Pathophysiology of Cerebral Ischemia

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Abstract

The purpose of this manuscript is to briefly review the pathophysiology of cerebral ischemia. Ischemic thresholds are well-defined in lower animals. The concept of the ischemic penumbra may include regions of brain around deeper regions of ischemia but has also been defined in terms of brain salvageable by reperfusion or by pharmacological therapies. The principal pathophysiological processes in cerebral ischemia are energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity, and free radical-mediated toxicity. The underlying biochemical processes are similar regardless of the amount of brain that is made ischemic or the duration of ischemia. The relative contributions of each process are believed to vary significantly especially in relation to the level of cerebral blood flow. Neurons may die by necrosis or apoptosis. In the core of an infarct where blood flow is very low, the predominant process is energy failure and rapid necrotic cell death. Reperfusion of ischemic tissue produces an influx of inflammatory cells and of oxygen that can cause increases in oxygen-derived free radicals. Free radicals are also important in prolonged ischemia. There is interest in changes in gene expression after ischemia. Induction of heat shock proteins suggests that gene expression changes may protect neurons from death. Changes in gene expression also may initiate apoptosis or other detrimental processes. Although advances have been made, there are still no proven pharmacological therapies to rescue ischemic human neurons. Such therapies do appear to be on the horizon.

Key words: apoptosis, cerebral ischemia, cerebral infarction, excitotoxicity, free radicals, intracellular calcium

Introduction

Cerebral ischemia may be thought of as focal or global, transient or permanent, and by how severely cerebral blood flow (CBF) is reduced. The underlying biochemical processes are similar regardless of the amount of brain that is made ischemic or the duration of ischemia although the relative contributions of each process are believed to vary significantly in relation to these factors and especially the level of CBF. The principal processes are energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium ([Ca²⁺]), excitotoxicity, and free radical-mediated toxicity. Neurons may die by necrosis or apoptosis. In the core of an infarct where CBF is very low, the predominant process is energy failure and rapid necrotic cell death. Reperfusion of ischemic tissue produces an influx of inflammatory cells and of oxygen that can cause increases in oxygen-derived free radicals. Most of what follows is based on animal models, the applicability to human stroke of which is debated by some but suffice it to say that they represent the current state of the art for investigation of such processes.

Thresholds of Ischemia, the Ischemic Penumbra, and Reperfusion

I. Ischemic thresholds

In most models of focal cerebral ischemia, the middle cerebral artery is occluded for minutes to hours, or sometimes permanently, and there is a moderately severe reduction in CBF in this territory. In models of global or forebrain ischemia, the flow reduction is more severe but of shorter duration since long durations of ischemia to such large areas of brain would be fatal. These differences affect the rate of development of pathophysiological changes and their relative importance and could account for why different therapies help different types of ischemia. In global ischemia, the short duration generally does not produce major microcircula-
tory problems whereas such changes are important in focal ischemia especially in the penumbra. In focal ischemia, there tends to be a central area of severe ischemia (the umbra) where infarction occurs rapidly — within 30 minutes in rats and 30 to 60 minutes in monkeys — and an area around this that has some degree of blood flow (the penumbra). How rapidly infarction occurs is related to how low flow goes so that no flow for a few minutes leads to infarction but a flow of 12 ml/100 g/min can be tolerated after middle cerebral artery occlusion in monkeys for 2 or less hours.26,47

Brain electrical activity is lost if CBF falls below 16-18 ml/100 g/min.47 This is the threshold for loss of neuronal electrical function below which neurons are ischemic but not necessarily infarcted or dead. Below this level of CBF synaptic function is lost. It is believed that restoration of flow, even after long times, could restore function of these neurons. There is a second threshold with loss of neuron ion homeostasis below 10-12 ml/100 g/min.6 This threshold is near the level where energy production in the form of high energy phosphates (mainly adenosine triphosphate [ATP]) falls behind energy use. Below this threshold, efflux of K+ and influx of Ca2+ occurs and there is rapid neuronal death or infarction. These two thresholds are relatively constant across species, being only slightly higher in rats and gerbils compared with primates but representing the same percentage reductions in flow from normal.46,47 Classical studies carried out by several groups showed that in monkeys, infarction developed after 1 to 3 hours with flows of 10-12 ml/100 g/min and after permanent arterial occlusion with flows of 17 to 18 ml/100 g/min.5,12,26 There are other thresholds that are generally at higher flows and that are for development of edema and failure of protein synthesis.49

II. Ischemic penumbra

Since collateral flow around an ischemic area was thought to produce a graded decrease in flow towards a more densely-ischemic core, the concept was initially brought forth that there was a volume or ischemic penumbra around the core where there was loss of electrical activity but maintenance of ion homeostasis.47 The neurons here theoretically could be rescued by reperfusion. The anatomical identification of the penumbra has been difficult especially in primates and man where the transition from infarct to normal brain seems to be very sharp, implying a very small penumbra.46,47 The zone is larger in cats and rats which might account for the efficacy of some treatments for ischemia in these models and their failure in man although there are undoubtedly other factors at play. Because of such anatomic difficulties, Siesjö47 defined the penumbra not by CBF or pathophysiological criteria but as the area of infarct that is salvageable with some pharmacological therapy (pharmacological penumbra) or by early reperfusion (reperfusion penumbra).

III. Reperfusion

Reperfusion plays an important role in the pathophysiology of cerebral ischemia. In rats, there is a large difference between infarct size after 30 minutes of middle cerebral artery occlusion compared with permanent middle cerebral artery occlusion. This suggests that a penumbra exists where early reperfusion saves neurons that would otherwise die. The times over which infarct sizes evolve vary between species. In general, however, reperfusion must be within 3 or 4 hours. Reperfusion is necessary if there is to be any hope of saving penumbral-type neurons but it may also produce deleterious effects. There may be hemorrhage into the infarcted brain. There is a sudden increase in oxygen and white blood cells. Toxic oxygen-derived free radicals may be generated and polymorphonuclear leukocytes may adhere to endothelial cells causing microcirculatory occlusions. This may contribute to the no-reflow phenomenon, which is the inability to reperfuse infarcted areas or a delayed decrease in CBF that occurs after reperfusion.2,12 Free radicals and inflammatory mediators may contribute to breakdown of the blood brain barrier. The ischemic arterioles are maximally dilated and may have impaired autoregulation so that reperfusion causes CBF in excess of normal (reactive hyperemia). These factors may contribute to water and osmoles in the blood pouring into the ischemic brain and aggravating brain edema. An attractive but complex therapeutic option would be to preload the blood with pharmacological agents to prevent these processes before reperfusion is established.

Pathophysiology

It is widely held that energy failure is the primary event underlying much of the pathophysiology of ischemia. Energy failure and the secondary processes that occur after ischemia and contribute to neuronal death are discussed independently but they are interrelated and dependent on each other to some extent. For example, most of the effects of increased [Ca2+], are secondary to energy failure and [Ca2+] in turn triggers many reactions leading to generation of free radicals. Free radicals aggravate processes leading to increased [Ca2+]. Loss of ion homeostasis contributes to energy failure, glutamate-mediated excitotoxicity, and increased [Ca2+], which in turn
leads to more excitotoxicity. The relative contribution of each process to brain damage depend on whether the etiology is hypoglycemia, hypoxia, or ischemia. Siesjo\textsuperscript{47,48} suggested that selective neuronal vulnerability, which is observed in hypoglycemic coma, epilepsy, and after brief ischemia, is related to increased $[\text{Ca}^{2+}]$. Infarction is due to acidosis and free radicals, and reperfusion problems in stroke are due to inflammatory mediators that involve increased $[\text{Ca}^{2+}]$, free radicals, and lipid mediators.

I. Energy failure and loss of ion homeostasis

The key process that occurs with severe reductions in CBF is energy failure. There is a lack of oxygen and glucose supply to the brain, which are the necessary substrates for synthesis of ATP. There are no stores of oxygen or glucose in the brain and since ATP consumption continues, there develops a net loss of ATP and probably the other nucleotide triphosphates. This occurs within 60 seconds of complete ischemia and probably over longer times with lesser degrees of CBF reduction. Energy failure leads to several deleterious processes.\textsuperscript{47,51} Protein synthesis is decreased during ischemia because it requires ATP. Continuing protein synthesis is necessary to maintain the cytoskeleton and other regulatory proteins within the cell. Lack of ATP and oxygen causes metabolism of glucose by anaerobic rather than aerobic glycolysis, resulting in lactic acid production and intra- and extracellular acidosis. Anaerobic glycolysis also produces much less ATP than aerobic glycolysis through the Krebs’s cycle and the electron transport chain. ATP is required to maintain ionic gradients across cell membranes. Normally, ATP-driven (Na$^+$/K$^+$-adenosine triphosphatase [ATPase] and Ca$^{2+}$/ATPase) pumps drive Na$^+$ and Ca$^{2+}$ out of neurons and K$^+$ in. These are active pumps that consume over 50% of neuronal ATP.\textsuperscript{3} There are also passive ion leaks — Na$^+$ and Ca$^{2+}$ passively leak into neurons down concentration gradients of about 10:1 and 10,000:1 (outside:inside), respectively. K$^+$ and Cl$^-$ tend to leak out. Passive ion leaks may be increased during ischemia by depolarization of neurons and by generalized increases in membrane permeability. Loss of ATP with pump failure will thus aggravate Na$^+$ and Cl$^-$ influx and K$^+$ efflux by increased leakage and decreased pumping. This depolarizes the cell membrane and causes increases in $[\text{Ca}^{2+}]$, and glutamate release. Additional problems arise because these abnormal ion fluxes may induce more ATP-consuming pump activity to try to restore ionic gradients. Furthermore, extracellular K$^+$ rises and water accumulates in the neurons because it follows osmotic gradients created by the Na$^+$ and Cl$^-$ influx. This produces neuronal swelling or cytotoxic edema. Increased extracellular K$^+$ also may be taken up by glial cells, followed by an obligatory amount of water, leading to edema in astrocytes. A continuing supply of water if there is some low level of CBF might aggravate this compared with if there were no CBF at all.

II. Excitotoxicity

Depolarization of synaptic membranes with increases in $[\text{Ca}^{2+}]$ is a physiological signal transduction mechanism for many intracellular processes including release of neurotransmitters. During ischemia, an abnormal and probably prolonged depolarization of neurons, as well as the changes in $[\text{Ca}^{2+}]$, homeostasis, cause release of nonphysiological amounts of many neurotransmitters including glutamate.\textsuperscript{46-48,51} The release of other neurotransmitters such as dopamine, γ-amino-butyric acid, acetylcholine, and aspartate is also affected.\textsuperscript{3,10} Synthesis of dopamine, acetylcholine, and other neurotransmitters requires ATP, oxygen, and/or functioning of the Kreb’s cycle, so is likely to be decreased by ischemia.\textsuperscript{40} The termination of action of some neurotransmitters is dysfunctional in ischemia because it requires reuptake into cells, which requires an intact sodium gradient.

Glutamate is the major excitatory neurotransmitter in the brain and the one that has received the most attention in ischemia research. It can act on at least five receptors — low- and high-affinity kainate, N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and quisqualate. Activation of the first four is associated with changes in ion flux across the cell membrane and thus are called ionotropic. The quisqualate receptor is not linked to an ion channel and is a metabotropic receptor. Kainate and AMPA receptors seem to be linked to the same type of ion channel that probably causes Na$^+$, K$^+$, and H$^+$ influx and depolarizes the membrane. Membrane depolarization in this way, as well as opening of NMDA receptors which allow entry of Ca$^{2+}$ as well as Na$^+$ and K$^+$, all lead to increased neuronal $[\text{Ca}^{2+}]$. The activation of glutamate receptors is a normal physiological mechanism in which the $[\text{Ca}^{2+}]$ increase is terminated by physiological control mechanisms and the reuptake of excess glutamate. In ischemia, physiological controls and reuptake are disrupted by energy failure and loss of ion homeostasis (principally the Na$^+$ gradient), as discussed above, so that the increased $[\text{Ca}^{2+}]$ may persist. Abnormal intraneuronal Ca$^{2+}$ levels are one postulated mechanism by which glutamate is toxic in ischemia.\textsuperscript{11} Others are in-
creased metabolic demand caused by increased neurotransmitter release and increased use of ATP trying to take up excess neurotransmitters and restore membrane ion gradients.46)

The evidence for a role of excitotoxicity and glutamate release in cerebral ischemia is the demonstration of neuronal toxicity when glutamate is injected into the brain and the efficacy of glutamate receptor antagonists at reducing infarct size in ischemia.47,48,51) Glutamate receptor antagonists have little effect on dense global forebrain ischemia or on the core of focal ischemic lesions. Under these circumstances there is maximal depolarization and Ca2+ probably enters cells through multiple pathways so that blocking one such pathway has little effect. Some NMDA antagonists, however, decreased infarct size in transient focal ischemia by decreasing damage in the penumbral area.7)

III. Calcium homeostasis

[Ca2+]i is an important intracellular second messenger that regulates many intracellular processes such as differentiation, growth, gene expression, channel and synaptic function, and cytoskeletal stability. The concentration of free [Ca2+]i, thus is under stringent control within cells and is kept about 10,000 times lower (about 10−7 mol/l) than that in the extracellular space (10−3 mol/l). Tymianski and Tator47 noted that [Ca2+]i is modulated by the balance of several processes: influx and efflux across the cell membrane, release and uptake from intracellular stores, and binding to intracellular proteins (Ca2+ buffering). Influx may be through voltage-gated or agonist-operated Ca2+ channels. Ca2+ is extruded from neurons by Ca2+-ATPase and Na+/Ca2+ exchange. Release from intracellular stores is also controlled by channels opened by inositol trisphosphate and possibly by Ca2+ itself. Reuptake into stores is by a Ca2+-ATPase. Ischemia disrupts these control mechanisms at multiple points. Ischemia depolarizes neurons, causing Ca2+ entry to increase and glutamate to be released. Activation of glutamate channels may lead to further Ca2+ entry. Depolarization with loss of the transmembrane Na+ gradient reverses the direction of operation of the Na+/Ca2+ exchanger so that Na+ is pumped out of the cell and Ca2+ is pumped in. [Ca2+]i will rise because there is no ATP to drive sequestration or efflux. Since multiple pathways of Ca2+ influx and efflux are disrupted, it is not surprising that drugs such as voltage-gated Ca2+-channel antagonists that block only one possible path of Ca2+ entry have minimal efficacy in cerebral ischemia. These drugs may have other mechanisms of action that are helpful such as dilation of collateral vascular channels to increase CBF to the ischemic area and favorable effects on blood rheology.13,25)

Normal physiological stimuli do not cause a rise in [Ca2+]i that mediates the effects listed above. Physiological [Ca2+]i increases are usually brief and there is evidence that they need only occur very locally within small regions of the cell. For example, the localization of voltage-gated Ca2+ channels in synaptic terminals mediates localized increases in axon terminal [Ca2+]i that lead to neurotransmitter release. When [Ca2+]i rises to nonphysiological levels and/or for abnormal times during ischemia, there may be pathological processes initiated that lead to cell death. This was thought to occur whether [Ca2+]i rose by influx through voltage-gated channels or by other pathways. It is now believed that the source of the increased [Ca2+]i is important in determining the pathological effects. The same amount of Ca2+ entering through voltage-gated channels may not be as detrimental as if it enters through channels gated by glutamate.11,51) Part of the evidence for this comes from studies of neurons in vitro and from pharmacological studies that showed that while NMDA-receptor antagonists decreased infarct size in transient focal ischemia, voltage-gated Ca2+-channel antagonists such as nimodipine were less effective.15,31,53) This suggests an interplay between the excitotoxicity mentioned above, and [Ca2+]i. In addition to the route of Ca2+ entry or release, it is likely that the time over which Ca2+ is elevated and the maximal concentration to which it is elevated are also important. Depletion of cell ATP and loss of ion homeostasis may lead to higher and more prolonged increases in [Ca2+]i that have more opportunity to activate too many enzymes, overwhelm normal buffering and sequestration mechanisms, and cause pathological processes to begin.

How do abnormal increases in [Ca2+]i kill neurons? There may be nonphysiological activation of Ca2+-dependent enzymes such as proteases, phospholipases, protein kinases, plasmalogens, guanylate cyclase, nitric oxide (NO) synthases, calcineurins, and endonucleases. Proteases such as the calpains can break down the neuronal cytoskeleton causing membrane blebbing and inhibition of axonal transport.45) These enzymes normally participate in remodeling the cell cytoskeleton which is required for normal cell function. Proteases may accelerate the deleterious effects of ATP depletion on the cell skeleton. Phospholipases such as phospholipase A2 and phospholipase C are activated by increased [Ca2+]i. This causes increased production of free radicals and vasoactive and inflammatory substances. Phospholipase A2 metabolizes ethanolamine phosphoglycerides, phosphatidyl choline, and

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other cell membrane lipids to their lyso-forms. Platelet-activating factor, a cytokine that mediates adherence of leukocytes to endothelium and activates platelets is also formed. There are platelet-activating factor receptors in the brain and these may mediate inflammation after ischemia, impair microcirculatory flow, and thus contribute to generation of toxic free radicals, all of which may increase brain damage. A role for these processes is suggested by the efficacy of platelet-activating factor antagonists in ameliorating transient focal and global ischemia in several models.38

Phospholipase C metabolizes phosphatidylinositol biphosphate to diacyl glycerol and inositol triphosphate. Inositol triphosphate can increase \([\text{Ca}^{2+}]\) by release from intracellular stores. Diacyl glycerol is metabolized to arachidonic acid which is a primary substrate for the actions of cyclooxygenase and 5-lipoxygenase. These enzymes produce the eicosanoids (prostaglandins, thromboxanes) and leukotrienes. They require oxygen and, therefore, may be important in regions with some perfusion or during reperfusion. Leukotrienes and their metabolic precursors, the hydroperoxyeicosatetraenoic acids, attract leukocytes, constrict vessels, and increase their permeability. They may therefore contribute to breakdown of the blood brain barrier and to brain edema as well as promoting inflammation. Most of the prostaglandins are vasoconstricting and prothrombotic. Prostacyclin does the reverse but its formation may be inhibited by free radicals formed during ischemia.47 These concepts are based in part on experiments showing that inhibitors of thromboxane formation or of eicosanoid formation in general improve CBF after reperfusion in models of focal and global transient ischemia.39,40

Protein kinases are enzymes that phosphorylate structural and regulatory proteins such as cell receptors and membrane channels. Phosphorylation can alter protein function and abnormal phosphorylation of multiple cellular proteins may disrupt many normal cell functions. Attention has focussed particularly on protein kinase C which requires diacetyl glycerol for activation. It is responsible for normal cellular functions but may wreak havoc on cell function when activated for prolonged times and/or to excessive levels during ischemia. It phosphorylates proteins including probably cell membrane channels which may contribute to their dysfunction and to loss of ion homeostasis. Protein kinase C may mediate some of the detrimental effects of glutamate since glutamate activation of NMDA channels may activate protein kinase C and this can increase \([\text{Ca}^{2+}]\).

Expression of some genes is regulated by \([\text{Ca}^{2+}]\), and could be disturbed by loss of normal \([\text{Ca}^{2+}]\) control. Abnormalities could trigger expression of immediate early genes such as c-fos and c-jun that were shown to be activated in ischemia.1,27,40 Theoretically, pathological elevations in \([\text{Ca}^{2+}]\), could also activate incorrect gene expression pathways leading to apoptosis of some neurons after ischemia. Similarly, activation of \([\text{Ca}^{2+}]\)-dependent endonucleases may be important in apoptosis.1,50

The proposed central role of \([\text{Ca}^{2+}]\) in ischemic brain damage led one group of investigators to try to reduce \([\text{Ca}^{2+}]\), by using drugs that chelate \([\text{Ca}^{2+}]\). This was based on the observation that most of the \([\text{Ca}^{2+}]\) entering neurons is buffered by intracellular proteins such as calmodulin, calbindin, and parvalbumin. It is thought that the normal function of these proteins and of \([\text{Ca}^{2+}]\) buffering is to help localize transient \([\text{Ca}^{2+}]\) increases within cells and to then rapidly lower levels after the desired physiological effect has been activated. Favorable effects of \([\text{Ca}^{2+}]\) chelation were found in models of focal ischemia.

IV. Acidosis

Normally glucose is broken down during oxidative phosphorylation to two pyruvate, two ATP, and two reduced nicotinamide-adenine dinucleotide. Pyruvate enters the Kreb's cycle and electron transport chain to use \(\text{O}_2\) and produce \(\text{CO}_2\), \(\text{H}_2\text{O}\), and 36 ATP. During ischemia there is a lack of \(\text{O}_2\) for the Kreb's cycle and electron transport chain so that pyruvate becomes preferentially reduced to lactate. The net result is acidosis and less ATP production. Siesjö47,48 listed four mechanisms of acidosis-induced neuronal damage: edema formation, inhibition of mitochondrial respiration, inhibition of lactate oxidation, and inhibition of \(\text{H}^+\) extrusion. Acidosis affects \([\text{Ca}^{2+}]\) because it displaces \([\text{Ca}^{2+}]\) from intracellular binding sites. Increased \([\text{Ca}^{2+}]\) impairs mitochondrial function because they preferentially sequester \([\text{Ca}^{2+}]\) rather than produce ATP. Acidosis may increase blood brain barrier permeability.

The severity of acidosis depends on preischemic glucose levels as well as how low CBF goes and the degree of ATP failure. It is clear that in experimental cerebral ischemia, hyperglycemia aggravates infarction after transient global ischemia. This is probably because more glucose will produce more lactate. In view of the above, this should occur only if ATP levels also fall — this would be most likely in the core of the infarct where CBF is lowest. It is assumed in humans that increased glucose is detrimental after ischemia and that normoglycemia should be maintained.

In models of permanent focal ischemia the effects of hyperglycemia have been more variable. The
ischemic core would be unlikely to be affected since this tissue undergoes necrosis regardless of glucose level. In the penumbra, hyperglycemia could convert selective neuronal necrosis to necrosis by aggravating acidosis. In the penumbra during transient focal ischemia, hyperglycemia could have the same effect which would manifest as a shortened time for potential recovery in the penumbra.

V. Free radicals

A free radical is any molecule with an unpaired electron in its outer orbital. This generally renders the molecule extremely reactive and able to act as an electron acceptor or donor in reactions with other molecules that then alter the chemical structure of the other molecule. Most toxic free radicals are oxidizing agents, that is they remove electrons from critical molecules within cells. The primary evidence supporting a role for free radicals as cellular toxins is that cells possess enzymes (glutathione peroxidase, superoxide dismutase, catalase) and small molecules (glutathione, ascorbic acid, α-tocopherol) whose sole function is to metabolize certain free radicals to render them unreactive. These free radical scavengers are found in cells because normal cell reactions, such as electron transport, eicosanoid synthesis, purine metabolism, catecholamine autoxidation, and the action of enzymes such as cytochrome P450 reductase, generate free radicals.

Some conditions, such as reperfusion after ischemia, are associated with increased production of free radicals. The influx of oxygen with reperfusion leads to production of superoxide and hydroxyl free radicals as well as hydrogen peroxide that is not a radical but is reactive and can participate in reactions leading to production of free radicals. Reperfusion also supplies inflammatory cells that can generate free radicals. Reperfusion is not necessary for increased free radical production; it can occur under conditions of reduced oxygen supply such as during ischemia. It is believed that free radicals are important in ischemia of long duration and that their effects are mainly on the microvasculature. Experimental studies of free radical-scavenging drugs show that such agents decrease infarct size and edema in focal ischemia but they are generally less effective in ameliorating damage after short periods of global ischemia. The four electron reduction of oxygen as it passes along the electron transport chain in mitochondria generates superoxide radicals and hydrogen peroxide. During ischemia, there is increased leakage of these reactive oxygen species allowing them to participate in other reactions. Superoxide dismutase normally catalyzes the breakdown of superoxide to hydrogen peroxide which is further metabolized to water and oxygen by catalase or glutathione peroxidase. Another reaction believed to form free radicals is the xanthine oxidase catalyzed conversion of hypoxanthine and xanthine to xanthine and uric acid with formation of superoxide and hydrogen peroxide. The subsequent reaction of these oxygen species to form the more reactive hydroxyl radical is catalyzed in the presence of iron that may become available when released from intracellular transferrin stores by acidosis and other reactions promoted during ischemia. NO, itself a free radical, can produce peroxynitrite and hydroxy radicals when it reacts with superoxide. Hydroxyl and peroxynitrite radicals are highly reactive and capable of oxidizing cell protein, lipid, and nucleic acid. Each radical can participate in chain reactions that produce more radicals and in the process damage important cellular proteins, nucleic acids, and lipids. The Ca++-dependent activation of lipases releases more free fatty acids that participate in deleterious free radical chain reactions such as lipid peroxidation.

In addition to pharmacological approaches to modify free radical reactions in ischemia, evidence from use of transgenic and knockout mice has suggested a role for free radicals in ischemia. Genes and their protein products may have different roles depending on the stage of development of the organism. An animal that can survive without a protein that plays some role in the physiology of the adult organism may have developed alterations in other genes that render it susceptible in different ways to ischemia or other insults, independent of what the original function was of the gene that was knocked out or expressed in excess. In transgenic mice expressing copper-zinc superoxide dismutase, infarct volume, and cerebral edema are reduced after transient but not permanent focal ischemia. Results with knockout mice lacking copper-zinc or manganese superoxide dismutase are consistent with these findings in that these mice have increased infarct sizes after transient focal ischemia. Transgenic mice over-expressing BCL-2 develop smaller infarcts after permanent focal ischemia. BCL-2 is the mammalian homologue of the antiapoptosis gene, ced-9, that was identified in the nematode, Caenorhabditis elegans. It interferes with apoptosis in neurons by acting as an antioxidant.

There seems to be little question that at some point in the ischemic injury cascade free radicals participate in and contribute to the damage. The increased production of free radicals during ischemia may overwhelm normal cell defenses. The question
remains, however, about the extent to which free radicals contribute primarily to neuronal death in ischemia, and to which they are simply markers of cell death since there is substantial free radical production as a consequence of cell death. Much of the data implicating free radicals is derived from correlations between ischemic damage and levels of free radicals and the reduction in infarct size by treatment with inhibitors or scavengers of free radicals. In the latter circumstance, however, the drugs may have an effect because free radicals produced by dead cells cause more damage and not necessarily because free radical mechanisms are involved in the primary processes that killed the cells. Finally, since NO is produced in endothelial cells and since reperfusion introduces oxygen primarily in the blood vessels themselves and in the microcirculation, free radicals have been implicated in endothelial cell damage and the microvascular obstructions after ischemia (no-reflow phenomenon), and with endothelial cell damage.

VI. NO

NO is released from endothelial cells and activates guanylate cyclase in smooth muscle cells, increasing cyclic guanosine monophosphate, and causing smooth muscle relaxation. The enzyme responsible for NO synthesis, NO synthase, has since been identified in other cells such as macrophages and neurons. The NO synthases found in endothelial cells are constitutively expressed and dependent on intracellular Ca"⁺ for function whereas the NO synthase in macrophages is inducible by stimuli to the cells and is independent of Ca"⁺. In neurons, NO may function as a neurotransmitter. It is believed that NO itself is not toxic but that it can participate in reactions activated during ischemia that lead to free radical formation. Excess superoxide radicals produced during ischemia can react with NO to form the oxidizing agent peroxynitrite. Intracellular free iron may increase after ischemia because of release from intracellular stores and this can contribute to free radical reactions with NO and oxygen-derived free radicals.

Mice in which the genes for NO synthase have been knocked out have been produced. The effect of these knockouts on infarct size needs to be interpreted after considering, in addition to the factors mentioned above, the potentially beneficial vasodilatory effect of NO and its possible participation in deleterious free radical reactions. Mice deficient in neuronal NO synthase have smaller infarcts than usual indicating that NO derived from the cells that normally contain neuronal NO synthase is deleterious. Knockout of endothelial NO synthase increases infarct size. Inducible NO synthase knockout mice have not been studied.

VII. Cytokines and inflammation

Polymorphonuclear leukocytes accumulate in and around cerebral infarcts starting 4 to 6 hours after transient ischemia and 12 hours after permanent ischemia. Their accumulation would be expected to be a typical tissue reaction to injury (inflammation) and to be accelerated by reperfusion which would supply more leukocytes. The inability to reperfuse the brain after global ischemia (no-reflow phenomenon) and the observed increase in CBF after reperfusion of focal ischemic brain that can be followed by a secondary decrease in CBF (delayed hypoperfusion) may be related to inflammation and the accumulation of leukocytes. The maximal vasodilation of ischemic arteries, presumably a homeostatic response to increase CBF, may aggravate hypoperfusion because vasodilation decreases blood flow velocity. Blood viscosity increases at low flow rates and may precipitate sludging of erythrocytes.

Reperfusion after ischemia causes up-regulation of intercellular adhesion molecules on vascular endothelial cells. These are the sites of binding to integrins (CD11/CD18) on leukocytes that are necessary for adherence of leukocytes to the endothelium and their subsequent migration into tissues to mediate the inflammatory response. Up-regulation of adhesion molecules may cause an overabundant recruitment of leukocytes which could decrease perfusion by blocking vessels directly, releasing vasoconstricting substances that contract the arteries, and killing endothelial cells. Leukocytes cross into the brain, where they degranulate, release cytotoxic enzymes such as myeloperoxidase and generate oxygen-derived free radicals. The evidence in support of these processes and their potentially deleterious consequences was reviewed and includes studies showing consistently that depleting an animal of leukocytes or administering monoclonal antibodies to integrins on leukocytes or to intercellular adhesion molecules reduces infarct size in situations where reperfusion occurs, such as in transient focal ischemia in rats, but not in models of permanent focal ischemia or severe transient ischemia.

The biochemical mechanisms mediating the recruitment of inflammatory cells into infarcts is beginning to be elucidated. In general, such responses involve cytokines, which are a family of low molecular weight proteins and glycoproteins that act on receptors like hormones. They include interferons and inflammatory cytokines (tumor necrosis factor-α and β, interleukins, macrophage-derived...
cytokines, growth factors, chemokines, and monokines). Expression of leukocyte integrins is up-regulated by various inflammatory mediators such as tumor necrosis factor-α, platelet-activating factor, and complement. The possible activation of pathways for synthesis of platelet-activating factor after ischemia is mentioned above and could serve as one stimulus for recruitment of leukocytes into infarcts. There is evidence that some cytokines such as tumor necrosis factor-α, interleukin-6, and interleukin-1β are increased within hours of onset of permanent or transient focal ischemia.

Although the detrimental effects of inflammation and presumably leukocyte infiltration into ischemic brain are believed to occur, some experiments suggested beneficial effects of such processes. Not all aspects of inflammation are necessarily detrimental, for example, inflammation may mediate important remodeling processes after infarction such as gliosis, removal of damaged tissue, stimulation of neovascular proliferation, and improved neuronal plasticity and regeneration.

VIII. Changes in gene expression

Cerebral ischemia tends to cause a decrease in protein synthesis that is predominantly secondary to decreased translation. The transcription and translation of some groups of genes is increased after ischemia. Those identified so far include immediate early genes, stress genes, and genes encoding growth factors and their receptors. Knowledge of the gene response to ischemia may be helpful since the changes presumably reflect processes involved in the death or potentially the survival of injured neurons.

The immediate early genes are induced rapidly and transiently in cells in vitro in response to physiological and pathological stimuli. Many immediate early genes produce proteins that are transcription factors so it is believed that they mediate a delayed genetic response to these stimuli by changing the expression of so-called long-term response genes. Different stimuli induce responses in different immediate early genes — for example, water deprivation selectively induces c-fos expression in the hypothalamus. Cerebral ischemia is followed by a more diffuse induction of multiple immediate early genes including c-fos, fos-B, c-jun, jun-B, jun-D, zif268, Krox 20, and nurr77. The duration and severity of the ischemia probably modifies the response since severe ischemia with rapid necrosis is associated with decreased protein synthesis and rapid cell death. Under these conditions, gene expression is irrelevant but important changes may occur in penumbral areas. In contrast, immediate early gene expression and production of protein products of the genes increases in the ischemic core when there is mild focal ischemia with reperfusion.

Genes for trophic factors and their receptors and for some neurotransmitters and their receptors were increased or decreased by cerebral ischemia. Some of these genes may be regulated by transcription factors encoded by immediate early genes because they have corresponding binding sites in their promoter regions. The role of these changes in ischemia is largely unknown at present although some growth factors seem to protect against permanent focal ischemia in rats.

Stress genes are a second class of genes induced by ischemia. These encode proteins such as heat shock proteins, heme oxygenase, glucose-regulated proteins, and ubiquitins. The heat shock proteins are induced during the same early time as immediate early genes and may protect neurons from ischemic damage.

IX. Apoptosis and necrosis

Neurons die after ischemia by necrosis or apoptosis. Necrotic cell death is characterized by early mitochondrial and cell swelling, rupture of the plasma membrane with release of cell contents, and inflammation. In contrast, apoptosis is cell death determined by transcription of specific genes and subsequent protein synthesis. There are characteristic biochemical and morphological changes. The deoxyribonucleic acid (DNA) is fragmented into characteristic lengths leading to DNA laddering on gels. There is chromatin condensation at the periphery of the nucleus, formation of apoptotic bodies, cell membrane blebbing, and fragmentation of the cell with preservation of mitochondrial and cell membrane integrity. There is no inflammation. The cell dies and is phagocytosed by macrophages. One way in which altered gene expression has been suggested to be important in cerebral ischemia is through modulation of apoptosis. Clearly the predominant process in the core of a region of ischemic brain is necrotic cell death but if there is milder ischemia in a penumbral region or when there is transient ischemia followed by reperfusion, then there could be mild cell injury that activates a gene response leading to apoptosis. This theory has gained some support from experimental stroke models that demonstrate that 90 minutes of middle cerebral artery occlusion in rats leads to an infarct 1 day later whereas 30 minutes of middle cerebral artery occlusion produces no damage after 1 day but an infarct develops over 3 days. The delayed infarction can be reduced by treatment with a glutamate antagonist and cycloheximide, a protein synthesis inhibitor.
more, mice over-expressing Bcl-2 have decreased infarct size after focal cerebral ischemia. Previous studies reported delayed neuronal necrosis days after ischemia but this may actually have been apoptosis.

Since changes in gene expression may increase or decrease a cell’s susceptibility to undergo apoptosis, activation of some genes may be beneficial whereas that of others may be detrimental. Little work has been done but some studies show neuroprotective effects of some growth factors in vivo and detrimental effects of others in vitro.

X. Other processes

Endothelins are potent vasoconstricting peptides. Immunoreactivity for endothelins in brain and their protein levels in cerebrospinal fluid have been reported to be elevated in focal ischemia models. Endothelin receptor antagonists were shown to be beneficial in increasing CBF and decreasing infarct size in such models. It is uncertain if this is secondary to vascular effects such as dilation of collateral pathways or to prevention of vasoconstriction during reperfusion, although the latter would be an unlikely mechanism in cases where benefit was shown in models of permanent middle cerebral artery occlusion.

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