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Immunopharmacology and inflammation

The type 2 diabetes drug liraglutide reduces chronic inflammation induced by irradiation in the mouse brain

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ABSTRACT

Chronic inflammation in the brain is found in a range of neurodegenerative diseases such as Parkinson's or Alzheimer's disease. We have recently shown that analogues of the glucagon-like polypeptide 1 (GLP-1) such as liraglutide have potent neuroprotective properties in a mouse model of Alzheimer's disease. We also found a reduction of activated microglia in the brain. This finding suggests that GLP-1 analogues such as liraglutide have anti-inflammatory properties. To further characterise this property, we tested the effects of liraglutide on the chronic inflammation response induced by exposure of the brain to 6 Gy (X-ray). Animals were injected i.p. with 25 nmol/kg once daily for 30 days. Brains were analysed for cytokine levels, activated microglia and astrocyte levels, and nitrite levels as a measure for nitric oxide production and protein expression of iNOS. Exposure of the brain to 6 Gy induced a pronounced chronic inflammation response in the brain. The activated microglia load in the cortex and dentate gyrus region of hippocampus ($P<0.001$), and the activated astrocyte load in the cortex ($P<0.01$) was reduced by liraglutide. Furthermore, the pro-inflammatory cytokine levels of IL-6 ($P<0.01$), IL-12p70 ($P<0.01$), IL-1β ($P<0.05$), and total nitrite concentration were reduced in the brains of animals treated with liraglutide. The results demonstrate that liraglutide is effective in reducing a number of parameters linked to the chronic inflammation response. Liraglutide or similar GLP-1 analogues may be a suitable treatment for reducing the chronic inflammatory response in the brain found in several neurodegenerative conditions.

1. Introduction

Chronic inflammation in the brain has detrimental effects on neurons. The release of pro-inflammatory cytokines and the generation of free radicals by activated microglia(Smith and Perry, 1995) has detrimental effects on cognition(Cunningham and Skelly, 2012), synaptic activity(Kotilinek et al., 2008), neuronal repair(Greig et al., 2004) and stem cell activation (Monje et al., 2003). Numerous neurodegenerative diseases such as Alzheimer's or Parkinson's disease display chronic inflammation responses in the brain which are at least in part the cause of neurodegeneration (Cole et al., 2004; Harkavyi et al., 2008; Lee et al., 2010).

Liraglutide (NN221, Victoza®), a GLP-1 mimetic, is in the market for once daily treatment of type 2 diabetes. It has an enhanced half-life of 13 h, is well tolerated and does not affect glucose levels in normoglycemic people (Vella et al. 2002, Agersø and Vicini, 2003).Liraglutide does not show any adverse reaction in mice and in open field, changes in motor activity, exploratory behaviour and anxiety levels were not observed in previous studies (Holscher, 2010; Porter et al., 2010;McClean et al., 2011). GLP-1 receptors are found on neurons in the brains of rodents and humans (Goke et al., 1995; Perry and Greig, 2005). They are predominately expressed on large neurons, on cell bodies and also on dendrites (Hamilton and Holscher, 2009). GLP-1 and analogues of GLP-1 including liraglutide have been shown to cross the blood brain barrier (Hunter and Holscher, 2012; Kastin et al., 2002; McClean et al., 2011) and increase cell growth, proliferation and repair, and inhibit apoptosis (Perfetti et al., 2000). Enhanced progenitor cell proliferation in the brain was also found (Hamilton et al., 2011; Li et al., 2010). GLP-1 analogues are not only neuroprotective, but also have anti-inflammatory activity. Both activated microglia and astrocytes express GLP-1 receptors and GLP-1 can prevent an endotoxin (LPS) induced increase in IL-1β (Iwai et al., 2006). Exendin-4, a GLP-1 analogue, can prevent LPS-induced cytokine and chemokine mRNA synthesis in both human and mouse monocytes (Arakawa et al., 2010) and also reduces an LPS induced increase in microvascular permeability (Dozier et al., 2009). In our recent study testing the effects of liraglutide on the main biomarkers in an APP/PS1 mouse model of Alzheimer's disease, we found that the synthesis of ß-amyloid, the aggregation of amyloid plaques, and the chronic inflammation

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response in the brain was much reduced and after chronic treatment with liraglutide enhanced memory and learning and increased synaptic functions were also observed (Holscher, 2010; McClean et al., 2011). This finding indicates that liraglutide has anti-inflammatory effects in the brain in chronic inflammation responses. However, as amyloid plaques induce an inflammation response, and the plaque load had been reduced in these APP/PS1 mice, it may well be that the reduction of the inflammation response in the brain was only due to the reduction of the plaque load. To investigate the anti-inflammatory properties in a more unequivocal way, we chose the x-ray induction of a chronic inflammation response in the brain as a model. Irradiation of the brain induces a defined inflammation response that can be used to study a reduction of the activation of microglia and astrocytes and their release of cytokines (Kalm et al., 2009; Monje et al., 2003).

2. Materials and methods

2.1. Mice

Twenty male C57BL/6 mice (6 months old) were used in the experiment. Animals were housed in single cages in a temperature controlled holding room (21.5 °C ± 1) with 12:12 h light and dark cycle. All experiments were carried out in accordance with the UK animals (Scientific Procedures) Act 1986.

2.2. Irradiation

To anaesthetize, mice were injected intraperitonially with a mixture of Hypnorm and Hypnovel (1:1) prepared in 2 parts of sterile water for injections (100 µl/30 g). Irradiation was carried out using CP160 Generator (160 kV, 18 mA) with a filter of 0.3 mm copper and 4 cm applicator. Two mice were irradiated at any one time. The mice were placed on a custom made jig with their head positioned on a wire to keep the head horizontal. Whole body of the mice was shielded with 6 mm thick lead except for an opening of 3 × 11 mm above the head. The X-ray beam was directed down to the head with source to skin distance of 31 cm. Dosimeter was performed using GDSTLD18 (Medray, Dublin) and the dose delivered was ~6 Gy. All control animals were anaesthetized but not irradiated. Animals were kept on a heating pad to avoid hypothermia and after recovery from anaesthesia were returned to their respective cages. Experimental timeline is shown in Fig. 1.

2.3. Drug treatment

Next day after irradiation animals were treated with either 0.9% NaCl or 25 nmol/kg liraglutide intraperitonially (i.p.) once daily for 30 days. A dose of 25 nmol/kg liraglutide was chosen as it was the best effective dose in a previous study on biomarkers in a mouse model of Alzheimer's disease (McClean et al., 2011). Body weights and food intake were recorded every day. At the end of 30 days, animals were perfused transcardially with ice cold phosphate buffer saline and the brains were removed, one hemisphere was snap frozen in liquid nitrogen and the other half was fixed in 4% ice cold paraformaldehyde.

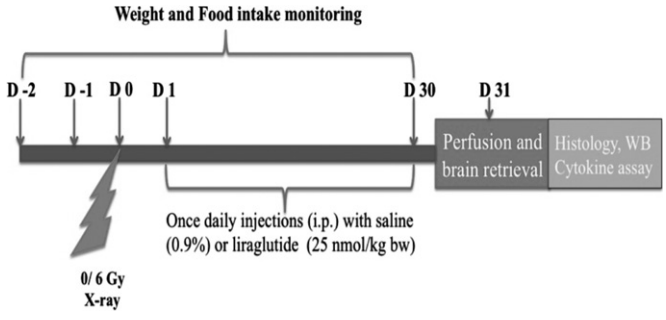

Fig. 1. Schematic diagram of experiment design. To evaluate the effect of irradiation on inflammation, animals, 5 per group, were irradiated (6 Gy) or sham treated on Day0. From Day1 till Day30 they were either injected with Liraglutide (25 nmol/kg) or saline. On Day 31 brains were harvested for histology, cytokine assay and nitrite levels.

2.4. Food and weight

Food intake and body weight of the animals was monitored by daily weighing animals and food pellet. By subtracting weight of consecutive days of food pellet in the cage, food intake was recorded. Water was available ad libitum.

2.5. Cytokine assay

Brain cytokine assay was performed using mouse proinflammatory 7-plex assay ultra sensitive kit from Meso Scale Discovery (MD, USA) according to manufacturer's protocol. In brief, the brains were weighed and then homogenised in Tris buffer saline (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X) with supplements of protease inhibitor cocktail. Homogenates were incubated for 20 min on ice and then centrifuged at 14,000 rpm at 4 °C for 10 min and supernatant used for quantification of cytokines as per manufacture's protocol. Bradford assay was done to quantify the total amount of protein. All measurements were done in triplicates and the final cytokine concentration was determined following normalisation with protein concentration in individual sample.

2.6. Determination of nitrite content

Concentration of Nitrite (a metabolite of Nitric oxide), in the supernatant of brain homogenates was determined using Griess Reagent kit (Invitrogen, UK) as per manufacture's protocol. The absorbance at 520 was read on a plate reader. Concentration of nitrite was calculated from 10-point sodium nitrite (Sigma) standard curve. All measurements were done in duplicate and values were normalised to total protein concentration in individual sample (Radenovic et al., 2003).

2.7. Western Blotting

Protein samples were denatured for 10 min in boiling water in lamelli buffer (0.125 M Tris HCL, pH 6.8, containing 4% SDS, 10% 2-mercaptorthanol, 20% glycerol and 0.004% bromophenol blue). The proteins were then separated by SDS-PAGE and transferred to nitrocellulose membrane (Invitrogen, UK). Blocking was done in 5% non fat milk and incubated in primary iNOS antibody (1:1000 Abcam, Cambridge, UK) and beta actin (1:5000) overnight at 4 °C. Membranes were washed three times in TBST (Tris/phosphate/saline/Tween) and incubated in horseradish peroxidase-conjugate secondary anti rabbit or anti mouse antibody (1:10,000, GE Healthcare) and signals were detected by Luminata forte chemiluminescence system (Millipore, MA). The blots were then exposed on X-ray films and bands were quantified using image J software (NIH, USA). Relative densities of bands were normalised to β-actin expression.

2.8. Histology

Brains in paraformaldehyde were transferred to 30% sucrose overnight and then snap frozen with Envirofreez™ (Sigma, UK) and using Leica cryostat, 40 µm thick coronal sections were cut at