Cannabinoid as a neuroprotective strategy in perinatal hypoxic-ischemic injury

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Abstract: Perinatal hypoxia-ischemia remains the single most important cause of brain injury in the newborn, leading to death or lifelong sequelae. Because of the fact that there is still no specific treatment for perinatal brain lesions due to the complexity of neonatal hypoxic-ischemic pathophysiology, the search of new neuroprotective therapies is of great interest. In this regard, therapeutic possibilities of the endocannabinoid system have grown lately. The endocannabinoid system modulates a wide range of physiological processes in mammals and has demonstrated neuroprotective effects in different paradigms of acute brain injury, acting as a natural neuroprotectant. Concerning perinatal asphyxia, the neuroprotective role of this endogenous system is emerging these years. The present review mainly focused on the current knowledge of the cannabinoids as a new neuroprotective strategy against perinatal hypoxic-ischemic brain injury.

Keywords: perinatal hypoxia-ischemia; brain injury; neuroprotective strategies; endocannabinoid system

1 Introduction

Perinatal hypoxic-ischemic brain injury remains a major cause of developmental disability in children, despite the improvements in perinatal care^[1–4]. Its incidence has been reported to be 0.2%–0.6% in term births^[5], and significant neurological sequelae occur in as many as 50%–75% of these children^[6]. After asphyxia, infants may suffer from long-term neurological sequelae such as cerebral palsy, mental retardation and epilepsy. Asphyxia is also associated with attention deficits and hyperactivity in children and adolescents^[7,8]. The high incidences of certain kinds of hypoxic-ischemic brain lesions^[9,10] can be partly attributed to the fact that the developing brain is especially

vulnerable to ischemia^[11–15].

To minimize as much as possible the neurological consequences from hypoxic-ischemic damage, neuroprotective strategies are urgently required, including rapid identification of affected neonates to allow timely institution of therapy, improved monitoring in the perinatal period and strict control in intensive care, and post-insult therapies to ameliorate the evolving injury^[16,17]. The period of time following hypoxia-ischemia when the therapeutic strategies can be efficacious in the reduction of brain damage, also called “therapeutic window”, is normally short, and therefore, rapid identification of children with a greater risk of suffering from neonatal hypoxia-ischemia is important. Following hypoxic-ischemic damage, this window may vary from 2 h to 6 h. Thus, the rapid identification would facilitate applications of diverse rescue strategies^[18].

The consequence of asphyctic damage depends on the severity, the intensity and the timing of asphyxia, as

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well as selective ischemic vulnerability and immaturity of the brain, determining that some cells die and others remain in a state of ischemic penumbra^[19–21]. Despite the recovery of cerebral blood flow, many of these cells die in a process referred to as delayed cell death, whose cellular expression is programmed necrosis (necroptosis) and apoptosis^[22–28]. It is these cells that appear to be more likely rescued, involving the search for therapeutic interventions that prevent the onset or interrupt the progression of destructive cascades.

2 Therapeutic strategies

As hypoxic-ischemic brain injury is often unpredictable, the primary approach is to develop post-insult therapies to ameliorate ongoing or secondary injury^[17,29]. In this regard, many studies have been carried out to identify new therapeutic strategies that diminish brain damage caused by hypoxia-ischemia^[30–32].

The employment of hypothermia as a therapy for neuronal rescue^[33] has received particular attention. Although studies using hypothermia as a therapeutic strategy in adults presenting cerebral infarction have shown encouraging results, the effectiveness of this strategy in neonatal ischemia still continues to be experimental^[34,35]. However, studies in newborns have shown different levels of neuroprotection from hypothermia, either through inhibiting apoptotic cellular death and DNA fragmentation following hypoxia-ischemia^[24,36] or through inducing a reduction in glutamate concentration at the synaptic space, a delay in the accumulation of intracellular calcium and a reduction in the production of nitric oxide^[37,38]. Nevertheless, its effectiveness differs among asphyctic children, being more effective in larger than in smaller infants^[39].

Other therapies are centered on diminishing brain damage arising from free radicals, thus employing antioxidant molecules such as allopurinol which inhibits xanthine oxidase^[40,41], and N-acetylcysteine which increases the intracellular level of glutathione and reduces apoptosis and inflammation^[42–45], to sequester free radicals. Other antioxidant-related drugs commonly used include erythropoietin,

which has antiapoptotic and angiogenic properties^[46] and provides neuroprotection and neurogenesis in neonatal rats^[47,48], and melatonin, which reduces brain damage and development of later sequelae^[49,50]. Furthermore, compounds with anti-inflammatory properties have also been tested, such as the second generation of tetracycline that inhibits the activation of microglia, resulting in an increase in neuronal survival^[51,52], and the statins^[53,54] which reduce the expression of interleukin-1 beta and Inter-Cellular Adhesion Molecule 1.

Since there is still no specific treatment for perinatal brain lesions due to the complexity of neonatal hypoxic-ischemic pathophysiology, interest has grown in the neuroprotective potentials of cannabinoids after perinatal asphyxia.

3 The endocannabinoid system

The newly discovered intercellular communication network called “endocannabinoid system”, with its important homeostatic and pathologic functions, has recently sparked interest in the possible applications of cannabinoids in research and clinical practice. This system consists of the target receptors (cannabinoid receptors and non-cannabinoid receptors), the exogenous and endogenous ligands, as well as the enzymes responsible for endocannabinoid biosynthesis, transport and degradation^[55–57].

3.1 Target receptors The main endogenous targets are the cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) receptors, both of which are G protein-coupled receptors^[58]. Endocannabinoids can also interact with others, such as TRPV1 (vanilloid) receptors^[59], the TASK-1 potassium channel, peroxisome proliferator-activated receptors^[60] and other unidentified receptors. Within the central nervous system, the CB1 receptor is primarily neuronal, located at presynaptic terminals, although there are examples of CB1 receptor functionally expressed in non-neuronal cells. The CB1 receptor is located preferentially in the basal ganglia, amygdale, molecular layer of the cerebellum and certain parts of the hippocampus, and to a lesser extent in I and IV layers of the cerebral cortex, while a limited number of receptors are found in the hypothala-

mus, brainstem and bone marrow. Apart from expression in brain, the CB1 receptor is also expressed in the spleen, heart, prostate, uterus, ovary, thymus and testicles^[61]. The location of CB2 receptor is more restricted, mainly at the peripheral level (the spleen, pharyngeal amygdalae, B lymphocytes and to a lesser extent, monocytes and T lymphocytes)^[61]. Recently, CB2 expression has been demonstrated in activated microglia and also in neurons, but its level is lower than that of CB1 receptor^[62-65].

3.2 Cannabinoids The endocannabinoid system receives its name from the cannabis plant *Cannabis sativa*, which contains different chemical compounds that cause the psychoactive and physiological effects of the plant, considered as cannabinoids^[66]. Currently, 60 different cannabinoids have been identified. To date, 3 kinds of cannabinimetic compounds have been described: phytocannabinoids (produced only by the cannabis plant), endogenous cannabinoids or endocannabinoids (synthesized in the brain and in certain peripheral tissues), and synthetic cannabinoids (produced by pharmaceutical companies for medical use).

Phytocannabinoids (also called herbaceous or classic cannabinoids) are substances that normally have a carbocyclic structure of 21 carbons and are formed generally by 3 rings of cyclohexene, tetrahydropyran and benzene. Within this group, $\Delta(9)$ -tetrahydrocannabinol (THC) which has the greatest psychoactive potency, $\Delta(8)$ -THC, cannabiol and cannabidiol are the most representative^[67].

Although other homologues with cannabinimetic activity have been described, the 2 most widely studied endocannabinoids are N-arachidonylethanolamide (anandamide, AEA)^[68] and 2-arachidonoyl-glycerol (2-AG)^[69,70]. These eicosanoids are endogenous compounds of lipid nature derived from polyunsaturated fatty acids with long chains, synthesized and released in moments of intense activity of the brain^[71]. Other recently described endogenous cannabinoids are 2-arachidonyl glyceryl ether (noladin ether)^[72], O-arachidonoyl-ethanolamine (virodhamine)^[73] and N-arachidonylethanolamine^[74,75].

Synthetic cannabinoids have been developed based on the chemical structures of phytocannabinoids in the aim

of understanding more about the pharmacology of these compounds. There are 2 options of pharmacological modulation of associated responses. The first one attempts to block the effects of endogenous ligands using antagonists of cannabinoid receptors. The mostly used antagonists are SR141716 or rimonabant^[76] and AM251 (CB1 receptor-selective antagonists), as well as SR144528^[77] and AM630 (CB2 receptor-selective antagonists). The second one attempts to potentiate the effects associated with endogenous receptors by using specific agonists such as ACEA (a CB1 receptor-selective agonist), AM1241 and JWH015 (CB2 receptor-selective agonists), or non-selective agonists like CP55940^[78], HU210 or WIN55212-2 (WIN)^[79]. Until the 1990s, all the known compounds acting as cannabinimetics were structurally derived from THC. This situation changed when a new family of aminoalkylindoles possessing cannabinimetic properties was produced, with WIN being the head of this group. WIN is a non-selective agonist of CB1 and CB2 receptors: it has a great affinity for both receptors, with a moderate predilection for CB2 (Ki: 1.9 nmol/L at CB1; Ki: 0.28 nmol/L at CB2) and develops an intrinsic activity relatively high in both of them^[67]. Through the employment of a radiolabeled aminoalkylindole [³H]WIN55212-2, a technique has been developed by which the cannabinoid receptors in the rat brain can be localized and characterized^[80,81].

3.3 Metabolic enzymes AEA is produced when needed from the hydrolysis of a pre-formed membrane phospholipid precursor N-arachidonoylphosphatidylethanolamine by the action of N-arachidonoyl phosphatidylethanolamine-phospholipase-D^[82,83]. In most tissues, hydrolysis of anandamide is catalyzed by fatty acid amide hydrolase (FAAH)^[84], but anandamide also acts as a substrate for palmitoylethanolamide-preferring acid amidase^[85,86], cyclooxygenase-2, lipoxygenases, and cytochrome P450^[87] to produce biologically active products.

2-AG is also synthesized on demand through the conversion of 2-arachidonate-containing phosphoinositides to diacylglycerols, which are then converted to 2-AG by the action of diacylglycerol lipase. The serine hydrolase monoacyl glycerol lipase (MAGL) is mainly responsible

for its metabolism *in vivo*, although it can also be metabolized by FAAH as well as by cyclooxygenase-2 and lipoxygenases^[83,84,88,89].

4 The endocannabinoid system in pre- and postnatal development

The endocannabinoids and their receptors are abundantly present from the early developmental stages and are important during the maturation of the nervous system and its functions. AEA can be detected from the early stages of the embryo^[90], but during the fetal period, 2-AG is present at much higher concentrations than AEA^[91]. Moreover, the fetal-postnatal expression pattern differs between the 2 endocannabinoids. AEA concentration gradually increases throughout development until an adult level is reached, whereas the fetal level of 2-AG is similar to those in young and adult brains^[92]. Concerning the CB receptors, similar expression patterns of CB1 receptor were found during human pre- and postnatal development in comparison to those reported in rat brain^[92,93]. In this respect, significant levels of these receptors are present in the fetal human brain from relatively early stages of development to the late gestational period. Thus, CB1 receptors are detected at week 14 of gestation in the human embryo and, after 6 weeks, selective expression of CB1 receptor is recorded in the limbic area CA of the hippocampus and in the amygdale^[94]. Consistent with the previous studies in rat models^[92], a progressive increase in the concentration of CB1 receptor is found in most human brain regions (frontal cortex, hippocampus, basal ganglia and cerebellum) from the fetal period to adulthood, with the CB1 receptors being functionally active at all stages of development^[95].

5 Cannabinoid signaling in neural cells

In general, the signaling of the endocannabinoid system is characterized by rapid synthesis of endocannabinoids, the activation of receptors and the not-least rapid breakdown of those^[96]. Also, a very important role of CB1 receptor in the brain is its participation in the retrograde system of signaling. This mechanism can be defined as the

communication through molecules (endocannabinoids in this case) arising in post-synaptic cells and diffusing into pre-synaptic cells^[96,97].

The synthesis of cannabinoids results from an intense or prolonged release of excitatory neurotransmitters^[98]. In response to the post-synaptic depolarization, voltage-dependent calcium channels open, inducing an increase in intracellular calcium level^[99,100] and activating the enzymes that synthesize endocannabinoids from lipid precursor molecules. This pathway can synergize with the activation of a set of glutamate metabotropic receptors as well as with G protein-coupled receptors bound to phospholipase C, which increases thereby the production of endocannabinoids^[100]. The endocannabinoids then leave the post-synaptic cells and activate the CB1 receptors on the pre-synaptic neurons. Through the activation of the G protein, the calcium pre-synaptic channels are inhibited, and neurotransmitter release is suppressed. Thus, endocannabinoids act as retrograde messengers, inhibiting the release of neurotransmitters via activation of CB1 pre-synaptic receptor, regulating many synapses and finally participating in the control of neuron survival^[101].

The activation of pre-synaptic CB1 receptor has in the inhibition of glutamatergic neurotransmission its main neuroprotective capacity^[102-105], avoiding massive accumulation of intracellular calcium, nitrogen and reactive oxygen species which would trigger the cell death^[106]. However, CB1 receptor may also inhibit GABAergic synaptic transmission^[107,108], causing a neurotoxic effect^[109]. Apart from the inhibition of the glutamatergic neurotransmission, the protective effect of cannabinoids in neural cells also includes its implication in diverse pathways of cell survival, such as the PI3K/Akt and some kinase pathways regulated by extracellular signals^[110]. CB1 also participates in some mechanisms involved in the control of neuronal functionality, cell migration and differentiation, the inhibition of protein kinase A through the adenylate cyclase-cAMP pathway, the activation of protein kinase cascades through mitogenes such as extracellular signal-regulated kinases, Jun N-terminal kinase and p38, as well as the generation of ceramide^[58,101].

6 Therapeutic potential of cannabinoid after hypoxia-ischemia

Although the exact mechanisms of the neuroprotective effect of cannabinoids are not completely understood, cannabinoids have been suggested to be involved in numerous processes, such as the modulation of calcium homeostasis and excitability^[111,112], regulation of immune and inflammatory responses^[113,114], activation of cytoprotective signalling pathways^[112], as well as modulations of synaptic plasticity, excitatory glutamatergic transmissions^[111] and their hypothermic and antioxidant properties^[115]. In this regard, the endocannabinoid system may also represent a pivotal neuroprotective mechanism both in chronic neurodegenerative disorders and in acute forms of neuronal injury.

The first cannabinoid tested in cerebral ischemic models was the cannabinoid agonist WIN55212-2. Nagayama *et al.*^[116] have shown that in both global and focal ischemia models, WIN significantly reduces the infarct volume and decreases the loss of hippocampal neurons. Accumulative studies have supported the neuroprotective effect of these compounds in both global^[117,118] and focal^[119-121] ischemia *in vivo*, showing that synthetic as well as natural cannabinoids can diminish the neuronal damage. In addition, CB1 knock-out mice^[122] show increased mortality from permanent focal cerebral ischemia and larger infarcts after transient focal cerebral ischemia, compared with the wild-type mice. On the other hand, other studies

were focused on the possible neuroprotective effect of diverse cannabinoid-related compounds after ischemia, such as endocannabinoids^[123-125], antagonists such as rimonabant^[126], and compounds that inhibit the degradation of them^[127,128].

Regarding the perinatal asphyxia, there has been a growing interest in cannabinoid-mediated neuroprotection, largely due to the fact that they modulate a wide range of relevant factors involved in hypoxic-ischemic brain damage. Various studies have elucidated that cannabinoids can inhibit intracellular calcium influx, reduce the release of glutamate and tumor necrosis factor- α (TNF- α), decrease stimulated inducible nitric oxide synthase (iNOS) expression, induce hypothermia, and exert immunomodulatory and antioxidant actions^[129-134].

Recently, we have found that administration of the cannabinoid agonist WIN decreases brain injury in a lamb model of perinatal asphyxia^[135]. In this study, we showed that WIN successfully prevented delayed cell death, as the number of TUNEL⁺ cells in hypoxic-ischemic fetal lambs treated with the cannabinoid agonist was similar as that in non-asphyxiated pups. Moreover, the beneficial effect induced by WIN administration was also extended to glial cells, with the maintenance of S100 protein levels (Fig. 1, unpublished data), an early and sensitive biochemical marker of cerebral injury in term newborn infants with hypoxic-ischemic encephalopathy^[136-140]. Another group reported that WIN also had a neuroprotective effect on 7-d-

Fig. 1 Representative light microphotographs of S100-stained cells from basal nuclei brain sections obtained after sham operation (A) or hypoxia-ischemia from lambs receiving vehicle (B) or 0.01 µg/kg WIN treatment (C) (unpublished data). WIN administration preserved the number of S100-positive cells (arrows). Scale bar: 100 µm.

old rat forebrain slices exposed to oxygen-glucose deprivation, based on the reductions of glutamate release, TNF- α release and iNOS expression^[133]. Lately, this group demonstrated that WIN successfully reduced brain injury^[141] and enhanced subventricular zone cell proliferation, oligodendrogenesis, white matter remyelination, and neuroblast generation in newborn rats^[142].

It has been recently revealed that CB2 receptors mediate the anti-inflammatory and immunomodulatory effects of cannabinoids^[143,144]. A number of investigations showed that activation of CB2 receptor has anti-inflammatory therapeutic potential in central nervous system diseases, such as multiple sclerosis, traumatic brain injury and Alzheimer's disease^[145-147]. Recently, it has been shown that CB2 receptor is also detected in resident inflammatory cells within the brain such as microglia^[62-65,148], and that hypoxia-ischemia induces its expression in the brain^[144,149]. Moreover, the use of CB2 receptor agonists has revealed promising results in different paradigms of neonatal hypoxic-ischemic brain injury^[133,150], reducing cell death, accompanied by modulations of glutamate release, cytokine production, and cyclooxygenase-2 and iNOS expression, supporting the hypothesis that the protective effect of CB2 receptor relies mostly on its anti-inflammatory effects. This provides new hints on its possible use as a neuroprotective target after perinatal asphyxia.

7 Conclusion

As has been summarized in this review, activation of the CB receptors leads to cellular changes that are extremely relevant to ischemic injury. Moreover, the recently discovered CB2 expression in neurons and microglia offers new opportunities for its modulation against brain inflammation. On the other hand, research interest is gradually shifting to the use of FAAH and MGL inhibitors in neurological disorders. By preventing the deactivation of endocannabinoids, these inhibitors may prove to have neuroprotective effects, avoiding the variable effects of exogenous cannabinoid agonists and the ubiquitous receptor activation. In summary, pharmacological properties of cannabinoids support the high interest in cannabinoids as a

future feasible strategy to be included in neonatal hypoxic-ischemic encephalopathy management.

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大麻素在围产期缺血缺氧性脑损伤的保护作用

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摘要: 围产期缺血缺氧一直是引起新生儿脑损伤的首要因素, 往往导致死亡或终生后遗症。由于新生儿缺血缺氧性脑损伤的病理复杂性, 目前还没有针对此病的特定疗法。因此, 寻找新的神经保护性疗法正日益引起研究者的关注。在哺乳动物体内, 大麻素系统能调节大范围的生理过程, 而且在不同类型的急性脑损伤中也具有神经保护作用。近几年的研究表明, 内源性大麻素系统在围产期窒息中也扮演着神经保护者的角色。本文主要就大麻素作为一种新的治疗策略在围产期缺血缺氧性脑损伤中的神经保护作用做一综述。

关键词: 围产期缺血缺氧; 脑损伤; 神经保护策略; 内源性大麻素系统