Original Paper

Hydralazine is a Suitable Mimetic Agent of Hypoxia to Study the Impact of Hypoxic Stress on *In Vitro* Blood-Brain Barrier Model

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Background/Aims: Understanding cellular mechanisms induced by hypoxia is fundamental to reduce blood-brain barrier (BBB) disruption. Nevertheless, the investigation of hypoxia on cellular pathways is complex with true hypoxia because HIF-1 α has a short lifetime and rapidly reverts back to a normoxic state. To overcome this difficulty, mimetic agents of the hypoxia pathway have been developed, including the gold standard CoCl₂. In this study, we proposed to compare CoCl₂ and hydralazine in order to determine a suitable mimetic agent of hypoxia for the study on the BBB. **Methods:** We studied the cytotoxicity and the impact of these molecules on the integrity of an *in vitro* BBB model by comparing them to hypoxia controls. **Results:** We showed that the impact of hypoxic stress in our *in vitro* BBB model is rather similar between hydralazine and CoCl₂. Chemical hypoxic stress led to an increase of BBB permeability either with CoCl₂ or hydralazine. Tight junction protein expressions showed that this chemical hypoxic stress decreased ZO-1 but not occluding expressions, and cells had set up a defence mechanism by increasing expression and activity of their efflux transporters. **Conclusion:** Our results demonstrated that hydralazine is a better mimetic agent and more suitable than CoCl₂ because it had the same effect but without the cytotoxic effect on *in vitro* BBB cells.

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The blood-brain barrier (BBB) is a physical and metabolic barrier which separates the central nervous system (CNS) from the blood circulation. It is a multicellular neurovascular unit composed of brain microvascular endothelial cells, astrocytes, pericytes, microglia and extracellular matrix [1, 2]. Brain endothelial cells control the transport of substances between

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brain and blood through transporters and tight junction (TJ) proteins [3]. TJ proteins regulate the transport of ions via the development of high transendothelial electrical resistance (TEER) as well as the transport of molecules [4, 5], which create a physical blockade to paracellular diffusion [6]. This specialized phenotype is fundamental for protection of the brain. Moreover, this protection is also ensured by efflux ABC family transporter proteins which are ATP dependant and expressed by endothelial cells, like P-glycoprotein (Pgp) and multidrug resistance proteins (MRPs). These active efflux proteins act as a barrier system which reduce the exposure of the CNS to drugs, by pumping compounds out of endothelial cells in blood [4]. These barrier properties are mostly induced and maintained by the close opposition between brain endothelial cells and astrocytes [7, 8]. Since the structural and functional integrity of transporters and TJ proteins are necessary for an intact BBB, alteration of these components is a key event in the BBB's impairment.

Hypoxia is a major stress factor which induces a BBB's disruption. Indeed, the brain is a major consumer of oxygen and is therefore sensitive to changes in oxygen levels [9, 10]. The cellular response to hypoxia is mainly driven through activation of the HIF-1 pathway [11]. Under normoxia conditions, oxygen regulates the HIF-1 α subunit, which is rapidly degraded by prolyl hydroxylation that targets its degradation in the proteasome. Hypoxia inhibits the prolyl hydroxylase domain leading to stabilization of the HIF-1 α subunit in the cytoplasm. Then it is translocated to the nucleus where it binds to hypoxia responsive elements in promoter regions of target genes involved in cellular adaptation to hypoxic stress and induces their expression [12].

Elucidation of the cellular and molecular mechanisms, induced by hypoxic stress, is complex with true hypoxia because HIF-1 α has a short half-life (almost five minutes) and is rapidly reverted. In this regard, a wide variety of prolyl hydroxylase domain (PHD) inhibitors, which lead to a stabilization of HIF-1 α , have been developed. These inhibitors allow to create a chemical hypoxic stress and represent a useful method to investigate the BBB's disruption by hypoxia. The PHD inhibitor usually used in studies is CoCl₂ [12–14]. Cervellati et al. used CoCl₂ to achieve stabilization of HIF-1 α because it inhibits PHD by blocking the catalysis of prolyl hydroxylases [15]. Unfortunately, CoCl₂ is a rather highly cytotoxic agent and prevents its use in *in vitro* studies to mimic prolonged cerebral hypoperfusion, such as in ischemic stroke. It is difficult to know whether the observed effect is due to chemical hypoxic stress induced by CoCl₂ or its cytotoxicity. Other inhibitors of PHD exist such as hydralazine. Hydralazine is a vasodilator used to treat severe hypertension, congestive heart failure, myocardial infarction and pre-eclampsia [16]. Hydralazine also shows a capacity to induce a transient and physiological HIF-1 α overexpression by inhibiting PHD activity [17]. In the literature, hydralazine was only used to mimic a hypoxic state in *in vivo* and *in vitro* cancer models [18]. Since hydralazine is already used in *in vivo* models, it is therefore interesting to test hydralazine as a mimetic agent of hypoxia because others mimetic agents, like CoCl₂, are rather toxic to be used on *in vivo* models. In a recent work, we validated hydralazine as a mimetic agent of hypoxia on an *in vitro* BBB model in comparison with true hypoxia [19].

In the present study, we wanted to determine a suitable mimetic agent of hypoxia which is not cytotoxic, reproducible and controllable. For that, we compared hydralazine and CoCl₂ to study the impact of prolonged chemical hypoxia on an *in vitro* transwell BBB model. We used a co-culture in contact model composed of the immortalized cell line bEnd.3 and the C6 cell line (which display astrocytic properties [20]). This approach allowed interaction between endothelial cells and astrocytic cells. Then impact of prolonged chemical hypoxic stress, induced by hydralazine or CoCl₂, was assessed by studying endothelial paracellular permeability with TEER measurements and absolute membrane permeability was determined with sodium fluorescein (Na-F) [21, 22]. Evaluation of transport was also investigated on expression and activity of two efflux transporters (Pgp and MRP-1) and on expression of two TJ proteins (ZO-1 and occludin). Our results showed that hydralazine represented a suitable and reproducible way to create an *in vitro* chemical hypoxic environment without cytotoxicity. Moreover we demonstrated that hydralazine induced the same effect on our cells than true hypoxia.

Materials and Methods

Chemicals and reagents

Hydralazine, CoCl_2 , BCECF-AM, probenecid, verapamil and Na-F were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). MTT (methyl-thiazolyl-tetrazolium) kit and DMEM (Dulbecco's Modified Eagle's Medium) were also purchased from Sigma. LDH release test was purchased from Promega Corporation (Madison, USA). Antibodies and reagents for the detection of HIF-1 α were products of R&D systems (Lille, France). All compounds for Ringer-Hepes buffer were purchased from Sigma.

Occludin and ZO-1 antibodies were purchased from Life Technologies (Saint Aubin, France), Pgp and MRP-1 antibodies were purchased from GeneTex (San Antonio, Texas, U.S.A) and Santa Cruz Biotechnology (Dallas, Texas, U.S.A), respectively.

bEnd.3 cells and C6 cells were obtained from the ATCC (Manassas, VA, U.S.A).

Cell culture inserts for 24-well (0.4 μm pore diameter size, transparent PET membrane) were purchased from Corning distributors (Sigma). EVOM voltohmmeter system was purchased from World Precision Instruments (Hertfordshire, UK).

In vitro cytotoxicity assay

Cytotoxicity of hydralazine or CoCl_2 was measured by the MTT method. Growing cells were seeded at 10,000 cells/well using a 96-well microplate that was supplemented with DMEM. Cells were allowed to grow for 48 h before they were exposed to drugs. Then cells were treated with various concentrations of hydralazine or CoCl_2 . Phosphate buffer saline (PBS) 1X was used to dissolve drugs. After the treated cells were incubated for 24 h, MTT solution was added and the plates were incubated at 37°C for 4 h. To dissolve formazan, 100 μl DMSO was added and the plates were measured at 570 nm with a spectrophotometer. The least cytotoxic values were determined by plotting the drug concentrations versus the survival ratio of treated cells.

Then we also evaluated cell death with the LDH release method to confirm the least cytotoxic concentrations given by MTT results. For that, cells were seeded in 96-well plates at 10,000 cells/well. 100 μM of hydralazine or CoCl_2 was added during 24 h. After the exposure period, a lysis solution was used in control wells to generate a maximum LDH release. Then cells were incubated with cytotox-ONE™ reagent during 10 min. A stop solution was added before recording fluorescence with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The average fluorescence values of the culture medium background (wells without cells) were subtracted from fluorescence values of experimental wells. Then percent cytotoxicity was given by the following equation:

$$\% \text{ cytotoxicity} = 100 \times \frac{(\text{experimental} - \text{culture medium background})}{(\text{maximum LDH release} - \text{culture medium background})}$$

Exposure to true hypoxia

Our *in vitro* BBB cells were exposed to 2% O_2 into a hypoxic chamber during 24h [19].

Effect of hydralazine or CoCl_2 treatment on hypoxia metabolic pathway

Effect of hydralazine or CoCl_2 treatment was validated by the study of HIF-1 α expression (the key regulation of hypoxic response) to determine the effective dose where HIF-1 α was induced. We also exposed cells to 2% O_2 as hypoxia controls. These controls allowed us to verify that hydralazine or CoCl_2 mimic hypoxic cellular environment. For that, cells were seeded onto a 96-well plate at a density of 10,000 cells/well. Then cells were treated with either hydralazine or CoCl_2 (50 and 100 μM ; the least cytotoxic doses defined by cytotoxic test) during 24 h. Finally, cells were fixed to the support with 4% formaldehyde and the expression of HIF-1 α was determined by whole cell-ELISA. For that, anti-HIF 1 α antibodies were added at 4°C overnight (diluted at 1/100), then cells were incubated with secondary antibodies for 2 h at room temperature (diluted at 1/500). Fluorescence was measured with a fluorescence spectrophotometer with 540 nm excitation and 600 nm emission wavelengths.

In vitro BBB model set up

The BBB model was composed of bEnd.3 cells (immortalized mouse brain endothelial cells) and C6 cells (rat malignant glioma cells). All cells were used before passage number 30 (passages 12-28 for bEnd.3

Fig. 1. Schema of the *in vitro* blood-brain barrier model.

cells and 10-30 for C6 cells), which corresponded to the time where these cells may begin to lose their BBB properties, as cited by the supplier and in a previous study [23].

Once the cells reached subconfluency, they were placed onto cell culture inserts for 24-well plates. Figure 1 shows a schematic description of the *in vitro* BBB model.

For the procedure of contact co-culture, the transwell filter was inverted and C6 cells were seeded onto the abluminal side of the filter at a density of 4×10^4 cells/cm². Then cells were placed at 37°C for 6 h (time necessary for the cells to adhere to the membrane of the insert). Afterward, the insert was flipped back and C6 cells were cultured for two days in DMEM. At the end of two days, bEnd.3 cells were seeded onto the luminal side of the transwell filter at a density of 4×10^5 cells/cm² and co-cultured with C6 cells for eight days.

TEER measurements

To characterize the formation of a tight endothelial cell monolayer, TEER was recorded using an EVOM resistance meter. One electrode was placed on the luminal side and the other electrode on the abluminal side. These two electrodes were separated by the endothelial layer. The TEER measurements of blank filters (without cells) were subtracted from those of filters with cells. Then the resulting value was multiplied by the membrane area to obtain the TEER measurements in $\Omega \cdot \text{cm}^2$.

Na-F permeability measurements

Endothelial paracellular barrier function was also evaluated by measuring the permeability of cells to Na-F (MW = 376 Da). First the medium was removed and cells were washed with Ringer-Hepes prewarmed buffer (5 mM Hepes, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃ and 2.8 mM glucose). Then Ringer-Hepes buffer containing 10 $\mu\text{g}/\text{ml}$ of Na-F was loaded onto the luminal side of the insert and incubated at 37°C for 1 h. Samples were removed from the abluminal chamber at 10, 20, 30, 40, 50 and 60 min and immediately replaced with fresh Ringer-Hepes buffer. The concentration of fluorescein was determined using a fluorescence multiwell plate reader (Ex (λ) 485 nm; Em (λ) 530 nm). Transendothelial permeability coefficient (Pe) was calculated as previously described by Deli et al. [24]. The increment in cleared volume was calculated by dividing the amount of compound in the receiver compartment by the drug concentration in the donor compartment. The volume cleared was plotted versus time and the slope estimated by linear regression analysis. Then the average cleared volume was plotted versus time, and permeability $\times$ surface area product value for endothelial monolayer (PSe) was calculated as follows:

$$\frac{1}{P_{\text{Sendothelial}}} = \frac{1}{P_{\text{Stotal}}} - \frac{1}{P_{\text{Sinsert}}}$$

$P_{\text{Sendothelial}}$ divided by the surface area (A) in cm² (0.33cm² for transwell-24) generated the endothelial permeability coefficient (Pe in 10⁻⁶cm.s⁻¹) and calculated as follows:

$$P_e = \frac{P_{\text{Sendothelial}}}{A}$$

Whole cell ELISA of Pgp, MRP1, ZO-1 and occludin

Co-cultured cells were washed with 1% BSA in PBS at pH 7.4 and fixed for 20 min at room temperature with 4% paraformaldehyde in PBS at pH 7.4. Then inserts were washed again and overlaid with 3% H₂O₂ in methanol for 30 min to block endogenous peroxidase, followed by 20% normal goat serum to block unspecific immunoglobulin binding. Cells were incubated all night at 4°C with either monoclonal mouse anti-Pgp (2 $\mu\text{g}/\text{ml}$), monoclonal mouse anti-MRP-1 (10 $\mu\text{g}/\text{ml}$), rabbit anti-ZO-1 (4 $\mu\text{g}/\text{ml}$) or rabbit anti-occludin (1 $\mu\text{g}/\text{ml}$) antibodies. Then cells were washed and secondary antibodies were added for 2 h at room

temperature (diluted at 1/500). After washing several times, 0.1% of o-phenylenediamine and 0.002% H_2O_2 in 0.05 M citrate buffer at pH 4.5 was added for 10 min at room temperature and in the dark. The colour reaction was measured with a spectrophotometer at 490 nm, as described by Cioni et al. [5].

Drug transporter functional assays

The functionality of Pgp and MRP-1 was tested by assessing the release of the substrate BCECF-AM (specific for Pgp and MRP-1) in the absence or presence of specific inhibitors such as verapamil for Pgp and probenecid for MRP-1. The co-cultured cells were washed and cultured in DMEM. Then cells were preincubated for 15 min in the absence or presence of inhibitors at 37°C (40 μ M verapamil and 1 mM probenecid; concentrations were determined previously [25]). Inhibitors were added in the luminal compartment to study the transport from the luminal to the abluminal side and conversely. The inserts were incubated with 1 mM of BCECF-AM for 1 h at 37°C. Finally, 100 μ l was sampled from each compartment and fluorescence was measured with a fluorescence spectrophotometer at 493 nm excitation and 515 nm emission wavelengths.

Statistical analysis

The values are expressed as the means $\pm$ s.e.m. Each test was repeated three times and made in triplicate ($n = 9$). Statistical analysis was performed using Student's t-test. One-way and two-way analyses of variance (ANOVA) followed by Tukey-Kramer's tests were applied to multiple comparisons. Statistical analysis was performed using Statview software. The differences between means were considered to be significant when P values were less than 0.05.

Results

In vitro cytotoxicity assay

bEnd.3 cells were exposed to a range of concentrations of hydralazine or $CoCl_2$ (25 – 300 μ M) according to the literature. We studied the effect of hypoxic stress with an incubation time of about 24 h because the impact of prolonged hypoxia on BBB is less described than short exposure and difficult to monitor with chemical agents like $CoCl_2$, which has an important cytotoxicity [15]. After 24 h of exposure to drugs, cytotoxicity activity was evaluated by the MTT method (Fig. 2A). IC_{50} values of drugs were 200 μ M for hydralazine and 100 μ M for $CoCl_2$, respectively. So the least cytotoxic concentrations for hydralazine and $CoCl_2$ are 50 and 100 μ M. We verified the cytotoxicity of these concentrations by evaluating cell death with the LDH release method (Fig. 2B). We showed that hydralazine had a percentage of cytotoxicity about $2.36 \pm 0.4\%$ for 50 μ M and $17.77 \pm 4.9\%$ for 100 μ M; whereas $CoCl_2$ had a percentage of cytotoxicity about $50.84 \pm 4.9\%$ for 50 μ M and $68.96 \pm 7\%$ for 100 μ M.

As expected, $CoCl_2$ exposure produced a high cytotoxicity. On the contrary, hydralazine was not cytotoxic and can be used for 24 h of exposure.

Effective dose of drugs induced HIF-1 pathway

Hydralazine and $CoCl_2$ are chemical agents which could mimic hypoxia since they inhibited PHD, which negatively regulates HIF-1. First we determined the effective dose for each drug which induced HIF-1 α overexpression. For that, bEnd.3 cells were exposed to 50 and 100 μ M of hydralazine and $CoCl_2$ during 24 h. The concentration 50 μ M of hydralazine and $CoCl_2$ did not induce HIF-1 α (data not shown). After 24-h treatment with 100 μ M hydralazine, the level of HIF-1 α significantly increased by 226% ($p < 0.05$); whereas 100 μ M of $CoCl_2$ significantly induced HIF-1 α after 24 h of treatment, with an increase of 239% ($p < 0.05$) (Fig. 3). Consequently, we used the concentration of 100 μ M in our studies. In parallel, cells were exposed to 2% O_2 and validated that hydralazine or $CoCl_2$ induced HIF-1 α in a same way as true hypoxia. Indeed, our results showed that the level of HIF-1 α significantly increased by 245% ($p < 0.05$).

Fig. 2. Cytotoxicity effect of drugs in bEnd.3 cells. Cells were incubated with various concentrations of hydralazine or CoCl₂ for 24 h. Cytotoxicity was measured by an MTT assay (A) and LDH release method (B). The results are presented as a mean value for triplicates.

Fig. 3. HIF-1 expression induced by each drug and true hypoxia. bEnd.3 cells were treated with 100 μM hydralazine, CoCl₂ or exposed to 2% O₂. Results were presented as mean value ± s.e.m (n = 9) and normalized. * P < 0.05 normoxia vs. exposure (hypoxia or drugs).

Fig. 4. TEER measurement after cells of the blood-brain barrier model were exposed to hydralazine, CoCl₂ or true hypoxia during 24 h. Results are presented as mean value ± s.e.m (n = 9). * P < 0.05 normoxia vs. exposure (hypoxia or drugs).

Impact of CoCl₂ and hydralazine on BBB integrity

On TEER measurements. At the optimal TEER measurement (D6 established at the laboratory), cells were exposed 24 h to either CoCl₂, hydralazine or true hypoxia. Figure 4 shows the impact of addition of these molecules on TEER in an *in vitro* BBB model. A decrease in TEER was observed after 24 h for each molecule. For CoCl₂, TEER significantly decreased by 55%. TEER values varied from 83 ± 2 to $37 \pm 4 \Omega \cdot \text{cm}^2$ ($p < 0.05$). For hydralazine, TEER significantly decreased by 58%. TEER values varied from 83 ± 5 to $34 \pm 4 \Omega \cdot \text{cm}^2$ ($p < 0.05$). Hypoxia controls showed that TEER significantly decreased by 46% ($p < 0.05$).

On membrane permeability. Absolute membrane permeability was determined after 24-h incubation with hydralazine, CoCl₂ or exposed to 2% O₂, and results are illustrated by Fig. 5. Coefficient permeability of fluorescein in the BBB model significantly increased from $6.06 \pm 0.6 \times 10^{-6}$ to $6.66 \pm 0.46 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$ for CoCl₂ ($p < 0.05$). Cells of the BBB model treated with hydralazine showed a significant increase in fluorescein permeability ($p < 0.05$). The permeability coefficient of fluorescein varied from $9.09 \pm 0.1 \times 10^{-6}$ to $1.57 \pm 0.3 \times 10^{-4} \text{ cm} \cdot \text{s}^{-1}$.

Fig. 5. Absolute membrane permeability measurement during 24 h of hydralazine, CoCl_2 or true hypoxia exposure versus normoxia. The membrane permeability of fluorescein is expressed in cm.s^{-1} . Results are presented as mean value $\pm$ s.e.m (n = 9). * $P < 0.05$ normoxia vs. exposure (hypoxia or drugs).

Fig. 6. Concentrations of ZO-1 and occludin after cells of the blood-brain barrier model were exposed to hydralazine, CoCl_2 or true hypoxia and determined by cell-ELISA. Results are presented as mean value $\pm$ s.e.m (n = 9). * $P < 0.05$ normoxia vs. exposure (hypoxia or drugs).

Moreover, the permeability coefficient of fluorescein significantly increased after exposure to true hypoxia ($2.98 \pm 0.9 \times 10^{-4} \text{ cm.s}^{-1}$). There was no significant difference between the three exposures.

On paracellular transport mediated TJ proteins occludin and ZO-1. Determination of occludin and ZO-1 expressions in our *in vitro* BBB model were established after exposure to hydralazine, CoCl_2 or true hypoxia (Fig. 6). Expression of ZO-1 significantly decreased after these exposures ($p < 0.05$). Expression of ZO-1 decreased by 37% with hydralazine, by 35% with CoCl_2 and by 46% with true hypoxia. However, there was no significant difference for occludin's expression with these three exposures. The concentration of occludin varied from 3.78 to $3.04 \pm 0.63 \mu\text{g/ml}$ with hydralazine, from 3.78 to $2.84 \pm 0.35 \mu\text{g/ml}$ with CoCl_2 and from 3.78 to $3.20 \pm 0.52 \mu\text{g/ml}$ with true hypoxia. There was no significant difference between these exposures.

On transendothelial transport mediated efflux transporters. Transendothelial transport in our model was evaluated with two efflux transporter pumps, Pgp and MRP-1. It was evaluated by studying the transport of a substrate of Pgp and MRP-1, *i.e.*, BCECF-AM, with a method developed in our laboratory [26]. BCECF-AM is cleaved by intracellular esterase into a fluorescent component BCECF. In a previous work, we demonstrated that pumps were more active on apical membranes of endothelial cells (transport A to B), since BCECF was more efflux [19]. So we investigated if the transport of BCECF from A to B was changed after hydralazine, CoCl_2 or true hypoxia exposure (Fig. 7A). We noticed that efflux transporters prevented entry of BCECF in cells, since an important transendothelial transport was observed after each exposure. A significant increase of extracellular BCECF was also observed after hydralazine exposure: $5.13 \pm 0.97 \mu\text{g/ml}$ versus $8.80 \pm 0.78 \mu\text{g/ml}$ ($p < 0.05$) together with CoCl_2 , where extracellular BCECF varied from $5.13 \pm 0.97 \mu\text{g/ml}$ to $8.88 \pm 0.41 \mu\text{g/ml}$ ($p < 0.05$). We also observed a significant increase of extracellular BCECF with true hypoxia ($9.91 \mu\text{g/ml}$). In parallel, we observed no significant difference between the two chemical agents and hypoxia controls. The relative importance between Pgp and MRP-1 was evaluated with two specific inhibitors of Pgp and MRP-1, *i.e.*, verapamil and probenecid, respectively. We observed no significant difference between these two transporters.

Fig. 7. (A) Release of BCECF during transport on apical membranes of endothelial cells, after exposure to hydralazine, CoCl₂ and true hypoxia. (B) Concentrations of MRP-1 and Pgp after cells of the blood-brain barrier model were exposed to hydralazine, CoCl₂ and 2% O₂ during 24 h. Results are presented as mean value $\pm$ s.e.m (n = 9). * P < 0.05 BCECF vs. inhibitors and * P < 0.05 normoxia vs. exposure (hypoxia or drugs).

In a second time, we studied the expression of Pgp and MRP-1 proteins in our *in vitro* BBB model after the three exposures, in order to understand the increase in the pump's observed activity (Fig. 7B). MRP-1 expression significantly increased after incubation with each exposure. For hydralazine MRP-1 increased by 91% (p < 0.05), by 136% (p < 0.05) for CoCl₂ and by 90% for true hypoxia (p < 0.05). There was no significant difference between hydralazine, CoCl₂ and hypoxia controls. Pgp expression significantly increased by 439% (p < 0.05), 502% (p < 0.05) and 371% for hydralazine, CoCl₂ and true hypoxia, respectively. There was no significant difference between these exposures.

This study on transendothelial transport mediated efflux transporters showed that the functionality of Pgp and MRP-1 increased after chemical and true hypoxic stress, which can be explained by an overexpression of Pgp and MRP-1 proteins.

Discussion

The BBB is a fundamental structure for the CNS because it confers a protection for the brain. This protection is mainly ensured by TJ proteins and efflux transporter proteins [27]. On the one hand, TJ proteins create a tightness barrier and reduce paracellular diffusion. On the other hand, efflux transporter proteins actively pump compounds out of endothelial cells to reduce the entry of potential cytotoxic substances. However, this BBB can be disrupted in some cases. Hypoxia is a key factor of the BBB's disruption and is involved in some neurological diseases. The cellular response to hypoxia is driven by the HIF-1 pathway [12]. Nevertheless, elucidation of cellular mechanisms induced by hypoxic stress is rather difficult with true hypoxia because HIF-1 α has a short half-life, is rapidly reverted and thus less reproducible. In this regard, we present in this study a suitable method of chemical hypoxic stress induction via mimetic agents of the hypoxia pathway. We decided to compare the capacity of a potential mimetic agent, hydralazine, with the classical agent used in the literature, CoCl₂ [14, 28], to determine the suitable mimetic agent for the study of chemical hypoxic stress on the BBB. Moreover, we validated their effects by comparing with hypoxia controls. This induction method was set up to understand cellular mechanisms involved by chemical hypoxic stress on an original *in vitro* BBB model. We exposed our model to hydralazine or CoCl₂ during 24 h to study the effect of prolonged chemical hypoxic stress on the BBB to mimic prolonged cerebral hypoperfusion, like in ischemic stroke. In a first step, we demonstrated that these two molecules were able to induce a chemical hypoxic state on our cells since we observed an increase of HIF-1 α protein expression, similar to the expression observed with hypoxia controls. Nevertheless, we showed that 100 μ M of CoCl₂ was the IC₅₀ for bEnd.3, meaning that at this concentration there were 50% dead cells and at a lower concentration there was no induction of HIF-1 α . Cervellati et al. explained that

this decrease of viability in cells induced by CoCl_2 must be due to activation of caspase-3, which leads to apoptosis [15]. This decrease in viability of cells could be a problem because we could not determine whether the observed effect was due to the inhibition of the PHD induced by CoCl_2 or its cytotoxicity. This has also been confirmed by other researchers using these agents [14, 15, 17]. Moreover, we observed the same result for HIF-1 expression after 24-h exposure to true hypoxia. So we decided to use this concentration to investigate the consequences of chemical hypoxic stress on our *in vitro* BBB model. Our results showed that chemical hypoxic stress, induced by hydralazine or CoCl_2 , significantly decreased TEER value and significantly increased membrane permeability to fluorescein. Taken together, these results demonstrated that the BBB lost its physical barrier properties after chemical hypoxic stress since ionic compounds (measured by TEER value) and molecules (measured by Na-F) could cross cell membranes.

To understand this loss, we investigated the expression of TJ proteins since they are in charge of these physical barrier properties. Chemical hypoxic stress significantly decreased ZO-1 but not occludin expression. This result confirmed those obtained by Engelhard et al. with a CoCl_2 and true hypoxia approach [12] and must be explained by the fact that ZO-1 has an important role in the development and barrier maintenance of the BBB [29]. However, an increase of VEGF via HIF-1 pathway decreased ZO-1 protein expression and lead to a rearrangement of ZO-1 in brain endothelial cells [30, 31]. It was shown that hydralazine induced a stabilization of HIF-1 α and increased VEGF secretion [17]. So, this suggested that HIF-1 α signalling impacts ZO-1 localization via VEGF pathway but has no effect on occludin localization. Animal experiments (knockdown for occludin) have shown that occludin was not essential for the establishment of TJ complexes, despite the fact that a loss of this protein was involved in many diseases. Occludin would participate in the regulation of the BBB and more particularly in the regulation of calcium flux through the BBB [32].

We then investigated the impact of prolonged chemical hypoxic stress on efflux transporters as they conferred a protection for the brain by rejecting potential dangerous compounds. We showed that Pgp and MRP-1 were significantly active since they rejected more BCECF during chemical hypoxic stress; but we noticed no significant difference between the two pumps. This result was shown in tumors cells which are rather hypoxic cells. In these cells, HIF-1 α was overexpressed and was associated with an increase of Pgp and MRP-1 activity, which explained a chemoresistance for these cells [33]. Moreover, this result confirmed those obtained with cells exposed to 2% O_2 . Finally, we showed that this increase in activity was linked to an overexpression of Pgp and MRP-1 proteins after chemical hypoxic stress. Taken together, these results demonstrated that cells established a defence mechanism to protect the brain from chemical hypoxic stress, but it would create a resistance to therapy, as it has been described for anti-epileptic drugs and chemotherapy [34]. This should be taken into consideration during the development of therapeutics.

To conclude, this study was undertaken to define a suitable mimetic hypoxia agent in order to investigate and understand cellular and molecular impacts of hypoxic stress on an *in vitro* BBB model. Our *in vitro* BBB model is composed of immortalized cell lines and presents a lower TEER values than primary cells, which reproduce more physiologically the cellular and structural characteristics of an *in vivo* BBB. Nevertheless, the permeability and the expression of TJ proteins respond to the expected characteristics of an *in vitro* BBB model [35]. Moreover, these immortalized cells allow to obtain a reproducible model, and to study the impact of hypoxic stress over time, which is not necessary the case with primary cells. Our results presented hydralazine as a suitable candidate to create a chemical hypoxic state in our *in vitro* BBB model, since it was able to induce HIF-1 α without any cytotoxic effect. Moreover, we showed that hydralazine induced the same effect on our *in vitro* BBB model than hypoxia controls. This result is in line with those described in the literature [17, 19]. CoCl_2 could be a useful tool to create a hypoxic state, but it was too cytotoxic for our cells. Hence induction of chemical hypoxic stress by hydralazine is a suitable mimetic agent of hypoxia to understand the early stages of the consequences of hypoxic stress on the BBB. This induction method has many advantages on true hypoxia because chemical hypoxic

stress is standardization controlled, easy to produce, reproducible and reversible. It is also less expensive. We are confident that this induction method by hydralazine will allow the understanding of cellular and molecular mechanisms activated at the BBB during hypoxic events like repeated hypoxic stress.

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Disclosure Statement

The authors declare no conflict of interest.

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