Modulation of nociceptin receptor and nociceptin/orphanin FQ mRNA expression in whole blood cultures

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Meinen lieben Eltern und Großeltern

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Abkürzungsverzeichnis

°C	Grad Celsius
µg	Microgram (10^{-6} g)
µl	Microliter (10^{-6} L)
µM	Micromolar (10^{-6} M)
A	Adenine
ANOVA	Analysis of variance
bp	Base pair
C	Cytosine
cDNA	Complimentary DNA
CNS	Central nervous system
CO_2	Carbon dioxide
Cp	Cross point
ddH_2O	Double distilled water
dNTP	Dideoxyribonucleoside triphosphate
Dyn17	Dynorphin17
E	Efficiency
EDTA	Ethylene diaminetetraacetic acid
E_R	Efficiency of reference amplification
E_T	Efficiency of target amplification
Fig.	Figure
FKS	Fetal calf serum
G	Guanin
GPCR	G-protein coupled receptor
h	Hour
HPRT	Hypoxanthine phosphoribosyltransferase gene
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-1ra	Interleukin-1ra
IFN-γ	Interferon gamma

LBP	LPS-binding protein
LPS	Lipopolysacchride
mAb	Monoclonal antibody
min	Minute
ml	Milliliter (10^{-3} L)
mRNA	Message RNA
ng	Nanogram (10^{-9} g)
N/OFQ	Nociceptin/orphanin FQ (the first amino acid F (phenylalanine) and last Q (glutamine))
NOP	Nociceptin receptor
ORL-1	Opioid receptor like-1
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
p	P valve
pmol	Picomolar
pp-N/OFQ	pre-pro-nociceptin/orphanin FQ
RNA	Ribonucleic acid
RGS19	Regulator of G-protein signalling 19
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SEA	Staphylococcal enterotoxin A
sec	Second
SEM	Standard error of mean
T	Thymin
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
U	Unit
UV	Ultra-violett

1. Zusammenfassung

Der Nociceptin-Rezeptor (NOP) gehört zur Gruppe der G-Protein-gekoppelten Rezeptoren und weist eine 60%-ige Strukturhomologie zu klassischen Opiodrezeptoren auf. Nociceptin/Orphanin FQ (N/OFQ), der endogene NOP-Ligand, ist an zahlreichen physiologischen Prozessen beteiligt. Bei weitgehender Sequenzhomologie zu klassischen Opioidpeptiden besitzt N/OFQ jedoch ein typisches eigenes pharmakologisches Profil. Menschliches Hirn- und Rückenmarksgewebe, aber auch Immunzellen exprimieren NOP and N/OFQ. Das lässt auf eine wichtige Rolle des N/OFQ-NOP-Systems sowohl für zentralnervöse als auch für immunologische Abläufe schließen. Eine Induktion von N/OFQ durch LPS in Zellkulturen von murinen sensorischen Neuronen und Astrozyten wurde beschrieben. Zahlreiche weitere Studien am Tiermodell untersuchen die Expression von NOP und N/OFQ in Zellen des Nervensystems. Deutlich weniger ist bekannt über Veränderungen in der Expression von N/OFQ und seines Rezeptors in menschlichen Blutzellen unter inflammatorischen Bedingungen.

Im Rahmen dieser Studie wurde Vollblut von 30 gesunden Probanden bis zu 24 Stunden mit Lipopolysaccharid (LPS) (10 ng/ml), Tumornekrosefaktor alpha (TNF- α) (3 ng/ml), Interleukin beta (IL-1β) (3 ng/ml), Interleukin-10 (IL-10) (50 ng/ml) oder Interferon gamma (IFN-γ) (10 ng/ml) inkubiert. Anschließend wurde die Expression von NOP- und N/OFQ-mRNA mittels RT-PCR analysiert und quantifiziert. Der Überstand wurde mit einem ELISA-Assay auf Konzentrationen von TNFα, IL-1β and IL-10 untersucht. Zusätzlich erfolgte eine Interventionsstudie mit neutralisierenden Antikörpern gegen TNF-α, IL-β, IFN-γ und IL-10, um mögliche modulierende Faktoren der LPS-Wirkung auf das N/OFQ-NOP-System zu untersuchen.

Die quantitative RT-PCR zeigte die konstitutive Expression von NOP und N/OFQ auf der mRNA-Ebene im peripheren Blut, die nach Stimulation mit LPS stark herabreguliert wurde. Die inflammatorischen Zytokine TNF-α, IL-β, IL-10 und IFN-γ führten gleichfalls zu einer Verringerung der Konzentration von NOP- und N/OFQ-mRNA. Die Blockade von LPS-induziertem TNF-α and IL-1β konnte den supprimierenden Effekt von LPS auf die NOP-Expression während der frühen inflammatorischen Phase (3h) teilweise antagonisieren.

Unsere Ergebnisse belegen eine Modulation der NOP- und N/OFQ-Expression durch inflammatorische Mediatoren im Rahmen der Immunreaktion. TNF- α und IL-1β könnten an der Regulation der LPS-induzierten Expression von NOP-mRNA im Vollblut während der akuten

Phase der Hyperinflammation beteiligt sein.

Der Mechanismus des modulierenden Einflusses inflammatorischer Zytokine auf das N/OFQ-NOP-System und die Bedeutung dieser Mechanismen für die Regulation von immunologischen Prozessen humaner Zellen sollte weiter untersucht werden.

2. Introduction

The nociceptin receptor (NOP) and its endogenous ligand nociceptin/orphanin FQ (N/OFQ) are widely distributed throughout the immune system as well as the nervous system and have been implicated in a wide range of biological functions. There is abundant evidence that the NOP and N/OFQ are correlated with immune responses suggesting the N/OFQ-NOP system play a role in immune systems. In recent years, a growing literature documented that the functional regulation between N/OFQ-NOP system and inflammatory mediators is bidirectional.

2.1 Nociceptin receptor

The nociceptin receptor (NOP), also known as opioid receptor-like-1 (ORL-1) receptor (Mollereau, 1994), LC132 (Bunzow, 1994) or ROR-C (Fukuda, 1994) was discovered in 1994 (Fukuda, 1994; Mollereau, 1994). As the fourth member of the opioid receptor family, NOP possesses overall 60% homology with the classic opioid receptors (Fukuda, 1994; Mollereau, 1994). Although NOP is a member of the G-protein coupled receptor (GPCR) superfamily with close homology to classical opioid (μ, δ and κ) receptors, native opioid peptides and synthetic agonists selective for μ, δ and κ receptors do not show significant affinity for NOP receptor (Meunier, 1997).

Among the μ, δ and κ and NOP, the highest homology is found in the first extracellular loop, transmembrane domains, as well as the intracellular loops and the carboxyl-terminal domain adjacent to the seventh transmembrane domain (Figure 1). Specific features that are retained in the four opioid receptors are the aspartate residues in transmembrane domains II and III as well as cysteine residues in extracellular loops II and III.

The gene coding for NOP is located on Ch20q13.33 in humans. This gene may be involved in the regulation of numerous brain activities, particularly instinctive and emotional behaviors. A promoter for this gene also functions as a promoter for another gene, regulator of G-protein signaling 19 (RGS19), located on the opposite strand. Two transcript variants encoding the same protein have been found for this gene.

There is also homology among the four receptors at the level of the gene. Conserved intron/exon boundaries are present in the sequences encoding the areas following the first and fourth transmembrane domains. However, unlike the opioid receptors, there is evidence that the NOP is alternatively spliced at these two intron/exon boundaries in various species (Figure 1) (Zaki, 1998).

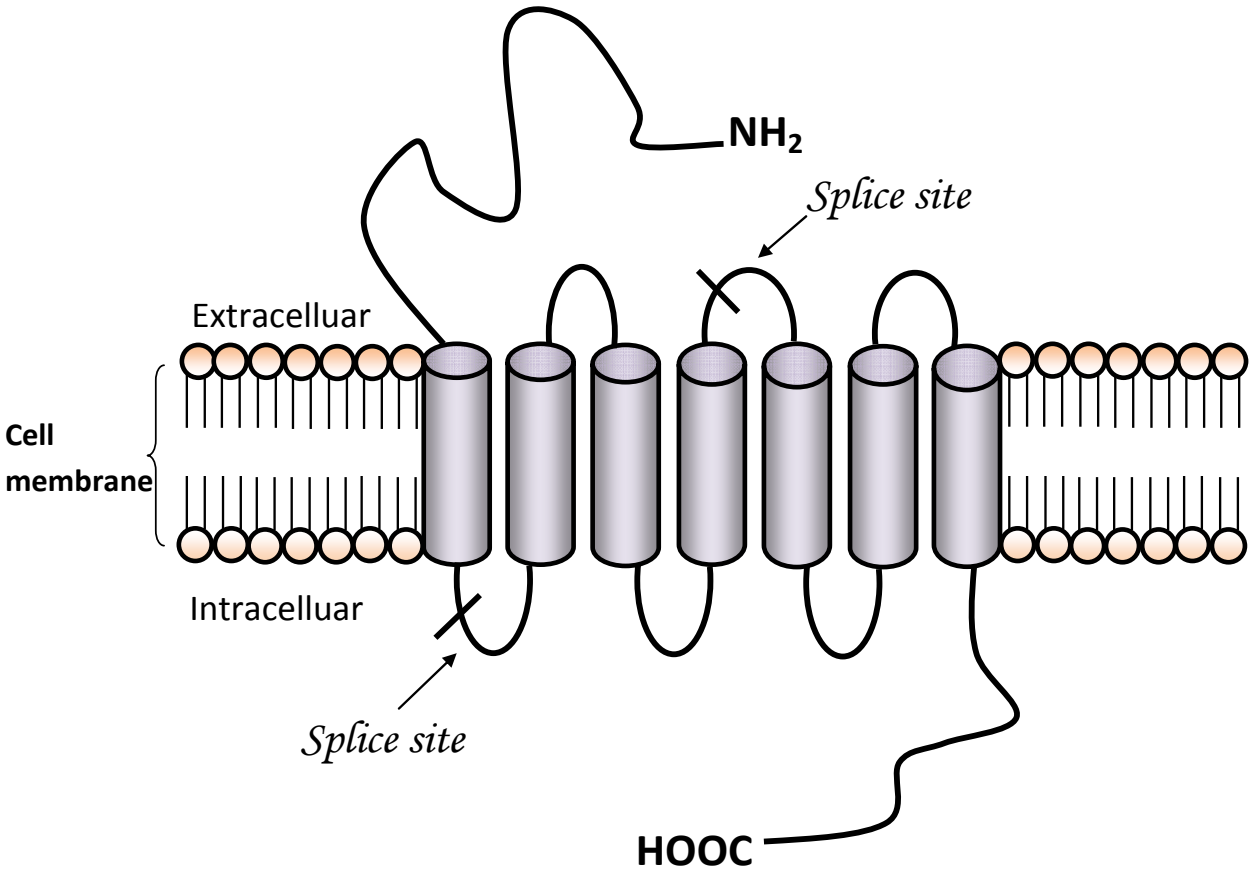

Figure 1 Structure of human nociceptin receptor.

NOP is mainly distributed in the brain and the central nervous system (CNS) (Meunier, 1997; Bigoni, 1999; Mollereau, 2000). Moreover, the presence of NOP receptor mRNA was reported in peripheral organs, such as intestine, skeletal muscle, vas deferens and spleen (Wang, 1994). Aside from the nervous system the immune system is one of the principal locations of the NOP synthesis (Miller, 2007). mRNA Transcripts have been detected in mouse splenic lymphocytes (Halford, 1995). In addition, human circulating lymphocytes and monocytes also express NOP mRNA as well as lymphocytic B and T and monocytic cell lines (Peluso, 1998; Wick, 1995). Recently, it was

shown that NOP mRNA is expressed in both $CD8^+$ T cells and $CD4^+$ T cells isolated from human PBMCs (Arjomand, 2002). Moreover, $CD3^+$ T cells were also able to express N/OFQ when the peripheral blood lymphocytes were activated with the phytohemagglutinin (PHA) mitogen (Arjomand, 2002).

The wide distribution and localization of the NOP mRNA and/or protein indicates that the receptor has the potential to modulate a variety of central processes. The NOP seems to be associated with a large number of physiological responses. It has been observed that NOP is involved in modulating pain mechanisms in the spinal cord and forebrain. Previous in vivo studies with NOP and its peptide analogs have demonstrated that NOP modulates a variety of biological functions, such as feeding, learning, diuresis, drug addiction, cardiovascular function, and locomotor activity and that it controls the release of neurotransmitters including serotonin and dopamine at peripheral and central sites (Mogil, 1996; Calo, 2000; Calo, 2002). Some researchers suggested that NOP may also be relevant in the treatment of CNS disorders including anxiety and drug abuse (Mogil, 1996; Ueda, 1997; 2000; Calo, 2002). Since the functional capacity of NOP was demonstrated by the ability of nociceptin to induce the chemotaxis of immune cells (Serhan, 2001; Trombella, 2005), and the expression of NOP may actually be greater on cells of the immune system (Pampusch, 2000), the receptor seems to play a pivotal role in the immune responses. Moreover, human immune cells also express the precursor protein for nociceptin and prepronociceptin, suggesting the presence of an intact NOP-nociceptin circuit entirely within the immune system (Fiset, 2003). Thus the N/OFQ-NOP system might be an important new molecular target for the treatment of various human disorders. The identification of potent and selective NOP agonists and antagonists could provide new classes of drugs for some human disorders involving pain and anxiety or for the treatment Parkinson's disease (Morari, 2006).

2.2 Nociceptin/orphanin FQ

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand of the NOP. This 17 amino acid protein has been isolated by two independent groups in 1995, and was named nociceptin by Meunier, because of its apparent pronociceptive properties (Meunier, 1995), and orphanin FQ named by Reinscheid to denote its relation to an orphan receptor and to specify its first amino acid F (phenylalanine) and last Q (glutamine) (Reinscheid, 1995). It is derived from the prepronociceptin protein, as are a further two peptides, nocistatin and NocII (Okuda, 1998). The gene coding for prepronoci-

ceptin is located on Ch8p21 in humans (Mollereau, 1996). As a classic neuropeptide, N/OFQ is present as a single copy flanked by basic proteolytic cleavage sites, in a larger, highly conserved, precursor (Meunier, 1995; Mollereau, 1996; Nothacker, 1996; Saito, 1995).

N/OFQ is a novel heptadecapeptide with N-terminal tetrapeptide reminiscent to that of the opioid peptides and strikingly homologous to the endogenous opioid Dyn17, a high-affinity κ-opioid receptor agonist, and BAM18, a high-affinity μ and κ-opioid recptor agonist (Figure 2) (Zaki, 1998). However, it binds to the NOP with high affinity and interacts poorly with the opioid receptors, in part because of the presence of a phenylalanine residue at position 1 of the peptide in place of the tyrosine of opioid peptides (Butour, 1997; Henderson, 1997; Meunier, 1997; Reinscheid, 1995). Furthermore, in vitro and in vivo effects induced by N/OFQ are not reversed by the opioid antagonist naloxone. Up to now, the analog [Phe1c(CH2-NH)Gly2]- nociceptin-(1–13)-NH2 is the only peptide reported to behave as an antagonist at the NOP (Guerrini, 1998). And this peptide has also been shown to have agonist properties both in vitro (Butour, 1998) and in vivo (Grisel, 1998; Xu, 1998).

Figure 2 Structural homology between N/OFQ, dynorphin17 (Dyn17) and BAM18. N/OFQ is strikingly homologous to the endogenous opioid peptides Dyn17and BAM18. The three peptides have a number of basic amino acid residues (red) after a highly conserved amino-terminal sequence (blue) and end with a carboxyl-terminal glutamine residue.

Studies demonstrated that N/OFQ is abundantly expressed in the brain and spinal cord and may have important roles in broad physiological functions, including the nervous system (central and peripheral), the cardiovascular system, the airways, the gastrointestinal tract, the urogenital tract and the immune system (Mogil, 2001; Chiou, 2008; Lambert, 2008). As described in Figure 3, N/OFQ is involved in a wide range of responses and thus has wide potential for drug development.

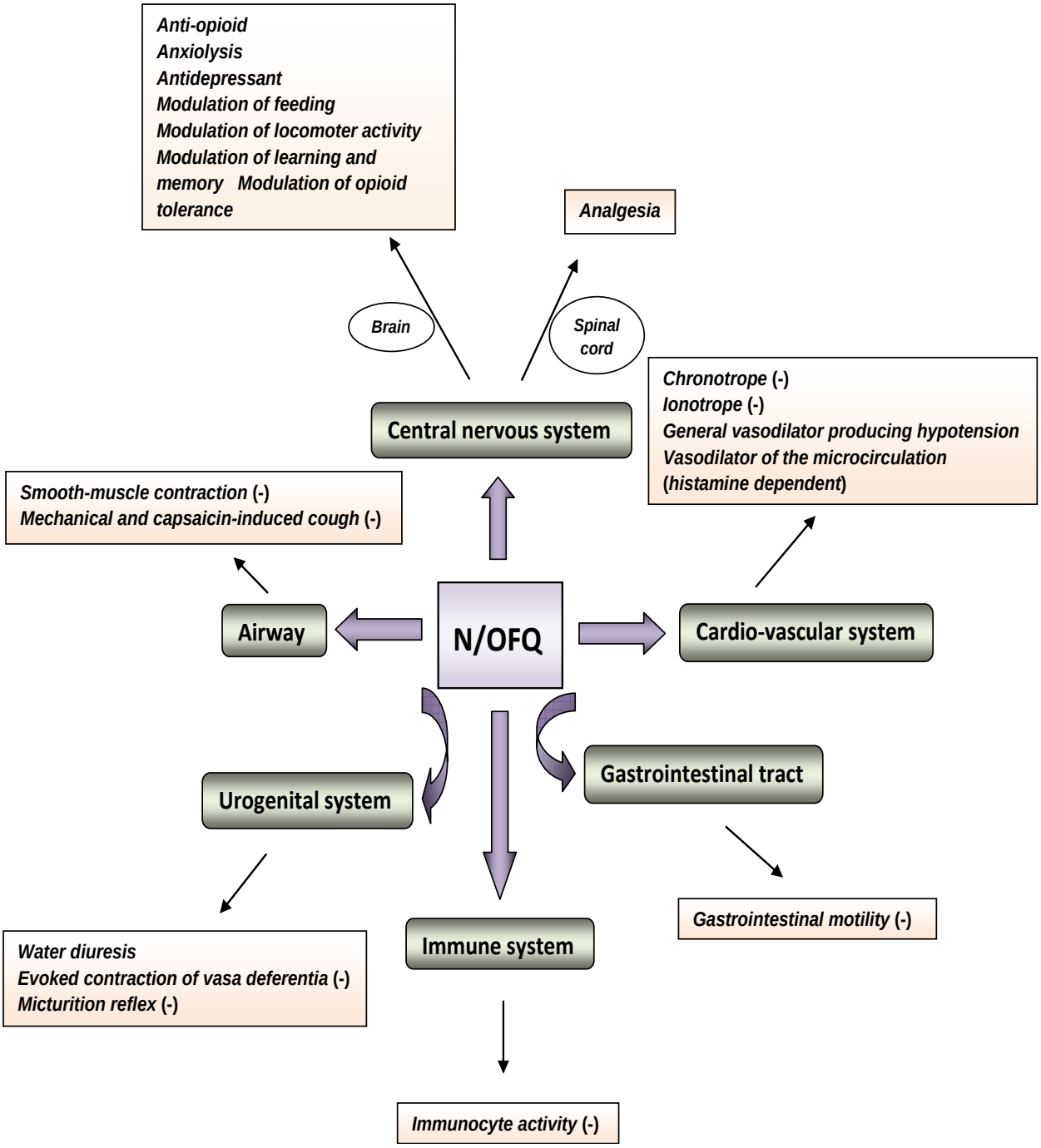

Figure 3 Pleiotropic effects of N/OFQ on major organ system. (-): inhibition

The effects in the nervous system are complex and have received much attention. It was suggested that the spinal N/OFQ is antinociceptive with many features that are common to the classical

members of the opioid family (Zeilhofer, 2003). Whereas, when given supraspinally, it reverses the effects of opioids and induces hyperalgesia (Zeilhofer, 2003). In the brain, this peptide produces hyperphagia and affects the responses to the stress, anxiety and locomotion (Mogil, 2001; Chiou, 2008).

Recently, accumulating evidence demonstrated that outside of the CNS, a major location of N/OFQ synthesis is the peripheral immune system. RT-PCR analyses support the capacity of various immunocyte populations to synthesize mRNA for the N/OFQ (Serhan, 2001). In addition, stimulated human polymorphonuclear cells rapidly secrete N/OFQ by exocytosis, suggesting that the neuropeptide is stored in preformed vesicles (Fiset, 2003). The fact that both NOP and N/OFQ are expressed in the human central nervous system as well as in immune cells at similar levels, indicating that the N/OFQ-NOP system may act as an important mediator of both nervous and immune responses in humans (Peluso, 1998; Serhan, 2001), led to the hypothesis that it is involved in the functioning of the brain-immune axis (Wick, 1995). In recent years, a number of separate lines of evidence showed that the N/OFQ-NOP system is correlated with immune responses, suggesting that NOP and N/OFQ may be potential regulatory elements in the immune system (Lambert, 2008; Waits, 2004). Moreover, NOP knockout prevents the development of colitis, thus indicating a further link between NOP-N/OFQ and the immune system (Kato, 2005).

2.3 Inflammatory mediators

2.3.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major structural feature of gram-negative bacteria and has been found to stimulate various immune cells by increasing their production of cytokines, generation of oxygen free radicals, facilitation of phagocytosis, and chemotaxis (Ziegler-Heitbrock, 1995; Guha, 2001). This potent proinflammatory agent promotes hyperalgesia and pain (Watkins, 1994; Kanaan, 1996; Reeve, 2002). In the immune system, the core components of the most thoroughly characterized LPS receptor complex are CD14, Toll-like receptor 4 (TLR4). Several results suggested that LPS might quickly trigger a cascade of inflammatory events that are self-perpetuating, establishing an activated state that is no longer dependent of LPS. Still other lines of evidence indicated that soluble mediators derived from LPS-stimulated macrophages are responsible for the lethal effect of LPS. In addition, LPS is a potent stimulus for the production of tumor necrosis factor alpha

(TNF-α), interleukin-1beta (IL-1β), interleukin-1ra (IL-1ra), interleukin-10 (IL-10) in the whole blood culture.

2.3.2 Inflammatory cytokines

Cytokines are small proteins produced by most cells in the body, which possess multiple biologic activities that promote cell-cell interaction. Abundant evidence suggests that cytokines play an important role in several physiological and pathological settings such as immunology, inflammation and pain (Benveniste, 1992; Theoharides, 2004). It is well documented that cytokines play a very important role in the immune responses. Furthermore, pro-inflammatory cytokines, such as TNF-α (Kiguchi, 2009; Scholl, 2009) and IL-1β (Li, 2009; Honore, 2009), as well as anti-inflammatory cytokines, e.g. IL-10 (Vale, 2003), have been shown to play a significant role in the modulation of pain threshold and could contribute to trigeminal nervefibers sensitization. LPS stimulates the synthesis and the release of cytokines in the whole blood. During the early hyperinflammatory phase induced by LPS in the whole blood system, maximum concentrations of cytokines such as TNF-α, IL-1β, IL-1ra and IL-10 can be detected (van Crevel, 1999).

2.3.2.1 TNF-α

TNF-α is a pleiotropic cytokine with diverse activities in inflammation, cell activation and migration. It is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. The primary role of TNF is the regulation of immune cells. TNF is also able to induce apoptotic cell death, to induce inflammation and to inhibit tumorigenesis and viral replication. Systemic exposure to TNF-α causes a syndrome of shock and tissue injury. In addition to its well-known role in septic shock, it has been implicated in the pathogenesis of chronic processes. It is the central mediators of immune regulation and of the pathophysiological changes associated with bacteremia and sepsis syndrome.

TNF-α is produced mainly by macrophages, but is also synthesized by a broad variety of other cell types, including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neuronal tissue. Large amounts of TNF are released in response to lipopolysaccharide.

2.3.2.2 IL-1β

IL-1β is a pro-inflammatory cytokines involved in immune defense against infection. It is produced by macrophages, monocytes and dendritic cells and forms an important part of the inflammatory response of the body against infection. IL-1β increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes, the cells that fight pathogens, to sites of infection and re-set the hypothalamus thermoregulatory center, leading to an increased body temperature. The production of IL-1β in peripheral tissue has also been associated with hyperalgesia (increased sensitivity to pain) and with fever (Morgan, 2004).

2.3.2.3 IFN-γ

IFN-γ is a cytokine critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFN-γ in the immune system stems in part from its ability to directly inhibit viral replication, but most importantly derives from its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer cells and natural killer T cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte effector T cells, once antigen-specific immunity develops (Schoenborn, 2007).

2.3.2.4 IL-10

IL-10 is a potent, anti-inflammatory cytokine, which is produced predominantly by activated macrophages and T cells. Originally demonstrated to inhibit cytokine production by macrophages (Fiorentino, 1991), numerous studies have shown that this cytokine plays a critical role in shaping the development of the immune response. It blocks class II major histocompatibility complex expression, inhibiting T helper cell type 1 effector cell development, and decreasing proinflammatory cytokine expression (Donnelly, 1999; Moore, 2001).

It is widely acknowledged that IL-10 plays a central role in down-regulating the inflammatory response through its ability to inhibit macrophage proinflammatory gene expression (Donnelly, 1999). Studies in IL-10 knockout mice have clearly documented overproduction of proinflammatory cytokines and the development of a chronic enterocolitis (Takakura, 2002; Berg, 1995; Kühn, 1993). The overexpression of IL-10 in macrophages results in an “autocrine deactivation” of the cells and in an impaired ability to clear pathogens (Lang, 2002a). Recently, gene expression profil-

ing studies in macrophages have demonstrated a rather limited profile of genes induced in response to IL-10 (Lang, 2002b; Williams, 2002). One of the genes most strongly induced by IL-10 is suppressor of cytokine signaling 3, which, like IL-10, has been implicated in the inhibition of macrophage responses to IFN-γ and IL-6 (Ito, 1999; Lang, 2003).

2.4 Hypothesis

NOP and N/OFQ are involved in a wide range of physiological responses. Recently, accumulating evidence suggested that N/OFQ-NOP system play a role in immunomodulation with the relationship between N/OFQ-NOP system and immune system being bidirectional. The hypothesis of the present study is that inflammatory cytokines are related to the regulatory effect of LPS on NOP and N/OFQ mRNA expression in a human whole blood culture system. So the purpose of this ex vivo study is to:

- Investigate the LPS regulatory effect on NOP and N/OFQ mRNA levels in human whole blood
- Evaluate effects of inflammatory cytokines on NOP and N/OFQ expression
- Determine whether changes in inflammatory cytokines are related to the modulating effects of LPS on NOP and N/OFQ mRNA expression.

3. Material and Methods

3.1 Equipment and Reagents

3.1.1 Equipments

Sterile syringe 5ml, 10ml and 20ml (Becton Dickinson, Germany)

3M Walter paper (Schleicher & Schuell, Germany)

24-well plate (Greiner Bio-One, Germany)

Biofuge fresco (Heraeus Instruments GmbH, Germany)

BioPhotometer 6131 (Eppendorf, Germany)

Butterly-23 (Venisystems)

Centrifugator (Heraeus, Germany)

CO_2 incubator (Heraeus, Germany)

Falcon Tube 15 ml and 50 ml (Becton Dickinson, Germany)

Filter tip (Biozyme, Germany)

Gel Doc 1000 Video Gel Documentation System (Bio-Rad Laboratory, USA)

GeneAmp PCR System 2400 (Applied Biosystems, USA)

GeneMapperTM Software (Applied Biosystems, USA)

Genetic Analyzer Sample Tube 0.5 ml (Applied Biosystems, USA)

Genetic Analyzer Septa for 0.5 ml Sample Tubes (Applied Biosystems, USA)

HP spectrophotometer (Hewlett-Packard, USA)

LightCycler Capillary (Roche, Germany)

LightCycler Carousel Centrifuge (Roche, Germany)

LightCycler Instrument (Roche, Germany)

MagnaPure LC Cooling Block (Roche, Germany)

Microtubes 0.2ml, 1.5ml and 2ml (Biozyme, Germany)

Microwave oven (Siemens, Germany)

Mini-Sub Cell Electrophoresis Systems (Bio-Rad Laboratory, USA)

Model 583 Gel GRYER (Bio-Rad Laboratory, USA)

PAXgene Blood RNA Tube (Qiagen/Becton Dickinson, Germany)

Pipette 10µl, 100µl, 1000µl (Eppendorf, Germany)

Polaroid MP4+ Instant Camera System and Polaroid 667 (Fabrique an Royanme-Uni, U.K)

PowerPac 200 Power Supply (Bio-Rad Laboratory, USA)
Round bottom tube (Greiner Bio-One, Germany)
Thermomixer 5436 (Eppendorf, Germany)

3.1.2 Reagents

10×TBE Buffer (SIGMA, USA)
100% Ethanol (Merck, Germany)
1st Strand cDNA Synthesis Kit (AMV) (Roche, Germany)
Agarose (Biozym, Germany)
Anti-IL-10 mAb (R&D, UK)
Anti-IL-1β mAb (R&D, UK)
Anti-IFN-γ mAb (R&D, UK)
Anti-TNF-α mAb (R&D, UK)
DMEM-medium (Gibco, Germany)
DMSO (Sigma, Germany)
Ethidium Bromide (SIGMA, USA)
Heparin (Ratiopharm GmbH)
HPRT primers (OPERON, Germany)
IFN-γ (R&D, UK)
IL-10 (R&D, UK)
IL-1β (R&D, UK)
IL-6-ELISA-CB Kit (R&D, UK)
IL-10-ELISA-CB Kit (R&D, UK)
IL-1β-ELISA-CB Kit (R&D, UK)
Isotype control IgG1 (R&D, UK)
Kodak X-OMAT 5000RA (Kodak)
LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche, Germany)
LightCycler® TagMan® Master (Roche, Germany)
LPS (SIGMA, Germany)
NOP primers (OPERON, Germany)
Nociceptin primers (TIB MOBIOL, Germany)

PBS 10×buffer (invitrogen, UK)

Penicillin/Streptomycin (Invitrogen, USA)

Photometer (Hewlett Packard)

QIAamp RNA Blood Kit (Qiagen, Germany)

RNeasy® Mini Kit (Qiagen, Germany)

RPMI 1640 (Biochrom, Germany)

SK-N-DZ cell (DSMZ ATCC/LGC, Promochem)

TNF-α (R&D, UK)

TNF-α-ELISA-CB Kit (R&D, UK)

Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany)

Universal ProbeLibrary #73 (Roche, Germany)

UV-Stratalinker (Stratagene)

3.2 Blood donors

30 healthy volunteers (13F/17M) between ages of 26 and 50 years were included in the present study. The study was approved by the local ethics committee and either the participants or a legal custodian gave written informed consent.

Exclusion criteria were:

- Lack of informed consent
- Age younger than 18 years
- Concomitant infectious, neoplasmatic or autoimmune diseases
- Administration of antibiotic or immunostimulating agents for at least 30 days before entering the study
- Pregnancy

3.3 Blood culture

In all experiments, blood samples were drawn from healthy individuals between 07:00 and 08:00 a.m. All experiments were performed using 24 well culture plates. The following stimuli were used: LPS (*E.coli* serotype 0127:B8; Sigma, Steinheim, Germany); TNF-α, IL-1β, IL-10 and IFN-γ (all from R&D system, Germany); anti-TNF-α monoclonal antibody (mAb), anti-IL-1β mAb, anti-IL-10 mAb, anti-IFN-γ mAb and isotype control IgG1 (all from R&D system, Germany). Stimuli were dissolved in RPMI 1640 to the expected concentrations and added shortly before heparinized blood (900µl) into the respective culture wells. After the indicated times of incubation in a humidified atmosphere (95% air/5% CO_2 at 37 ºC), blood was centrifuged at 1500 ×g for 5 min and the supernatant was stored at -80°C until final analysis.

3.3.1 Dose-response effect of different inflammatory mediators

Blood from four healthy volunteers was analyzed for NOP and N/OFQ mRNA expression after co-incubation with various concentrations of TNF-α, IL-1β, IL-10 and IFN-γ for 3 h or LPS for 6 h. The final concentrations ranged from 0.5 to 10^4 pg/ml LPS; 1 to 10 ng/ml TNF-α and IL-1β; 0.5 to 50 ng/ml IL-10; 1 to 20 ng/ml IFN-γ. Quantitative RT-PCR was used for detecting the mRNA expression of NOP and N/OFQ.

3.3.2 Whole blood incubation with different inflammatory mediators

According to the results of dose-response experiments, heparinized blood was stimulated with final concentrations of LPS (10 ng/ml), TNF-α (3 ng/ml), IL-1β (3 ng/ml), IL-10 (50 ng/ml) or IFN-γ (10 ng/ml), respectively. Incubation time varied between 0, 3, 6 and 24 hours. For control purposes, blood samples were simultaneously cultured without any stimulation. Subsequently, at the end of each incubation time, blood was harvested and centrifuged at 1,500 ×g for 5 minutes. The supernatant was stored at -80°C until final analysis.

3.3.3 Intervention study

Blood from 10 volunteers was used for the intervention study. From the results of LPS dose-effect dependency on NOP and N/OFQ, the concentration of LPS 50 pg/ml was used (Figure 9). Neutralizing antibodies to human TNF-α or IL-1β or IL-10 or IFN- γ or TNF-α and IL-1β were added to the whole blood prior to the addition of LPS (50 pg/ml) at a final concentration of 5μg/ml, respectively. In addition, isotype control IgG1 was chosen to estimate the non-specific binding of target antibodies due to fragment, crystallizable (Fc) receptor binding or other protein-protein interactions, according to the immunoglobulin type of the antibodies used in the present study.
To completely antagonize the cytokines induced in LPS-challenged (LPS 50 pg/ml) whole blood neutralizing antibodies to human TNF-α or IL-1β or IL-10 or IFN- γ or TNF-α and IL-1β (all from R&D Systems, UK) were added at final concentration of 5 μg/ml prior to addition of LPS. At the same time blood incubated with LPS (50 pg/ml) and isotype control IgG1 (5 μg/ml) and blood without any stimulation or only treated with LPS was also cultured. NOP and N/OFQ expression were assessed throughout the 24 h culture (0, 3, 6 and 24 h). Blood incubated with isotype control IgG1 and LPS was served as the active control.

3.4 Total RNA isolation

Whole blood was collected at each point of incubation. Total RNA was isolated from whole blood by means of QIAamp RNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then dissolved in diethylpyrocarbonate-treated water and stored at -80°C until further analysis. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer.

3.5 cDNA synthesis

cDNA was synthesized from 8.2 µl (approximately 500 ng RNA) with polymerase chain reaction (PCR) template using 1st Strand cDNA Synthesis Kit (AMV). The reaction mixture was prepared with the components listed below (Table 1).
The reaction was incubated at 25°C for 10 minutes, 42°C for 60 minutes, and 99°C for 5 minutes and then cooled to 4°C for 5 minutes. The cDNA product was stored at -20 ºC for the further analysis.

Table 1 Reaction mix preparation for the cDNA synthesis

Component	**Vol. (µl)**	**Final**
10x Reactions-Puffer	2	1x
25 mM MgCl2	4	5 mM
dNTP-Mix	2	1 mM
Random Primer p(dN)6	2	3,2 µg
RNase Inhibitor	1	50 U
AMV Reverse Transkriptase	0.8	≥ 20 U
RNA	8.2	-
Total volume	**20**	

3.6 Calibrator

3.6.1 SK-N-DZ cell line culture

SK-N-DZ cell line was cultured with DMEM-medium, in which 10% FKS and 1% Penicillin/Streptomycin was added under sterile conditions. Cells were grown in a humidified atmosphere in 95% air/5% CO_2 at +37 ºC.

3.6.2 RNA isolation

Total RNA was extracted from SK-N-DZ cells using of RNeasy® Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instruction, then dissolved in diethylpyrocarbonate-treated water and stored at -80°C until further analysis.

3.6.3 cDNA synthesis

RNA was transcribed to cDNA in 20-µl reaction volume using the Tramscriptor First Strand cDNA Synthesis Kit (Roche, Germany) with Achored-oligo $(dT)_{18}$ primer, 50 pmol/µl following the manufacturer's instructions.

Procedure

Anchored-oligo $(dT)_{18}$ primer was used in the reverse transcription. In each single reaction template-primer mix was prepared with 1 µg total RNA, 0.5 µl Anchored-oligo $(dT)_{18}$ Primer (50 pmol/µl) and variable volume of PCR-grade H_2O to make total volume 13 µl. Subsequently, the template-primer mixture was denatured by heating the tube for 10 min at 65 ºC in a thermal block cycler.

Following component of the RT mix were added to the plate-primer mix (Table 2):

Table 2 RT mix preparation for the cDNA synthesis

Component	Vol. (µl)	Final Concentration
Transcriptor Reverse Transcriotase Reaction Buffer, 5x conc.	4	1x (8 mM $MgCl_2$)
Protector Rnase Inhibitor, 40 U/µl	0.5	20 U
Deoxybycleotide Mix, 10 mM each	2	1 mM
Transcriptor Reverse Transcriptase	0.5	10 U
Total volume	**20**	

Mix the reagents in the tube carefully. Depending on the primer used and the length of the target mRNA, incubate the RT reaction for 60 min at 50 ºC and 5 min at 85 ºC. Then the reaction was stopped by placing the tube on ice and the cDNA products were stored at -20 ºC.

3.7 Quantitative real-time PCR

The relative levels of NOP and N/OFQ mRNAs in cultured whole blood were determined by RT-PCR. NOP, N/OFQ and the house-keeping gene human hypoxanthine phosphoribosyl-transferase (HPRT) were conducted in separate capillaries as duplicates on a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany), with threshold cycles for each sample being compared to a standard curve. The calibrator used in the real-time PCR was cDNA prepared from the SK-N-DZ cell line. Analysis of melting point for each sample revealed the presence of only one amplified product. For each gene of interest, the sample data were expressed in arbitrary units based on the standard curve. Quantitative determination of human NOP and N/OFQ mRNA levels relative to a reference gene and was normalized to the amount of the Calibrator RNA using the LightCycler Relative Quantification Software. Moreover, Amplified PCR products were resolved by electrophoresis in the 1.5% or 4% agarose gel containing 0.5 μg/ml ethidium bromide, and visualized under UV illumination.

3.7.1 Standard curve

In the quantitative RT-PCR experiments, the LightCycler compares the amplification of target nucleic acids in an unknown sample against a standard curve prepared with known concentrations of the same target. The standard samples are amplified in separate capillaries but within the same LightCycler run.

The standard curve is the linear regression line through the data points on a plot of crossing point (threshold cycle) (Cp) versus logarithm of standard sample concentration.

In the present experiments, the concentration chosen for the standard curves matches the expected concentration range of each target. Standard curves were generated from five samples, which were prepared by serial 10-fold dilutions for NOP and HPRT, 2-fold serial dilutions for N/OFQ (Figure 4).

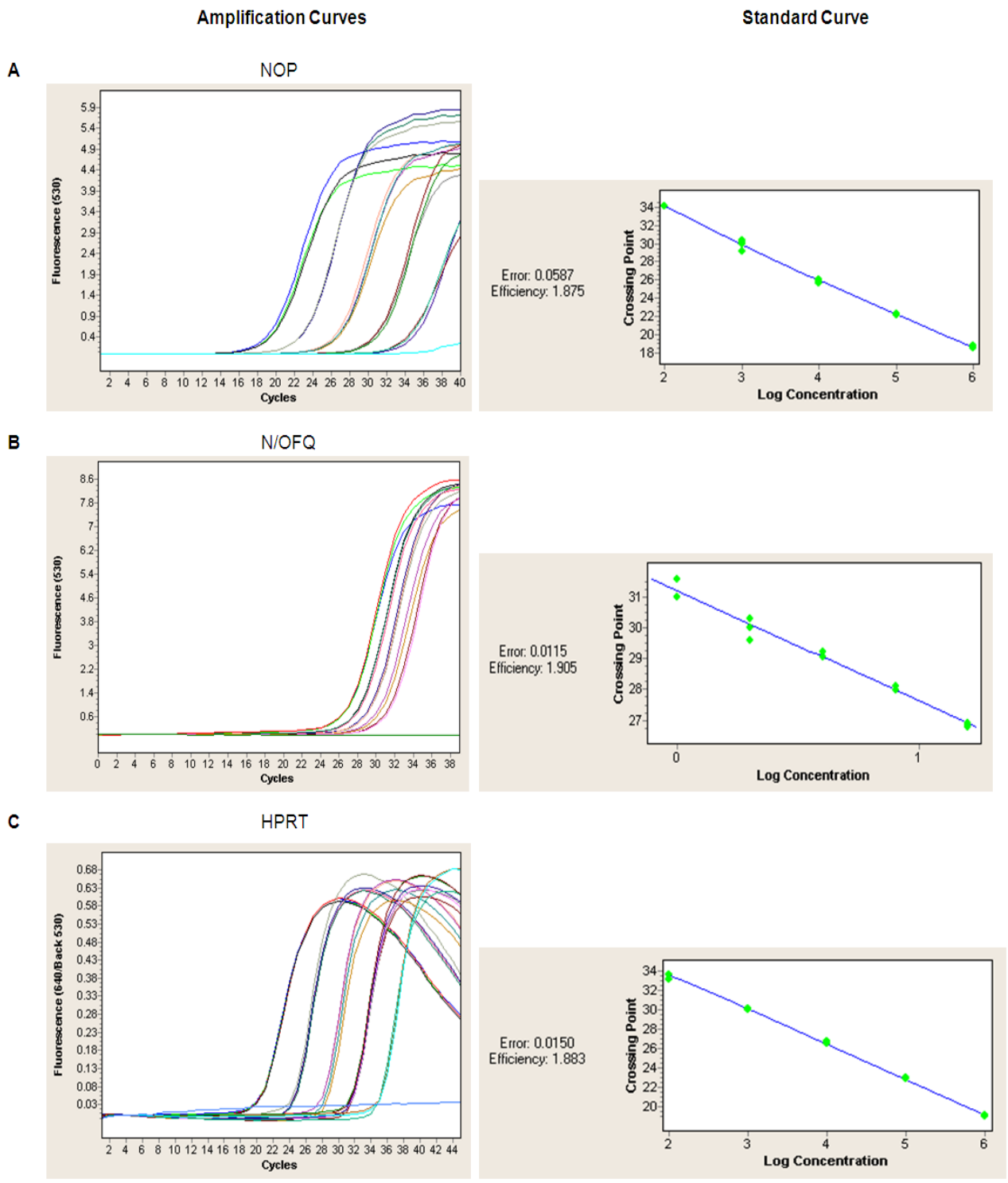

Figure 4 Standard curves for NOP (A), N/OFQ (B) and HPRT (C) were generated from five samples, which were prepared by serial 10-fold dilutions for NOP and HPRT, 2-fold serial dilutions for N/OFQ.

All the samples were tested in triplicate and the average values were used for quantification. The LigheCycler software calculates the slope for each standard curve.
Since the crossing point values of the unknowns will be converted to concentration using the data derived from the standards, the amplification efficiency (E) of the standard and the target must be identical. Since the LightCycler software calculates the slope for each standard curve, the PCR efficiency may be calculated from the following formula:

$$E = 10^{-1/slope}$$

3.7.2 Housekeeping gene

A housekeeping gene is typically a constitutive gene that is transcribed at a relatively constant level. The housekeeping gene's products are typically needed for maintenance of the cell. Housekeeping genes are used as internal standards in quantitative polymerase chain reaction since it is generally assumed that their expression is unaffected by experimental conditions.
HPRT plays an important role in the purine salvage pathways, where it mediates the recycling of hypoxanthine and guanine into the usable nucleotide pools. The HPRT gene is reported as a constitutively expressed housekeeping gene (Pernas-Alonso, 1999). HPRT RNA levels are very low, 1 to 10 molecules per cell (Steen, 1990) which makes it suitable as an endogenous mRNA control in RT-PCR for highly sensitive quantification of low copy or rare mRNAs (Rey, 2000; Specht, 2001; Sellner, 1996). To detect the relative quantification of the NOP and N/OFQ expression in the cultured whole blood, HPRT was used as the housekeeping gene in the present study.

Procedure

The cDNA fragments were amplified for the HPRT with sequencing primers (Table 3).

Table 3 Sequences of primers used in HPRT analyses

Primer	Sequence (5′→3′)	Size of products
HPRT 1 (forward)	TgACCTTgATTTATTTTgCATACC	102 bp
HPRT 2 (reverse)	CgAgCAAgACgTTCAgTCCT	

The PCR reaction mix was performed using LightCycler® TagMan® Master Kit (Table 4)

Table 4 Reaction mix preparation for HPRT gene detection

Reagent	Vol. (µl)	Final
H_2O (PCR grade)	13.4	-
Probe #73	0.2	-
Primer: HPRT 1	0.2	0.2 µM
Primer: HPRT 2	0.2	0.2 µM
LightCycler® TagMan® Master	4	
cDNA	5	-
Total volume	**20**	

Table 5 PCR Program for HPRT detection

Cycle number	Temperature Target (°C)	Hold Time (sec)	Slope (°C/sec)	Acquisition Mode	Program
1	95	600	20	None	Initial denaturation
45	95	10	20	None	Amplification
	60	30	20	None	
	72	1	20	Single	
1	95	0	20	None	Melting curve
	50	15	20	None	
	98	0	0.1	Continuous	
1	40	30	20	None	Cooling

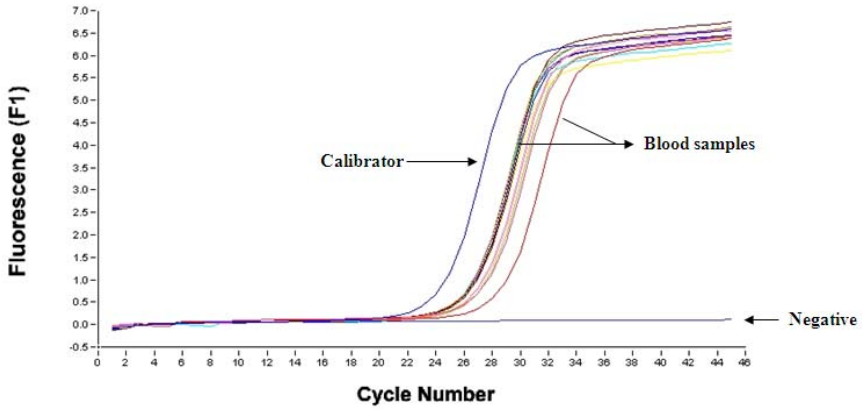

Figure 5 HPRT amplification curves. cDNA from SK-N-DZ cell line was used as calibrator in each PCR run. Samples were cDNAs obtained from blood cells of healthy volunteers.

3.7.3 Target gene

3.7.3.1 NOP

cDNA fragments were amplified for the NOP with the following sequencing primers (Table 6).

Table 6 Sequences of primers used in NOP analyses

Primer	Sequence (5′→3′)	Size of products
NOP 1 (forward)	TgCCgTTCTgggAggTTATCTA	404 bp
NOP 2 (reverse)	TTAgggTgAAggTgCTggTgA	

Procedure

Reaction mix preparation

The PCR reaction was performed using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I. The reaction mixture was prepared as Table 7.

Table 7 Reaction mix preparation for NOP gene detection

Reagent	Vol. (µl)	Final
H_2O (PCR grade)	13	-
Primer: NOP 1	0.5	0.5 µM
Primer: NOP 2	0.5	0.5 µM
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	4	1×
cDNA	2	-
Total volume	**20**	

Table 8 PCR Program for amplification of NOP

Cycle number	Temperature Target (°C)	Hold Time (sec)	Slope (°C/sec)	Acquisition Mode	Program
1	95	600	20	None	Initial denaturation
45	95	5	20	None	Amplification
	60	10	20	None	
	72	20	20	None	
	88	0	20	Single	
1	95	10	20	None	Melting curve
	40	60	20	None	
	99	0	0.2	Continuous	
1	40	30	20	None	Cooling

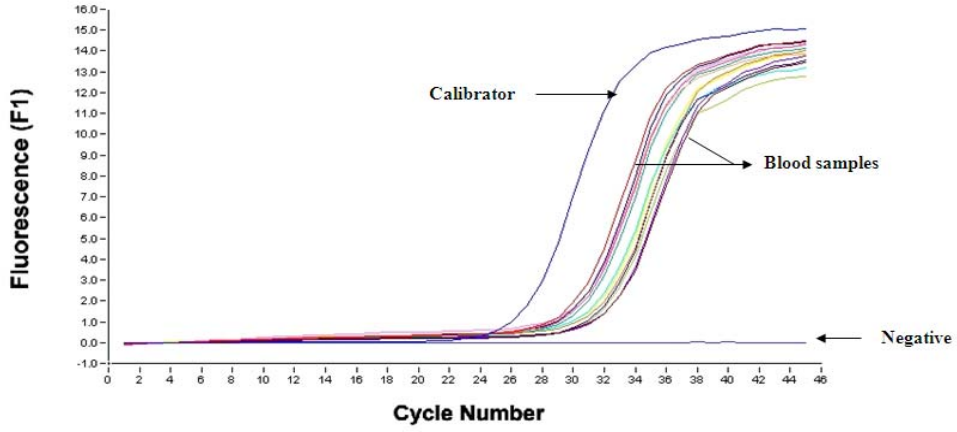

Figure 6 NOP amplification curves. cDNA from SK-N-DZ cell line was used as calibrator in each PCR run. Samples were cDNAs obtained from blood cells of healthy volunteers.

3.7.3.2 N/OFQ

cDNA fragments were amplified for the N/OFQ with the following sequencing primers (Table 9).

Table 9 Sequences of primers used in N/OFQ analyses

Primer	Sequence (5′→3′)	Size of products
Noci 1 (forward)	CCTgCACCAgAATggTAATg	108 bp
Noci 2 (reverse)	gCTgAgCACATgCTgTTTg	

Procedure

Reaction mix preparation

The PCR reaction was performed using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I. The reaction mixture was prepared as Table 10.

Table 10 Reaction mix preparation for N/OFQ gene detection

Reagent	Vol. (μl)	Final
H_2O (PCR grade)	13	-
Primer: Noci 1	0.5	0.5 μM
Primer: Noci 2	0.5	0.5 μM
LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	4	1×
cDNA	2	-
Total volume	**20**	

Table 11 PCR Program for amplification of N/OFQ

Cycle number	Temperature Target (°C)	Hold Time (sec)	Slope (°C/sec)	Acquisition Mode	Program
1	95	600	20	None	Initial denaturation
40	95	10	20	None	Amplification
	60	10	20	None	
	72	5	20	Single	
1	95	0	20	None	Melting curve
	65	15	20	None	
	95	0	0.1	Continuous	
1	40	30	20	None	Cooling

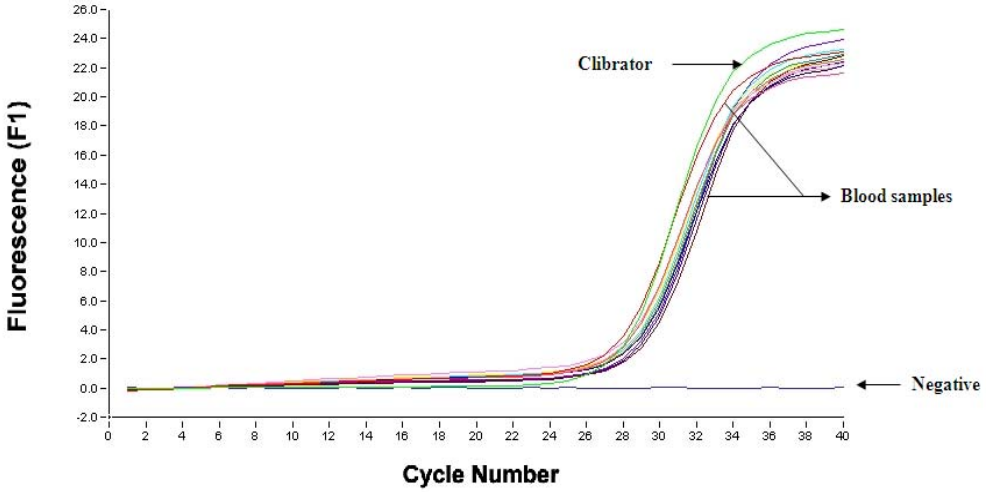

Figure 7 N/OFQ amplification curves. cDNA from SK-N-DZ cell line was used as calibrator in each PCR run. Samples were cDNAs obtained from blood cells of healthy volunteers.

3.7.4 Relative quantification

The relative quantification results were calculated with the LightCycler relative quantification software (Roche). The relative amount of a target and a reference gene is determined for each sample and one calibrator integrated in each LightCycler run. The calculation of the relative amount of any target or reference gene was based on the crossing point of a sample and the efficiency of the PCR reaction. For the calculation of the final result only the Cp-values obtained by the LightCycler analysis software were required.

After the relative ratio of target to reference for each sample and for the calibrator was calculated the target/reference ratio is then divided by the target/reference ratio of the calibrator. Thus, the normalized ratio was calculated with the following formulas:

$$\textbf{Normalized Ratio} = \frac{\text{concentration target (sample)}}{\text{concentration reference (sample)}} : \frac{\text{concentration target (calibrator)}}{\text{concentration reference (calibrator)}}$$

$$\Longrightarrow \qquad \textbf{Normalized Ratio} = E_T^{CpT(C)-CpT(S)} \times E_R^{CpR(S)-CpR(C)}$$

In the present experiments, the coefficient files were created by PCR amplification of NOP, N/OFQ and HPRT in a series of diluted cDNA (relative standard curve) in triplicates. Data of real-time PCR, including calibrator and samples, were imported into the Relative Quantification Software and analyzed with the Fit Coefficients File. NOP and N/OFQ mRNAs expression was calculated with the formula above. The normalized ratios (arbitrary units) calculated directly reflected the expression level of NOP and N/OFQ mRNA.

3.8 Enzyme-linked immunosorbent assay (ELISA)

Supernatants collected after the indicated time points were detected for cytokine concentrations. For determining the concentrations of TNF-α, IL-1β and IL-10 in the plasma, the TNF-α-ELISA-CB Kit, IL-1β-ELISA-CE Kit and IL-10-ELISA-CB Kit (R&D, UK) and the ELISA-Reader (Bio-Rad Lab, USA) were employed.

3.9 Statistics

Statistical analysis was performed using Statistica 6.0 and GraphPad Prism 4. Data were presented as mean±SEM or mean±SD. Results of up to 24 h blood culture with diverse inflammatory mediators and area under the time concentration curve (AUC) for NOP and pN/OFQ expression of the different treatment groups were compared by Mann-Whitney U test. Paired t test was used for comparison of NOP and pN/OFQ expression under co-incubation with different stimulants. Results were corrected for multiple testing. A $p<0.05$ was regarded as statistically significant.

4. Results

4.1 Spontaneous NOP and N/OFQ expression in whole blood

NOP and N/OFQ mRNA expression in peripheral blood cells from healthy volunteers were determined by quantitative RT-PCR. Both, the receptor and its ligand were constitutively detected at the mRNA level in the peripheral blood from all healthy volunteers enrolled in the present study (Figure 8).

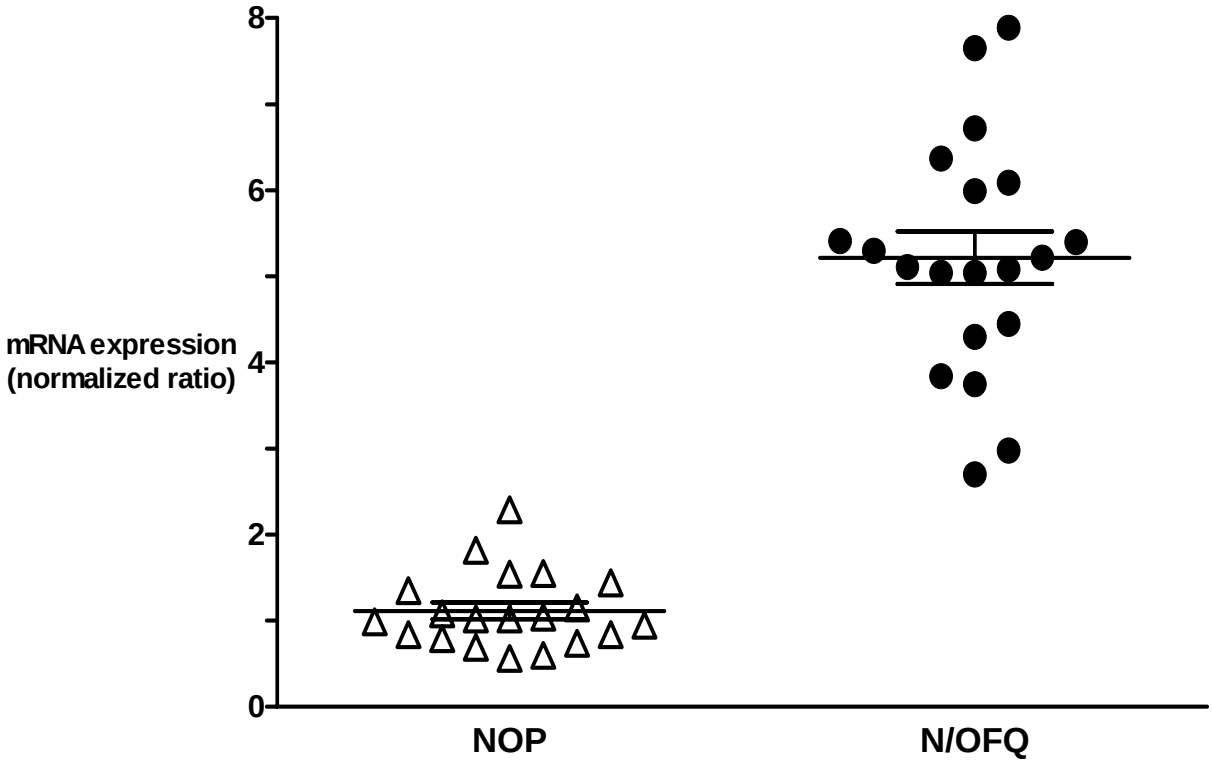

Figure 8: NOP (Δ) and N/OFQ (●) mRNA expression in whole blood from twenty healthy volunteers was detected using quantitative RT-PCR. Bars represent mean±SEM.

4.2 LPS modulated NOP and N/OFQ mRNA expression

4.2.1 LPS Dose-effect

Whole blood was incubated with increasing concentrations of LPS (0.5-10^4 pg/ml) for 6 h. The results showed that NOP and N/OFQ expression was inhibited in the whole blood stimulated by

LPS and the down-regulation was dose dependent. Dose-response analysis showed that the concentration of 10ng/ml was maximally effective for NOP and N/OFQ expression (Figure 9).

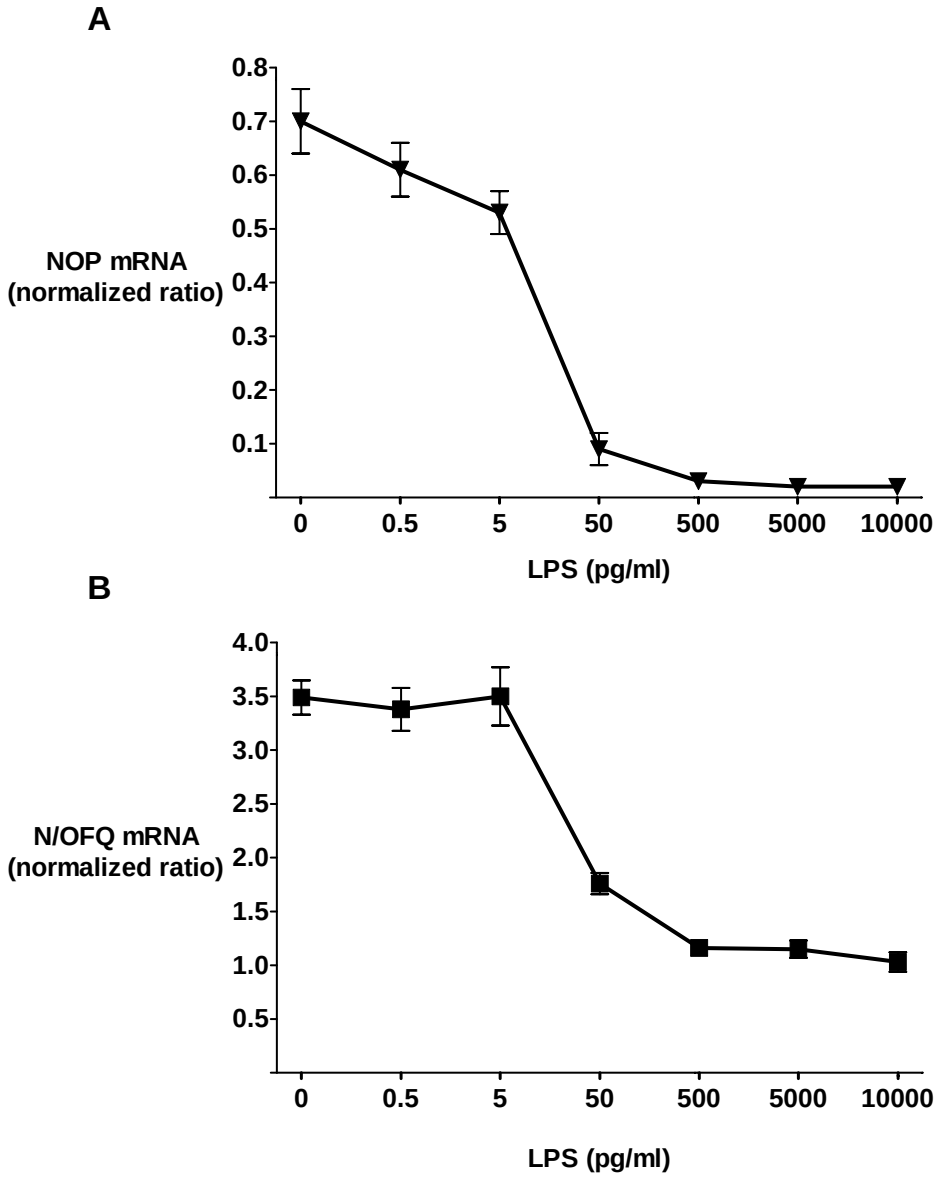

Figure 9: NOP (A) and N/OFQ (B) mRNA expression (mean±SEM) in whole blood from 4 healthy individuals after incubation with increasing concentrations of LPS (0.5 to 10^4 pg/ml) for 6 h.

4.2.2 Time course of LPS effect on NOP and N/OFQ expression

To determine the time course of LPS effect on NOP and N/OFQ expression, whole blood was in-

cubated in the absence or presence of the LPS (10 ng/ml) for 0, 3, 6 and 24 h, respectively. Using quantitative RT-PCR, NOP and N/OFQ expression were detected in all the samples from the respective time points.

Results showed that both of the NOP and N/OFQ mRNAs were expressed constitutively across the indicated incubating time points. The expression of NOP and N/OFQ was decreased in the presence of LPS across time (Figure 10).

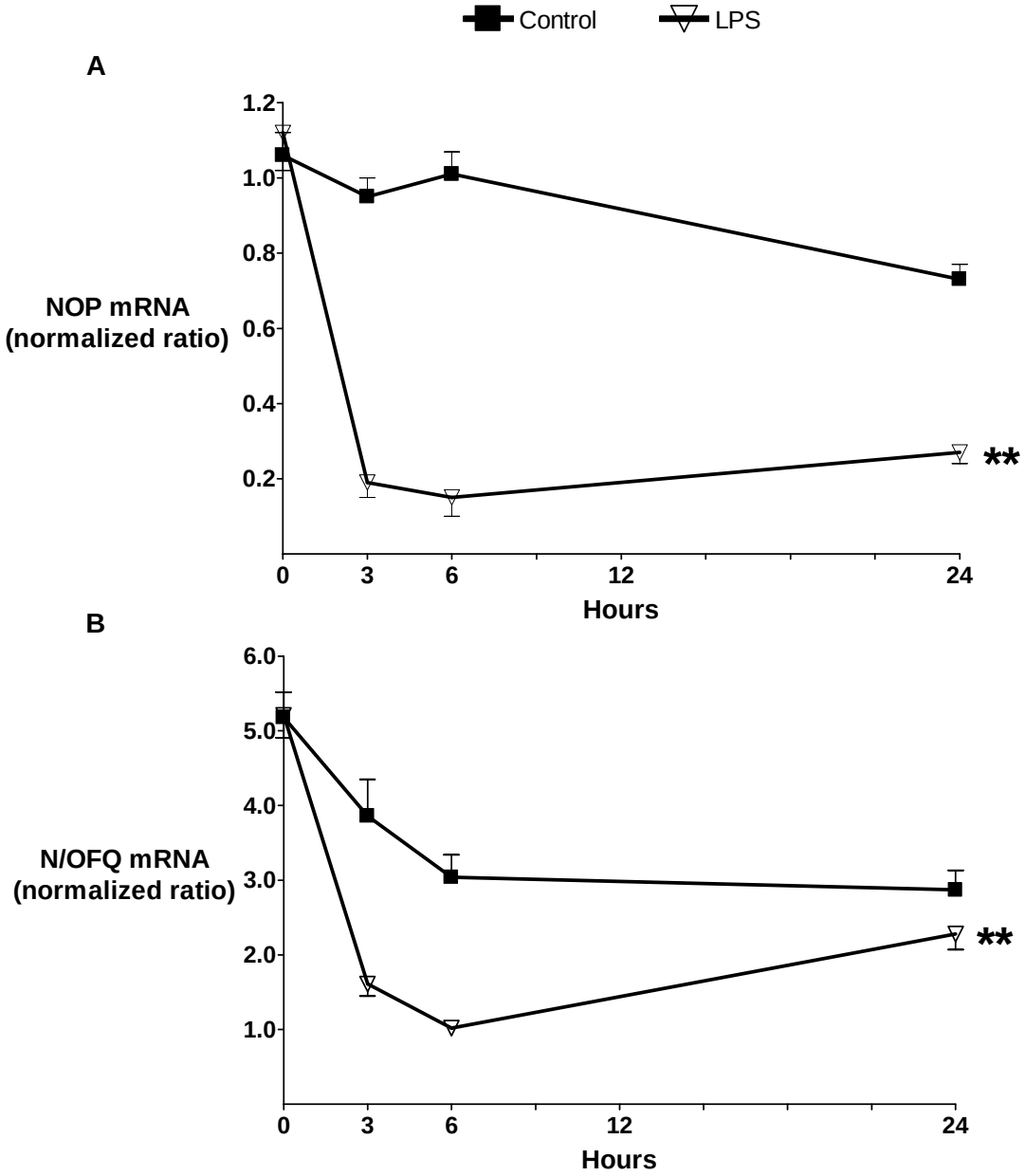

Figure 10 NOP (A) and N/OFQ (B) mRNA expression in whole blood stimulated with LPS 10 ng/ml for 0, 3, 6 and 24 h. Data present mean±SEM (n=20). Whole blood cultured without stimulation served as control. Statistics were analyzed with Mann-Whitney U test, ** $p<0.01$.

The NOP mRNA expression was inhibited further at 3 h and 6 h, compared with 24 h, with each value significantly different compared with the respective control ($p<0.01$, Figure 10A, Figure 11A). For N/OFQ, the expression was down-regulated after incubation with LPS at 3 h and reached the lowest measures at 6 h, with a significant difference compared to the respective controls ($p<0.01$, Figure 10B, Figure 11B). After 3, 6 and 24 h incubation, LPS elicited a 78%, 85% and 60% decrease in NOP mRNA levels, when compare to respective controls. For N/OFQ mRNA expression level a 55%, 62% and 20% suppression were observed at 3, 6 and 24 h compared to respective controls.

Interestingly, self suppression was observed in both of the NOP and N/OFQ expression in the blood samples without any stimulation during the 24 h incubation. This phenomenon was more obvious in the expression of N/OFQ than NOP (Fig 10). Comparing with the samples at the 0 h point, NOP expression in the untreated samples was decreased 10%, 5% and 31% after 3, 6 and 24 h, respectively (Figure 10A). For N/OFQ the respective values were 25%, 41% and 45% (Figure 10B).

4.3 Cytokines production in LPS-challenged whole blood

LPS is a potent stimulus for the production of TNF-α, IL-1β, IL-10 in whole blood and, thus, the concentrations of these cytokines were measured in the supernatants of the blood cultures. Before stimulation as well as after incubation without stimuli, TNF-α, IL-1β and IL-10 were at a very low concentration (TNF-α, IL-1β) or could not be detected (IL-10). After the blood cells were treated with LPS 10 ng/ml, concentrations of TNF-α, IL-1β and IL-10 increased (Table 12).

Table 12 Cytokines induced by LPS 10 ng/ml in whole blood from 20 healthy volunteers. TNF-α, IL-1β and IL-10 (mean±SD) were measured in supernatants after whole blood was incubated at 37°C for 3, 6 and 24 h.

Cytokine (pg/ml)	**Before stimulation**	**LPS stimulated**		
		3 h	**6 h**	**24 h**
TNF-α	1.4±2.84	7078±3404	10754±4941	4321±3437
IL-1β	2.14±4.9	915.5±981.7	6392±3211	7536±3438
IL-10	0	0	201.2±283.2	3279±1242

4.4 Inflammatory cytokines modulated NOP and N/OFQ mRNA expression

4.4.1 Inflammatory cytokines dose-effect dependence

To determine whether the cytokines effect the expression of NOP and N/OFQ and whether there

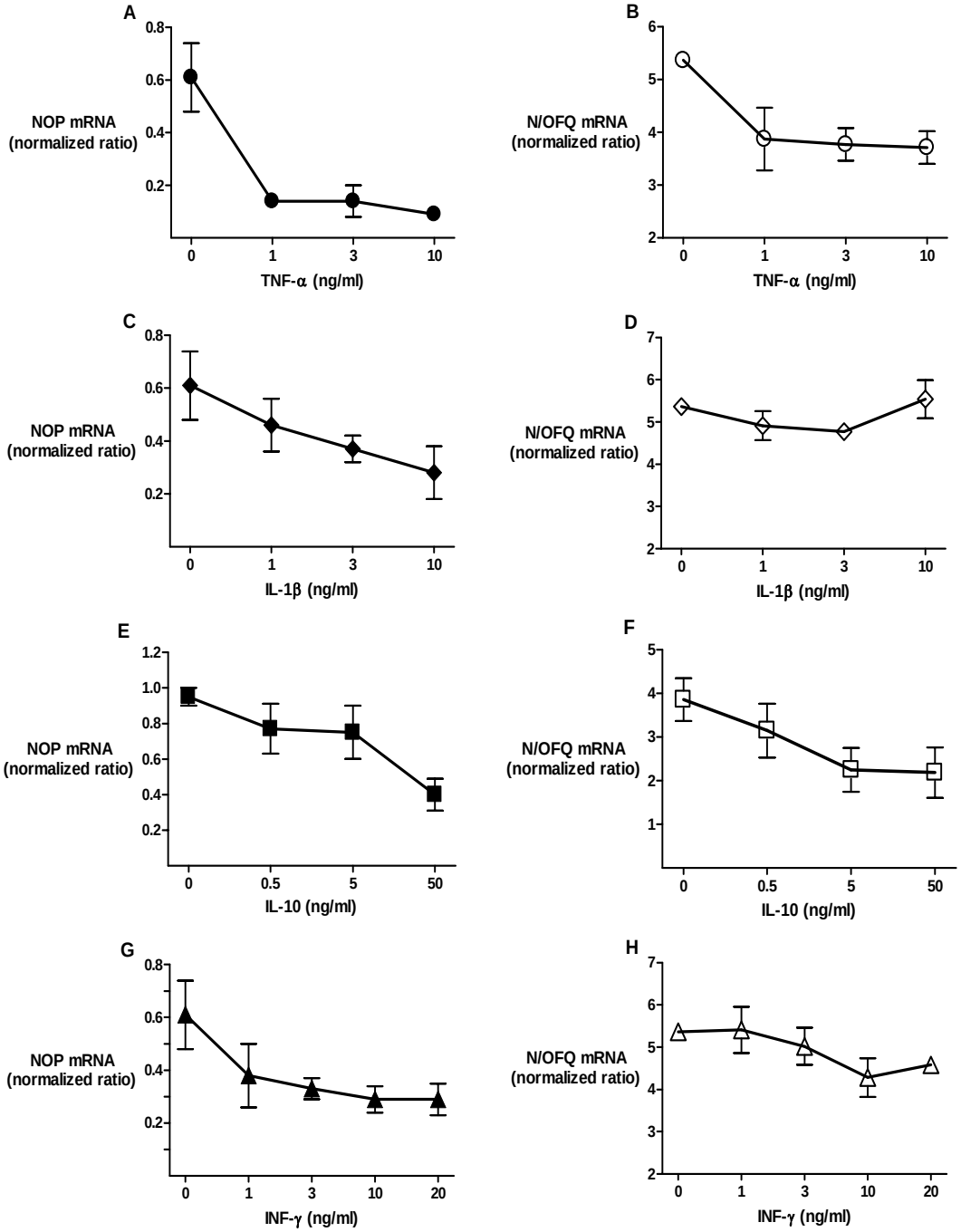

Figure 11 NOP and N/OFQ expression (mean±SEM) after co-incubation with different cytokines for 3 h (n=4). A, B: TNF-α (1-10 ng/ml); C, D: IL-1β (1-10 ng/ml); E, F: IL-10 (0.5-50ng/ml); G, H: IFN-γ (1-20 ng/ml).

is dose-dependent effect, the expression of the receptor and its ligand was investigated in whole blood stimulated with different concentrations of TNF-α, IL-1β, IL-10 and IFN-γ (Figure 11).
Since the previous experiments indicated that LPS down-regulated the expression of NOP and N/OFQ after 3 hours, an incubation time interval of 3 h was chosen for the next series of experiments. The different concentrations for each cytokine were chosen according to previously published data (van Crevel, 1999) and the present ELISA results.
Whole blood from four healthy volunteers was treated with increasing concentrations of TNF-α (1-10 ng/ml), IL-1β (1-10 ng/ml), IL-10 (0.5-50 ng/ml) or IFN-γ (1-20 ng/ml). Results showed that all studied cytokines have a dose dependent effect on NOP and N/OFQ mRNA expression (Figure 12). According to these dose-response curves and the ELISA results, TNF-α 3 ng/ml, IL-1β pg/ml, IL-10 50 ng/ml and IFN-γ 10 ng/ml were used for the subsequent experiments.

4.4.2 Time course of inflammatory cytokines effect on NOP and N/OFQ expression

To further characterize the mediators involved in the regulation N/OFQ and NOP expression, the effects of the inflammatory cytokines TNF-α, IL-1β, IL-10 and IFN-γ was investigated. Culture periods of 0, 3, 6 and 24 h were chosen. Results showed that inflammatory cytokines also modulated expression of NOP and N/OFQ to a varying extent, but less potently than LPS (Figure 12).
After stimulation with different inflammatory cytokines, comparison by Mann-Whitney U test showed a significant decrease of NOP expression for TNF-α ($p<0.001$), IL-10, IFN-γ ($p<0.001$) and IL-1β groups ($p=0.03$) when compared to the control (Figure 12A).

For N/OFQ expression a trend to down-regulation was observed for all the groups treated with diverse inflammatory cytokines, however, level of significance was not met when compared to the control group (Figure 12B).

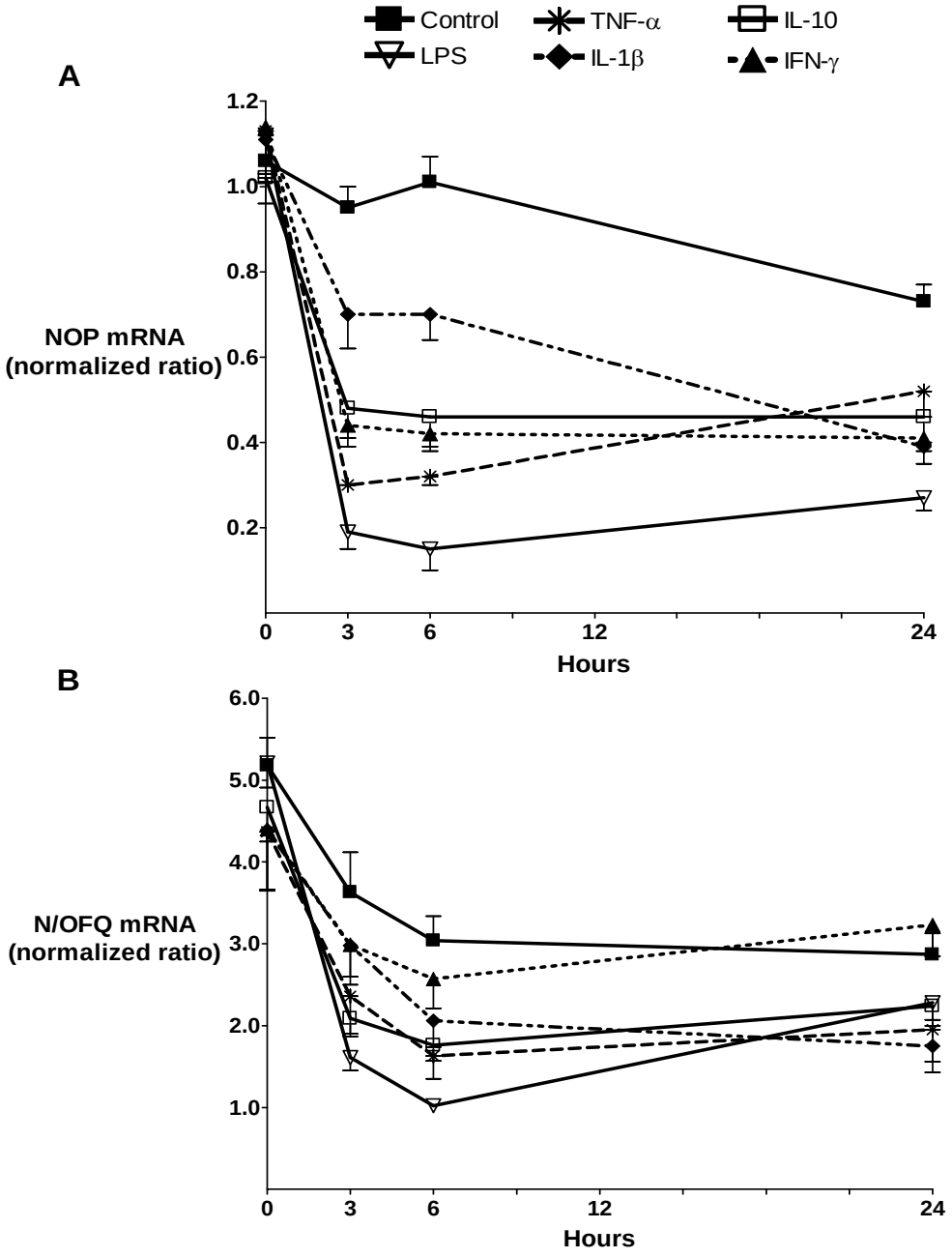

Figure 12 Time course of NOP (A) and N/OFQ (B) mRNA expression (mean±SEM) stimulated with LPS 10 ng/ml (n=20), TNF-α 3 ng/ml (n=7), IL-1β 3 ng/ml (n=7), IL-10 50ng/ml (n=16) or IFN-γ 10 ng/ml (n=6) for 0, 3, 6 and 24 h.

AUCs of NOP and N/OFQ expression for the different treatment groups under stimulations for up to 24 h were calculated. Data are shown in Table 13.

Table 13 AUCs for the course of expression of NOP and N/OFQ (normalized ratio) in a 24 hours blood culture presented as means±SEM.

Whole blood culture	**AUC**[a] ORL expression	**p**[b]	**AUC**[a] N/OFQ expression	**p**[b]
Without stimulation	21.59±1.05		76.06±5.81	
LPS 10 ng/ml	6.26±0.78	<0.001	44.60±3.18	<0.001
IL-10 50 ng/ml	11.98±1.39	<0.001	51.88±4.32	0.018
TNF-α 3 ng/ml	10.58±0.88	0.002	48.38±8.50	n.s.
IL-1 β 3 ng/ml	14.64±1.17	0.032	52.94±7.91	n.s.
IFN-γ 10 ng/ml	11.17±0.59	0.002	71.59±8.89	n.s.

a: AUC: Area under the time concentration curve, b: p values from Mann-Whitney U test, n.s.= no significant

4.5 Blockade of TNF-α and IL-β partly prevented LPS effect on NOP and N/OFQ expression

As the inhibition of NOP and N/OFQ expression was observed both with LPS and diverse cytokines, we asked whether the suppression effect by LPS alone was mediated by endogenous cytokines.

To explore the functional relevance of cytokines in mediating the response of blood cells to LPS, we investigated the effect of anti-TNF-α mAb, anti-IL-β mAb, anti-IL-10 mAb and anti-IFN-γ on the expression of NOP and N/OFQ in the LPS stimulated whole blood.

After a 3 h interval of incubation, the LPS induced deteriorated NOP expression could be partly antagonized by co-incubation of antibodies against TNF-α or IL-1β or both. The NOP expression of anti-TNF-α mAb, anti-IL-β mAb and the combination of these two antibodies was 1.6 ($p<0.001$), 1.3 ($p<0.01$) and 1.7-fold ($p<0.001$) higher compared to that of the isotype control group (Figure 13A).

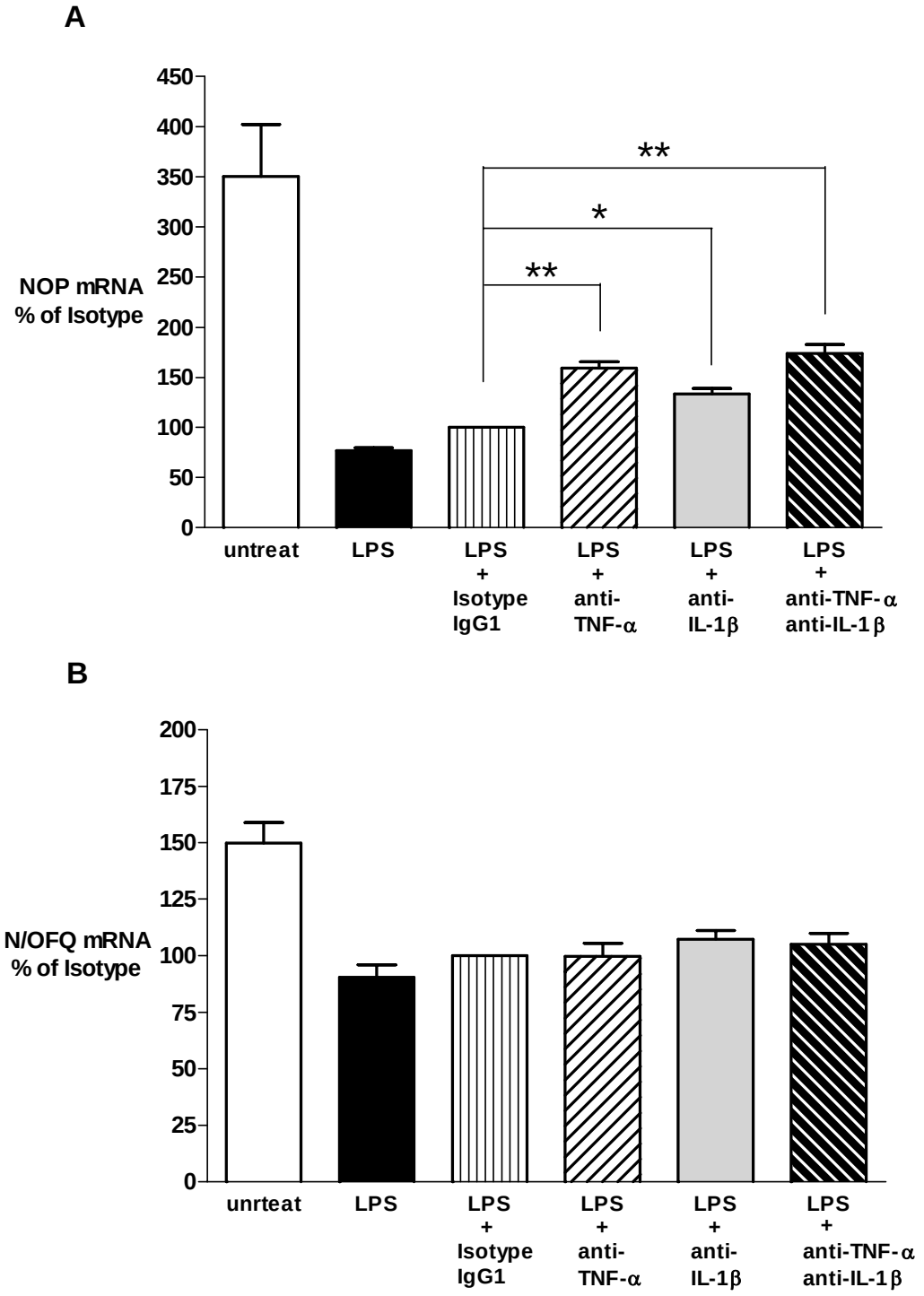

Figure 13 NOP (A) and N/OFQ (B) expression (mean±SEM) co-cultured with LPS 50 pg/ml, LPS + anti-TNF-α mAb 5 μg/ml, LPS + anti-IL-1β mAb 5 μg/ml, LPS + anti-TNF-α mAb 5 μg/ml + anti-IL-1β mAb 5 μg/ml, LPS + isotype IgG1 5 μg/ml or without any additional substances (untreated control) for 3 h. n=10. Paired t-test, * $p<0.01$; ** $p<0.001$.

However, these preventive effects were not observed at the later incubation time points of 6 and 24 hours, respectively (data not shown).

In contrast, for N/OFQ expression, there was only a very weak trend of inhibition of LPS effects at 3 h, without any statistical significance (Figure 13B). For the 6 h and 24 h time points anti-TNF-α and anti-IL-1β mAbs did not display any antagonistic effects on LPS induced down-regulation of N/OFQ expression (data not shown).

For both, anti-IL-10 mAb or anti-IFN-γ mAb, there was no antagonistic effect on LPS mediated down-regulation on NOP and N/OFQ expression observed at each respective incubation time point (data not shown).

5. Discussion

The purpose of the present study was to evaluate the expression of NOP and N/OFQ mRNA in whole blood cultures under inflammatory conditions ex vivo and explore the possible mechanisms involved in the modulation of the NOP system. The present series of experiments demonstrated that both, NOP and N/OFQ expression is modulated by LPS as well as by TNF-α, IL-1β, IFN-γ and IL-10 in this experimental setting. Cytokines are one of the possible factors involved in the regulation on the NOP and N/OFQ expression in the LPS-challenged whole blood.

5.1 N/OFQ-NOP system and inflammation

Although NOP and N/OFQ are mainly distributed in brain and CNS, recent researches explored that the immune system is one of the principal locations aside from the nervous system. Moreover, it is reported that both genes are expressed in the central nervous system as well as in immune cells at similar levels. Therefore, it was suggested that the N/OFQ-NOP system may play an important role in the immune responses. Previous investigations revealed that peripheral blood mononuclear cells transcribe mRNA encoding NOP (Williams, 2007), and the receptor and its ligand are expressed and functional in cell of the immune system (Fiset, 2003). In addition, the NOP protein was detected on the cell surface of all types of white blood cells without any age or sex dependent differences (Krüger, 2006). The ability of immune cells to produce N/OFQ (Fiset, 2003; Williams, 2008a) and the existence of NOP on circulating lymphocytes and monocytes (Wick, 1995; Peluso, 1998; Waits, 2004) suggested an immunomodulatory role of this N/OFQ-NOP system. Administration with N/OFQ dose-dependently induced polymorphonuclear chemotaxis to the injection site and human polymorphonuclear cells secreted N/OFQ rapidly when stimulated with exocytosis (Serhan, 2001), suggesting that the neuropeptide is stored in preformed vesicles and the presence of an intact N/OFQ-NOP circuit entirely within the immune system (Fiset, 2003). Moreover, high initial nociceptin concentrations in non-survivors of sepsis add further evidence to the hypothesis of a considerable up-regulation of the NOP system in inflammatory states (Williams, 2008b).

5.2 Evaluating NOP and N/OFQ expression in whole blood cultures

It was reported that N/OFQ is expressed in human PBMC (Williams 2008a) and the functional relevance of immunological N/OFQ is dependent on expression of functional NOP. Studies showed that NOP transcripts are distributed in human normal circulation monnocytes, lymphocytes, neutrophils and human lymphoid cell lines. NOP mRNA is expressed in both $CD8^+$ T cells and $CD4^+$ T cells isolated from human PBMC's. Moreover, N/OFQ precursor mRNA has been localized to the $CD19^+$ B cell subset of unstimulated human peripheral blood lymphocytes and the $CD3^+$ T cell following activation with PHA.

Up to now, numerous studies focused on the regulation of inflammatory mediators on NOP or N/OFQ expression in nervous system, but seldom investigated the relevant of modulation of NOP and N/OFQ expression in immune responses in whole blood cultures. Examination of the time course of NOP and N/OFQ expression response to individual mediators, such as LPS or cytokines, has not previously been reported in whole blood. In the current series of experiments, LPS and diverse cytokines were studied in ex vivo whole blood cultures. Specifically, it was investigated whether cytokines induced by LPS stimulation accounted for variations in NOP and N/OFQ mRNA expression.

Whole blood culture retains all blood components including serum and maintains the different cell types at their in vivo ratios with non-cellular components in their nature environment. Therefore, stimulating whole blood with inflammatory mediators can mimic the inflammatory condition that is closer to the pathophysiological phenomena of the individual. Furthermore, whole blood culture can be performed immediately after sampling and with small volumes of blood.

The present results demonstrated that both of NOP and N/OFQ are constitutively detected in mRNA levels in the peripheral blood samples from all of the healthy volunteers. This is in accord with the previous study (Williams 2008a). The expression of NOP and N/OFQ was modulated in the whole blood under the inflammatory responses. The finding that the NOP and N/OFQ expression is modulated by inflammatory mediators in whole blood suggests that the N/OFQ-NOP system is involved and might play a role under the inflammatory responses in the whole blood system.

5.3 LPS regulates NOP and N/OFQ expression

LPS is a potent pro-inflammatory agent and its co-administration to whole blood cultures served as a model of a bacterial endotoxin challenge. It has been well documented that the LPS-stimulated whole blood system has been used widely as a model to investigate the production of inflammatory mediators during sepsis (Foca, 1998). The cellular recognition of LPS is initiated by a cascade of three LPS receptors, LPS-binding protein (LBP), CD14 and the Toll-like receptor 4 (TLR4)/MD-2 complex (Triantafilou, 2002). LBP is acutely induced in serum by infection and can extract LPS from the membranes of invading bacteria, or vesicles derived from them (Tobias, 1997). LPS acts as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages. In addition, LPS activates many transcription factors and induces many types of mediators, which also play an important role in the "LPS challenge". It was revealed that transcription factors not only act downstream of signaling cascades related to biological stimuli, but also can be downstream of signaling cascades involved in environmental stimuli.

According to our previous study and the current dose-effect dependency of LPS, the concentration 10 ng/ml for LPS was used to evaluate the modulation of NOP and N/OFQ mRNA expression in response to inflammatory stimuli. The present results showed that both of the NOP and N/OFQ expression was strongly down-regulated by LPS in the whole blood, and it reached the maximum degree of inhibition after the blood cells were treated for 6 hours. In addition, LPS suppressed NOP more than N/OFQ when compared with the untreated blood samples at each incubating time point, respectively. Interestingly, it was observed that both of the NOP and N/OFQ expression in the samples without any treatment was also decreased during the 24 h incubation. And this self suppression was more obvious in the N/OFQ expression crossing the incubation. The possible explanation for the phenomena might be that the metabolism of the cells in the whole blood system also effects the expression of the NOP and N/OFQ. NOP and N/OFQ mRNA expression is effected by multiple factors. In untreated whole blood samples the expression of the N/OFQ might depend more on cell conditions, such as the cell metabolism, cell density and cell quality etc., which may have also influenced N/OFQ mRNA levels and may be responsible for part of this observed variability.

Inconsistent with our results in whole blood cultures, studies using astrocytes (Buzas, 2002) or sensory neurons culture (Acosta, 2008) showed that N/OFQ expression was up-regulated by treatment with LPS. Furthermore, the MAP kinase pathways and NFκB were identified to be the two

major mechanisms by which LPS regulated the expression of N/OFQ in astrocytes. In addition, both of the NOP and N/OFQ mRNA expression was reported to be induced after mice thymus challenged with Staphylococcal enterotoxin A (SEA) in (Goldfarb, 2006) or human PBMC stimulated with PHA (Wick, 1995).

Interestingly, in contrast to the trend in thymus, the amount of splenic mRNA for NOP and N/OFQ was significantly reduced under the challenge of SEA in mice. The author suggested that the inverse relationship was due to variable demands imposed on the N/OFQ-NOP system or may reflect changes in the movement of lymphocytes. Moreover, using the severe stressor model rats, the inhibition of NOP mRNA transcripts in cerebral cortex, hippocampus and hypothalamus was reported (Zhao, 2002). However, experimental conditions in these studies were different from ours and measurement of NOP and N/OFQ mRNA in peripheral whole blood were not performed. In summary, the regulation of NOP and N/OFQ by inflammatory mediators did not show consistent modes in different tissue or cell lines. Overall, data on NOP and N/OFQ are still inconsistent and the bidirectional results, mainly from animal studies and the different culture systems, make it difficult to interpret the present findings.

5.4 Inflammatory cytokines regulate NOP and N/OFQ expression

Cytokines are small proteins produced by most cells in the body, which possess multiple biologic activities that promote cell-cell interaction. Several lines of evidence suggested that cytokines play an important role in several physiological and pathological settings such as immunology, inflammation and pain (Benveniste, 1992; Theoharides, 2004). Earlier studies showed that the elevated cytokines were associated with the hyperalgesic effects of nerve (Ignatowski, 1999) and inflammation (Souter, 2000; Sweitzer, 1999). Some pain conditions were suggested due to the increase of cytokines or the imbalance of the pro- and anti-inflammatory cytokines (Anderberg, 1998; Uçeyler, 2008). Treatment with TNF-α (Watkins, 1995) and IL-1β (Oka, 1996) as well as administration with LPS (Maier, 1993), have all been shown to produce hyperalgesia. Moreover, IL-1 receptor antagonism attenuated the hyperalgesia induced by LPS (Maier, 1993; Oka, 1996), indicating that IL-1 may be one of the mediate factors in the LPS-induced hyperalgesia.

To investigate the effects of cytokines on the expression of N/OFQ-NOP system in the whole blood cultures, blood cells was treated with diverse exogenous cytokines, such as TNF-α, IL-1β, IL-10 and IFN-γ, respectively. The results revealed that inflammatory cytokines caused a decrease

in expression of NOP and N/OFQ from the unstimulated whole blood to varying extent, but less potently than LPS.

5.5 Cytokines involved in the regulation of N/OFQ-NOP system by LPS

It was well known that challenge with LPS caused the induction of the inflammatory cytokines TNF-α, IL-1β, IL-10 and IFN-γ in whole blood. Exogenous cytokines modulated NOP and N/OFQ expression as well as LPS in whole blood culture. In addition, it was revealed that the activation of second messenger pathways by LPS and TNF-α and IL-1β is similar. Given that it is reasonable hypothesis whether the high levels of endogenous cytokines presented in the whole blood cultures challenged with LPS participate in the regulation of N/OFQ and NOP expression.

There was considerable literature demonstrating that the level of circulating TNF increases rapidly in human subjects injected with endotoxin. TNF reaches peak elevation 60 to 90 minute following treatment, and the peak levels of IL-1β occur at s 3 to 4 hours (Cannon, 1990; Michie, 1988). Still other lines of evidence indicated that soluble mediators induced by LPS-stimulated macrophages are responsible for the lethal effect of LPS. Among these mediators TNF-α appears to be the central agent, which is produced earlier than most other cytokines after LPS challenge and is secreted in large amounts by activated macrophages (Cope, 1998; Beutler, 1995). Furthermore, the IL-1 levels are suppressed by anti-TNF therapy suggesting that IL-1 is under the control of TNF in some inflammatory model (Fong, 1989).

Our ELISA results showed that both TNF-α and IL-1β are induced during the 3 h after whole blood is treated with LPS, whereas IL-10 is detected after 6 h incubation. This is in line with the previous studies (van Crevel, 1999). Moreover, the present intervention study further supported the evidence that cytokines are the mediate factors regulating the NOP expression involved in the LPS-stimulated whole blood system. The observation that neutralizing anti-TNF-α mAb and anti-IL-1β mAb partly blockade the suppression effect of LPS on the NOP expression at the early culture time point (3 h) is consistent with the kinetics of the cytokine production induced by LPS. Taken all together, TNF-α and IL-1β are the two main cytokines induced by LPS in the whole blood system at the early inflammatory phase (3 h), which are seems to play a role in inhibiting the expression of NOP during this period. While after the whole blood was cultured for 6 h and 24 h, other inflammatory mediators and a large number of cytokines, including IL-10, IFN-γ and IL-6, are abundantly derived from the blood cells as well as the TNF-α and IL-1β. The net effect often

exceeds the additive effect of each cytokine, when kinds of cytokines are induced by the LPS in the whole blood system. Thus, the observed inhibition of the NOP expression is due to the comprehensive effects of all of the diverse cytokines and other inflammatory mediators in the blood at these time points (6 and 24 h). On the other hand, the partial blockade effects of anti-TNF-α mAb and anti-IL-1β mAb indicated that cytokines are only part of the factors participating in the modulation on the NOP expression in the LPS-induced whole blood.

Interestingly, these neutralizing effects are not observed for the N/OFQ expression, except a weak trend of blockade effect was shown at the 3 h point. However, it didn't reach the statistical significance. According to the present results, the reason of this phenomenon is still not clear at this point. This possibly due to the small sample size enrolled in the current intervention study. In addition, the obvious self suppression of N/OFQ also makes it difficult to estimate its current intervention results. Furthermore, there might be other dominant factors or/and more complex mechanisms involved in the regulation of N/OFQ expression in the LPS-induced whole blood system.

5.6 Relationship between N/OFQ-NOP system and immune system

It has been well documented that the N/OFQ-NOP system acts with important effects in brain and neuronal cells. Recently, numerous studies demonstrated that this system also played an important role in immune function.

Reasearches suggested that N/OFQ modulates several immune parameters including proliferation of human PBMC (Peluso, 2001; Waits, 2004), monocyte (Trombella, 2005) and neutrophil chemotaxis (Serhan, 2001) and mast cell histamine release (Kimura, 2000). In addition, the functional capacity of NOP was demonstrated by the ability of nociceptin to induce the chemotaxis of immune cells (Serhan, 2001; Trombella, 2005). A recent study demonstrated that expression of NOP may actually be greater abundance in cells of the immune system than opioid receptor (Pampusch, 2000). Investigation into the N/OFQ-NOP system suggested a role for NOP as a down-regulator of immune function (Finley, 2007). These studies were based on the observed effects of exogenously applied N/OFQ, whereas the functional significance of endogenous N/OFQ in immune responses is less well understood.

In the current study we addressed the regulation of the expression of endogenous N/OFQ and its receptor NOP in ex vivo whole blood cultures. The results suggested that inflammatory mediators, such LPS and cytokines, suppressed the expression of NOP and N/OFQ in the whole blood system.

LPS was reported to induce N/OFQ in rat astrocytes culture (Buzas, 2002) and sensory neurons (Acosta, 2008). Moreover, the pro-inflammatory cytokines, TNF-α and IL-1β both caused a marked increase in secretion of N/OFQ in splenocytes (Miller, 2007). The activation of the lymphocytes with PHA results in at least a 10-fold induction of the AT7-5EU message in human PBMC, suggesting that NOP may have an important immunological function with the (Wick, 1995). Furthermore, Goldfarb and his co-works found that endogenous and exogenous N/OFQ modulate the cytokine response and mRNA levels of NOP and prepro-N/OFQ were altered in thymus and spleen after SEA challenge (Goldfarb, 2006).

These studies, combined with the results from the present investigations, suggest that the N/OFQ-NOP system can influence immune function and is itself influenced by immune stimuli. The relationship between the N/OFQ-NOP system and the immune system is bidirectional.

6. Prospect

The basic findings of NOP and N/OFQ mRNA in peripheral blood cells have previously been reported. The present study is the first to report changes in the mRNA expression of N/OFQ and NOP as a result of an in vitro immune challenge. The current results indicate that cytokines are those immuno-modulatory factors that regulate the expression of NOP and N/OFQ in the LPS-stimulated whole blood system. At the very least, this reinforces the candidacy of TNF-α and IL-1β as the potential variables attenuating the expression of NOP and N/OFQ during the early inflammatory phase induced by LPS in whole blood.

Our present findings suggest that inflammatory mediators regulate NOP and N/OFQ expression providing some support for the hypothesis that the N/OFQ-NOP system is involved in inflammation. Further work is required to identify to which extent NOP and N/OFQ are involved in the immune function. Further potential mechanisms and pathways influencing the N/OFQ-NOP system via inflammatory mediators have to be investigated. Moreover, the relevance of these findings in immune-related diseases and inflammatory processes like sepsis has yet to be determined.

7. Summary

The nociceptin receptor (NOP) is a member of G-protein-coupled receptor superfamily and possesses overall 60% homology with the classic opioid receptors. Nociceptin/orphanin FQ (N/OFQ), the endogenous ligand for NOP, is involved in a wide range of physiological responses. It shares sequence homology with classical opioid peptides but with a distinct pharmacological profile. Both NOP and N/OFQ were shown to be abundantly expressed in the brain and spinal cord, and may have important roles in broad physiological functions. The fact that N/OFQ and NOP are expressed in the central nervous system as well as in immune cells at similar levels indicates that the N/OFQ-NOP system may act as an important mediator of both nervous and immune responses in humans. It was reported that lipopolysaccharide (LPS) induced N/OFQ expression in the rat sensory neurons and astrocytes cultures. Although numerous animal studies extensively investigated in the expression of NOP and N/OFQ in the nervous system, much less is known about changes of N/OFQ and its receptor expression in human blood cells under the inflammatory condition.

In this experimental setting peripheral blood from 30 healthy volunteers was cultured for up to 24 hours with LPS 10 ng/ml, tumor necrosis factor alpha (TNF-α) 3 ng/ml, interleukin-beta (IL-1β) 3 ng/ml, interleukin-10 (IL-10) 50 ng/ml or interferon-gamma (IFN-γ) 10 ng/ml. NOP and N/OFQ mRNA levels were detected by quantitative RT-PCR. The supernatants of the whole blood culture were harvested and assayed for TNFα, IL-1β and IL-10 concentrations by Enzyme-linked immunosorbent assay (ELISA). In addition, intervention studies with neutralizing antibodies to TNF-α, IL-β, IL-10 and IFN-γ were performed to investigate the mechanism possibly involved in the modulation of LPS on the N/OFQ-NOP system.

Quantitative RT-PCR results showed that both NOP and N/OFQ were constitutively detected at the mRNA level in the peripheral blood. The expression of NOP and N/OFQ was strongly down-regulated by LPS. Inflammatory cytokines, i.e. TNF-α, IL-β, IL-10 and IFN-γ, also attenuated NOP and N/OFQ mRNA levels to varying extents. Blockade of the LPS-induced TNF-α and IL-1β partially antagonized the down-regulating effects of LPS on NOP expression during the early inflammatory phase (3 h).

The present results suggest that both, NOP and N/OFQ expression are modulated by inflammtory mediators. The effects of TNF- α and IL-1β might be related to the regulatory effect of LPS on NOP mRNA expression during the hyperinflammatory phase.

The mechanism that regulates the N/OFQ-NOP receptor system via cytokines and the role of this system in the regulation of immune response need to be further defined.

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