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Free Radicals and Brain Damage in the Newborn

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Abstract
Newborns and particularly preterm infants are at high risk of oxidative stress and they are very susceptible to free radical oxidative damage. Indeed, there is evidence of an imbalance between antioxidant- and oxidant-generating systems which causes oxidative damage. The brain may be especially at risk of free radical-mediated injury because neuronal membranes are rich in polyunsaturated fatty acids and because the human newborn has a relative deficiency of brain superoxide dismutase and glutathione peroxidase. The brain of the term fetus is at higher risk of oxidative stress than that of the preterm fetus, as a consequence of its higher concentration of polyunsaturated fatty acids and the maturity of the N-methyl-*D*-aspartate receptor system at term. There seems to be a maturation-dependent window of vulnerability to free radical attack during oligodendrocyte development. Early in its differentiation, the oligodendrocyte may be vulnerable because of active acquisition of iron for differentiation at a time of relative delay in the development of certain key antioxidant defenses in the brain. Excess free iron and deficient iron-binding and -metabolizing capacity are additional features favoring oxidant stress in premature infants. Free radicals may be generated by different mechanisms, such as ischemia-reperfusion, neutrophil and macrophage activation, Fenton chemistry, endothelial cell xanthine oxidase, free fatty acid and prostaglandin metabolism and hypoxia. Reactive oxidant production by these different mechanisms contributes in a piecewise manner to the pathogenesis of perinatal brain injury.

Introduction

Free radicals are highly reactive chemical molecules containing one or more unpaired electrons. They donate or take electrons from other molecules in an attempt to pair their electrons and generate a more stable species. Oxygen-derived free radicals, collectively termed reactive oxygen species (ROS), are normally produced in living organisms. When overproduced, they are important mediators of cell and tissue injury [1, 2]. Free radicals are relatively unstable and certain enzymes and small-molecular-weight molecules with antioxidant capabilities have a protective effect [3]. There is therefore a critical balance between free radical generation and antioxidant defenses. Oxidative stress in vivo is a degenerative process caused by the overproduction and propagation of free radical reactions. Free radical reactions lead to the oxidation of lipids, proteins and polysaccharides and to DNA damage (fragmentation, apoptosis, base modifications and strand breaks), and therefore have a wide range of biologically toxic effects [4, 5]. Intracellular and extracellular antioxidant systems protect against free radical-induced damage. Transferrin (Tf), ceruloplasmin, vitamin C, vitamin E, uric acid, bilirubin, sulfhydryl groups and other unidentified antioxidants contribute to the total antioxidant capacity of extracellular fluids [6].

Oxidative stress exists and tissue damage is possible when there are low levels of antioxidants or increased free radical activity [1]. Newborns and particularly preterm infants are at high risk of oxidative stress and they are very susceptible to free radical oxidative damage [4]. Indeed, there is evidence of an imbalance between antioxidant- and oxidant-generating systems which causes oxidative damage [7]. At birth, the newborn encounters an environment much richer in oxygen (PO_2 100 Torr) than the intrauterine environment (20–25 Torr). This 4- to 5-fold increase is exacerbated by the low efficiency of natural antioxidant systems in the newborn, especially the preterm newborn [8]. Neonatal plasma has an antioxidant profile with low levels of glutathione peroxidase, superoxide dismutase, β-carotene, riboflavin, α-proteinase, vitamin E, selenium, copper, zinc, ceruloplasmin, Tf and other plasma factors [9–11]. The brain may be

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especially at risk of free radical-mediated injury, because neuronal membranes are rich in polyunsaturated fatty acids and because the human newborn, especially if preterm, has a relative deficiency of brain superoxide dismutase and glutathione peroxidase [12]. The brain of the term fetus is at higher risk of oxidative stress than that of the preterm fetus as a consequence of its higher concentration of polyunsaturated fatty acids and the maturity of the N-methyl-*D*-aspartate receptor system at term [13, 14]. There seems to be a maturation-dependent window of vulnerability to free radical attack during oligodendrocyte development [15]. Early in its differentiation, the oligodendrocyte may be vulnerable because of active acquisition of iron for differentiation at a time of relative delay in the development of certain key antioxidant defenses in the brain [16, 17].

Excess free iron and deficient iron-binding and -metabolizing capacity are additional features favoring oxidant stress in premature infants [18, 19]. Free radicals may be generated by different mechanisms, such as ischemia-reperfusion, neutrophil and macrophage activation, Fenton chemistry, endothelial cell xanthine oxidase, free fatty acid and prostaglandin metabolism and hypoxia [20–24].

Hypoxia-Asphyxia

Hypoxia-asphyxia plays a key role in the perinatal period. Although the consequences of hypoxia-asphyxia can be easily observed, the specific pathologic processes preceding the onset of irreversible cerebral damage are not well understood and appear to be a combination of several complex mechanisms [25]. Early events in the hypoxia-induced response trigger tyrosine phosphorylation cascades involving many enzymes and substrates. Studies performed in guinea pig cerebral cortical synaptosomes suggest that hypoxia remodels the signaling pathway by inducing quantitative and qualitative changes in protein phosphorylation [26]. Many experimental studies have demonstrated free radical production and oxidative damage due to hypoxia in fetal life [27–29]. We recently demonstrated a direct relation between the degree of hypoxia and the severity of oxidative damage in plasma of newborn infants at birth [30].

In the developing brain, hypoxia results in an increase in anaerobic metabolism, leading to a rapid rise in levels of lactic acid and oxygen free radicals [31, 32]. Mitochondrial oxidative metabolism, nitric oxide (NO), phospholipid metabolism, iron, proteolytic and inflammatory pathways are other potential sources of intracellular free radicals and ROS [33–35]. Free radicals may cause lipid peroxidation of immature myelin sheaths and lipid peroxides are themselves free radicals [36].

Mitochondrial Production of Free Radicals

ROS are normally generated in a continuous manner by the mitochondrial respiratory chain [33, 37]. In normal mitochondria, oxygen is reduced to water by cytochrome C oxidase in four consecutive one-electron steps. Thus, the production of superoxide radicals ($O_2^{\cdot-}$) occurs during the operation of complex I and complex II in the mitochondrial electron transport chain, and at least at the level of coenzyme Q as a result of the semiquinone state ($UQ^{\cdot-}$) of ubiquinone donating electrons to molecular oxygen [38]. Mitochondria have an efficient antioxidant system composed of superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione, NAD(P) transhydrogenase, NADPH, vitamins E and C, thiol peroxidases such as SP-22 and mitochondrial respiration itself [39–41]. Superoxide radicals produced by the respiratory chain are readily dismutated by mitochondrial superoxide dismutase, producing H_2O_2 [42]. Under conditions in which mitochondrial superoxide generation increases, or when antioxidant systems are depleted, H_2O_2 may accumulate, leading to a condition of mitochondrial oxidative stress. In this situation, H_2O_2 may react with mitochondrial Fe^{2+}, resulting in the formation of the highly reactive hydroxyl radical (HO) via the Fenton reaction [43].

The basal rate of mitochondrial superoxide generation may be altered by different physiological or pathological conditions [37]. Specifically, when mitochondria are loaded with Ca^{2+}, uncoupling of mitochondrial respiration from ADP phosphorylation increases mitochondrial production of ROS [44]. Thus, accumulation of ROS and oxidative stress is initiated early during hypoxia-ischemia due to the dramatic increase in cytosolic Ca^{2+} concentrations, which upsets mitochondrial handling of Ca^{2+}. Normal Ca cycling across the inner mitochondrial membrane serves to regulate mitochondrial enzymes such as pyruvate dehydrogenase and α-oxyglutarate dehydrogenase [35]. However, when intracellular Ca increases over the set point for net calcium accumulation or when the calcium release pathway is stimulated by prooxidants, cycling may become excessive and lead to increased ROS production, loss of mitochondrial membrane potential, structural alterations of the inner mitochondrial membrane and inhibition of adenosine 5′-triphosphate (ATP) synthesis [35]. ROS generation increases due to disorganization of the mitochondrial respiratory chain, since most components of this system are integral inner mitochondrial membrane proteins. Mitochondria are particularly sensitive to hypoxic injury and play a central role in both apoptosis and necrosis [45]. Severe mitochondrial damage releases a flood of Ca^{2+} and ROS into the cytosol, leading to the disruption of plasma membrane integrity and cell damage [35]. They may also directly activate caspase-9 and trigger apoptotic cell death [46].

Role of Energy Metabolism and Calcium

The normal functioning of the brain is essentially dependent on an adequate oxygen supply to maintain energy metabolism. At the cell level, cerebral hypoxia-ischemia sets in motion a cascade of biochemical events commencing with a shift from oxidative to anaerobic metabolism, which leads to an accumulation of NADH, FADH and lactic acid and H^+ ions [47] (fig. 1). Anaerobic glycolysis does not provide sufficient energy, resulting in the depletion of high-energy phosphate reserves, including ATP [48]. During moderate hypoxemia, the fetus maintains adequate levels of ATP by speeding up the rate of anaerobic glycolysis, but an acute reduction in the fetal oxygen supply leads to the breakdown of energy metabolism in a few minutes [49–51]. The Na^+/K^+ pump stops working through lack of energy. The transcellular ion pump fails, leading to loss of membrane potential and an influx of Na^+, Ca^+ and Cl^-. Intracellular accumulation of Na^+ and Cl^- ions leads to swelling of the cells as water enters by osmosis (cytotoxic cell edema) [52]. Calcium buildup in the cytoplasm occurs by other mechanisms besides massive influx due to the extreme extraintracellular concentration gradient. Calcium enters the cytosol by the activation of voltage-dependent channels [25]. Calcium is released by mitochondria stimulated by the increase in intracellular Na^+ and free fatty acids. It is also released by the endoplasmic reticulum through the depletion of ATP [53]. It enters through agonist-dependent channels such as amino-hydroxyl-methyl-isoxa-

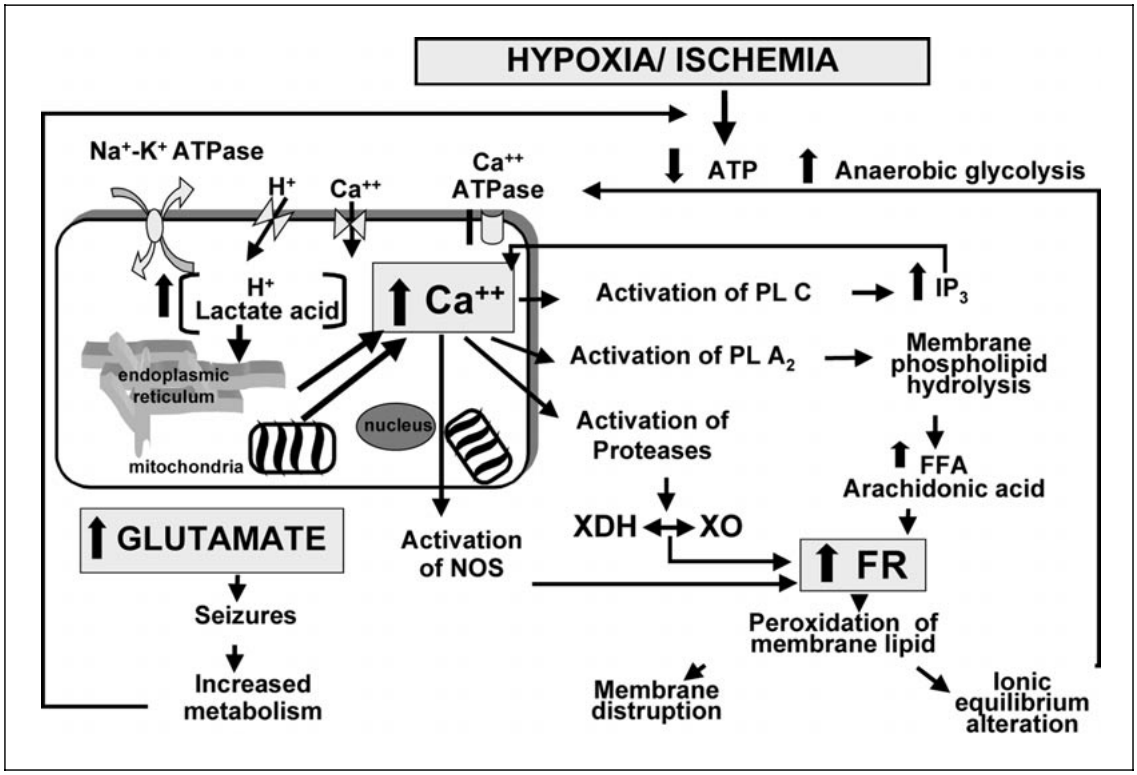

Fig. 1. Cellular mechanisms of free radical production during hypoxia. PL = Phospholipase; IP_3 = inositol triphosphate; XDH = xanthine dehydrogenase; XO = xanthine oxidase; FFA = free fatty acids; FR = free radicals.

zole propionate, kainate and N-methyl-*D*-aspartate receptors [54]. The intracellular buildup of calcium has many consequences. One damaging effect is the activation of phospholipases A_2 and C [55]. These reactions lead to membrane phospholipid hydrolysis, producing free radicals, disrupting cell and organelle membranes, increasing permeability and altering ionic distribution. Phospholipase C also catalyzes reactions leading to the production of inositol triphosphate, a second messenger that releases calcium from the endoplasmic reticulum, and diacylglycerol, which decreases calcium-sodium exchange [53, 56]. Both reactions further augment calcium concentrations in the cell and amplify its deleterious effects, creating a vicious circle that ultimately destroys the cell.

Free Radicals during the Reperfusion Phase

During cerebral ischemia, the cutback in oxidative phosphorylation rapidly diminishes reserves of high-energy phosphates [48]. High levels of adenosine and hypoxanthine accumulate in a few minutes. During reperfusion, these metabolic products are further metabolized by xanthine oxidase to xanthine and uric acid [57], resulting in their build up in blood and tissues such as the brain [58]. The activity of xanthine oxidase in the resting brain is very low [59], but during cerebral ischemia, a massive conversion of xanthine dehydrogenase to xanthine oxidase takes place, regulated by the calcium-dependent protease calpain [60]. The breakdown of hypoxanthine by xanthine oxidase in the presence of oxygen produces a flood of superoxide radicals [61, 62]. These are then converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide and tissue iron can then combine to form hydroxyl radicals by the Haber-Weiss reaction [63]. Xanthine oxidase concentrates in endothelial cells lining the cerebral microvasculature, targeting the blood-brain barrier for oxidative attack [64]. Accelerated arachidonic acid metabolism in brain tissue and leukocyte activation after ischemia also produce large quantities of oxygen radicals [21].

Although there is more evidence that blood vessels are the main source of free radicals in cerebral ischemia-reperfusion, neurons also generate superoxide in response to anoxia by activated neutrophils and microglia [65]. Free radicals impair transmembrane enzyme Na^+/K^+-ATPase activity, especially in cortical synaptosomal membranes, resulting in persistent membrane depolarization and excessive release of the excitatory amino acid glutamate [29, 66]. Cerebellar granule cells produce superoxide when exposed to the excitatory amino acid N-methyl-*D*-aspartate [67]. Besides being neurotoxic, glutamate is also toxic to oligodendroglia, via free radical effects [68]. Glutamate enters the cell in exchange for cystine. Intracellular cystine depletion is followed by a drop in glutathione levels and the cells die of oxidative stress [68].

Studies have shown that free radicals are also implicated in initiating delayed cell death by apoptosis after cerebral ischemia [69, 70]. An enormous amount of data suggests that oxidative stress plays a role in the initiation of apoptosis [70–73], but it is not yet clear

exactly how ROS trigger this response. Like Ca^{2+}, oxidative stress may either promote or inhibit apoptosis, depending on the degree of the insult [74]. Kim et al. [75] demonstrated that hydrogen peroxide and NO directly inhibit caspase activity in vivo. The close relationship between oxidative stress and mitochondrial function also suggests that overproduction of ROS leads to ATP depletion and apoptosis [76]. Oxidative stress may also disrupt intracellular Ca^{2+} homeostasis, inhibiting apoptosis [77].

Nitric Oxide

NO is a free radical synthesized by NO synthase (NOS) in endothelial cells and neurons in response to rises in intracellular calcium concentrations [78]. NOS produces NO, citrulline and water from arginine, NADPH and oxygen [79]. NO and superoxide radicals combine to produce peroxynitrite, which spontaneously decomposes to form hydroxyl radicals, nitrogen dioxide and NO_2^+ [80]. Three types of NOS are known: neuronal NOS (NOS 1), inducible NOS (NOS 2) and endothelial NOS (NOS 3) [81–83]. However, NOS occurs in a wide variety of other cell types. Although NOS 1 is the principal neuronal form of NOS and the predominant form in the nervous system, all three forms of NOS are reported to be expressed in some populations of neurons. In addition, NOS 1, NOS 2 and possibly NOS 3 have been detected in astrocytes, and NOS 1 in oligodendrocytes and microglia. The activity of all three forms of NOS increases during ischemia; NOS 1 and 3 within minutes and NOS 2 after several hours [84].

Since there is no oxygen available during ischemia, NO cannot be synthesized until the reperfusion phase. Likewise, many superoxide radicals are produced in mitochondria by xanthine oxidase and other pathways during and especially after ischemia. During reperfusion, NO and superoxide radicals combine to produce peroxynitrite, leading to the formation of more potent radicals [85, 86]. Other potentially damaging metabolites of NO include the nitrogen dioxide radical NO_2 and nitryl chloride, formed by the reaction of nitrite, an end product of NO metabolism, with hypochlorous acid, itself produced by the action of myeloperoxidase in neutrophils [87, 88]. Experimental studies have demonstrated that the initial NO-mediated vasodilation and enhanced perfusion that result from the activation of NOS 3 are neuroprotective, at least during the first 2 h of ischemic insult [89]. However, the overall effects of enhanced NOS 1 and NOS 2 activity after ischemia are detrimental [90].

Iron Toxicity

Iron is a versatile and highly reactive element. By virtue of its two common valences, iron (II) (ferrous) and iron (III) (ferric), it has access to a wide range of redox potentials spanning the standard redox potential range from ± 300 to -500 mV [91]. This property underlies its essential biological role in oxygen transport and many electron transfer reactions [92].

Normally, iron is safely sequestered in transport proteins such as Tf and lactoferrin and stored in proteins such as ferritin (Ft) and haemosiderin [93]. The plasma copper-containing protein ceruloplasmin acts in concert with the above proteins, catalyzing the oxidation of reactive ferrous ions to less reactive ferric ions which bind Tf [94, 95]. In healthy adults, plasma Tf is approximately one third loaded with iron, and the protein retains a considerable ability to bind iron salts. Tf can bind 2 mol of iron per mole of protein, and when iron is correctly loaded on its high-affinity binding sites, it is not available as a growth factor for tissues, or as a prooxidant factor [96, 97].

Since iron ions cannot exist in plasma, the term 'free iron' has been introduced to indicate a low-molecular-mass iron form, free of high-affinity binding to Tf [96]. Free iron seems to occur in plasma, complexed to citrate, lactate or phosphate or loosely bound to albumin or other proteins [98]. During situations of iron overload and low plasma pH, as occurs during ischemia, Tf releases its iron and chelatable forms of Fe (iron ions or redox-active complexes of iron) escape sequestration in biological systems, producing free radicals [99, 100]. These free radicals may release even more iron by mobilizing it from ferritin. This may lead to a cascade of iron release and free radical production, causing extensive cell damage [101].

We recently observed higher intraerythrocyte free iron levels in infants with asphyxia [102]. Iron may be released from hemoglobin in erythrocytes as result of oxidative stress [103]. Since the erythrocyte is a target of extracellular free radicals, free iron release may follow extracellular oxidative stress caused by superoxide anion release due to phagocyte activation [104]. Intraerythrocyte free iron concentrations appear to be a reliable marker of red cell oxidative stress and an indicator of the risk of oxidative injury in other tissues. Indeed, free radicals are linked to neonatal oxidative stress and are involved in severe diseases such as retinopathy, bronchopulmonary dysplasia, intraventricular hemorrhage and hypoxic-ischemic encephalopathy [4, 105]. In these oxidative stress-related pathologies, iron is released from iron stores and may cause cell damage by lipid and protein peroxidation. The highest values of lipid and protein peroxidation have been found in hypoxic newborns. The more severe the hypoxia, the higher the intraerythrocyte free iron release, free radical production and oxidative damage [30, 106].

The newborn infant is very susceptible to free iron-induced oxidative damage [107]. Plasma from a high percentage of normal term and preterm neonates has recently been shown to contain free iron, as if Tf were fully loaded with iron [108–110]. The iron-binding capacity of cerebrospinal fluid was also found to be low (low Tf concentrations), and high concentrations of vitamin C and low concentrations of ceruloplasmin in cerebrospinal fluid suggested that most of the iron was in its active ferrous form [111, 112].

Since iron-positive reactive glia collect near damaged tissue, iron accumulation is a sensitive indicator of injury [113]. In damaged areas, there is an increase in iron-positive reactive glia starting about 8 h after recovery and an earlier (4–8 h after hypoxia-ischemia) increase in microglia, especially around cortical blood vessels [114]. The perivascular distribution of iron reaction products is a consistent finding 1 week after recovery. The consequences of selective vascular injury include secondary ischemia and blood-brain barrier disruption. After asphyxia in newborn infants, there is an increase in intraerythrocyte and plasma free iron, significantly correlated with neurodevelopmental outcome [109]. Leakage of plasma free iron into the brain through a damaged barrier may occur and is particularly damaging, as it is taken up directly by cells in a manner that is independent of Tf. Additional sources of free iron could be iron released by heme catabolism and iron released from storage protein by oxidative stress. During ischemia-reperfusion, ROS are generated by mitochondrial dysfunction, exocytotoxic insult, metabolism of arachidonic acid, inflammation and stimulation of NOS and xanthine oxidase [115]. Oxidative stress may also result from iron delocalization induced by the superoxide anion, acidosis and anoxia [102, 106].

Acidosis during cerebral ischemia potentiates oxidative neuronal death resulting from impaired antioxidant enzyme functions and increased intracellular free iron levels [116]. Enhanced proteolytic activity occurring in injured tissue also releases iron from storage proteins [117]. When non-protein-bound iron gains access to the extracellular space, its uptake by cells is enhanced by intracellular calcium and paradoxically also by increased levels of intracellular iron [118].

The toxicity of iron is inversely proportional to the availability of ferritin to sequester and detoxify ferrous ion, and directly proportional to the quantity of hydrogen peroxide available to produce hydroxyl radicals by the Fenton reaction [99, 119].

After hypoxia, the expression of Tf receptors (TfRs) on brain macrophages increases [120]. Hypoxia-reoxygenation is known to increase iron content and iron release in the extracellular space, causing injury to the periventricular white matter where microglial cells are known to preponderate [121]. It has been suggested that TfRs are involved in acquiring excess iron from the extracellular spaces, probably for storage; they therefore presumably help protect the brain from the toxic effects of excess iron. Normal axonal transport of brain iron has also been reported to be disrupted in anoxia-ischemia, leading to increased accumulation of iron in the white matter [121]. The increased expression of TfRs along with accumulation of iron in microglial cells is a protective mechanism to facilitate the active uptake of excess iron that may be released by iron-rich oligodendrocytes, or may accumulate due to disruption of its normal transport after hypoxic insult [119].

Role of Infections

Ischemia and subsequent reperfusion can set off an inflammatory reaction in the brain [122, 123]. IL-1, IL-6, transforming growth factor and fibroblast growth factor appear to be formed in activated microglia [65, 124]. They are thought to mediate the migration of inflammatory cells in reperfused tissue. Increased expression of the adhesion molecules P- and E-selectin and ICAM-1 on endothelial cells and integrins on leukocytes cause granulocytes to attach to the endothelium, migrate through the vessel wall and accumulate in the interstitium [68, 125, 126]. There, after further activation by cytokines, they synthesize oxygen radicals, especially superoxide radicals, that proceed to damage neuronal tissue. So, reperfusion injury has been attributed to free radicals produced by neutrophils at the site of damage, but this is only part of the story. Neutrophils, which are activated by C5a and IL-8 released by ischemic tissue [127], damage reperfused tissue by other mechanisms in addition to free radical production, and the damaged tissue has other sources of free radicals besides neutrophils [128].

Recent clinical studies suggest that perinatal brain damage is closely associated with intrauterine infection before or at birth [129–131]. However, it remains unclear whether fetal brain damage due to endotoxemia is the result of cerebral hypoperfusion caused by circulatory decentralization or is caused directly by endotoxins on cerebral tissue. Lipopolysaccharide (LPS)-induced effects on fetal circulation seem to play a central role in the development of fetal brain damage due to intrauterine infection [132]. A direct toxic effect of LPS on immature brain tissue seems unlikely; however, delayed activation of LPS-sensitive pathways involved in apoptosis-like cell death and damage limited to a small subgroup of cells, such as oligodendrocyte progenitors, cannot yet be excluded [132].

Conclusions

The relationship between free radical production and perinatal brain damage is complex. It is clear that free radical damage results from many pathogenic influences; hypoxia, ischemia-reperfusion, neutrophil and macrophage activation, Fenton chemistry, endothelial cell xanthine oxidase, phospholipid metabolism, NO, mitochondrial oxidative metabolism, iron and proteolytic pathways are all implicated. Reactive oxidant production by these different mechanisms contributes in a piecewise manner to the pathogenesis of perinatal brain injury, but each mechanism is only one of the many factors responsible. Each step in the oxidative cascade has become a potential target for therapy. The multiplicity of pathways and processes involved suggests that there is considerable potential for additive or synergistic benefit from combined therapies.

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