Cannabidiol Reduces Brain Damage and Improves Functional Recovery After Acute Hypoxia-Ischemia in Newborn Pigs

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ABSTRACT: Newborn piglets exposed to acute hypoxia-ischemia (HI) received i.v. cannabidiol (HI + CBD) or vehicle (HI + VEH). In HI + VEH, 72 h post-HI brain activity as assessed by amplitude-integrated EEG (aEEG) had only recovered to $42 \pm 9\%$ of baseline, near-infrared spectroscopy (NIRS) parameters remained lower than normal, and neurobehavioral performance was abnormal (27.8 ± 2.3 points, normal 36). In the brain, there were fewer normal and more pyknotic neurons, while astrocytes were less numerous and swollen. Cerebrospinal fluid concentration of neuronal-specific enolase (NSE) and S100 β protein and brain tissue percentage of TNF α (+) cells were all higher. In contrast, in HI + CBD, aEEG had recovered to $86 \pm 5\%$, NIRS parameters increased, and the neurobehavioral score normalized (34.3 ± 1.4 points). HI induced histological changes, and NSE and S100 β concentration and TNF α (+) cell increases were suppressed by CBD. In conclusion, post-HI administration of CBD protects neurons and astrocytes, leading to histological, functional, biochemical, and neurobehavioral improvements. (*Pediatr Res* 70: 272–277, 2011)

Cannabinoids (CBs) have emerged as valuable tools for reducing brain damage after hypoxia-ischemia (HI) in newborns (1). Several effects of CBs such as their anti-inflammatory and antioxidant effects, their vasoactive effects, the reduction of Ca²⁺ influx and of glutamate release, and the modulation of nuclear factor (NF)- κ B activity account for their observed neuroprotective action (1). Usually, CB₁ agonists have been considered the best neuroprotective CBs for both the mature (1) and immature (2) brain. Nevertheless, some concern has arisen lately regarding a possible deleterious effect of CB₁ overactivation in the immature brain (3). These findings have prompted further investigation of the neuroprotective effect of non-CB₁ agonists in the immature brain.

In this regard, our group recently reported that the phytocannabinoid cannabidiol (CBD) successfully reduces immediate brain damage when administered to newborn piglets after HI (4). CBD is the major nonpsychoactive constituent of

Cannabis sativa; the lack of psychoactive effects derives from the lack of significant binding to CB₁ receptors (5–7). In immature mice brain, it has been shown that some of the neuroprotective effects of CBD are because of interaction with CB₂ and adenosine receptors (8). Recently, it has been reported that CBD increases brain levels of adenosine through inhibition of uptake (9). In the newborn piglet model, administration of CBD after HI reduces immediate brain damage by modulating cerebral hemodynamic impairment and brain metabolic derangement and preventing the appearance of brain edema and seizures; these neuroprotective effects are not only free from side effects but also associated with some beneficial cardiac, hemodynamic, and ventilatory effects (4). That study, however, involved only an extremely short follow-up (6 h) and did not include behavioral outcomes. Only studies with follow-ups longer than 24 h post-HI and including neurobehavioral assessment are thought to be valid candidates to be transferred to asphyxiated babies (3,10).

In the present work, we studied the neurobehavioral impairment associated with the derangement of brain activity and metabolism induced by HI in piglets, investigating whether such damage was reduced by CBD administration. Furthermore, in this occasion, we studied HI piglets for longer, up to 72 h post-HI insult. This period is commonly selected in this model (10) because biochemical, neuropathological, and neurobehavioral consequences of HI are well established over that interval of time, so the neuroprotective effect of a given strategy can be properly assessed (11,12). In addition, we gained some insight into the mechanisms of CBD neuroprotection in HI piglets. Special attention was paid to a possible protective effect not only on neurons but also on astrocytes, because glial cell protection is now considered indispensable to provide sustained and successful neuroprotection (13).

Received January 7, 2011; accepted March 29, 2011.

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Supported by grants from the Spanish fund for health research FIS-PI060839 and FIS-PS0900434 [F.J.A.], FIS-PI061085 and FIS-PS09/01900 [J.M.-O.], and FIS PS09/02326 [E.H.]; Basque Government GCI-07/79, IT-287-07 [E.H.]; and the Carlos III Health Institute (RD08/0072: Maternal, Child Health and Development Network) within the framework of the VI National Plan for R&D+i (2008–2011).

Abbreviations: aEEG, amplitude-integrated electroencephalography; CB, cannabinoid; CBD, cannabidiol; CBF, cerebral blood flow; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; HI, hypoxia-ischemia; MDA, malondialdehyde; NBS, neurobehavioral scale; NIRS, near-infrared spectroscopy; NSE, neuronal-specific enolase; nTHI, normalized tissue hemoglobin index; TOI, tissue oxygenation index; VEH, vehicle

METHODS

Animal preparation. The experimental protocol, which met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and was approved by the Ethical Committee for Animal Welfare of the Gurutzetako Ospitalea, has been extensively described elsewhere (4). Briefly, 1- to 3-d-old piglets previously anesthetized and paralyzed with a perfusion of fentanyl, propofol, and midazolam in dextrose 5% (0.004, 3, and 0.5 mg/kg/h, respectively) and vecuronium (3 mg/kg/h) administered through an ear vein were intubated and mechanically ventilated (Bourns BP200; CA). The femoral artery was cannulated to monitor blood pressure (Ominare CMS24; HP, Göbblingen, Germany) and to obtain blood samples. Blood oxygen saturation was monitored by transcutaneous pulse oxymetry. Blood gases and glycemia were regularly checked out to adjust the ventilator settings and/or to add dextrose or vasoactive drugs to correct deviations from appropriate levels. A cerebrospinal fluid (CSF) sample was obtained at the end of the experiment. Rectal temperature was maintained between 37.5 and 38.5°C with heat lamps.

Experimental procedure. HI was induced by clamping both carotid arteries with vascular occluders and lowering the fraction of inspired oxygen to 8–10% over 20 min. Fifteen and 240 min later, HI piglets received i.v. 1 mL of CBD 0.1 mg/Kg (HI + CBD, $n = 10$, age 1.3 ± 0.3 d, and weight 1.7 ± 0.1 kg) or vehicle (VEH) (saline) (HI + VEH group, $n = 12$, age 1.3 ± 0.3 d, and weight 1.6 ± 0.1 kg). The CBD (Tocris Bioscience, Bristol, United Kingdom) was dissolved in Emulphor/ethanol/saline 1:1:18 (4). Other piglets were similarly anesthetized and intubated but were kept with normal oxygen levels and their carotids were not clamped (SHAM, $n = 7$, age 1.7 ± 0.5 d, and weight 1.7 ± 0.1 kg). Half of the animals in each group were kept ventilated with sedation and analgesia up to 6 h after HI or the equivalent period in the SHAM group. Then, a CSF sample was obtained, immediately frozen in liquid nitrogen and stored at -80°C until its use. The remaining animals were allowed to spontaneously recover after HI and fed with artificial piglet formula milk until 72 h after HI. At the end of the experiment, anesthetized piglets were killed with potassium chloride i.v. Then, their brains were perfused *via* the carotid arteries with cold heparinized saline and removed. One hemisphere was immediately frozen in liquid nitrogen and stored at -80°C until its use; the other hemisphere was fixed with 4% paraformaldehyde, and then stored at 4°C .

Data acquisition and analysis

Neurophysiological assessment. The tissue oxygenation index (TOI), expressed as percentage, and normalized tissue Hb index (nTHI) were continuously monitored using a near-infrared spectroscopy (NIRS) system (NIRO-200; Hamamatsu Photonics KK, Joko Cho, Japan). The NIRS sensor was placed on the skull frontoparietally at the midline and fixed with bandages. Brain activity was monitored using a two-channel bedside amplitude-integrated EEG (aEEG) monitor (BRM2; BrainZ Instruments, Auckland, New Zealand) using five needle electrodes at C3–P3 and C4–P4 (International 10–20 system).

NIRS and aEEG were continuously recorded throughout the period of mechanical ventilation and sedation and analgesia. In awake piglets, both measurements were performed for 15-min long periods every 12 h with the animal being gently restrained. Muscle artifacts were excluded from the aEEG analysis.

Neurobehavioral scale. Also with the animals awake, a neurological examination was performed every 12 h using an adapted standardized approach for scoring piglets (12). This Neurobehavioral Scale (NBS) considers a range of items related to vigilance (mental status and behavior), cranial nerves (pupils and vestibulo-ocular reflex), reflexes (stepping—walking on forelimbs with hind limbs elevated, and righting—ability to move or roll over from the lying on their back), motor performance (tonus and standing), and coordination (walking and feeding behavior). The global NBS ranges from 10 to 36 (normal).

Biochemical analysis. Samples from frozen brain hemispheres were disaggregated in microplates using a 70- μm cell-strainer (BD Falcon; Becton Dickinson, San Jose, CA) in 0.25% trypsin-EDTA. The cell suspensions were fixed in 70% ethanol and incubated overnight with mouse-anti-porcine TNF α (1:500) and then incubated with secondary antiserum (Alexa Fluor anti-mouse 1:200; Molecular Probes, Invitrogen, CA) for 1 h and analyzed by flow cytometry (Epics Elite, Coulter Electronics; Hialeah, FL) to determine the percentage of TNF α (+) cells and the mean fluorescence intensity of positive cells. Cell suspensions similarly processed but without primary antiserum served as controls. Other brain samples were homogenized with PBS and EDTA to quantify by ELISA (OxiSelect TBARS Assay Kit; Cell Biolabs, San Diego, CA) the concentration of malondialdehyde (MDA; expressed in nM

per μg of DNA). The concentration of neuronal-specific enolase (NSE) and S100 β protein in CSF samples were also measured by ELISA (CanAg NSE and S100; Fujirebio, Diagnostics, Sweden).

Histological analysis. Fixed brain hemispheres were cut into sections 5 mm in width and embedded in paraffin. Coronal sections (4 μm) were cut and mounted on a glass slide for staining. To determine early neuronal necrosis, consecutive pairs of brain sections were stained by the Nissl method (4). Areas of 1 mm² in the central three lobes of the parietal cortex at 3 mm in the posterior plane, as shown in a stereotaxic atlas of pig brain (14), were examined, focusing on layers II–III, by an investigator blinded to the experimental group using an optical microscope (400 $\times$) and a grid of 50 compartments; the mean of three compartments was calculated. Apparently normal neurons were identified by the presence of typical nuclei with clear nucleoplasm and a distinct nucleolus surrounded by purple-stained cytoplasm. Neurons were defined as damaged when no distinction could be made between the nucleus and cytoplasm (pyknotic or necrotic). To determine the presence of apoptotic cells, brain sections were stained with TUNEL (ApoTag In Situ Apoptosis Detection Kit; Millipore, MA) (15). Finally, glial fibrillary acidic protein (GFAP)(+) cells were identified in the same areas by immunohistochemistry. Tissue sections were washed in PBS and incubated with the GFAP-Cy3 conjugated antibody (1:1000; Sigma Chemical Co.-Aldrich, Madrid, Spain) at 4°C overnight. After incubation with the secondary antibody, sections were washed in PBS and mounted in aqueous medium with Vectashield (Vector Laboratories, Burlingame, United Kingdom). Visualization and photography of the samples was carried out with a confocal Nikon Eclipse CI coupled to a Nikon 90i microscope and DXM1200F camera (Nikon; Haarlem, Netherlands). The areal percentage of GFAP-immunoreactive processes and cell bodies was calculated by using the ImageJ 1.43s software (National Institutes of Health, Bethesda) (16). By dividing this figure by the number of GFAP(+) cells, a mean size of astrocytes was obtained.

Statistical analysis. SPSS 15.0.0 software was used for all statistical analyses. Median values have been compared using the Kruskal-Wallis analysis of ranks with the Dunn's *post hoc* test for multiple comparisons. A p value < 0.05 was considered to be significant. All data are presented as means $\pm$ SE.

RESULTS

aEEG studies. In SHAM, aEEG amplitude remained stable throughout the 72 h-experimental period. The HI episode induced a severe decrease in the aEEG amplitude to $20 \pm 2\%$ of the baseline. In HI + VEH, this was followed in the first 24 h by a modest improvement to $33 \pm 5\%$ (Fig. 1), but little further progress was seen; accordingly, 72 h after HI, the aEEG amplitude stood at $42 \pm 9\%$ of the baseline value ($p < 0.05$ versus SHAM). By contrast, in HI + CBD, the aEEG amplitude recovered to $60 \pm 12\%$ 24 h post-HI with further improvement; thus, 72 h after HI, the aEEG amplitude was back to $86 \pm 5\%$ of the baseline ($p < 0.05$ versus HI + VEH; Fig. 1).

NIRS studies. Compared with SHAM, in HI + VEH, there was a severe decrease in TOI 6 h after HI (Fig. 1). In the following days in this group, TOI firstly increased and later decreased again. Specifically, 72 h post-HI TOI was around 10% lower in HI + VEH than in SHAM. Administration of CBD led to a sustained increase in TOI from the first 6 h after HI (Fig. 2). Indeed, TOI in HI + CBD was similar to SHAM from 24 to 72 h after HI.

In HI, the nTHI remained similar to in SHAM during the first 48 h after HI (Fig. 2). At 72 h post-HI, the nTHI fell to values lower than SHAM in HI + VEH, whereas in HI + CBD, the nTHI increased (Fig. 2).

Neurobehavioral examinations. All animals showed a normal NBS before the experimental procedure, scoring 36, and the SHAM group maintained a normal score throughout the entire study period (Table 1). However, the HI + VEH animals showed profound neurobehavioral impairment 24 h

after HI, their score being 60% lower than SHAM; all items were affected, excepting vestibulo-ocular reflexes (Table 1). In the following 24 h, there was a modest improvement, mainly in the vigilance and behavioral items, whereas in reflexes and

motor items, the improvement was negligible; overall, their NBS was then 33% lower than SHAM (Table 1). However, these animals did not improve to a similar degree in the following 24 h. By contrast, CBD administration after HI led to a faster recovery, such that 24 h after HI, the NBS was 39% lower than SHAM; the improvement in vigilance and feeding was particularly impressive, with the scores on these items becoming similar to SHAM, and this improvement was also seen in some motor items (Table 1). What is more, neurobehavioral improvement continued in the following days, with the NBS of HI + CBD being just 15% lower than SHAM 48 h after HI and similar to SHAM at 72 h (Table 1).

Biochemical analysis. Flow cytometry on the brain tissue showed the percentage of TNF α (+) cells was more than 2-fold higher in HI + VEH 6 h after HI than in SHAM, whereas in HI + CBD, the percentage of TNF α (+) cells was similar to the SHAM value (Table 2). Mean fluorescence intensity was similar in all groups (5.5 ± 2.6 , 4.8 ± 1.0 , and 4.3 ± 1.0 arbitrary units for SHAM, HI + VEH, and HI + CBD, respectively, NS). By contrast, no significant differences were observed in concentration of MDA 6 h after HI (Table 2). No significant differences were detected between any of the groups 72 h post-HI (data not shown).

Concentrations of NSE and S100 β protein had also increased 6 h after HI in CSF from HI + VEH and, once again, in HI + CBD the HI-induced increases were much smaller (Table 2).

Histological studies. Nissl staining of brain slices obtained 6 h after HI revealed a decrease in the number of apparently normal neurons in cortex of HI + VEH (653 ± 6 versus 371 ± 21 cells/mm² for SHAM and HI + VEH, respectively, $p < 0.05$). In addition, a number of pyknotic neurons were seen, mainly in the deeper layers of the cortex. In HI + CBD, this loss of normal neurons was blunted and the appearance of pyknotic cells reduced; specifically, the density of normal neurons were 482 ± 21 cells/mm² ($p < 0.05$ versus HI + VEH and versus SHAM). In HI + VEH, the number of normal neurons observed had not increased 72 h post-HI

Figure 1. Changes in aEEG (A), TOI (B), and nTHI (C) induced by HI (●: HI + VEH, $n = 12$) and their modifications by the administration of CBD (○: HI + CBD, $n = 10$), when compared with nonasphyxiated newborn piglets (□: SHAM, $n = 7$). Results expressed as mean $\pm$ SEM (*) $p < 0.05$ vs SHAM. (**) $p < 0.05$ vs HI + VEH.

Figure 2. Histological study of brain from newborn piglets 72 h after a hypoxic-ischemic insult, treated with VEH (■: HI + VEH) or CBD (□: HI + CBD), when compared with nonasphyxiated newborn piglets (□: SHAM). (A) Nissl staining. Arrows indicate necrotic neurons. (B) TUNEL staining. Arrows indicate TUNEL+ cells. (C) GFAP immunostaining. Arrows indicate GFAP+ cells (astrocytes). In HI + VEH, astrocytes appear swollen. Photographs show representative examples from each group (original magnification, $\times 200$). Bar scale: 50 μm . Bars represent the mean $\pm$ SEM of all studies. (*) $p < 0.05$ vs SHAM. (**) $p < 0.05$ vs HI + VEH.

Table 1. Results of the neurobehavioral score

	24 h			48 h			72 h		
	SHAM	HI + VEH	HI + CBD	SHAM	HI + VEH	HI + CBD	SHAM	HI + VEH	HI + CBD
Mental status	4	1.8* (0.3)	3.2*† (0.4)	4	2.8* (0.5)	3.8† (0.1)	4	3.3 (0.5)	4.0 (0)
Behavior	4	0.8* (0.1)	1.8*† (0.5)	4	1.4* (0.4)	3.0† (0.5)	4	2.5* (0.5)	3.8† (0.1)
Pupils	3	2.0* (0.3)	3.0† (0)	3	2.2* (0.3)	3.0† (0)	3	2.8 (0.1)	3.0 (0)
Vestibulo-ocular reflex	3	3	3	3	3	3	3	3	3
Stepping	3	1.4* (0.2)	1.6* (0.3)	3	2.0* (0.3)	2.3* (0.3)	3	2.1* (0.2)	2.6*† (0.2)
Righting	2	1.1* (0.1)	1.3* (0.2)	2	1.4* (0.2)	1.6* (0.1)	2	1.8 (0.2)	1.8 (0.1)
Muscle tone	4	2.1* (0.4)	2.5* (0.3)	4	3.2* (0.3)	3.2* (0.3)	4	3.1 (0.2)	3.8 (0.1)
Standing	4	1.1* (0.1)	2.2*† (0.5)	4	2.1* (0.3)	2.8* (0.5)	4	2.3* (0.4)	3.5† (0.4)
Walking	4	1.0* (0)	2.3*† (0.3)	4	2* (0.3)	3*† (0.3)	4	2.3* (0.5)	3.6† (0.3)
Feeding	5	2.5* (0.3)	4.5† (0.4)	5	3.7* (0.6)	5 (0)	5	4.3 (0.7)	5.0 (0)
Global	36	14.5* (0.2)	25.5*† (0.4)	36	24.1* (2)	30.8*† (2.3)	36	26.8* (2.3)	34.3† (1.4)

Neurobehavioral score performed 24, 48, or 72 h after a HI insult to newborn piglets, treated with vehicle (HI + VEH) or cannabidiol (HI + CBD). Values are presented as mean (SEM).

* $p < 0.05$ vs SHAM.

† $p < 0.05$ vs HI + VEH.

Table 2. Biochemical studies

	SHAM (n = 3)	HI + VEH (n = 6)	HI + CBD (n = 5)
Brain tissue			
TNF α (+) cells (%)*	20.6 (4.4)	41.5† (2.1)	28.7§ (2.7)
MDA (nM/ μ g)‡	47.8 (11.5)	52.2 (6.6)	46.2 (7.4)
CSF			
NSE (μ g/L)‡	2.1 (0.2)	55.1† (8.1)	13.1†§ (7.6)
S100 β (μ g/L)‡	1.1 (0.1)	8.3† (0.5)	3.5†§ (0.6)

Samples obtained 6 h after a HI insult from newborn piglets, treated with vehicle (HI + VEH) or cannabidiol (HI + CBD). Values are presented as mean (SEM).

* Flow cytometry.

† $p < 0.05$ vs SHAM.

‡ ELISA.

§ $p < 0.05$ vs HI + VEH.

(613 ± 36 versus 404 ± 42 cells/mm² for SHAM and HI + VEH, respectively, $p < 0.05$), but the number of pyknotic cells had increased (percentage of pyknotic cells out of the total amount of stained cells: $26.5 \pm 6.8\%$; Fig. 2A). By contrast, in HI + CBD, the number of normal neurons in the cortex had increased 72 h post-HI to a density similar to that in SHAM (654 ± 32 cells/mm², NS versus SHAM and $p < 0.05$ versus HI + VEH) and the percentage of pyknotic cells remained negligible and again similar to SHAM (1.3 ± 0.6 and $2.6 \pm 0.9\%$ for HI + CBD, both $p < 0.05$ versus HI + VEH; Fig. 2A). The histological beneficial effect was extended to hippocampus (pyknotic cells 72 h post-HI: 1.9 ± 1.4 , 23.2 ± 6.2 , and $2.3 \pm 0.8\%$ for SHAM, HI + VEH, and HI + CBD, respectively, $p < 0.05$).

TUNEL staining showed no TUNEL(+) cells in any group 6 h after HI (data not shown). However, 72 h after HI in cortex from HI + VEH, the number of TUNEL(+) cells was more than 6-fold higher than in SHAM (24.2 ± 3.8 versus 153.7 ± 87.9 cells/mm² for SHAM and HI + VEH, respectively, $p < 0.05$; Fig. 2B). Notably, administration of CBD after HI dramatically reduced the number of TUNEL(+) cells (46.3 ± 10.4 cells/mm², $p < 0.05$ versus HI + VEH and versus SHAM; Fig. 2B).

GFAP immunohistochemical staining did not reveal any differences between groups in the number or morphology of astro-

cytes 6 h after HI (data not shown). However, 72 h after HI, the number of astrocytes in HI + VEH cortex had fallen (110.7 ± 7.6 versus 92.1 ± 3.1 cells/mm² for SHAM and HI + VEH, respectively, $p < 0.05$), and surviving astrocytes appeared swollen and smaller than those from SHAM (mean size 825 ± 58 versus 459 ± 68 μ m² for SHAM and HI + VEH, respectively, $p < 0.05$; Fig. 2C). By contrast, in HI + CBD, astrocytes were similar to SHAM in number (116.9 ± 5.1 cells/mm², NS versus SHAM and $p < 0.05$ versus HI + VEH) and in shape and size (mean size 703 ± 85 μ m², NS versus SHAM, $p < 0.05$ versus HI + VEH; Fig. 2C).

DISCUSSION

The present work supports the hypothesis that administration of CBD after a HI insult affords robust neuroprotection in the immature brain. We have reported recently that post-HI administration of CBD to newborn piglets produces beneficial effects in the very early hours after HI, as reflected by the improvement of brain activity studied by aEEG and metabolism studied by NIRS (4). Here, we report that these beneficial effects are sustained 72 h after HI and that they are associated not only with histological improvement but also with neurobehavioral normalization.

The HI insult led to a profound decrease in aEEG amplitude, which reflects the severity of the insult (17) and strongly predicts poor outcome in humans (18). Depressed aEEG amplitude in the hour following HI is a good predictor of poor outcome in piglets (19). In HI + VEH, the modest recovery of aEEG amplitude observed in the first 24 h did not continue, and these animals were observed to not return to normal cerebral function, as reported (11). Just after HI, the aEEG amplitude was as depressed in HI + CBD than in HI + VEH. Nevertheless, brain activity improved in HI + CBD in the first 24 h and progressed further in the following days, so that 72 h after HI the aEEG amplitude reached around 90% of the baseline values. These data are consistent with a robust neuroprotective effect of CBD. The lack of progress of aEEG amplitude in HI + VEH was associated with a fall in TOI and nTHI from 24 to 72 h after HI. However, the increase in aEEG amplitude in HI + CBD was associated with TOI and nTHI

increases. The TOI represents the tissue saturation of oxygen, while nTHI provides an absolute measure of total Hb in brain tissue, changes in which reflect changes in cerebral blood volume (CBV) (20). In the context of the aEEG, the neurobehavioral and the histological findings, the observed changes in TOI and nTHI likely suggest that the poor outcomes in HI + VEH were associated with a deterioration of brain metabolic activity and cerebral blood flow (CBF) that was not recovered 72 h post-HI. In agreement, depressed brain metabolism and CBF, assessed by NIRS, has been reported in piglets just after HI, related to depressed aEEG amplitude (19). However, the beneficial effect of CBD seems to occur in association with the stabilization of brain metabolic activity and CBF. These conclusions have to be viewed with caution, as we did not measure CBF directly; although TOI and nTHI correlate with CBF and CBV they are not accurate markers of these parameters (20). However, in a rat model of stroke in which direct CBF measurement by laser Doppler flowmetry was performed, it was found that CBD administration increased CBF throughout the entire ischemic period (21).

Our results from aEEG and NIRS studies correlated with the histological analysis. HI led to severe neuronal damage, as reflected by the increase of NSE concentration observed in CSF 6 h post-HI in HI + VEH. The increase of NSE concentration in CSF is an early marker of HI neuronal damage in newborns and is highly predictive of abnormal outcome (22). In agreement with this, in HI + VEH, there was a loss of viable neurons together with an increase of cell death, features starting to appear just 6 h post-HI and being even more evident at 72 h. Apoptosis is a key feature of HI damage in the immature brain, appearing shortly after insult and coexisting with necrosis (15). Treatment with CBD prevented the HI-induced NSE increase in CSF. In line with this, cell death was successfully reduced by CBD 72 h post-HI. In brain slices of newborn mice exposed to oxygen-glucose deprivation (OGD), CBD is known to reduce lactic dehydrogenase efflux and caspase-9 production, markers of necrotic and apoptotic processes, respectively (8). Interestingly, the protective effect of CBD was not limited to neurons but extended to astrocytes. HI increased the levels of S100 β in CSF 6 h postinsult, as observed in HI + VEH. The amount of S100 β protein released into CSF dramatically increases after HI insults, predicting an adverse outcome (23). It comes mostly, although nonexclusively, from astroglial cells (23). Consistent with this, in HI + VEH astrocytes were reduced in number and size and appeared swollen, a feature related with enhanced excitotoxicity (13). This reduction in astrocyte number and size, because of the decrease of astrocytic processes, has been observed in piglet brain in the first few days after HI preceding the gliotic response, which in those animals occurred >96 h after HI (16). In our experiments, administration of CBD prevented the HI-induced increase of S100 β in CSF. The modulation of such an increase is a good marker of effective neuroprotection (23). Accordingly, CBD treatment led to a preservation of the number, size, and morphology of astrocytes. The protective effect of CBD on astrocytes is of great importance, because the preservation of the functional integ-

rity of astrocytes is now considered the key for sustained neuroprotection (13,16).

It is noteworthy that the beneficial effect of CBD after the HI insult in our experiments was not only neurophysiological and histological but had also a clinical expression. As assessed by means of a standardized NBS, CBD administration dramatically improved piglet neurobehavioral performance as early as 24 h after the HI insult. This improvement progressed in the following days, so that at 72 h the NBS in HI + CBD was similar to in SHAM. In HI + VEH, there was a modest recovery in neurobehavioral performance in the first day after HI, but this recovery did not continue in subsequent days. The HI + CBD had better scores than HI + VEH on items related to different aspects of performance such as vigilance (behavior), motor performance (standing), and coordination (walking), which suggests that the beneficial effect of CBD on brain function was general and not focused on particular brain areas. Normalization of the neurological assessment in the first days after HI is one of the best predictors of good outcome in asphyxiated newborns (24). Nevertheless, because we are unable to maintain piglets for >3 d in our facilities, we could not assess whether that CBD-induced neurobehavioral recovery is sustained in the long term. Thus, further experiments testing the long-term effect of CBD administration after a HI insult using models, such as rodents, allowing studies over these extended periods, are warranted.

Little is known about the mechanisms of CBD neuroprotection (5–7). In this work, we observed that the anti-inflammatory effect of CBD contributed to its beneficial effect against HI brain damage. Specifically, CBD blunted the HI-induced increase of TNF α (+) cells in piglet brain. In immature mice brain slices exposed to OGD (8), it has been observed that the increase in TNF α production is prevented by CBD. TNF α production increases in the brain as early as 1 h after HI insult, decreasing after 24–48 h (25). Accordingly, we did not observe any difference between groups in the proportion of TNF α (+) cells 72 h after HI. The increase of TNF α production after HI correlates with the extent of tissue injury and with the clinical outcome (25). Indeed, modulation of the inflammatory response is considered a critical component of neuroprotective strategies (25). In addition, because CBD has demonstrated neuroprotective effects by modulating oxidative stress (26), we investigated the effect of CBD on brain concentration of MDA. However, we failed to demonstrate any difference between groups. It has been previously reported that oxidative stress markers are not increased in brain tissue from hypoxemic piglets in the first hours after resuscitation (27). We cannot rule out, however, that our results are because of insufficient sensitivity of the technique used (ELISA on brain homogenate) to detect differences in MDA brain concentration or to an inappropriate timing of sample collection (6 or 72 h after HI) in this study.

In conclusion, CBD administration after HI to newborn piglets led to a neuroprotective effect as reflected by aEEG, NIRS, histological, biochemical, and neurobehavioral studies. A significant beneficial effect was apparent as soon as 24 h after HI and continued at later times. CBD neuroprotection included both neurons and astrocytes and was related to an

anti-inflammatory effect. Overall these data strongly support the interest on CBD as a possible neuroprotectant in asphyxiated infants. Thus, further studies on long-term beneficial and detrimental effects of CBD administration, temporary therapeutic window, and CBD pharmacology in newborns are required.

Acknowledgment. We thank Elena Gastiasoro for technical assistance, David Hallett for reviewing the manuscript, and Julián Romero for invaluable advice.

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