid, tocopherol, retinol</td>
</tr>
<tr>
<td></td>
<td>Oxidation products</td>
<td>3-nitrotyrosine, 8-hydroxy-2-deoxyguanosine, F2-isoprostanes, oxidised phospholipids</td>
</tr>
<tr>
<td>Blood-brain barrier injury</td>
<td>Extravasation serum components</td>
<td>Albumin ration cerebrospinal fluid/serum</td>
</tr>
<tr>
<td>Proteases</td>
<td>Matrix metalloproteinases (esp. MMP-9)</td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td>Tumor necrosis factor, interleukins, monocyte chemoattractant protein-1, vascular endothelial growth factor</td>
<td></td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>Intercellular adhesion molecule-1, vascular cell adhesion molecule-1, selectins</td>
<td></td>
</tr>
<tr>
<td>Acute phase reactants</td>
<td>C-reactive protein, fibrinogen, leukocyte count (esp. neutrophils)</td>
<td></td>
</tr>
<tr>
<td>Hemostatic activation</td>
<td>Coagulation factor VII and VIII, tissue factor, tissue factor pathway inhibitor, anti-thrombin III, thrombin-antithrombin III complex, prothrombin fragment F1.2., fibrinopeptide A, procarboxypeptidase U, carboxypeptidase U</td>
<td></td>
</tr>
<tr>
<td>Fibrinolysis</td>
<td>Tissue plasminogen activator, D-dimer, plasminogen activator inhibitor-1, plasmin-alpha2 plasmin inhibitor complex</td>
<td></td>
</tr>
<tr>
<td>Platelet activation</td>
<td>β-thromboglobulin, platelet factor 4, P-selectin</td>
<td></td>
</tr>
<tr>
<td>Endothelial function</td>
<td>Von Willebrand factor, thrombomodulin</td>
<td></td>
</tr>
<tr>
<td>Vasoactive markers</td>
<td>Vasodilatation</td>
<td>Atrial natriuretic peptide, brain natriuretic peptide</td>
</tr>
<tr>
<td></td>
<td>Vasoconstriction</td>
<td>Endothelin-1</td>
</tr>
</tbody>
</table>

with sufficient specificity and sensitivity has been identified to justify its use in routine clinical practice (80). This can largely be attributed to peculiar difficulties that have to be tackled in biomarkers research for ischemic stroke, including the complexity of the ischemic cascade, the presence of a blood-brain barrier which
substantially slows the release of biomarkers in blood and the limited specificity (81). Many potential blood markers of cerebral ischemia are also elevated in conditions that not rarely co-occur with stroke (for instance myocardial infarction) or mimic stroke (like brain infection). In order to optimize research in this field, recommendations for the design and reporting of studies on diagnostic blood biomarkers in stroke have been published (80).

CONCLUSIONS

Tissue damage following acute focal cerebral ischemia results from multiple complex pathophysiological processes, all of which are targets for the development of better stroke therapeutics and diagnostics. Acute stroke care focuses on rapid restoration of the cerebral blood flow by lysing or mechanically removing an arterial thrombus and on neuroprotection, which aims to reduce the intrinsic vulnerability of the penumbra. The complex pathophysiology of acute ischemic stroke and the failure of many promising therapeutics in clinical trials may indicate that the time for multi-target approach has come. Similarly, biomarkers reflecting relevant events in the ischemic cascade would be useful to improve the accuracy of acute stroke diagnosis and to predict stroke outcome more reliably. Obviously, many mechanisms and cascades leading to ongoing ischemic injury have been elucidated but a number of crucial issues remain unsolved, both at the bench and at the bedside.

ACKNOWLEDGEMENTS

The studies were financed by the Fund for Scientific Research-Flanders (F.W.O.-Vlaanderen, Belgium), the Institute Born-Bunge, the agreement between the Institute Born-Bunge and the University of Antwerp; the Interuniversity Attraction Poles (IAP) program P6/43 of the Belgian Federal Science Policy Office, Belgium and the Medical Research Foundation Antwerp.
LIST OF REFERENCES


67. Schaller B, Graf R. Cerebral ischemia and reperfusion: the pathophysiologic concept as a