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THE INFLUENCE OF INSULIN-LIKE GROWTH FACTOR-1 AND ENDOTHELIN-1 ON INFLAMMATORY CYTOKINES SECRETION FROM GLIAL CELLS

VPLIV INZULINU PODOBNEGA RASTNEGA DEJAVNIKA-1 IN ENDOTELINA-1 NA IZLOČANJE VNETNIH CITOKINOV IZ CELIC GLIJE

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Statement

I declare, that I conducted my work to the best of my knowledge and carried out my master’s thesis work independently under the mentorship of Prof. Dr. Mojca Kržan, dr. med. and co-mentorship of Prof. Dr. Ron Kooijman.

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ABSTRACT

Insulin-like growth factor-1 exerts neuroprotective effects in various experimental models of ischemic stroke. Efficient treatments, that would benefit the majority of stroke patients, are lacking. Insulin-like growth factor-1 could be a good treatment option, due to its ability to interfere with many pathways in the ischemic cascade. Endothelin-1, a strong vasoconstrictor, is believed to be released from reactive astrocytes in multiple sclerosis patients and responsible for a reduction in cerebral blood flow, which may contribute to the pathology of multiple sclerosis. The endothelin-1 receptor antagonist bosentan increased the perfusion in the brain, so reduction of endothelin-1 levels in the brain could be beneficial for patients.

The purpose of our study was to determine, whether insulin-like growth factor-1 modulates cytokine production by direct effects on microglia or astrocytes during neuroinflammation. Additionally, we wanted to find out, which side effects endothelin receptor antagonists may have on the neuroinflammation, by studying the effect of endothelin-1 on cytokine production. For our research we used the commercially available murine BV2 microglia and 1321N1 human astrocytic cell line. Western blot assays were used to detect functional IGF-1 receptors by assessing the phosphorylation of Akt and Erk kinases. The cells were exposed to a pro-inflammatory stimulus and treated with insulin-like growth factor-1, or they were pre-treated with endothelin-1, and later exposed to a pro-inflammatory stimulus. Cytokines released in the supernatant were measured by enzyme linked immunosorbent assay (ELISA), and the effects on the mRNA levels were assessed with real time quantitative polymerase chain reaction. Microglial BV2 cells express functional IGF-1 receptors signaling through the MAPK-pathway. After 5-hour incubation time with BV2 cells, insulin-like growth factor-1 significantly increased TNF-α production, when exposed to lipopolysaccharide, and significantly reduced TNF-α release, when exposed to interleukin-1β. After 24 h of pre-incubation with endothelin-1, BV2 cells secreted significantly less TNF-α in response to interleukin-1β. We conclude, that insulin-like growth factor-1 modulates cytokine production and its pro- or anti-inflammatory effects depend on the type of stimulation. Therefore, additional studies should be done preferably in ischemic environment. Our results conclude, that endothelin-1 shows anti-inflammatory effect on neuroinflammation, but its cytotoxicity remains to be determined.

KEY WORDS: Insulin-like growth factor-1; Endothelin-1; Cytokines; Microglia; Astrocytes

Mikroglija so rezidentni makrogafi centralnega živčnega sistem in so odgovorni za imunsko obrambo možganov. Njihova posebnost je hitra aktivacija v primeru patoloških sprememb ali poškodb v centralnem živčevju, kot so možgansko vnetje, ishemija, možganski tumorji ali nevrodegenerativnost. Ker so te celice prve, ki se odzovejo na možgansko poškodbo, so privlačile veliko pozornosti v predkliničnih študijah in predstavljajo zelo pomembno tarčo za nove terapevtske pristope k preprečevanju možganske poškodbe. S fagocitiranjem tujih celic in ostankov celic ohranjajo ravnovesje v možganih. Aktivirana mikroglija izloča vnetne citokine in prispeva k vnetju, izloča pa tudi nevroprotektive faktorje in protivnetne citokine ter tako spodbuja obnovo tkiva. Aktivacija mikroglije pripomore k fagocitozi nekrotičnih in apoptotičnih nevronov ter spodbuja nevrogenezo.
Astrociti so najštevilčnejši predstavniki nevroglije in so odgovorni za vzdrževanje intaktnosti krvno-možganske pregrade. Poleg tega so odgovorni za metabolično preskrbo nevronov in urejanje pretoka krvi v možgane. Poleg teh fizioloških vlog pa so astrociti vpleteni tudi v patološke procese. Išemija povzroči tako imenovano reaktivno gliozo. Reaktivni astrociti pripomorejo k možganskemu vnetju z izločanjem citokinov, kot so TNF-α, IL-1β, IL-6.


Namen magistrske naloge je ovrednotiti terapevtsko uporabo inzulinu-podobnega rastnega dejavnika-1 v primeru možganske kapi in učinke modulacije endotelina-1 v primeru multiple skleroze. Za našo raziskavo smo uporabili komercialno dostopno nesmrtno mišjo.
celično linijo BV2 in humano astrocitno celično linijo 1321N1. Želeli smo ugotoviti, če inzulinu-podoben rastni dejavnik-1 vpliva na izločanje citokinov z neposrednim učinkom na mikroglijo in astrocite med možganskim vnetjem. Poleg tega smo želeli ugotoviti, kakšne neželene učinke bi antagonist receptorja za endotelin-1 imel na možgansko vnetje v primeru multiple skleroze. Merili smo vpliv endotelina na izločanje citokinov iz aktiviranih celic. Celice smo nasadili na ploščo z 12 (1321N1) ali 24 (BV2) vdolbinicami in jih inkubirali čez noč pri 37 °C, 5 % CO₂ in 95 % zraka. V primeru IGF-1 smo jih naslednji dan stimulirali s citokini za 6 ur ali z lipopolisaharidom za 5 ur. Nato smo jim zamenjali raztopine in jih tretirali z IGF-1 ali 24 ur. S prenosom po Westernu smo najprej preverili funkcionalnost kinaz Akt in Erk v celicah BV2. V primeru endotelina-1 smo celice najprej inkubirali z endotelinom-1 za 24 ur, nakar smo jih stimulirali s citokini skupaj z endotelinom-1 nadaljnje 6 ur. Za določanje koncentracije citokinov TNF-α, IL-1β in IL10 v supernatantu smo uporabili encimsko imunoadsorpcijski test (ELISA). Poleg tega smo izmerili izražanje genov za TNF-α, IL-1β, IL10, TGF-β in iNOS na transkripcijski ravni s kvantitativno reakcijo verižnega pomnoževanja s polimerazo v realnem času. Naši rezultati so pokazali funkcionalnost kinaz Erk v celičah BV2 in potrdili delovanje receptorjev IGF-1 na celičah mikroglije. Ugotovili smo, da je IGF-1 po 5-urni inkubaciji značilno povečal raven sproščenega TNF-α v supernatantu celic BV2, ko so bile te stimulirane z lipopolisaharidom, in značilno znižal koncentracijo sproščenega TNF-α, ko so bile te stimulirane z interferkinom-1β. Po 24-urni predtretaciji je endotelin-1 značilno znižal koncentracijo TNF-α v supernatantu z IL-1β stimuliranih celičah BV2. Zaključimo lahko, da IGF-1 učinkuje neposredno na mikroglijo, saj mikroglija izraža aktivne receptorje Erk, katerih aktivacija je ključnega pomena za učinek antiapoptotičnega IGF-1. Ali ima IGF-1 vnetni ali protivnetni učinek na mikroglijo, je odvisno od načina aktivacije. V prihodnje predlagamo, da bi tiskaverni bili izvedeni v ishemičnem okolju. Za endotelin-1 predvidevamo, da ima protivnetni vpliv na izločanje TNF-α iz celic aktivirane mikroglije, vendar zaradi visokih vrednosti laktat dehidrogenaze tega še ne moremo potrditi. Če se protivnetni učinek endotelina-1 potrdi, potem bi to lahko pomenilo, da bi uporaba antagonist receptorja za endotelin-1 lahko s povečanim pretokom krvi skozi možgane koristila pacientom z multiplo sklerozo, vendar obenem prispevala k možganskemu vnetju.

**Ključne besede:** Inzulinu-podoben rastni dejavnik-1; Endotelin-1; Citokini; Mikroglija; Astrociti
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GNDF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>Iba-1</td>
<td>Ionized calcium binding adaptor molecule-1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor-binding proteins</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteases</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rt-PA</td>
<td>Recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>Sdha</td>
<td>Succinate dehydrogenase complex flavoprotein subunit A</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</tbody>
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1  INTRODUCTION

1.1 Stroke

Stroke is one of the leading causes of mortality and disability globally (1). With an ever-aging society, stroke has a strong negative socio-economic impact (2). Stroke is a cerebrovascular event, caused by a reduction in blood flow to the brain (1). According to American Heart Association, 87% of strokes are ischemic, caused by a sudden occlusion of a blood vessel supplying the brain by a thrombus or embolus (3,4). This leads to direct loss of oxygen and glucose supply to the cerebral tissue (3). The remaining 13% of strokes are caused by a hemorrhage of a blood vessel (intracerebral or subarachnoid) (4).

After ischemic stroke, depending on the level of the remaining blood supply, there are two different regions of damage. The core of the insult, with a very low level of blood supply and the area surrounding the core, called penumbra. In the core of the infarct, blood flow is reduced to < 10 to 25% of the normal blood supply (5). This leads to almost complete energy failure and necrosis, which happens within minutes (1). The surrounding penumbra is hypoperfused, with more blood supply coming from adjoining arteries. As a result, this part of brain can still be saved, if reperfusion is restored quickly (2, 6). Since penumbra can stay viable for 16-48 hours after stroke (7), rescuing this brain tissue presents an extremely important therapeutic approach for neuroprotective therapy and it is an opportunity to reduce post-stroke disability (3). This finding is the basis for thrombolytic therapy of stroke (5). At the moment, reperfusion using dissolving thrombus by recombinant tissue plasminogen activator (rt-PA) is still the only available drug for treatment of the ischemic stroke. Regaining perfusion of the brain is an obvious solution to the decrease in cerebral blood flow. But not a lot of patients benefit of this treatment option, due to its limited time window for treatment and many contraindications for thrombolysis (1,8,9). Thrombolytic therapy with rt-PA needs to be administered within 4,5 hours after stroke onset at the latest (2). This short time window, and the fact that in cases of severe stroke, rt-PA administration does not lead to significant improvement (which could be because of reperfusion injury), we are looking for new treatment options, that could help more people (1). In addition, additive treatments combined with rt-PA could also be desirable (1).
1.1.1 The ischemic cascade

a) Early events

The ischemic cascade starts within minutes after the loss of blood flow to a region of the brain, which includes biochemical events leading to a breakdown of cell membranes and neuronal death in the core of the insult (3). Decreased cerebral blood flow disturbs the ionic homeostasis due to a loss of energy production, which leads to increased glutamate release and intracellular calcium concentrations causing excitotoxicity (6). Overload in glutamate release and neuronal death in the core of the insult (3). Decreased cerebral blood flow disturbs the ionic homeostasis due to a loss of energy production, which leads to increased glutamate release and intracellular calcium concentrations causing excitotoxicity (6). Overload in glutamate release, the main excitotoxic neurotransmitter (1), is not compensated by astrocytes, due to compromised glutamate uptake by astrocytes. Increased levels of extracellular glutamate evoke the overactivation of ionotropic receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) and subsequent augmentation of calcium channels opening (1). High concentrations of calcium trigger intracellular phospholipases and proteases, which causes degradation of membranes and proteins (10). Water relocates from extracellular to the intracellular space due to the osmotic gradient. Additionally, glutamate receptors facilitate sodium and water influx, which leads to cell swelling and edema (10). Ischemic cells high in calcium, sodium and adenosine diphosphate (ADP) can stimulate mitochondrial oxygen radical production, causing oxidative stress (1). Reactive oxygen species (ROS) can impair lipids, proteins, nucleic acids, especially if reperfusion occurs (1, 2, 5). Targeting early events in the ischemic cascade presents a big challenge, because of the difficulty of reaching stroke patients in time for acute therapy (1).

b) Late events including inflammation

Later events start a few hours to a few days after initial ischemia and include apoptosis and neuroinflammation. They consist of microglial activation, release of cytokines, chemokines and the infiltration of peripheral leukocytes (5). These events exacerbate the starting ischemic damage, they continue several hours to days after stroke and are responsible for most of the penumbral tissue death (5). Later events offer a wide window for neuroprotection (2, 5). In the ischemic core, cells die mostly because of necrosis, while in the surrounding penumbra, cells undergo delayed death by apoptosis (5). Neuroinflammation plays a fundamentally important role in the pathological events of an ischemic stroke. Reducing the neuroinflammation is beneficial and reduces the cerebral ischemic damage (6). Even though, it may seem contradictory, ischemia-reperfusion may
aggravate neuronal injury by generating ROS and activating inflammatory response. At the same time, in order to save neurons in the penumbra, reperfusion is of utmost importance (2). Additionally, blood-brain barrier integrity might be compromised and neuronal death by necrosis and apoptosis may still be in action (1). Cells that secrete cytokines are activated microglia, astrocytes, endothelial cells and neurons (5). After a few days to weeks after cerebral ischemia, peripheral immune response cells produce and secrete pro-inflammatory cytokines, which contribute to cerebral inflammation (11). In later events of ischemic cascade, high amounts of chemokines are produced, which leads to peripheral leukocyte infiltration and augmentation of the blood-brain barrier (BBB) permeability (11). Infiltrated neutrophils produce free radicals and matrix metalloproteases (MMPs), which exacerbates cerebral damage and brain perfusion (12).

Targeting late events, like neuroinflammation could lead to effective treatment, but in clinical trials, anti-inflammatory drugs did not show successful results (13). Which opposed a hypothesis, that neuroinflammatory response after stroke is not just deleterious, but is also important for the later endogenous repair of the brain (2, 5). At the moment, it is believed that components of the ischemic cascade have detrimental and neuroprotective roles at different time points after stroke onset.

1.1.2 Microglia and their role in ischemic stroke

Microglia is known to be the resident macrophages of the central nervous system (CNS) and they are responsible for the brain’s innate immune defense (14). Their distinctive attribute is fast microglial activation due to pathological changes or injuries in the CNS, like inflammation, trauma, ischemia, brain tumors or neurodegeneration (14). Beside their ability to eliminate cell debris by phagocytosis, activated microglia promote tissue repair by secreting growth factors (1, 14). Being the first responders in brain injury, these cells attracted a lot of attention in pre-clinical studies. Microglia represent an important target for new therapeutic approaches to prevent stroke damage (14, 15). Almost all pathological reactions in the brain include glial cells (16). Activated microglia are inflammatory mediators of many neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease, Alzheimer's disease (17, 18). In the healthy adult brain, microglia constantly maintain the healthy brain (19) and contribute to synapse remodeling and neurogenesis (20). In contrast, microglial activation, due to secreted inflammatory cytokines and
inducible nitric oxide synthase (iNOS), leads to neuroinflammation and tissue damage (21).

Inactivated microglial cells consist of a small cell body called soma, with ramified branches, extremely sensitive to any sort of change in the brain homeostasis (2, 22). The cells are activated within minutes after acute ischemic stroke onset and are immediately detectable in the core and penumbra. Upon activation, microglia undergo morphological changes and become amoeboid, round shaped, which is a characteristic of a macrophage (1). Their numbers peak at day 2-3 after stroke onset and can last up to several weeks (1). Activated microglia can be divided into two phenotypes and therefore play a dual role in the ischemic injury (23). Phenotype M1 is identified as inflammatory and deleterious microglia. This phenotype releases proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL) -1β, ROS via NADPH oxidase, nitric oxide (NO) and also neurovascular proteases such as matrix metalloproteinase (MMP-9). Phenotype M2 is called anti-inflammatory, neuroprotective microglia (1, 23). They produce less NO and release anti-inflammatory cytokines like interleukin (IL) -10 and neurotrophic factors like glial cell line-derived neurotrophic factor (GNDF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) (1, 23). Whether microglial activation is harmful due to releasing inflammatory factors or is in fact beneficial is a question, that has been discussed for a long time (24). After ischemic injury, it is believed, that activated microglia converts from temporary neuroprotective M2 in the acute phase of ischemia to detrimental M1 polarization in the latter phase (23). While M1 contribute to neuron loss by ROS production, M2 upregulates anti-oxidative mechanisms (ROS reduction, production of glutathione). By phagocytizing necrotic and apoptotic neurons, they make space for new neurons and therefore support and maintain survival of neurons (23). Through production of neurotrophic mediators IGF-1 and TGF-β, activated microglia also promote neurogenesis, neuron stem cells being responsible for tissue repair (23). Focusing on promoting M2 microglial polarization presents an important therapeutic approach to prevent neuronal loss after the ischemic injury. There is evidence, that selective removal of proliferating resident microglia, results in a significant increase in infarct size, increase in apoptotic cells, mostly neurons and a decrease in IGF-1 levels (25). This proves a neuroprotective potential of activated microglia, as well as IGF-1’s importance as a neurotrophic molecule (25). The conflicting effect of activated microglia...
on stroke outcome can be due to the time and the degree of cytokine expression (1).
Microglia are activated via CD14, followed by toll-like receptor 4 (TLR4) stimulation (21).
Activated microglia are detected by the expression of ionized calcium binding adaptor
molecule-1 (Iba-1). This is one of the most useful proteins to differentiate microglia by
immunohistochemistry (26).

1.1.3 Astrocytes and astrogliosis in ischemic stroke

Astrocytes are the most abundant cell type in the CNS (27) and "astrocytes are the
principal housekeeping cells of the nervous system" (28). These cells are star-shaped glial
cells in the brain and spinal cord. Their supportive role is the maintenance of the blood-
brain barrier integrity. Furthermore, they are responsible for brain homeostasis, including
ion and water homeostasis. Astrocytes are also responsible for scavenging transmitters,
that are released during synaptic activity and for releasing neurotrophic factors (27). Nerve
growth factor (NGF), bFGF, BDNF, TGF-β are released under normal circumstances and
they influence neuronal survival (27). Increased expression of these growth factors in
reactive astrocytes can stimulate neurite outgrowth and neurogenesis (29). They express
neurotransmitter receptors and their response to neural activity is characterized by an
increase in cytosolic Ca²⁺ (30). Astrocytes are responsible for the metabolic support of
neurons (28), regulating cerebral blood supply to meet neuronal glucose and oxygen
requirement (31). Their importance is intensified by the fact, that glutamate cannot be
produced by neurons. This is due to the lack of mitochondrial enzyme glutamine
dehydrogenase (28). Since glutamate doesn’t pass the BBB, excitatory transmission
remains up to glutamate produced by astrocytes (32). Although it is not yet clear, how
ischemia affects astrocytes, it is possible that the loss of supportive astrocyte functions
(failure of glutamate uptake in the ischemic penumbra, vascular control, water
homeostasis), contribute to excessive neuronal loss during ischemia. Therefore, neuron
survival can depend on surrounding astrocytes (28). Astrocyte changes are one of the first
changes in the brain after an ischemic attack. In the core of the insult the astrocytes are
necrotic, while they are still viable in penumbra, but they quickly undergo astrocytosis
(33).
**Figure 1:** Astrocytes in healthy tissue, in moderate and severe astrogliosis.

When astrocytes become activated following an ischemic injury, they express an increased intermediate filament glial fibrillary acidic protein (GFAP) and they undergo "reactive astrogliosis". This astrocyte activation consists of many structural and functional characteristics and it is a response to many CNS pathologies, as infection, trauma, neurodegenerative disease and ischemia, which in severe cases can progress to a scar formation (35). Astrocytes contribute to brain inflammation by expressing major histocompatibility complex (MHC), secretion of inflammatory factors, like cytokines, chemokines and iNOS (21). iNOS astrocytic expression is traceable within several hours after stroke, and peaks by day 2 or 3 (36). Astrocytes, that are stimulated by ischemic insult, can produce TNF-α and TNFβ, IL-1, -6, -10 and interferons (37). While IL-6 and TNF-α have been reported to promote demyelination, leukocyte infiltration and BBB disruption (37), they also show protective effects in ischemic and excitotoxic injury. Cytokines have pleiotropic cell-type specific properties (27). An important fact is, that there are clear differences in complexity of astrocytes, human astrocytes being larger and more complex than astrocytes in rodents (38).

### 1.1.4 Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is a 7,7-kDa single chain polypeptide of 70 amino acids (39). It is a growth-promoting pleiotropic peptide (40), involved in fetal and postnatal development, as well as in controlling tissue homeostasis through regulation of cell proliferation and programmed cell death (9, 41). IGF-1 is in the brain involved in regulation of proliferation of oligodendrocytes, it promotes myelin production in oligodendrocytes, neurite outgrowth and survival of neurons and glial cells (40). IGF-1 also shows metabolic effects, such as stimulating of protein synthesis and inhibition of
protein degradation (41). It is also an important differentiation factor for skeletal muscle and neuronal cells. The majority of IGF-1 is produced in the liver, but it is also produced in non-hepatic tissues (42, 43). Next to endocrine effects of liver-produced IGF-1, this growth factor also exerts autocrine and paracrine effects in different tissues including the CNS (41).

The IGF-1 system exists of three ligands of the insulin-related peptide family (insulin, IGF-1 and IGF-II), their receptors (insulin, IGF-I and IGF-II receptor) and a group of six IGF-I binding proteins (IGFBPs). IGF-I receptor is a glycoprotein on the cell surface. IGF-I binding stimulates intrinsic heterotetrametric receptor tyrosine kinase, constituted of two extracellular α subunits with ligand-binding activity. Ligand binding to the α subunits leads to the activation of the intrinsic tyrosine kinase (colored orange in figure 2) of the two transmembrane β subunits, which hold receptor activity. IGF-1 binding elicits the autophosphorylation of tyrosine residues of the β subunit, which serves to recruit the adaptor molecule insulin-receptor substrate (IRS-1 and -4) (41). This phosphorylation activates the two key IGF-I receptor signaling cascades: the phosphatidylinositol-3 kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) signaling pathway (38, 44). Activation of these two signal transduction cascades induces anti-apoptotic signals by phosphorylation of protein kinase B (Akt) and extracellular signal-regulated kinase (Erk1 and 2), isoforms of the mitogen-activated protein kinase MAPK family. This leads to inhibition of caspase cascade and following apoptosis (41). Downstream targets of Akt are involved in gene expression regulation, protein translation, metabolism, cell cycling, oxidative stress, autophagy and cell survival. P44-42 MAPK is involved in growth, gene transcriptions and proliferation (38, 45).
The IGFBPs have different structures, binding characteristics, localization and functions (41). They bind IGF-1 and IGF-II with a higher affinity than the IGF-I receptor. Therefore, 99% of the circulating IGF-1 is bound to the binding proteins and these binding proteins extend IGFs half-life and regulate the delivery to the tissues. That can result in either IGFBPs preventing IGF binding to the receptors or they could extend the IGF effect by controlling a slow release in the target cells (41). IGF-1 is in the serum mostly found as an IGF-1, IGFBP-3 and acid-labile subunit complex, which presents an important carrier for IGF-1 (46). Proteolytic cleavage of IGFBPs regulates the level of free IGF-1 which is able to bind to IGF-1 receptors (47). It was discovered, that intravenous administration of tPA after acute ischemic stroke resulted in temporary increased free IGF-1 serum levels (70%) after 1-hour infusion. This could be explained by tPa or plasmin causing limited proteolysis of IGFBP-3. The reduced affinity of IGFBP-3 cleavage products for IGF-1 leads to increased concentrations of bioavailable IGF-1 (47). This shows, that a combination therapy of tPA and IGF-1 could be interesting. IGF-1 receptor gene expression is seen on nearly every cell type and has also been detected in many tissues (40). Therefore, IGF-1 binding can stimulate an antiapoptotic effect in many cells (9).

Apoptosis is characterized as a programmed cell death and is responsible for the development and appropriate functioning of multicellular organisms. Through apoptosis,
unwanted or harmful cells are removed (41). The breakdown is very well controlled and not harmful to other cells. The cell content does not leak into the extracellular space, cell fragments called apoptotic bodies are recognized and phagocytized by macrophages. Cells that undergo necrosis, a process, which is not so well regulated, cause an acute inflammatory response (41). Next to necrosis that is happening in the ischemic core, cells in the surrounding penumbra undergo predominantly apoptosis (6, 48), which contributes to the brain injury after ischemic stroke (41). Apoptosis, which occurs hours or days after stroke, is a big reason for neuronal cell death (3, 5, 41). The mechanism of neuroprotection often involves inhibition of apoptosis.

1.1.5 IGF-1 as a potential neuroprotective drug in acute ischemic stroke

PI3K-Akt and Ras-raf-MEK-ERK signaling pathways play important roles in the regulation of apoptosis and through these pathways, IGF-1 exerts its antiapoptotic effect (41). Their synergistic actions were also reported (targeting different antiapoptotic targets) (41). IGF-1 interferes with multiple control points of apoptosis. IGF-1 can modulate the regulation of mitochondrial membrane permeability through antiapoptotic Bcl-2 proteins, whose expression is arranged by Akt. This inhibits both the intrinsic and extrinsic apoptotic pathway (components shown in figure 3) and keeps membranes integrity, by inhibiting its depolarization and the release of cytochrome c (41).

Figure 3: Components of the intrinsic and extrinsic apoptotic pathways. Apoptogenic proteins are marked with purple, while antiapoptogenic are marked with green color (41).
IGF-1 affects the inhibition of the caspases, which are involved in signal transduction and initiation of apoptosis and signaling of death-inducing receptors. Bcl-2 family proteins and caspase inhibitors are the main players in apoptosis regulation (41).

The brain itself promotes endogenous IGF-1 expression after brain injury. But this is detectable only after 24 h while maximum levels are reached after 5 days (49). IGF-1 receptors and binding proteins are highly induced within damaged brain areas (49). As neurons and other cell types die after a couple of hours or days after the insult due to apoptosis, exogenous IGF-1 can reduce the damage, in fact, in both gray and white matter, if administered within a few hours after the insult (49). Intracerebroventricular infusion of IGF-1 showed beneficial effects with decreased infarction rate and neuronal loss in adult rats, where stroke was induced by unilateral hypoperfusion (50). Reduced neuronal loss is a result of inhibition of neuron apoptosis, regulated by IGF-1 (41). IGF-1 also exerts neuroprotective effects in other neurodegenerative diseases. For example, in amyotrophic lateral sclerosis by inhibiting motor neuron apoptosis through the PI3K-Akt pathway (41). Also, in case of Alzheimer disease, IGF-1 has an effect on the prevention of apoptosis in the brain via regulation of apoptosis-inducing pathological factors (41).

A characteristic, that differentiates IGF-1 from other drugs that failed in clinical trials, is the fact that this growth factor targets more pathways in the ischemic cascade (9). IGF-1 protects white and gray brain matter, which is very important for prospective clinical trials, since the white mater in human brain makes up about half of the total brain volume, in comparison to 10 % in rodents (9). *In vitro* tests showed, that IGF-1 prevented glutamate-, nitric oxide- and hydrogen peroxide-induced apoptosis in motor and sensory neurons (51). IGF-1 stimulates *in vitro* proliferation and differentiation of neural and oligodendrocyte progenitors, which means it contributes to the regeneration after ischemic injury (9). It was also reported, that IGF-1 stimulates remyelination, by stimulating myelin expression and its effect on oligodendrocytes (52).

In 2013, department of Pharmacology in Brussels established, that subcutaneous administration of IGF-1 results in a significantly reduced infarct volume in normotensive rats, when administered 30 min after ischemic stroke induction (53). Endothelin-1 was used to induce transient middle cerebral artery occlusion (MCAO) for this study’s rat stroke model (53). Effects of IGF-1 were assessed 24 hours after the insult. IGF-1 didn’t seem to affect the activation of astrocytes (no change in GFAP levels) or modulate the
blood flow in the rat striatum. Apart from that, IGF-1 effects on microglial cells were assessed, since activated microglia have been shown to protect neurons against ischemic damage (53). Microglial activation in the cortex of hypertensive rats was significantly reduced by IGF-1, while no change was observed in normotensive rats (53). Microglia in hypertensive rats show different characteristics, being less prone to activation during neuroinflammation, than microglia in normotensive rats (54). In this study, IGF-1 does not seem to modulate the activation of microglia and astrocytes. Thus, the conclusion is, that IGF-1 neuroprotection is not mediated by activation of microglia (53). In 2016, our group determined that systemically injected IGF-1 exerts its neuroprotective action by binding to central IGF-1 receptors in the brain, after crossing the blood-brain barrier (55). IGF-1 also decreased the reduction of NeuN-positive cells, NeuN being a marker for neurons. IGF-1 may enter the brain parenchyma via opening of the blood-brain barrier as a result of ischemia (53). Pre-treatment with IGF-1 did not show any change in ameliorating brain damage after the insult. This is probably due to limited intracerebral penetration into the uninjured brain (49). However, transportation from serum across the BBB of the majority of IGF-1 in the brain, is believed to also be mediated by IGF-1 binding to megalin/low-density lipoprotein receptor-related protein-2 (LRP2) on the endothelial cell surface (56).

The most recent study in our department, re-opened the discussion whether microglia could mediate neuroprotection by IGF-1 in ischemic stroke (57). Additionally, IGF-1 modulation of pro- and anti-inflammatory factors in microglia was addressed. The group of researchers concludes, that IGF-1 modulates neuroprotection in ischemic stroke mainly by reducing pro-inflammatory factors in the ischemic brain, influencing microglial transition from pro-inflammatory to anti-inflammatory phenotype. They also concluded, that systemic administration of IGF-1 has long-term neuroprotective effects, since they tested IGF-1 neuroprotective effectiveness 72h after ischemic insult. IGF-1 treatment led to a reduced expression of IL-1β and iNOS mRNA in the ischemic hemisphere. This coincided with a decreased expression of Iba-1 (microglial activation marker) and reduced iNOS expression in microglia extracted from the ischemic hemisphere. These discoveries suggest, that microglia are the primary target for IGF-1 in the brain (57).
1.2 Multiple sclerosis

Multiple sclerosis (MS) is the leading cause of inflammatory neurological disability in young population in North America and Europe (58). By now, we know two types of disease: relapsing-remitting MS and primary progressive MS. The first type is typical for majority of MS patients. It is characterized as alternating occurrence of neurological disability and recovery, that eventually changes to a secondary-progressive course (58, 59). The second type shows a fast decline in neurological functions and with no signs of recovery in-between (58). MS is a chronic inflammatory disease of the CNS, characterized by inflammatory demyelinating lesions and axonal degeneration occurring throughout the white matter. The latter represents the main determining factor for progressing disability in MS (60, 61, 62). Auto-immune response has an important role in the development of demyelinating lesions. This is because of T-cell mediated inflammatory reaction towards myelin in the CNS. Conventionally, it was considered as an auto-immune disease, but the exact pathology is not yet completely known (63, 64). Some inflammatory events are however known. Lymphocytes and microglial activity were detected in the area of MS lesions. An increase in production of neurotoxic factor prostaglandin E2 (PGE2) and NO (17) can be detected in this area, due to enhanced cyclooxygenase 2 (65) and inducible nitric oxide synthase (iNOS) expression (66) by microglia. Axonal injury is said to start at disease onset and corresponds with the inflammation in the lesions. It is believed, that inflammatory demyelination affects axon degeneration during relapsing-remitting MS (62). Demyelination and inflammation are indicators of active MS lesions, apart from reactive astrogliosis, phagocytic activity and oligodendroglial loss (62). Non-functional astrocytes in MS could cause axonal degeneration. By compromising the astrocytic sodium potassium pump, this could lead to decreased sodium-dependent glutamate uptake. White matter astrocytes are also believed to be insufficient in β2 adrenergic receptors (61). They participate in stimulating glycogenolysis and suppressing inducible nitric oxide synthase (NOS2) (61). Increased nitric oxide, glutamate toxicity may lead to axonal mitochondrial metabolism impairment, which could cause axonal degeneration (61).

1.2.1 Cerebral hypoperfusion in MS

There are many hypotheses on the etiology of MS. In this master’s thesis, we focused on one possible cause of the disease.
Cerebral blood flow (CBF) is characterized as the volume of blood, that distributes through a given volume of brain parenchyma per unit of time (67). It is believed, that a reduced CBF contributes to the pathology of MS. It is impaired in cases of early diagnosed relapsing-remitting MS as well as in primary progressive MS (68). The exact mechanism of reduced CBF is not known. The cerebral hypoperfusion could be mediated by high levels of cerebral circulating vasoconstrictive peptide endothelin-1. This hypoperfusion in MS patients could be linked with chronic hypoxia, focal lesion formation, diffuse axonal degeneration, cognitive dysfunction, neuroinflammation and fatigue (63). Axonal degeneration with decreased metabolic demands does not seem to be the cause for cerebral hypoperfusion. In fact, hypoperfusion of the brain can lead to neurogenerative changes, which also include axonal degeneration (63, 68). White matter is reported to be more susceptible to chronic hypoperfusion than gray matter, due to axonal and myelin component involvement (69). Restoring CBF could be a new therapeutic target in treating MS (63).

### 1.2.2 Endothelin-1

Endothelins (ET) are 21-amino acid cyclic peptides. The endothelin family includes three isoforms, ET-1, -2, and -3 (70). ET-1 was the first discovered in primary culture of porcine aortic endothelial cells and recognized as a very powerful endogenous vasoconstrictor (71). ET-1 is the only isoform, that is released from blood vessel endothelial cells (70). ETs exert their actions via ETA and ETB receptors. They are both subtypes of seven-transmembrane G-protein coupled receptors. Amino acid sequences of both receptors vary between humans and other species (70). ETA can be found on vascular smooth muscle cells, mainly of brain vessels (70). They mediate vasoconstriction, which is caused by the increase in the cytosolic Ca²⁺ (72). ETB receptors are found on endothelial cells. They mediate vasodilation via PI3K/Akt pathway, producing NO that exerts relaxation of the vascular smooth muscle cells (70). ETB outweight the ETA receptors (70). ET-1 immunostaining in the normal CNS has been found in some neurons, epithelial cells of the choroid plexus and endothelial cells of the microvasculature (73). ET-1 expression is normally not traceable in glial cells (74). Astrocytes in the normal CNS express only a very low ETA and ETB receptor detection (75). However, in different pathological conditions, reactive astocytes express high levels of ET-1 and mostly ETB receptors (76).
1.2.3 Enhanced astrocytic ET-1 expression and its role in MS

When resting astrocytes transform to reactive phenotype due to different elicitor, they are characterized by hypertrophy and overexpression of GFAP (77). One of the most important harmful effects of the ET-1 released from reactive astrocytes (shown in figure 4), is the strong vasoconstrictor activity (78). When released from astrocytic end-feet surrounding blood vessels, it can impact the cerebral microvasculature, which can lead to acute or chronic, focal or global cerebral hypoperfusion (70). Chronic cerebral hypoperfusion caused in rats has shown, that it causes oxidative stress, consequently brain energy failure, leading to neuronal death. It is assumed, that mitochondrial failure, due to hypoperfusion, is accountable for reactive oxygen species production. And this leads to oxidative stress (79). Additionally, it is also accountable for myelin breakdown, apoptosis of oligodendrocytes, axonal degeneration, inflammatory reactions and astrogliosis (80).

**Figure 4:** Overexpression of ET-1 in astrocytes. This leads to activation of ETA receptors, causing constriction of arteriolar myocytes. Production of matrix metalloproteinase, VEGF and reduction in astrocytic aquaporin-4 water channels directs an elevated permeability of BBB. Inflammation is caused due to chemokines and cytokines and excitotoxicity due to decreased expression of astrocytic glutamate transporter EAAT-2. Potential beneficial effects exist because of BDNF, GNDF and neurotrophin-3 production (70).

Higher levels of endothelin-1 were discovered in cerebrospinal fluid of relapsing remitting MS patients, as well as elevated levels of NO in MS patients in a stable and acute phase (81). Study at UZ Brussel in 2013 showed, that the excessive ET-1 seems to be released to the cerebral circulation from the brain (60). Jugular/peripheral vein ET-1 ratio was
increased in MS patients (1.4) in comparison to the controls (1.0). Jugular vein takes the deoxygenated blood from the brain back to the heart and presents the main pathway of venous outflow from the brain (60). CBF values, were generally reduced by 20 % in MS patients in comparison to the control group. They were measured with noninvasive arterial spin labeling. CBF in MS patients improved after administrating the ET-1 receptor antagonist bosentan, in fact, the values were comparable to healthy controls. Additionally, ET-1 immunohistochemistry on postmortem white matter brain MS cases located high ET-1 immunostaining in reactive astrocytes in MS plaques. Plaques were characterized by demyelination, reactive astrocytes, encompassed by phagocytic macrophages (60). ET-1 that is produced from reactive astrocytes can evoke long-lasting vasoconstriction, by reaching intracerebral arterioles. Normal astrocytes don’t show ET-1 immunoreactivity (82), which was confirmed in the study at UZ Brussel as well (60).

Reduced CBF in MS seems to be a crucial part of the disease, regardless of its course (relapsing or progressive) (60). It is reported, that ET-1 levels are increased in reactive astrocytes in numerous brain pathologies, for example acute ischemic stroke, Alzheimer's disease and viral infections (70).
2 RESEARCH AIM

The general purpose of the *in vitro* research is to address the mechanisms of neuroprotective action for stroke and multiple sclerosis treatments. We will address the therapeutic use of the insulin-like growth factor-1 (IGF-1) system in ischemic stroke and the effects of endothelin-1 (ET-1) modulation in MS.

1.) Neuroprotection by IGF-1 *in vivo*

Augmentation of local IGF-1 levels in the brain leads to neuroprotection in stroke and several disorders of the CNS. We aim to find out the mechanism, how glial cells respond to the neuroprotective agent IGF-1. We hypothesize, that IGF-1 can modulate the pro- and anti-inflammatory cytokine production by direct effects on microglia or astrocytes during neuroinflammation. We will first investigate whether direct effects of IGF-1 on microglia are possible by testing functionality of IGF-1 receptors on cultured microglia. Taken together, we hypothesize, that microglia modulate neuroinflammation through functional IGF-1 receptors.

2.) Neuroprotection by antagonist of ET-1 receptor in *vivo*

Since blocking the effect of ET-1 by using endothelin receptor antagonist bosentan in multiple sclerosis patients increases the perfusion of the brain, reduction of ET-1 levels in the brain could represent a treatment option for these patients. In order to predict possible side effects of the reduction in ET-1 levels via neuroinflammation, we will measure the *in vitro* effects of ET-1 on the secretion of cytokines. We hypothesize, that ET-1 increases the secretion of pro-inflammatory cytokines.

We will culture microglial murine BV2 cells and human 1321N1 astrocytes, which will be after pretreatment with cytokines exposed to IGF-1 or ET-1. Functionality of Akt and Erk kinases in BV2 cells will be assessed by immunoblotting using phospho-specific antibodies against phosphorylated kinases. Inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokine levels in the supernatant of these cell cultures will be detected by enzyme-linked immunosorbent assay (ELISA). Effects at the mRNA level for cytokines TNF-α, IL-1β, IL10, TGF-β expression and iNOS will be assessed using RT-qPCR.
3 MATERIALS AND METHODS

3.1 Materials

Table I: Laboratory equipment

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<th>Type</th>
<th>Company</th>
</tr>
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<td>CELLSTAR® Cell Culture Flasks</td>
<td>Sterile with a red standard cap 250 mL</td>
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<td>CELLSTAR® Cell Culture Plate</td>
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<td>Greiner Bio-One</td>
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<td>Cell culture multi-well Plate</td>
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<td>Thermo Fisher Scientific</td>
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<td>CELLSTAR® tubes</td>
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<td>Optical Adhesive Covers</td>
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### Table II: Chemicals and media

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**Table III: Prepared solutions**

| PBS – Phosphate buffered saline (20x) | 137 mM NaCl, 2,7 mM KCl, 10 mM Na2HPO4x2H2O, 1,8 mM KH2PO4, [pH 7,4] |
| Wash buffer | PBS 20x, 0,1 % Tween 20, dH2O |
| Blocking solution for Western blot | 5 % milk powder in wash buffer |
| Stop solution 1M H2SO4 for ELISA | 5,52 mL H2SO4 (96,5 %) in 100 mL H2O |
| Electrophoresis buffer 10x | Tris-base 25 mM, SDS 0,1 %, Glycine 192 mM, ddH2O |
| Sample buffer | Tris-HCl (1 M, pH 6,8), Glycerol, SDS, Mercaptoethanol, Bromophenol Blue, ddH2O |
| Transfer buffer | Tris 25 mM, Glycerine 192 mM, Methanol 20 %, ddH2O |

**3.2 Cell lines**

For our *in vitro* research we used BV2 cell line, derived from neonatal murine microglia, which was immortalized after infection with a v-raf/v-myc recombinant retrovirus. It is the most common cell-line used as a substitution for primary microglia cultures (83). These cells grow semiadherently, loosely attached and within suspension. The BV2 cells are attached on the bottom of the cell culture dish, usually in clusters, and some are floating in the medium, normally single cells not clusters. Cell lines were a gift, BV2 cell line came from IRCCS AOU San Marino – IST as passage 6 and 18 and they were not combined.
Different results were discovered on BV2 cell lines depending on the passage number, and our department continued using both of them separately for experiments.

1321N1 is a human astrocytoma cell line, isolated as a sub clone of the cell line 1181N1, which was derived from the parent line U-118 MG, isolated from a human malignant glioma (ECACC 86030402). These cells grow as adherent in the culture. 1321N1 cell line came from Dr. Müller (University of Bonn).

![Image](image_url)

**Figure 5**: BV2 p18 (A) and 1321N1 cell line (B). After 24-hour incubation time at 37 ºC, 5 % CO₂ and 95 % air, following cell passaging. Pictures were taken with Axioskop 40 microscope (Zeiss).

### 3.3 Experimental methods

#### 3.3.1 Cell passaging

Cell passaging is a splitting technique, that allows cells to have more space to grow and to survive under cultured conditions for a longer period of time. The right time to perform cell passaging is when the cells are 90-100 % confluent. Protocols are different for BV2 and 1321N1 cell line, due to their growth mode and rate of growth. All cell processes were performed under the laminar flow in sterile environment.

Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with glutamax with 10 % heat inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.75 μg/mL of amphotericin B. While medium, PBS and trypsin were being heated in a water bath at 37 ºC, the cells were checked under the microscope to confirm 90-100 % confluence.

The medium from the semi-adherent BV2 cell line flasks, was transferred to a 50 mL cell culture Falcontube. Trypsin/EDTA 0.05 % solution with PBS was added (7 mL for 250 mL flasks), so the adherent cells could detach. The flasks were incubated at 37 ºC, 5 % CO₂
and 95 % air for 5 min. To detach all the cells from the wall of the flasks, the cells were being mixed by pipetting up and down. After that, they were added to a Falcontube containing medium and centrifuged for 10 min at 1400 rpm. The supernatant was removed, the cell pellet was dissolved in 1 mL cell culture medium. Cell solution was diluted 1/5 with cell culture medium and incubated at 37 °C, 5 % CO₂ and 95 % air for further cell proliferation. The total amount of medium with cells put into the incubator was always 25 mL. The medium from the 1321N1 flasks was removed with a vacuum pump. These cells are only adherent, therefore only dead cells were removed with the pump. While flasks with trypsin/EDTA were in the incubator at 37 °C, 5 % CO₂ and 95 % air for 5 min, an excess volume of medium was added to a Falcontube (50 mL). Trypsin-cell solution was transferred to a Falcontube with medium and centrifuged for 10 min at 1400 rpm. Supernatant was removed with a vacuum pump and the pellet was resuspended in 1 mL of medium.

The desired number of cells was either put on the plate for an experiment (counted in a Bürker counting chamber, described later) or added to the marked passage flasks with fresh medium and then incubated at 37 °C, 5 % CO₂ and 95 % air for further cell proliferation.

3.3.2 Cell freezing

After cell counting and centrifugation of the cell suspension for 10 min at 1400 rpm, the supernatant was carefully removed and 1 mL of ice cold, freshly prepared mixture of 10 % DMSO in FBS per 1 million cells was added to resuspend the cell pellet. 1 mL with 1 million cells was transferred to each cryovial and stored in a container with liquid isopropanol at -80 °C overnight. The next day the cryovials were put into liquid nitrogen container.

3.3.3 Cell thawing

The process of cell thawing was preferably as short as possible. The cryovials were removed from the liquid nitrogen and heated in a water bath at 37 °C. They were then transferred to pre-labeled flasks, already filled with fresh warm medium (24 mL). The flasks were put into the incubator for 24 hours at 37 °C and 5 % CO₂. The next day we checked the cells under the microscope, to see if they were attached to the flask and the medium had to be replaced to remove the DMSO.
3.3.4 Cell counting

Experiments were performed in a 12- or 24-well plate. We needed 1 million cells per well. In order to have a proper number of cells per well, they need to be counted and appropriately divided on the plate. After the cells were detached and centrifugated, the supernatant was carefully removed and 1 mL of fresh warm medium was added. A small amount of sample (10 μL) was used to make a 10x or 100x dilution with trypan blue solution (0.2 %), depending on the number of cells. Bürker counting chamber was used for cell counting. It consists of two chamber sides and a glass cover on the top, leaving space for the cell mixture between the bottom of the chamber and the cover. Approximately 10μL of the cell solution gets absorbed into the space by capillarity and the counting chamber was placed under the microscope. Each chamber side consists of 9 large squares and each large square consists of 16 smaller squares (figure 6B). At least 100 cells needed to be counted in each chamber side in the same large squares. Cells overlapping the top and the left lines were included in the counting, while the ones overlapping the bottom and right lines weren’t (as seen in figure 6A). Only the living, not colored cells were counted.

![Figure 6: Squares in the Bürker counting chamber. Picture A) shows the cells included in counting (black), and the ones not included (white). Picture B) shows 9 large squares.](image)

Total amount of cells was determined by this following calculation:

\[
\text{Amount of cells/mL} = \frac{\text{average cells (both chamber sides) \times DF (dilution factor) \times 10^4}}{\text{volume factor} \times 1 \text{ mL solution}}
\]
3.3.5 Platting the cells for the experiment

a) IGF-1 treatment of BV2 p6 and p18

Microglial cells were detached and counted (see protocol for cell passaging and cell counting). Cells were seeded at 1x10^6 in 24-well plates in growth medium (2 mL in total in each well) and cultured overnight at 37 ºC, 5 % CO₂ and 95 % air. On the same day, solutions were prepared and frozen until the next day (see table V). The next day, medium was removed from the plates into tubes, which were centrifuged for 10 min at 1400 rpm. The supernatant was discarded and the pelleted cells were dissolved in the medium with cytokines or without (only with cytokine vehicle) and added to the right wells. 1 mL of each solution was always added into the well at each step. After 6 h incubation, the process of centrifuging the removed medium was repeated. A new solution containing IGF-1 or not was added. Before adding IGF-1, the wells were washed with PBS, to completely remove all the serum. The cells were incubated for 24 hours, and then harvested together with the supernatant. Cells were detached from the wells by pipetting up and down. The entire mix of supernatant and cells was put into vials, which were then centrifuged for 5 min at 1500 rpm. Supernatant was removed into another vial, both supernatant and cell pellet were then stored at -80 ºC until further assay. The same experiment was also performed using 5 h incubation time with cytokines or lipopolysaccharide (LPS), and 5 h incubation time with IGF-1.

b) IGF-1 treatment of 1321N1

The cells were seeded at 1x10^6 cells in 12-well plates, allowing them to have more space to attach. 1321N1 cell line is adherent, therefore there are no living cells in the medium. When changing the medium with cytokines solution and IGF-1 treatment, medium is carefully removed by the vacuum pump and replaced with a new solution.

c) ET-1 treatment of BV2 p6 and p18

Solutions with or without endothelin-1 were added in the wells first for 24 hours. The wells weren’t washed with PBS in between as in experiments with IGF-1, since we didn’t change the culture medium. The way of changing and adding solutions remained the same as with IGF-1 treatment of BV2. Solutions with either endothelin-1, cytokines or endothelin-1 with cytokine were added in the second step for 6 hours.
d) ET-1 treatment of 1321N1

The cells were seeded at 1x10^6 cells in 12-well plates. Centrifugation was excluded during the process, the order of added solutions was the same as with BV2 cell line (c).

### 3.3.6 Solution preparation for experiments with IGF-1 and ET-1

**Table IV**: Medium for experiments with IGF-1 and ET-1

<table>
<thead>
<tr>
<th>Medium for solutions with IGF-1 (serum-free)</th>
<th>Medium for solutions with ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI + 50 U/mL PS, 2.5 μg/mL Fungizone, 0.1% BSA (IGF-1 free), 12.5 μg/mL transferrin, 30 nM Selenium, 2.5 mM HEPES, 2 mM glutamine (mixed and filtered through 0.22 μm filter)</td>
<td>DMEM + GlutaMAX™ + 10 % Fetal Bovine Serum, 1 % Antibiotic/Antimycotic, 1 % Fungizone</td>
</tr>
</tbody>
</table>

**Table V**: Composition of solutions for IGF-1 treatment

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>LPS-free H₂O in medium with serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β stimulation</td>
<td>IL-1β + LPS-free H₂O or without in medium with serum</td>
</tr>
<tr>
<td>LPS is dissolved in PBS, therefore when used, our vehicle contained PBS instead of LPS-free H₂O.</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>PBS in serum-free medium</td>
</tr>
<tr>
<td>IGF-1 250ng/mL</td>
<td>IGF-1 + PBS in serum-free medium</td>
</tr>
<tr>
<td>IGF-1 1000ng/mL</td>
<td>IGF-1 in serum-free medium</td>
</tr>
</tbody>
</table>

**Table VI**: Composition of solutions for ET-1 treatment

<table>
<thead>
<tr>
<th>Placebo</th>
<th>UP H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 100ng/mL</td>
<td>LPS-free H₂O + ET-1 + UP H₂O</td>
</tr>
<tr>
<td>ET-1 500ng/mL</td>
<td>LPS-free H₂O + ET-1</td>
</tr>
<tr>
<td>IL-1β stimulation</td>
<td>IL-1β + UP H₂O + LPS-free H₂O</td>
</tr>
<tr>
<td>Vehicle</td>
<td>UP H₂O + LPS-free H₂O</td>
</tr>
<tr>
<td>ET-1 100ng/mL + IL-1β</td>
<td>IL-1β + ET-1 + UP H₂O + LPS-free H₂O</td>
</tr>
<tr>
<td>ET-1 500ng/mL + IL-1β</td>
<td>IL-1β + ET-1 + LPS-free H₂O</td>
</tr>
</tbody>
</table>

IGF-1 and ET-1 concentrations were chosen based on previous experiments performed in our laboratory (94).
3.3.7 Western blotting

For immunoblotting we analyzed microglial cells, BV2 p18. The cells were seeded at 500,000 cells in 24-well plates in growth medium and cultured overnight at 37 °C, 5 % CO₂ and 95 % air. Using this method, the functionality of IGF-1 receptors was tested in each group. First 2 groups of cells received placebo treatment for 3 and 10 min. Next 2 groups received IGF-1 in the concentration of 1000 ng/mL for 3 and 10 min. The last two groups were first stimulated with cytokine IL-1β (30 ng/mL) for 6 hours, and then treated with 1000 ng/mL of IGF-1 for 3 and 10 min. After harvesting, the vials were centrifuged at 1500 rpm for 5 min. The supernatants were removed and the cells were frozen at -80 °C until sample preparation for western blot (WB).

On the day of cell harvesting, the gels for WB were prepared and stored overnight at 4 °C, covered with a wet towel, preventing them to dry out. According to the size of our proteins of interest, we prepared a 12.5 % solution of running gel, that we used for the gel preparation. Medium used in the experiment (serum-free medium) was added to each sample to reach 30 μL, to which 30 μL of sample buffer was added. The samples were boiled in water for 10 min, cooled down and centrifuged briefly. Holders with two sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were put into the electrophoresis system and filled with electrophoresis buffer. Samples (25 μL of each) and molecular weight size markers (7 μL) were loaded on the gel. Electrophoresis was performed at 110 V until the blue line reached the bottom, which took approximately 90 minutes.

After electrophoresis was completed, the gels were immediately put from the electrophoresis container into a cold (4 °C) transfer buffer. After protein separation, proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) membrane. The membrane was cut in desired size and activated in methanol for at least 30 min, before it was put in cold transfer buffer. The transfer sandwich was prepared, which consisted of a sponge, 2 filter papers, gel and PVDF membrane, 2 filter papers and sponge, which were all pre-soaked in transfer buffer. Any air bubbles between the gel and PVDF membrane had to be squeezed out. The sandwich was transferred in an electrophoresis system, filled with transfer buffer, which was placed on ice to maintain low temperature (4 °C). The transfer ran for 90 minutes at 90 V.
After transfer, the membranes were put in a 5% milk blocking solution (Table III) for 1 h at room temperature on a shaker. After that, the membranes were incubated in a 1:1000 dilution of primary antibody in 5% bovine serum albumin (BSA) in wash buffer overnight on a shaker at 4 °C. The next day, membranes were washed with wash buffer 3x15 minutes at room temperature on a shaker, followed by 1-hour incubation with 1:5000 dilution of secondary antibody in blocking solution on a shaker. The washing step was repeated (3x15 min), and after that membranes were put into a dark cassette. They were incubated for 1 minute with a freshly prepared ECL mixture (750 μL of each of the two solutions), carefully shaking so the membrane was covered all the time. The membrane was dried and taken to the dark room in the cassette, where a film was put on the plastic, covering the membrane. The exposure time was adjusted to each primary antibody, so the bands were nicely visible. The entire process was performed in the dark, with red safelights. After specific exposure time, the film was put in the developer, followed by stop solution, fixator and then water. The films were rinsed with water and evaluated. The membranes were then washed in the washing buffer for 15 min, then incubated in strip solution for 15 min at room temperature and washed again 3x15 min. The process was repeated for other specific antibodies for protein of interest. The films were scanned to the computer and resultant bands were quantified with program Image J densitometry. Rectangles were drawn around the bands. The peaks appeared, the lines were indicated over the base line, and the areas (values) obtained were copied into an excel file. The ratio of the protein of interest was calculated as a ratio of area of phosphorylated protein normalized to the area of total protein.

\[
\text{Sample } x = \frac{p\text{ERK 42}}{\text{total ERK 42}} \quad \text{Sample } x = \frac{p\text{ERK 44}}{\text{total ERK 44}} \quad \text{Sample } x = \frac{p\text{Akt}}{\text{total Akt}}
\]

3.3.8 Measuring cytokine concentration in the supernatant with Enzyme-linked Immunosorbent Assay (ELISA)

Mouse and human TNF-α, IL-1β, IL10 uncoated ELISA kits were used for the assay. Sandwich ELISA was used for the quantitative measurements of cytokine levels in samples. 100 μL of capture antibody with specificity for the cytokine of interest was immobilized on the polystyrene microplate and left overnight at 4 °C. Next day, the unbound capture antibody was washed 5x with 250 μL of PBS/Tween 0.1%. The plate was blocked with 200 μL ELISPOT (supplied in each kit) for 60 min at room temperature.
and shaking. During this time, fresh standard samples were prepared (containing a known concentration of recombinant cytokine: 1000, 500, 250, 100, 40, 16, 8 pg/mL). 100 μL of standards in duplicates and samples were added and any analyte present was bound by the immobilized antibody. The plate was incubated overnight at 4 °C. Unbound protein was washed away 5x with 250 μL of PBS/Tween 0.1 %, after which 100μL biotinylated detection antibody was added for 60 min at room temperature on the shaker. The unbound detection antibody was washed away with PBS/Tween 0.1 % (5x250 μL), followed by incubation of 100 μL streptavidin-horseradish peroxidase (HRP) for 30 min at room temperature on the shaker. HRP enzyme binds to the detection antibody and the excess was washed away. A chromogenic tetramethylbenzidine (TMB) substrate solution (100 μL) was added to the wells (incubated for 15 minutes at room temperature in the dark), which was chemically converted by the enzyme bound to the detection antibody. A blue color developed in proportion to the amount of cytokine, that was present in the sample. The reaction was stopped with the addition of 50 μL 1M H₂SO₄, turning a blue color to yellow one. The absorbance was measured spectrophotometrically at 450/540 nm using a microplate reader.

Four parameter logistic curve fit was used to detect protein concentrations in the samples. Protein concentration was obtained from freshly prepared standard dilution curve. Known concentrations were plotted on the X axis (1000, 500, 250, 100, 40, 16, 8 pg/mL), measurements OD-OD blank (average of 2 values obtained from the microplate reader for each standard concentration – average of 2 blank values) were plotted on the Y axis. Analysis was performed with GraphPad Prism by transforming x values using x=Log(x), followed by nonlinear regression (curve fit) and sigmoidal, 4 PL, x is log(concentration) analysis. Values for top, bottom, Hill slope and EC₅₀ were given and were used for cytokine concentration calculation according to this formula:

\[
y = Bottom + \frac{Top - Bottom}{1 + 10^{[\log(EC50-x)\cdot Hill \ slope]}}
\]

\[
a = \log \left( \frac{top-bottom}{y-bottom} \right) - 1
\]

\[
b = \log (EC50) - \frac{a}{Hill \ slope}
\]

\[
b = \log (x) = \log (EC50) - \frac{a}{Hill \ slope} \quad x = 10^b
\]

Using Excel, we calculated value a (y=OD-OD blank for our unknown sample obtained spectrophotometrically), a/Hill slope and value b. Values x were our cytokine concentrations.
concentrations, results were expressed in pg/mL. R squared was checked for each standard curve, to evaluate its fit.

### 3.3.9 RNA isolation

RNeasy Mini Kit from Qiagen was used for RNA isolation. Vials containing frozen cells from the experiment were taken out of the freezer (-80 °C) and pelleted by centrifuging for 1 min at 8000g. All the supernatant had to be carefully removed by aspiration, otherwise the remaining cell-culture medium could inhibit lysis and affect the conditions for binding of RNA to the membrane. The process of RNA isolation was performed according to the manufacturer’s instructions (Rneasy Mini Handbook, 2012, Sample & Assay Technologies). Eluted high-quality RNA was then put in the freezer at -80 °C and kept there for further steps. Before reverse transcribing the mRNA, the concentration of mRNA needed to be measured in each sample. Samples were taken out of the freezer and kept on ice during the measurement. Concentration was measured from the absorbance at 230nm on the spectrophotometer (Nanodrop ND-1000). 2 μL of each sample were used for the measurement. Based on the ratio between absorbance at 260 and 280 nm, we can evaluate the purity of the mRNA, which is required to be > 1.9. For the subsequent step, the same amount of the starting material was used (400 ng/μL).

### 3.3.10 Reverse transcription into complementary DNA

RNA was reverse transcribed into first-strand complementary DNA (cDNA) with a GoScriptTM Reverse Transcription System (Promega). Depending on the measured concentration of experimental RNA in each sample, the corresponding volume of the isolated RNA (400 ng/μL) was mixed with 8 μL of primer mix (random: oligo(dT)15 primers = 1:1) and nuclease-free water up to 20 μL in sterilized vials. The vials were heated in a 70 °C heat block for 5 minutes and then directly put on the ice for at least 5 min. During this time, a reverse transcriptase mix (60 μL) was prepared. Reverse transcriptase is very unstable and needed to be kept on ice the entire time. Reverse transcriptase mix for 1 reaction consisted of 16 μL GoScriptTM 5x Reaction Buffer, 10 μL MgCl2, 4 μL PCR Nucleotide Mix, 2 μL Recombinant RNasin Ribonuclease Inhibitor, 4 μL GoScriptTM Reverse Transcriptase and 24 μL Nuclease-free water. The vials with RNA were briefly centrifuged and then 60 μL of reaction mix was added to each sample vial with RNA (total 80 μL). The vials were put back into the reverse transcription
machine, annealed in a heat block at 25 °C for 5 min, and extended in a heat block at 42 °C for one hour. The reverse transcriptase was inactivated in a heat block at 70 °C for 15 minutes. Vials with cDNA were put into the freezer at -20 °C until used for qPCR reaction. (Go Script Transcription System. Quick protocol, Promega)

### 3.3.11 Amplification of cDNA

The quantitative polymerase chain reaction (qPCR) was performed in 96-well plates using an ABI 7900HT Real-time PCR system. cDNA was amplified using the SYBR green master mix. The stability of the Succinate dehydrogenase complex flavoprotein subunit A (SDHA) expression was tested in all of our treatment groups, checked for its stability and chosen as our housekeeping gene. Gene expressions of TNF-α, IL-1β, TGF-β, IL10 and iNOS were tested. For the reaction in each well, 15 μL of reaction mix was prepared by combining 4μL of nuclease-free water, 0.5 μL of forward primer and 0.5 μL of reverse primer of a specific gene, and 10 μL of SYBR green mix. After that, we added 5 μL of sample specific cDNA. The plate was covered with an adhesive cover and centrifuged for 1 min at 900g. After centrifugation, the plate was loaded into the machine, using a following program: hold at 95 °C for 10 min (stage 1), denature at 95 °C for 15 s and anneal/extend at 60 °C for 1 min (40 amplification cycles – stage 2). SYBR green dye was used to detect PCR products by binding to each new copy of double-stranded DNA formed during PCR. The result is an increase in fluorescence intensity in proportion to the amount of DNA copies. The disadvantage of the SYBR green dye is that we can get false positive signals, as it binds to any double-stranded DNA. Since it also binds to nonspecific (primer-dimer) double-stranded DNA sequences, this may affect the quality of amplification data. In order to detect primer dimers, melting curve (dissociation) was run. Primer-dimer can be detected clearly (figure 7), since they have a lower melting temperature (84). Threshold cycle values (Ct) present cycle number at which the fluorescence reaches the fixed threshold.

ΔCt was calculated by subtracting the average Ct value of gene of interest from the average of Ct value of the reference gene SDHA for each separate group of interest. This ΔCt was used to calculate values $2^{-\Delta Ct}$, which are presented on y axis on our graphs. The statistical analysis between all the groups were performed on $2^{-\Delta Ct}$ values.
3.3.12 Statistical analyses

All data are presented as mean ± standard error of the mean (SEM). Statistical comparison between two groups was evaluated with unpaired Student t-test. The comparison between multiple groups was evaluated using one-way ANOVA followed by the Dunnett’s post-hoc (multiple comparisons) or Turkey’s test. Statistical analysis was done using GraphPad Prism. Results were considered statistically significant when p<0.05.
4 RESULTS

4.1 Activation of insulin-like growth factor-1 receptor signaling pathways

Western blot was performed to address the phosphorylation of Akt and Erk using phospho-specific antibodies against Erk-P and Akt-P. Erk shows 2 bonds at 42 and 44 kDa. Both bonds were evaluated separately. We wanted to see if there is any difference between receptor activation if the cells are activated or not, therefore 2 groups were stimulated with IL-1β. Phosphorylation of Akt (p/T) is the highest after 3- and 10-min incubation with only IGF-1, but there is also an increase in activated cells treated with IGF-1 in comparison to placebo after 3 min. Phosphorylation of Erk is the highest after 3 min, for cells that were pre-stimulated with IL-1β and this shows a significant difference when compared to placebo. Difference between placebo and IGF-1 treatment after 3 min shows a non-significant increase in Erk activation after IGF-1 incubation. There is also a significantly higher Erk phosphorylation seen, when the cells were pre-stimulated with IL-1β in comparison to only IGF-1 treatment. After 10 min, we don’t see any significant differences when compared to placebo, but the activation of Akt and Erk is still seen.
Figure 8: Activation of IGF-1 receptors on microglial cells BV2 p18. Histograms are showing pAkt and pERK staining normalized to the staining for total Akt and Erk, respectively. n=3 for placebo samples, and n=4 for treated groups. *p<0.05, **p<0.01, ns=not significant. One-way ANOVA was used for statistical analysis with Turkey’s and Dunnett’s multiple comparisons test.

4.2 Insulin-like growth factor-1 effect on the microglial and astrocytic secretion of cytokines in the supernatant

In the study we investigated effect of IGF-1 on the production of pro-inflammatory cytokines TNF-α, IL-1β and anti-inflammatory cytokine IL10 in cytokine or lipopolysaccharide (LPS)-stimulated BV2 microglia and 1321N1 astrocytes.
In order to mimic the neuroinflammatory state happening in the brain following ischemic stroke (and MS, when accessing ET-1 effect on neuroinflammation), we stimulated microglia and astrocytes with cytokines and LPS. Microglia activates in cases of neuronal cell death, cytokine release, inflammation or infection (85). In cases of cytokine stimuli (TNF-α, IL-1β) or LPS, this microglial activation ends in sequence of well-known outcomes. This includes changes to cellular morphology, ramified body changing to a round shape (85). LPS is a major component of the outer membrane of Gram-negative bacteria (86) and is used to activate immune cells.

The cells were first stimulated with 100 ng/mL of IL-1β. TNF-α production on the protein level in supernatant was measured by ELISA, which resulted in high TNF-α production, with a concentration difference depending on passage number of BV2 cell line (p6 and p18). When stimulated with TNF-α, the cells did not release any IL-1β, or it was below detection limit. Therefore, we latter used IL-1β to stimulate our cells, using different concentrations. Concentrations we used, were based on previous concentrations used in our laboratory.

First experiment was performed on BV2 p6 cell line, where we stimulated the cells with IL-1β for 6 h, after that the solutions were changed and incubated for 24 h with 250/1000 ng/mL IGF-1 or placebo. There was a slight decrease in TNF-α release within IGF-1 treatment group for both concentrations in pre-stimulated groups in comparison to placebo (average concentration 298.02 reduced to 266.61 pg/mL for IGF 250 ng/mL, and 299.61 reduced to 238.21 pg/mL for IGF-1 1000 ng/mL in cells pre-stimulated with IL-1β), but this difference was not significant. Interestingly enough, the highest TNF-α detection was actually in the placebo group (469.48 and 377.12 pg/mL respectively). The same stimulation and treatment were performed in BV2 p18 cell line, and showed no significant difference in our activated groups between a placebo and IGF-1.

Due to no significant effect seen after 24 hours treatment incubation, different stimulation and time points were used for our next experiment. BV2 p18 cell line was stimulated with LPS 100 ng/mL for 5 h and then treated with IGF-1 for 5 h. There was no significant increase seen in TNF-α production upon LPS activation itself compared to placebo control. But there was a significant increase in TNF-α production in LPS stimulated groups treated with 1000 ng/mL IGF-1, showing a pro-inflammatory effect after 5 hours.
Figure 9: *Effect of IGF-1 on TNF-α production in BV2 p18 cell line after 5 hours of treatment in LPS pre-stimulated cells.* The cells were first incubated with either the LPS vehicle (first three groups) or stimulated with LPS (100 ng/mL), which were our groups of interest. After 5 h activation, the solutions were replaced with placebo or IGF-1 (250 and 1000 ng/mL) for 5 h. (n=4) for all groups. *p<0.05, ns=non-significant.

Figure 10: *Effect of IGF-1 on TNF-α production in BV2 p18 cell line after 24 hours of treatment in LPS pre-stimulated cells.* The cells were first incubated with either LPS vehicle or stimulated with LPS (100 ng/mL), which were our groups of interest. After 5 h activation, the solutions were replaced with placebo or IGF-1 (250 and 1000 ng/mL) for 24 h. n=4 for all groups.
These short-term effects (together 10 hours) were compared with parallel 5 h stimulation with LPS, following 24 h IGF-1 incubation experiment, where TNF-α production is elevated and there is no IGF-1 effect seen. Additionally to TNF-α, we also measured IL-1β release, when cells were stimulated with LPS. After 10 hours (5 h LPS stimulation + 5 h IGF-1 incubation), IL-1β concentrations were all under detection limit (under 8 pg/mL), and after 24 h IGF-1 incubation we see higher cytokine production (between 20 and 37 pg/mL), but a lot of variation, and no significant effect.

Since we saw a significant proinflammatory effect with 5 h IGF-1 incubation, we did an experiment on BV2 p18 cell line, using lower stimulation with 30 ng/mL IL-1β for 5 h and then accessed the results after 5 h incubation with IGF-1. We wanted to see if after 5-hour incubation with IGF-1 we would see the same pro-inflammatory effect, regardless of the stimulus. IGF-1 treatment showed a reduction in TNF-α production in IL-1β activated cells, a significant reduction with 250 ng/mL and non-significant with 1000 ng/mL. IGF-1 shows an anti-inflammatory effect, but not in a dose-dependent manner.

**Figure 11:** Effect of IGF-1 on TNF-α production in BV2 p18 cell line after 5 hours of treatment in IL-1β pre-stimulated cells. The cells were first incubated with either the IL-1β vehicle (first 3 groups) or stimulated with IL-1β (30 ng/mL), which were our groups of interest. After 5 h incubation, the solutions were replaced with placebo or IGF- (250 and 1000ng/mL) for 5 h. Each group had 500.000 cell per well. *p<0.05, ns=non-significant.
IGF-1 induced production of anti-inflammatory cytokine IL-10 was measured as well in all
the experiments with IGF-1. IL-10 release was not detected on the supernatant or it was
under detection limit in all experiments we performed with IGF-1.

The same type of experiment was done in 1321N1 astrocytic cell line. Cells were
stimulated with cytokines for 6 hours, and after that incubated with IGF-1 250/1000 ng/mL
for 24 hours. There is no TNF-α, IL-1β or IL10 detected by ELISA. Astrocytes were later
stimulated with LPS 100 and 1000 ng/mL, combination of cytokines and LPS 100/1000
ng/mL and combination of cytokines (TNF-α + IL-1β each 100 ng/mL) for 6 hours. There
was no cytokine release detected in the supernatant by ELISA.

4.3 Effect of insulin-like growth factor-1 treatment on pro- and anti-inflammatory
mRNA expression in microglia

We looked at IGF-1 modulation of mRNA expression of inflammatory cytokines TNF-α,
IL-1β, inducible nitric oxide synthase (iNOS) and anti-inflammatory TGF-β and IL10 in
microglial cells (BV2 p18). We used the cells from experiment shown in figure 11. Cells
were stimulated with 30 ng/mL IL-1β for 5 hours, and then incubated with placebo or IGF-
1 for an additional 5 hours. We looked at gene expression difference in the groups, that
were pre-stimulated with IL-1β. We did not find a statistical difference for TNF-α
expression. IL-1β expression was elevated in activated BV2 cells treated with IGF-1
compared to placebo, but the increase in expression did not reach significance. Expression
of iNOS was statistically non-significantly down regulated after treatment with IGF-1, with
a bigger reduction in the non-activated cell groups, which was probably due to the fact,
that we measured gene expression only in 2 samples in the group not stimulated with
cytokine and treated with only IGF-1 1000 ng/mL. We can also see, that IL-1β and iNOS
mRNA were detectable in normal culture conditions (figure 12).
**Figure 12**: Effect of IGF-1 on IL-1β, iNOS and TGF-β gene expression in microglial cells BV2 p18. n=4 for all the groups, except n=2 for group IGF-1 1000 ng/mL. Y axis presents $2^{-\Delta Ct}$. $\Delta Ct = Ct$ (gene of interest) – Ct (reference gene).

IGF-1 250 ng/mL increased TGF-β expression in the cell group activated with IL-1β, compared to placebo in the activated group, but this was not significant. We also looked at anti-inflammatory IL10 expression, and there was almost no expression seen.
4.4 Endothelin-1 effect on the microglial and astrocytic secretion of cytokines in the supernatant

BV2 p6 cell line was pre-incubated with placebo or endothelin-1 for 24 h. Cytokines were added later for 6 hours, and they were combined with ET-1 in our treated activated group. TNF-α expression in the supernatant was overall much lower (maximum 76.51 pg/mL in the group activated only with IL-1β), which is normal for BV2 p6, and we saw a statistically significant increase in TNF-α production upon IL-1β activation (figure 13 A and B). We saw a non-significant reduction in TNF-α production in the group pre-incubated with ET-1 (100 and 500 ng/mL) + IL-1β compared to group only stimulated with IL-1β. The same experiment was repeated on BV2 p18 cell line, where we see higher TNF-α release (stimulation with IL-1β leads to the average of 612.6 pg/mL TNF-α) and this activation upon IL-1β was statistically significant (figure 14, p<0.0001). ET-1 100 ng/mL significantly reduced TNF-α production in the group pre-incubated with ET-1 and stimulated with IL-1β compared to TNF-α production in the group only activated with IL-1β (figure 14).

![Graph A](image1.png)

**Figure 13:** Effect of ET-1 on TNF-α production in BV2 p6 cell line after 24 hours of treatment in IL-1β (100 ng/mL) stimulated cells. *p<0.05, **p<0.01, ns=non-significant. A) Treated with 100 ng/mL of ET-1, and B) treated with 500 ng/mL of ET-1.
Figure 14: Effect of ET-1 on TNF-α production in BV2 p18 cell line after 24 hours of treatment in IL-1β (100 ng/mL) stimulated cells. Concentration of ET-1 was 100 ng/mL. *p<0.05, ****p<0.0001.

There was no significant difference between placebo and ET-1 500 ng/mL treatment in IL-1β pre-stimulated BV2 p18 cells.

Figure 15: Effect of ET-1 on TNF-α production in BV2 p6 cell line after 24 hours of treatment in IL-1β stimulated cells. A) Cells were pre-treated with ET-1 100 ng/mL. Student’s t-test was used for the statistical analysis; *p<0.05 and **p<0.01. B) BV2 p6 cell line, pre-treated with 100 and 500 ng/mL of ET-1 and stimulated with 30 ng/mL IL-1β. One-way ANOVA was used for statistical analysis with Dunnett’s multiple comparisons test; ns=non-significant.
Lower stimulation with 10 and 30 ng/mL IL-1β showed significant increase in TNF-α production upon activation (figure 15 A), and we saw a statistical reduction in TNF-α production in ET-1 (100 ng/mL) pre-incubated cells in comparison to placebo in cytokine (30 ng/mL IL-1β) stimulated group (A). Furthermore, we wanted to see if we would see a dose-dependent effect. Therefore, additional experiment was performed using 30 ng/mL for cytokine stimulation, and both 100 and 500 ng/mL of ET-1 were used for this test. We saw a non-significant increase in TNF-α production upon activation with IL-1β. There is a statistically non-significant reduction in TNF-α production, suggesting anti-inflammatory endothelin-1 effect, with a bigger reduction with higher concentration of ET-1 (figure 15 B). Although BV2 p6 cell line normally produced less cytokines in comparison to BV2 p18 cell line, we tested cell death in our second (B) experiment due to variation. Lactate dehydrogenase (LDH) values were measured in all groups after 24 h pre-incubation with placebo/ET-1, showing high LDH absorbance, but without any significant difference between the groups. LDH absorbance was measured again after 6 h incubation with only cytokine or cytokine + ET-1. When comparing LDH values to TNF-α production, we saw low cytokine release in the wells with a high LDH absorbance.

To examine, if ET-1 influences the production of cytokines in astrocytes, we used the same process as for microglial cells, ET-1 pre-incubation, followed by cytokine TNF-α or IL-1β stimulation. There was no TNF-α release detected, IL-1β was detected on ELISA, but under the detection limit (under 10 pg/mL).

4.5 Effect of endothelin-1 treatment on pro- and anti-inflammatory gene expression in microglia
Figure 16: Effect of ET-1 (100 and 500 ng/mL) on IL-1β, iNOS, TGF-β and TNF-α gene expression in microglial cells BV2 p18. Cells were pre-incubated with ET-1 100 (A) or 500 ng/mL (B) for 24 h, and then stimulated with cytokines TNF-α and IL-1β for 6 hours. **p<0.01. Y axis presents $2^{-\Delta Ct}$, $\Delta Ct= Ct$ (gene of interest) – Ct (reference gene).

For this analysis, cells from the experiment in figure 14 (BV2 p18) were used. IL-1β mRNA was expressed only when stimulated with IL-1β and showed a reduction in the expression when pre-treated with ET-1 100 and 500 ng/mL, but the difference didn’t happen in the dose-dependent manner, nor was it statistically significant. ET-1 increased iNOS expression when pre-treated with ET-1 and activated with TNF-α in comparison to not-treated TNF-α activated cells. This effect was seen in both experiments with ET-1, using 100 and 500 ng/mL. Expression of iNOS was non-significantly upregulated when pre-treated with ET-1 100 ng/mL in comparison to control when stimulated with IL-1β, and was down-regulated with 500 ng/mL of ET-1 pre-incubation. There is big variation in these two groups, therefore is possible that there is no effect. Pre-treatment with ET-1 100 and 500 ng/mL upregulated TGF-β expression in TNF-α stimulated cells, while the expression was down-regulated by ET-1 in BV2 cells activated by IL-1β. TNF-α is upregulated in the cells activated with both cytokines, and there is a statistically significant increase in TNF-α expression with ET-1 500 ng/mL pre-incubation in IL-1β stimulated cells in comparison to cell group activated with IL-1β.
5 DISCUSSION

Effects of the neuroprotectant IGF-1 on neuroinflammation in ischemic stroke

Neuroinflammation has a crucial role in the progression of the ischemic events and exacerbates the primary ischemic damage. Additionally, it is responsible for the majority of tissue death in the penumbra (5). Microglia are the major resident immune cells of the brain (21) and they are the first cells to get activated after ischemic stroke (14). Depending on their phenotype, activated microglia can secrete harmful mediators and contributes to harmful events following ischemic stroke (M1). They can also release protective neurotrophic factor and are known for phagocytic clearance of cell debris, which leads to neuron protection (M2) (23). Astrocytes become activated following ischemic stroke as well and take part in contributing to the neuroinflammation by secreting inflammatory cytokines (21).

Our in vitro study for IGF-1 was based on the previous results acquired in vivo. The most recent study at our department by Serhan et al. (57) showed, that IGF-1 in vivo not only reduced the infarct size, but also significantly reduced mRNA expression of iNOS and IL-1β in the ischemic hemisphere of rats with an ischemic stroke. Furthermore, they discovered, that IGF-1 modulates the mRNA expression of inflammatory mediator iNOS in microglia extracted from ischemic hemisphere, which was significantly reduced by IGF-1 treatment. Therefore, this group of researchers proposed IGF-1 stimulated neuroprotection in ischemic stroke occurs mainly by reducing inflammatory factors in the ischemic brain. Reducing inflammation significantly decreases cerebral ischemic damage (5), therefore, the ability of IGF-1 to reduce proinflammatory cytokine production in activated microglia would be very beneficial.

To confirm, that IGF-1 down-regulates inflammatory cytokine expression in microglia and to assess whether microglia are the direct target for IGF-1, we performed experiments with cultured microglia and additionally astrocytes. These experiments also allow us to study in more detail the effects of IGF-1 in the expression of several cytokines in different glial cells.

Two main IGF-1 receptor signaling pathways involved in antiapoptotic effects of IGF-1 were checked for their functionality, by measuring the phosphorylation of specific down-stream kinases belonging to either of the two pathways. The significant increase of p42 and
p44 phosphorylation indicates that the microglial BV2 cells express functional IGF-1 receptors signaling through the MAPK-pathway. Although the increase in Akt (protein kinase B) phosphorylation did not reach significance, a role for the PI3K pathway may not be excluded. This implies that neuroprotective effects of IGF-1 found in vivo in ischemic brain could be carried out by microglia. Interestingly, phosphorylation of Erk (extracellular-regulated kinase) showed no statistical significance after 10-min incubation. A mechanism of a negative feedback could explain the difference between 3- and 10-min incubation results, since one would expect higher phosphorylation over time.

Having confirmed the functionality of IGF-1 receptors, we assessed the effects of IGF-1 on the release of cytokines. TNF-α, IL-1β and IL10 were measured in the supernatants of the cells. At the protein level IGF-1 significantly increased or decreased the release of TNF-α depending on the type of stimulation. Our results show, that 5-hour incubation with IGF-1 in LPS pre-stimulated cells (5 hours) resulted in an increase in TNF-α production, in a dose dependent manner. Increasing TNF-α production could have a beneficial effect on the outcome of stroke. According to a study by Lamberts et al. (87), microglial-derived TNF has a neuroprotective role in focal cerebral ischemia in mice by being crucial for the survival of neurons. The significance of its role was highlighted by the fact, that there was approximately a 50 % increase in ischemic tissue injury in mice that were missing microglial-derived TNF (87). TNF-α shows pleiotropic effects in the ischemic brain with neurotoxic and neuroprotective effects (2). Initial increase in TNF-α is seen 1-3 hours and a second peak at 24-36 hours after ischemia onset (21). Our results could mean, that the short-term effect of IGF-1 is to increase TNF-α production and that this could have neuroprotective effects. However, this effect was not seen in IGF-1 5-hour incubation in IL-1β stimulated BV2 cells, where IGF-1 decreased TNF-α production. Overexpression of TNF-α is harmful to stroke outcome (1), therefore IGF-1 ability to reduce TNF-α production, would be beneficial as well. As microglia, cytokines too, have a dual role in the influence on stroke, therefore their effect may depend on the time and degree of the expression (1).

The results of the IGF-1 effect on the expression at the transcriptional levels indicate, that IGF-1 has the tendency to stimulate the mRNA expression of both inflammatory (IL-1β) and anti-inflammatory cytokines (TGF-β) in microglia. But the results are not statistically significant. Our results don’t confirm the statistically significant reduction in iNOS mRNA
expression by IGF-1, that Serhan et al. (57) measured in vivo in the ischemic hemisphere of rats, as well as in microglia isolated from the hemisphere mentioned. We only see a statistically non-significant reduction in iNOS mRNA expression. There is also a non-significant increase in TGF-β mRNA expression, which was also up-regulated in microglia isolated from the ischemic hemisphere of rats (57). TGF-β is believed to improve the recovery of the ischemic stroke (21), so augmentation by IGF-1 would be favorable. Additionally, Lu et al. (88) showed, that microglia-secreted TGF-β1 could protect cultured microglia, astrocytes and neurons in cases of ischemia-like events.

A similar study was performed by Brabazon et al. (85) on BV2 cell line, where the cells were activated with pro-inflammatory LPS (125 ng/mL) for 1 hour, and then exposed to insulin administration. Insulin is a promising treatment of Alzheimer’s disease and traumatic brain injury (85) and its receptors trigger the same signaling pathways as IGF-1. Insulin and IGF-1 can both bind and activate both insulin and IGF-1 receptor, showing 84% homology in the tyrosine kinase domain and 45-65% in the ligand binding domain (28), so their anti-inflammatory modulation of microglia could be similar. The effects were assessed after 24 hours and it was concluded, that insulin significantly reduced NO, ROS and TNF-α production, as well as significantly reduced iNOS expression (85). This study and our study imply that microglia can be a direct target for IGF-1. However, the question that remains is how IGF-1 effects on microglia induce neuroprotection.

Modulation of microglial IL-10 production by IGF-1 was also assessed, since this anti-inflammatory cytokine is mostly produced by microglia and astrocytes. It inhibits inflammatory cytokine production, and peaks on the third day after stroke onset (2). Brain injury after stroke can be reduced, if IL-10 is administered after MCAO (2). IGF-1 in our experiments did not induce any IL-10 production. Together with no significant increase found in vivo by IGF-1 in the ischemic hemisphere of rats (57), we can assume, that IGF-1 doesn’t modulate anti-inflammatory IL-10 production.

Despite no IL-1β detection in the supernatants of activated BV2 cells or treated cells with IGF-1, it still remains to be established, if IGF-1 could modulate this inflammatory cytokine expression. An interesting observation in the study from Lee et al. (89) is, that microglial IL-1β was mostly detected in cell lysates, peaking at 16 h in 100 ng/mL LPS stimulated microglia, that was isolated from second-trimester human fetal brains. Minimal values were detected in the supernatant. This is in accordance with our results, as we
detected low amounts of IL-1β secretion after LPS stimulation. Incubation time with LPS in our experiment was 5 h, in comparison to 24 h incubation in the study mentioned. There are also differences in cytokine expression in human and mouse microglia, thus direct comparison can’t be done, yet the pattern seems to be similar. Since cytokines peak at different time points, it remains to be established, what the perfect incubation time and concentrations are. A proper activation of the cells is needed, which would show, if IGF-1 modulate any anti-inflammatory effects. Activated microglia are responsible for the predominant part of early produced IL-1β, followed by astrocytes (2). IL-1β mRNA increase has been documented 15-30 min after ischemia, followed by protein increase after a few hours (21). IL-1β has been reported to be an endogenous pyrogen, and contributes to the aggravation of the neuronal loss (2). Therefore, it is important to establish, if IGF-1 could modulate microglial production of IL-1β, since the ability to reduce IL-1β secretion by microglia would benefit the stroke outcome. Especially because in vivo, Serhan et al. (57) found, that IGF-1 significantly reduced mRNA expression of IL-1β by microglia. Their results also show a non-significant decline in GFAP mRNA in the ischemic rat brain by IGF-1. They suggested, that IGF-1 could directly reduce IL-1β mRNA expression in astrocytes, or this reduction could be indirectly mediated by microglia (57).

As a further experiment, we suggest a microglia-astrocyte co-culture. First of all, 1321N1 astrocytes alone did not react to any type of stimulation (LPS or cytokines), nor they reacted to IGF-1 and ET-1 treatment alone, or together with cytokines. At least cytokines were not detected on the supernatant by ELISA. This pattern was also discovered in a study by Lee et al. (89), where no mRNA and protein expression for IL-1β, IL-6 and TNF-α was detected in LPS-stimulated astrocytes isolated from second-trimester human fetal brains. But in their study, IL-1β managed to induce mRNA and protein for TNF-α. However, protein levels were barely seen after 4 h, rapidly increased after 8 h and peaking at 16 h. After 24 h TNF-α detection already decreased (89). Therefore, our 6 h stimulation time was possibly not long enough to detect secreted cytokines. A co-culture with microglia-astrocytes might activate astrocytes, since microglia strongly respond to LPS stimulation and these cells could then direct a response in astrocytes, via production of IL-1β (89). Additionally, using this co-culture and this mean of activation, it would be possible to determine, if IGF-1 might via microglia indirectly affect the reduction in astrocytic IL-1β expression or if IGF-1 directly modulates this reduction on astrocytes. Before further experiments on 1321N1 astrocytoma, the functionality of IGF-1 receptors
should be checked. It was reported, that insulin/IGF-1 signaling pathway is present and functional in human primary astrocytes, therefore IGF-1 could mediate its effect via astrocytes (38).

We propose a couple of future experiments in microglia. The effect of IGF-1 on NO and iNOS production remains to be established. Additionally, Akt and Erk phosphorylation should be tested in activated cells (IL-1β or LPS) and compared. The goal is to answer the question how IGF-1 in activated cells affects receptor expression of these two down-stream kinases compared with activated cells, to further define if IGF-1 modulates inflammatory mediators in microglia via these two mentioned pathways. Microglia exists in two phenotypes (23). We used a M1 phenotype model, which expresses pro-inflammatory factors, by activating TLR receptors, leading to activation of transcription factors nuclear factor kappa-B (NF-κB) and signal transducer and activator of transcription 1 (STAT1) (90). It would be interesting to see, if IGF-1 would have a phenotype-specific effect. M2 phenotype can be stimulated by IL-4 or IL-13 to produce protective factors, by activating STAT6 (90). IGF-1 should be added, to see if IGF-1 could increase neurotrophic factors production, which would benefit stroke outcome by inducing tissue repair. Selective M2 phenotype activation is believed to be controlled by the enhanced ratio of transcriptional action of cAMP response element-binding protein (CREB) versus NF-κB. The balance of these two is significantly affected after cerebral ischemia, which is involved in the regulation of pro-inflammatory mediators (23). Levels of p-CREB are down-regulated and activity of NF-κB is elevated (23). It should be examined, if IGF-1 could affect this balance and therefore polarize microglia to M2 phenotype.

**Effect of the vasoconstrictor ET-1 on neuroinflammation in multiple sclerosis**

The potential effect of ET-1 on neuroinflammation was investigated. BV2 cells significantly increased TNF-α production upon IL-1β activation. ET-1 significantly down-regulated the secretion of TNF-α by activated cultured microglia, but this effect was not always significant. This suggests that ET-1 exerts anti-inflammatory effects. It remains a possibility, that inhibition of TNF-α production is due to endothelin-1’s cytotoxic effect in cytokine stimulated BV2 microglia. Lactate dehydrogenase (LDH) test was performed to determine whether endothelin-1 exerts any cytotoxic effects. LDH is a stable cytoplasmatic enzyme, that is released from all the cells in the case of plasma membrane damage (91). We evaluated the membrane integrity of the cells as a function of the amount of LDH.
leakage into the medium. We measured LDH values in the supernatant of the cells, that we used for the experiment shown in figure 15 B). Due to higher spectrophotometrically measured absorbances in the wells, where TNF-α production was low, ET-1 anti-inflammatory effect can’t be confirmed and cell toxicity needs to be excluded to confirm these effects. The results on the mRNA level are indicative of anti-inflammatory effects of ET-1, by down-regulating IL-1β upon activation with IL-1β and up-regulating TGF-β when stimulated with TNF-α. And they are also indicative of inflammatory effect of ET-1 by down-regulating TGF-β with IL-1β stimulation, up-regulating TNF-α expression and up-regulating iNOS expression upon TNF-α activation. The effects on the mRNA levels may depend on the way the microglia have been stimulated. Effects measured in the supernatant were not accompanied by the reduction in the expression of TNF-α at the transcriptional levels. Pre-treatment with 500 ng/mL of ET-1 upon IL-1β activation shows a significant increase in TNF-α expression, compared to only IL-1β activation. When the effects of ET-1 on the mRNA level don’t match the effect at the protein level in the culture medium, we have to consider possible effects on translation of the mRNA or the secretion into the medium. A reduction in protein levels can be due to inhibition of translation or secretion. As a further experiment, anti-inflammatory ET-1 effect should be confirmed by testing the effect of endothelin-1 receptor antagonist bosentan. Additionally, ET-1 effect on IL-1β production in cell lysates should be assessed as well.

Advantages and disadvantages of working with cell lines in vitro

In all the experiments with IGF-1, the activation by IL-1β and LPS doesn’t result in a significant increase in TNF-α compared to our control. In contrast, the question arises, why do we see so much cytokine TNF-α production in our cells when they don’t get activated by LPS or IL-1β. The cells we were working with, were genetically changed and many transformed cells have altered gene expression levels. Therefore, basal cytokine expression is not unusual. Since microglial cells are very sensitive to any type of change and they have characteristics of a macrophage, it is possible that the change of solutions or cell death caused activation and secretion of TNF-α. Our assumption is, that because the cells are incubated with IGF-1 in serum-free medium, they are starving, and therefore we have more dead cells, which then activate other microglia and produce so much cytokine TNF-α. The reason to use serum-free medium is the fact, that serum contains some growth factors, among which IGF-1, which would then interfere with cells when stimulating them
with IGF-1. In fact, cells should be starved overnight prior to activation and treatment, according to the literature (92).

The serum-free medium influence is confirmed by statistically significant increase in TNF-α production by IL-1β stimulation in experiments with ET-1, which were performed on the same BV2 cell line in medium with serum. We can conclude, that type of culture medium and passage number can have an effect on regulation of cytokine expression. The passage number influence was noticed during BV2 cell cultivation, when we noticed unexpected changes in cell confluency sometimes in cell passages with a higher number. The reason, that the effects seen in our results were not always repeatable, could also be due to cell passage number. A study by Henn et al. (83) confirmed the suitability of the BV2 cell line, and that it shows similar characteristics to primary microglia, since there were some doubts regarding its applicability. Even though there are some differences, BV2 cells seem to be an acceptable replacement for primary microglia. The use of microglia-like cell line reduces the need for animal experiments and consequent cell preparation (83). The cells we were working with, were not exposed to the same microenvironment as they are in neuroinflammatory state in acute ischemic stroke and MS. In the past, experiments were performed in in vivo isolated primary astrocytoma and microglia in our department. This gave a more realistic insight, but these cells didn’t survive in culture media for more than 3 days. However, our results with BV2 cells still give a good first indication, but further experiments need to be done, to develop a good model to test effects of IGF-1 and ET-1 on inflammation. This could include optimization of stimulation and treatment incubation times, since especially concentrations of LPS used and incubation times vary from one study to another (85, 93). Additionally, optimization of culture media for cultivating and for performing experiments should be considered.
CONCLUSION

1.) Microglial BV2 cells have functional IGF-1 receptors, which confirms our hypothesis and implies, that these cells can serve as a target for the neuroprotective action of growth factor IGF-1.

2.) After a 5-hour incubation period with IGF-1, BV2 cells significantly increased TNF-α production, when primed with LPS, and significantly reduced TNF-α release, when primed with IL-1β.

3.) According to IGF-1 receptor functionality and cytokine production in BV2 microglia, we can partially confirm our hypothesis. IGF-1 modulates pro-inflammatory TNF-α production during neuroinflammation, but not anti-inflammatory cytokine production by microglia. The effect of IGF-1 on microglia may depend on the microenvironment the cells are in. In order to understand IGF-1 role in neuroinflammation during ischemic stroke, we suggest these experiments should be repeated and performed in an ischemic environment, using oxygen-glucose deprivation (OGD) to better mimic ischemic conditions in the cells.

4.) ET-1 significantly down-regulated the secretion of TNF-α by activated cultured microglia, suggesting that ET-1 is an anti-inflammatory factor. This does not confirm our hypothesis regarding ET-1.

5.) This implies, that treatment of MS patients with the endothelin-1 receptor antagonist bosentan to increase the perfusion in the brain, could lead to pro-inflammatory effects. Further tests have to be done to determine the consequences that treatment with endothelin receptor antagonist would potentially have on neuroinflammation for MS patients.
7 REFERENCES


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