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Omega-3 polyunsaturated fatty acids ameliorate neuroinflammation and mitigate ischemic stroke damage through interactions with astrocytes and microglia

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ABSTRACT

Omega-3 polyunsaturated fatty acids (PUFA n3) provide neuroprotection due to their anti-inflammatory and anti-apoptotic properties as well as their regulatory function on growth factors and neuronal plasticity. These qualities enable PUFA n3 to ameliorate stroke outcome and limit neuronal damage. Young adult male rats received transient middle cerebral artery occlusion (tMCAO). PUFA n3 were intravenously administered into the jugular vein immediately after stroke and 12 h later. We analyzed stroke volume and behavioral performance as well as the regulation of functionally-relevant genes in the penumbra. The extent of ischemic damage was reduced and behavioral performance improved subject to applied PUFA n3. Expression of Tau and growth-associated protein-43 genes were likewise restored. Ischemia-induced increase of cytokine mRNA levels was abated by PUFA n3. Using an *in vitro* approach, we demonstrate that cultured astroglial and microglia directly respond to PUFA n3 administration by preventing ischemia-induced increase of cyclooxygenase 2, hypoxia-inducible factor 1alpha, inducible nitric oxide synthase, and interleukin 1beta. Cultured cortical neurons also appeared as direct targets, since PUFA n3 shifted the Bcl-2-like protein 4 (Bax)/B-cell lymphoma 2 (Bcl 2) ratio towards an anti-apoptotic constellation. Thus, PUFA n3 reveal a high neuroprotective and anti-inflammatory potential in an acute ischemic stroke model by targeting astroglial and microglial function as well as improving neuronal survival strategies. Our findings signify the potential clinical feasibility of PUFA n3 therapeutic treatment in stroke and other acute neurological diseases.

1. Introduction

Stroke is the result of a permanent or transient focal occlusion of major brain arteries or their branches and represents a main cause of death and disability in the industrialized civilization. Brain damage and neuronal cell death following acute ischemia result from a series of complex pathophysiological processes that evolves in time and space beginning a few minutes after stroke onset and lasting for hours and days including secondary damage due to edema spreading even if reperfusion has already been revived. Cell dysfunction and tissue destruction are accompanied by local blood–brain barrier (BBB) breakdown followed by the invasion of peripheral immune cells, *i.e.* T-lymphocytes, macrophages and polymorph nuclear granulocytes. Beforehand, a massive early disturbance of ion homeostasis, calcium dysregulation, excitotoxicity, mitochondrial impairment together with reactive oxygen species (ROS) formation can be observed (Iadecola and Anrather, 2011; Dirnagl, 2012). The described pathomechanisms coincide with the activation, attraction and proliferation of astroglial and microglial cells. Astrogliosis and microgliosis are prevailing incidents in the penumbra during the initial stage of ischemia. Both glial cell types control and tune early and late neuroinflammatory responses resulting from oxygen and nutrient deprivation soon after the beginning of the ischemic phase (Dang et al., 2011). Although microglia is believed to play the most prominent role in the shaping of inflammatory responses after stroke, latterly astrocytes in the center of ischemic tissue disintegration are considered to actively sense hypoxia and trigger a battery of anti-inflammatory reactions (Ronaldson and Davis, 2012; Habib and Beyer, 2014; Habib et al., 2014). Importantly, both types of glial cells, the adjacent extracellular matrix, the endothelium and neurons form a "neurovascular unit" that represents a dynamic entity which shapes neuroinflammation in the setting of stroke (Dirnagl, 2012).

Recent studies have shown that omega-3 essential polyunsaturated fatty acids (PUFA n3) and in particular docosahexaenoic acid (DHA, 22:6, n-3) and to a lesser extent eicosapentanaenoic acid (EPA, 20:5, n-3) exert profound anti-inflammatory effects on the brain and

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protect brain tissue in experimental models of acute stroke in neonatal and adult animals and neuroinflammatory challenges besides being beneficial for brain development and cognitive function (Bazan, 2007; Belayev et al., 2009; Hoffman et al., 2009; Cole et al., 2010; Orr et al., 2013). Following short-term transient middle cerebral artery occlusion (tMCAO), rodents with DHA substitution and higher brain DHA levels revealed reduced infarct areas and cellular inflammatory responses as well as attenuated leukocyte infiltration and concomitantly fewer microglial cells (Belayev et al., 2009; Lalancette-Hebert et al., 2011; Orr et al., 2013). Several hours after stroke, the resident microglial cells become activated, accumulate in the vicinity of the lesion site and in the penumbra region and start proliferating (Kriz and Lalancette-Hebert, 2009; Dang et al., 2011; Lalancette-Hebert et al., 2011). This defines the post-ischemic treatment window with DHA as 3–5 h. There is also good evidence that consumption of fish and fish products (fish oil contains large amounts of DHA) is positively associated with a reduced risk of ischemic events in the CNS and cardiovascular disease (Pascoe et al., 2014). There are several proposed cellular mechanisms which could explain the safeguarding role of PUFA n3 under neuropathological conditions in the brain (Orr et al., 2013). DHA affects growth factor regulation which may be responsible for increased neurite growth and synapse formation (Kim et al., 2011). Coevally, DHA is anti-inflammatory in non-neuronal and neural tissues targeting for instance cyclooxygenases (COX) and cytosolic phospholipase A_2 ($cPLA_2$) as well as leukocyte infiltration and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation (Marcheselli et al., 2003; Orr et al., 2013). Such effects occur brain-intrinsically but it has also been shown that DHA dampens systemic inflammatory responses (Sijben and Calder, 2007).

In the present study, we aimed at demonstrating the neuroprotective potency of PUFA n3 in an experimental stroke rat model (transient middle cerebral artery occlusion, tMCAO), its efficacy in restoring motoric and sensory behavioral defects as well as morphological injury, and analyzing its influence on the expression of stroke-associated inflammatory gene markers. By adopting an *in vitro* hypoxia approach, we intended to curtail cell type-specific effects which might explain neuroprotective mechanisms at the subcellular level.

2. Material and methods

2.1. Animals

In vivo experiments were performed with 12 week old male Wistar rats (300–350 g, Charles-River, Germany) which were maintained in a pathogen-free and climate-controlled environment with access to water and food *ad libitum* and handled according to the rules of "Care of Animal Subjects" (North Rhine-Westphalia, Germany). The animals underwent routine cage maintenance once a week and microbiological monitoring.

2.2. Surgery and tMCAO

Artery occlusion was induced by unilateral intraluminal occlusion of the middle cerebral artery for 1 h as previously described (Dang et al., 2011; Ulbrich et al., 2012). Rats were anesthetized by inhalation of 5% isoflurane (Abbott, Ludwigshafen, Germany) for 2 min and maintained at 1.5–2% using a face mask. Rectal temperature was maintained at 37.0 ± 0.5 °C during surgery with a temperature-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). Regional cerebral blood flow (CBF) was measured using Laser-Doppler flowmetry (Moor Instruments VMS-LDF2, Axminster, UK) on the ipsi- and contralateral side before, during, and after transient occlusion. The animals that did not show a CBF reduction of at least 50% of baseline levels or died directly after ischemia induction were excluded from further processing. For placing the laser probes, parietal bones were thinned 5.5 mm lateral and 1 mm posterior to the Bregma, and the Laser Doppler flow probes were fixed after adequate signal intensity (100 perfusion units) was detected. After adjusting the rat in a supine position and following a small midline neck skin incision, the external jugular vein was exposed and catheterized for subsequent blood sampling/administration of 500 μL of the respective emulsion or placebo solution. The catheter was passed subcutaneously and exteriorized through a small incision at the neck of the animal and affixed to a plastic pedestal (Plastics One, Roanoke, VA) mounted inside a harness system.

Subsequently, the common carotid arteries (CCA), internal carotid artery (ICA), and the external carotid artery (ECA) were exposed, and the vagal nerve carefully preserved. Then, the ECA was transiently clipped and the CCA was ligated at a proximal site. Directly before insertion of the catheter into the ICA, baseline values of CBF given as BPU were measured. A commercial silicon coated monofilament (Doccol Corporation, USA) was subsequently introduced from the lumen of the distal CCA at the bifurcation until an immediate drop in CBF baseline occurred. After 1 h of transient occlusion, CBF was restored (reperfusion phase) by removing the monofilament. The sham-operated animals underwent the same anesthesia and surgical procedures with the exception of MCA occlusion.

2.3. PUFA n3 substitution

Rats subjected to tMCAO were randomly assigned to receive 500 μL of PUFA n3, Lipofundin MCT (Lipo MCT) or NaCl (duration of each application 3 min). Lipo MCT served as placebo control, since this emulsion contains a comparable amount of triglycerides compared to PUFA n3 without substituted omega 3 fatty acids. All oil/NaCl emulsions were intravenously applied in the deeply anesthetized animals through a permanent ipsi-lateral jugular vein catheter immediately after 1 h occlusion and 12 h later. To avoid coagulation of the catheter tip within the jugular vein, the tube was flooded with highly diluted heparin (1:1000 in physiological NaCl). Blood sampling was always performed immediately before the application of oil emulsions and before sacrificing the animals. The extracted volume for blood analysis (fatty acids) was replaced by the same volume of physiological NaCl at body temperature immediately after blood sampling. All used solutions were warmed up to body temperature (38 °C) before application. All emulsions were 20% oil-in-water emulsions. The emulsions therefore contain 200 g glycerides (oil) per L.

The following oil emulsions were used [conc. per L]:

	Lipo MCT	PUFA n3
Soybean oil (LCT)	100 g	–
MCT	100 g	20 g
PUFA n3		180 g
DHA	–	Approx. 43 g
EPA	–	Approx. 65 g

LCT, long chain triglycerides; MCT, medium chain triglycerides, eicosapentanaenoic acid (EPA), docosahexaenoic acid (DHA).

By applying two times 500 μL per rat (300 g) post-stroke, each animal received a dosage of approx. 140 mg/day/kg (DHA) and 220 mg/day/kg (EPA).

2.4. Analysis of fatty acids in blood plasma

Blood samples (500 μL) were taken initially after 24 h from the tMCAO-, PUFA n3- and Lipo MCT-substituted groups before the animals were sacrificed through the permanent jugular vein catheter which was flushed before with a small volume (100 μL) physiological NaCl. Fatty acid analysis was commercially performed by Lipidomix GmbH (Berlin, Germany, www.lipidomix.de). The analysis of all fatty acids in the