Neuropharmacology xxx (2015) 1–10

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Intranasal delivery of progesterone after transient ischemic stroke decrease mortality and provides neuroprotection

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ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form
1 June 2015
Accepted 2 June 2015
Available online xxx

Keywords:
Intranasal
Steroid
Progesterone
Corticosterone
Neuroprotection
Stroke

ABSTRACT

Progesterone is a potential neuroprotective agent for cerebral stroke. One of the STAIR's recommendations is to test different routes of delivery of therapeutic agents. Here, we investigated the neuroprotective efficacy of intranasal delivery of progesterone in oleogel. Male mice were subjected to transient middle cerebral occlusion (MCAO) for 1 h. Mice received intranasal or intraperitoneal administrations of progesterone (8 mg/kg) at 1, 6, and 24 h post-MCAO. Plasma and brain levels of steroids were measured by gas chromatography-mass spectrometry 2 and 24 h after the last administration of progesterone. Behavioral and histopathological analyzes were performed at 48 h post-MCAO. For blood-brain barrier (BBB) permeability analysis, mice received one intranasal administration of progesterone or placebo at reperfusion and Evans Blue and sodium fluorescein extravasations were assessed at 4 h post-MCAO. Two hours after its nasal administration, progesterone reached elevated levels in brain and plasma and was bioconverted to its 5α-reduced metabolites and to 20α-dihydroprogesterone. However, brain levels of progesterone and its metabolites were about half those measured after intraperitoneal injections, whereas levels of 11-deoxycorticosterone and corticosterone were 5-times lower. In contrast, after 24 h, higher levels of progesterone were measured in brain and plasma after intranasal than after intraperitoneal delivery. Intranasal progesterone decreased the mortality rate, improved motor functions, reduced infarct, attenuated neuronal loss, and decreased the early BBB disruption. This study demonstrates a good bioavailability, a prolonged absorption and a good neuroprotective efficacy of intranasal delivery of progesterone, thus potentially offering an efficient, safe, non-stressful and very easy mode of administration in stroke patients.

1. Introduction

Stroke is a major cause of death and neurological disability (Feigin, 2005). Currently, the only FDA-approved treatment for acute stroke, thrombolysis with tissue plasminogen activator (rt-PA), can only be used in less than 5% of patients because of its narrow therapeutic window and risks (Wardlaw et al., 2012). Therefore, it is necessary to develop new alternatives or complementary strategies, and there is great interest in neuroprotective ones (Majid, 2014).

A number of studies have demonstrated neuroprotective efficacy of progesterone in experimental models of stroke, and in particular after MCAO (Gibson et al., 2008; Liu et al., 2012a; Schumacher et al., 2007; Stein, 2008; Wong et al., 2013). However, they have only investigated the efficacy of intraperitoneal and subcutaneous administrations (Wong et al., 2013), which would not be the most appropriate mode of administration in stroke patients since progesterone injection has a short half-life in both plasma and brain. Before clinical trials, refining of treatment protocols is needed. One of the STAIR's recommendations is

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http://dx.doi.org/10.1016/j.neuropharm.2015.06.002
0028-3908/

to evaluate different administration routes and to develop the one which better mimics what is feasible and practical in clinical settings. Intranasal delivery is one of the most attractive routes. It has gained significant attention as a convenient, efficient and noninvasive method for the administration of drugs including steroids (Dhuria et al., 2010; Ducharme et al., 2010; Kim et al., 2012; Lu et al., 2011; Pires et al., 2009; Rhim et al., 2013; Van den Berg et al., 2004; Wang et al., 2013). Recently, a comparison of intranasal administration and intravenous injection of progesterone in mice has shown that the bioavailability in blood 2 h after intranasal administration was 14%, the blood levels being 7.35 times lower than after intravenous administration. Brain levels of progesterone after intranasal administration did not vary significantly over time (2–120 min) while levels decreased with time after the intravenous injection. Furthermore, intranasal delivery of progesterone decreased anxiety (Ducharme et al., 2010).

These characteristics of the intranasal administration (low level in plasma, stability of levels in brain over 2 h, therapeutic potential) suggest that intranasal administration of progesterone is a promising route for the delivery of progesterone after stroke. Furthermore, in addition to the pharmacokinetic advantages, nasal application could be rapidly and easily performed in stroke patients without the need for special equipment or qualification. Thus, progesterone could be administrated even before reaching the hospital. This could represent a great advantage, as mitigate the initial pathological processes is very important in stroke. This route of administration should also be evaluated in other experimental models of stroke including hemorrhagic stroke, as intraperitoneal and subcutaneous administrations of progesterone have been shown to be beneficial in subarachnoid hemorrhage model (Yan et al., 2013) and also in transient MCAO model followed by delayed treatment with rt-PA (Won et al., 2014).

Progesterone acts after binding to intracellular progesterone receptors (PR) or membrane receptors (mPR and PGRMC1) and is also actively metabolized. Indeed, progesterone can be converted to 5α-dihydroprogesterone (5α-DHPROG) by the steroid 5α-reductases, then to 3α,5α-tetrahydroprogesterone (3α,5α-THPROG, allopregnanolone) by the 3α-hydroxysteroid dehydrogenase (3α-HSD). In addition, 5α-DHPROG can be reduced to 3β,5α-tetrahydroprogesterone (3β,5α-THPROG, isoallopregnanolone) (Supplemental Figure I). Like progesterone, 5α-DHPROG can activate gene transcription (Rupprecht et al., 1993) after binding to the intracellular PR, which have recently been shown to play a key role in the neuroprotective effects of progesterone after MCAO (Liu et al., 2012a). Although 3α,5α-THPROG has no affinity for the intracellular PR, it also exerts neuroprotective effects in experimental stroke, most likely by modulating $GABA_A$ receptors (Belelli and Lambert, 2005; Liu et al., 2012a; Sayeed et al., 2006). Progesterone can also be reduced to 20α-dihydroprogesterone (20α-DHPROG), but the significance of this pathway remains poorly understood.

In this study, we investigated whether the intranasal delivery of progesterone could be used therapeutically for stroke. In a transient MCAO mice model, we compared the relative efficiency of intranasal administration *versus* intraperitoneal injection for the delivery of progesterone to the brain. We then determined whether levels of progesterone reached in the brain by intranasal delivery have neuroprotective efficacy after MCAO. The formulation of progesterone used in this study, is the same that could be used in humans. It has been accepted for use in clinical studies in humans by the German health authority (BfArM) and has been shown to be efficient in mitigating sleep dysfunction in postmenopausal women (Steiger, 2013).

2. Experimental procedures

2.1. Animals and surgery

All procedures concerning animal care and use were carried out in strict accordance with national guidelines (authorization 94-345 to R.G., animal facility approval 94-043-13) and with French ethical laws (Act 87-848 and Act 2013-11) and European Communities Council Directives (November 24, 1986 86/609/EEC). C57BL6/129SvEv mice from our breeding colony were housed under standard conditions with a 12 h light–dark cycle and access to food and water ad libitum. Adult male C57BL6/129SvEv mice (3–4 months weighting 26–30 g) were used.

Transient cerebral ischemia was performed under ketamine (50 mg/kg) and xylazine hydrochloride (6 mg/kg) according to Liu et al. (2012a). Briefly, the left common carotid artery and the internal carotid artery were isolated and temporarily occluded. A nylon monofilament (Drennan; 83 μm diameter) coated with 'thermomelting' glue (4 mm long, 190 μm diameter) was introduced through an arteriotomy performed in the external carotid artery and advanced into the internal carotid artery to occlude the origin of the MCA. Occlusion of the MCA was controlled by monitoring the cerebral blood flow in the MCA territory with laser Doppler flowmetry (Moor Instruments, France). The criterion of exclusion in this study was set on the basis of the % of decrease of blood flow comparatively to baseline before MCAO: animals with less than 50% of decrease must be excluded. On this basis, no animals were excluded. The filament was withdrawn 1 h after occlusion to allow reperfusion, and the common carotid artery ligature was also removed. Body temperature was monitored throughout surgery by a rectal probe and maintained at 37 ± 0.5 °C with a homoeothermic blanket control unit (Harvard Apparatus, Edenbridge, Kent, UK). After surgery, the wound was sutured. The mice were returned to their cage maintained at 30 °C, with free access to food and water. In order to prevent dehydration, mice were injected subcutaneously with 0.5 ml of 0.9% NaCl just after reperfusion and then twice per day. All mice that survived were analyzed.

2.2. Progesterone treatments and experimental groups

For intranasal administration, progesterone was dissolved in a proprietary oleogel (4% in ologel composed of a viscous castor oil mixture; M et P Pharma AG, Emmetten, Switzerland). The stability of the oleogel containing progesterone has been investigated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidance for Industry "Stability Testing of New Drug Substances and Products", at 25 °C ± 2 °C/60% relative humidity (RH) ± 5% RH for 3 years and at 40 °C ± 2 °C/75% RH ± 5% RH for 6 months and has shown to be stable under these conditions. The pharmacokinetics of this formulation after intranasal administration to mice were recently reported (Ducharme et al., 2010).

For intraperitoneal injections, progesterone was dissolved in a small volume of ethanol and further diluted in sesame oil to obtain the desired final steroid concentrations (0.8 mg/ml) (Liu et al., 2012a). Progesterone was administered intranasally or intraperitoneally at the dose of 8 mg/kg. Nasal delivery was performed without anesthesia by direct application of progesterone oleogel into each nostril (1 μl/nostril/10 g of body weight) using a pipette.

Mice were randomly assigned to the different treatment groups. Throughout the analysis, experimenters were blinded to the experimental conditions of the mice. After MCAO, a number was randomly assigned to each mouse. The experimenter knew the number of the animal and not the group to which it belongs. The blind was lifted only when processing results. Two experiments were designed.

2.2.1. Experiment 1

It was designed to measure steroid levels in plasma and brain after intranasal or intraperitoneal delivery of progesterone. Mice (n = 42) were subjected to 1 h MCAO; 6 groups (n = 7) were designed: Three treatment groups and 2 times analysis. Three groups were composed: group 1 received no treatment; group 2 received intranasal progesterone; group 3 was injected intraperitoneally with the same dose of progesterone. Progesterone was administered at 1, 6, and 24 h after MCAO. Two times of analysis were chosen: mice were sacrificed either at 2 h after the last administration of progesterone (i.e. 26 h post-MCAO) or at 24 h after the last administration of progesterone (i.e 48 h post-MCAO). Mice were handled gently to minimize stress and were quickly decapitated by rapid cervical dislocation without anesthesia. Blood samples were taken and the brain dissected out of the skull on a bed of crushed ice and the ipsilateral ischemied hemisphere and contralateral hemisphere were separated. Plasma and brain samples were frozen on dry ice and stored at −80 °C until analysis. Among the 42 mice, 5 mice died. At the end of the experiment, 6 mice survived in all groups except for one group in which 7 mice survived. Brain and plasma levels of steroids were measured by gas chromatography/mass spectrometry (GC/MS) according to a validated protocol (Liu et al., 2012a).

Briefly, steroids were extracted from tissues and plasma samples from experiment 1 with methanol, and internal standards were added for steroid quantification: 2 ng [2H6]5-DHPROG (CDN Isotopes) for 5α-DHPROG, 2 ng 19-norprogesterone (Steraloids, Newport, Rhode Island) for progesterone and 3α,5α-THPROG and 3β,5α-THPROG, 2 ng DOC-d_8 for DOC, and 10 ng [$2H_8$] corticosterone (CDN Isotopes, Sainte Foy La Grande, France) for corticosterone. Unconjugated and conjugated steroids were separated by a previously described solid phase extraction and a

Please cite this article in press as: Fréchou, M., et al., Intranasal delivery of progesterone after transient ischemic stroke decrease mortality and provides neuroprotection, Neuropharmacology (2015), http://dx.doi.org/10.1016/j.neuropharm.2015.06.002