INVESTIGATION OF THE RECEPTORIAL AND MOLECULAR MEDIATORS OF SYSTEMIC INFLAMMATION IN DIFFERENT ANIMAL MODELS

Summary of the Ph.D. Thesis

Eszter Garaminé Pákai

Doctoral School of Clinical Medicine
Faculty of Medicine, University of Szeged
Director: Lajos Kemény, M.D., Ph.D., D.Sc.

Translational Medicine Program
Director: Péter Hegyi, M.D., Ph.D., D.Sc.

Supervisor:

András Garami, M.D., Ph.D.

Department of Thermophysiology, Institute for Translational Medicine
Medical School, University of Pécs

Szeged

2020

Publications related to the subject of the thesis

I. **Pakai E**, Tekus V, Zsiboras C, Rumbus Z, Olah E, Keringer P, Khidhir N, Matics R, Deres L, Ordog K, Szentes N, Pohoczky K, Kemeny A, Hegyi P, Pinter E, Garami A. The neurokinin-1 receptor contributes to the early phase of lipopolysaccharide-induced fever, IMMUNOLOGY 9 pp. 166, 15 p. (2018) **IF: 4.716**

II. **Pakai E**, Garami A, Nucci TB, Ivanov AI, Romanovsky AA. Hyperbilirubinemia exaggerates endotoxin-induced hypothermia, CELL CYCLE 14:8 pp. 1260-1267, 8 p. (2015) **IF: 3.952**

III. Banki E*, **Pakai E***, Gaszner B, Zsiboras Cs, Czett A, Bhuddi PRP, Hashimoto H, Toth G, Tamas A, Reglodi D, Garami A. Characterization of the thermoregulatory response to pituitary adenylate cyclase-activating polypeptide in rodents, JOURNAL OF MOLECULAR NEUROSCIENCE 54:3 pp. 543-554, 12 p. (2014) **IF: 2.343**

IV. Wanner SP, Garami A, **Pakai E**, Oliveira DL, Gavva NR, Coimbra CC, Romanovsky AA. Aging reverses the role of the transient receptor potential vanilloid-1 channel in systemic inflammation from anti-inflammatory to proinflammatory, CELL CYCLE 11:2 pp. 343-349, 7 p. (2012) **IF: 5.321**

V. Garami A, **Pakai E**, Oliveira DL, Steiner AA, Wanner SP, Almeida MC, Lesnikov VA, Gavva NR, Romanovsky AA. Thermoregulatory phenotype of the trpv1 knockout mouse: thermoeffector dysbalance with hyperkinesis, JOURNAL OF NEUROSCIENCE 31:5 pp. 1721-1733, 13 p. (2011) **IF: 7.115**

**Authors contributed equally to this work.*

Number of publications related to the subject of the thesis:	**5**	(3 first author)
Number of publications not related to the subject of the thesis:	**9**	
Number of book chapters:	**2**	
Cumulative impact factor:	**59.865**	

Table of Contents

List of abbreviations

1. Introduction and literature background 5

2. Aims of the study 8

3. Materials and methods 9

4. Results 11

    4.1. The role of bilirubin in systemic inflammation 11

    4.2. The role of transient receptor potential vanilloid-1 channels in thermoregulation under physiological conditions and in systemic inflammation 12

    4.3. Characterization of the thermoregulatory effects of pituitary adenylate cyclase-activating polypeptide 13

    4.4. The role of the neurokinin-1 receptor in systemic inflammatory processes 13

5. Discussion 14

6. Summary and conclusions 17

Acknowledgements 18

List of abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
CLP	cecal ligation and puncture
CNS	central nervous system
COX	cyclooxygenase
GGT	gamma-glutamyl transferase
HLI	heat loss index
i.c.v.	intracerebroventricular(ly)
i.p.	intraperitoneal(ly)
i.v.	intravenous(ly)
IL	interleukin
KO	knockout
LPS	lipopolysaccharide
MnPO	median preoptic nucleus
MPO	medial preoptic area
NK1	neurokinin-1
PACAP	pituitary adenylate cyclase-activating polypeptide
PG	prostaglandin
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SP	substance P
T_a	ambient temperature
T_{ab}	abdominal temperature
T_b	body temperature
T_c	colonic temperature
TNF	tumor necrosis factor
TRPV1	transient receptor potential vanilloid-1
T_{sk}	tail skin temperature
VO_2	oxygen consumption

1. Introduction and literature background

Systemic inflammation is a generalized pathological process, which can be clinically manifested in various forms, including sickness syndrome, systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, septic shock, and multiple organ dysfunction syndrome. The inflammatory response is a series of complex pathophysiological events that can result from infections, as well as from non-infectious causes, such as trauma, burns, and pancreatitis. According to a recent study, sepsis constitutes a global burden for health care, with an estimated 48.9 million incident cases and 11 million sepsis-related deaths worldwide in a year, moreover, it has an incidence rate of ~30% in patients admitted to the intensive care unit. It is important to mention, that sepsis can lead to death not only acutely, but it also increases the risk of death for five to eight years after the septic event. In the United States, the incidence of hospitalizations with sepsis increased by ~50% between 2003 and 2009, with estimated annual costs exceeding $17 billion nationally due in part to the extended hospitalization of septic patients. However, a more recent study describes that the incidence of sepsis and sepsis-related mortality has remained stable in the United States between 2009-2014.

As a systemic inflammation response, sepsis is often accompanied by changes of deep body temperature (T_b), which can be manifested as either fever or hypothermia in experimental animals, as well as in human patients. Based mainly on experimental data from animal studies it was proposed that fever and hypothermia can both develop as two definite adaptive mechanisms in sickness syndrome. Fever typically occurs at the onset of an infection, representing an active fight against the pathogen, while hypothermia is usually associated with progressed stage or severity of the disease and it aims to secure the vital systems of the host. On the one hand, the two adaptive strategies can develop consecutively (e.g., early phase fever followed by late phase hypothermia) as the severity of the disease progresses; on the other hand, hypothermia can be also one of the earliest developing events in animal models of endotoxin shock. A recent meta-analysis on this topic also supported these findings, showing that fever predicts lower, while hypothermia higher mortality rates compared with normal T_b, based on data from over 10,000 septic patients. It has to be noted that the prognostic value of hypothermia in sepsis does not automatically mean a negative effect of T_b itself. Instead, the association between hypothermia and mortality can simply reflect the higher prevalence of hypothermia in severe cases of sepsis. Despite the different pathological background of fever and hypothermia

in systemic inflammation, both the increase and the decrease of T_b are evaluated as significant signs in the clinical practice. In sum, because of its frequent occurrence and high mortality rates, as of today, systemic inflammation represents a highly important field in basic research as well as in clinical practice.

When the inflammation is triggered by an infectious insult, the inflammatory cascade is often initiated by an endotoxin, and then several signals activate the immune response. As a summary, many immune system cells hold receptors for common macromolecules present on invading organisms. For instance, CD14, a human protein expressed mainly by macrophages as part of the immune system, detects and binds lipopolysaccharide [(LPS), the major component of the outer membrane of Gram-negative bacteria] and activates Toll-like receptor 4, which triggers an intracellular multi-step process activating immune responses. In this immune response cytokines are released, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and other mediators, cumulatively referred to as the "inflammatory soup" and either attract other immune cells to the site of the inflammation, or act on other peripheral tissues to release further chemical mediators of inflammation. Among these mediators are the prostaglandins (PGs), which have a crucial role in mediating many aspects of systemic inflammation. PGs are derivatives of arachidonic acid, which is converted to PGH_2 by cyclooxygenase (COX) enzyme, which has two isoforms, COX-1 and COX-2. Various additional enzymes convert PGH_2 to end-products: other PGs (PGE_2 and PGD_2), prostacyclins or leukotrienes. PGE_2 acts on four different EP receptors, which are expressed in different parts of the central nervous system (CNS). In studies conducted on rodents, it has been shown that in response to a low dose of LPS fever develops mainly through the activation of COX-2 and that PGE_2 acts through EP3 receptors as the major mechanism in the development of the febrile response. In contrast with fever, when a high dose of LPS is injected to rats hypothermia develops, which is triggered mainly by the activation of the COX-1 enzyme. Receptorial and intracellular signaling cascades activated by the endogenous mediators are complex and involve lipid, peptide, gaseous, and other - as of today unidentified - messengers.

As common and prominent biochemical part of the inflammatory response, reactive oxygen species (ROS) are also produced. While production of ROS by different cells and tissues challenged with bacterial pyrogens is well documented, a little is known about causal roles of ROS in regulating T_b responses during inflammation and infection, mainly due to the

lack of effective tools for such investigations. Bilirubin, as a lipophilic molecule is generally considered as potentially toxic waste that needs to be excreted. However, some studies highlighted bilirubin as a powerful antioxidant that efficiently scavengers lipoperoxid radicals generated in either artificial or cellular membranes, and emphasizes its cytoprotective role. Hence, bilirubin might be used as a selective tool to inactivate water insoluble, lipid-generated ROS. In addition to ROS, studies have brought attention to the role that the transient receptor potential vanilloid-1 (TRPV1) channel, expressed on sensory neural fibers, may play a role in different inflammatory processes. TRPV1 is activated by diverse stimuli, including several ingredients of the inflammatory soup. Studies using either knockout (KO) (*Trpv1*$^{-/-}$) mice, a pharmacological blockade with capsazepine (TRPV1 antagonist) or desensitization with resiniferatoxin (TRPV1 agonist) have shown that TRPV1 plays an anti-inflammatory role in LPS-induced SIRS by, among other mechanisms, limiting the production of TNF-α, possibly via sensory nerves. However, most studies were conducted in young rodents. Because SIRS is considered a disease of the aged due to its markedly higher incidence in the older population, it is also important to clarify whether TRPV1 channels play a similarly prominent anti-inflammatory role in young and older populations. Performing experiments to elucidate the role of TRPV1 in systemic inflammation also implies the use of genetically modified (*Trpv1*$^{-/-}$) mice, but according to the literature, studies in genetically modified animals have failed to reveal a clear thermoregulatory phenotype in those mice. Therefore, it is essential to first clarify the thermoregulatory phenotype of the *Trpv1*$^{-/-}$ mice under normal conditions and so we could determine whether these mice are suitable for the study of the role of TRPV1 in systemic inflammation.

The activation of TRPV1 channels on neural endings (i.e., on capsaicin-sensitive afferent nerve fibers) also results in the release of an excess of inflammatory substances such as pituitary adenylate cyclase-activating polypeptide (PACAP)38, calcitonin gene-related peptide, and substance P (SP). PACAP38 deserves special attention, because the complex role of this, relatively newly discovered, peptide has been well established in various inflammatory processes. Moreover, the peptide and its receptors are broadly expressed in main thermoregulatory areas of the brain, including the preoptic area of the hypothalamus, which is an important site in the development of the fever response. In physiological studies, injection of PACAP38 into the CNS caused an increase of T_b in rodents, which was brought about by

elevation of non-shivering thermogenesis and increased locomotor activity, but it has remained unclear whether PACAP38 can lead to simultaneous activation of autonomic thermoeffectors, which would be in accordance with a fever-like response. To fill this gap and to study whether the release of PACAP38 from TRPV1-expressing neurons can be regarded as a mediator of systemic inflammation, as a part of our investigation we aimed to characterize the thermoregulatory effects of PACAP38 on deep T_b.

Since the neurokinin-1 (NK1) receptor, formerly known as the SP receptor, also plays an important role in mediation of local and systemic inflammatory processes, SP signaling has been presumed as mediator of fever. When rodents were treated with peptide SP (antagonist) analogs, the fever response to LPS was blocked, and similar attenuation of the LPS-induced fever was observed in rats after administration of NK1 receptor blockers. These studies strongly support that SP signaling contributes to the development of LPS-induced fever, but it has remained largely unknown which mediators of the febrile process are influenced by SP or its receptors. Alternative approaches, such as the use of KO mice, may help to better clarify which step(s) of the classical molecular mechanisms of fever are influenced by the NK1 receptor.

2. Aims of the study

The ultimate aim of the study was to discover new mechanisms, which may play a part in the mediation of systemic inflammation. Since systemic inflammation is often accompanied by alterations in deep T_b, in most experiments we used a thermophysiological approach to explore the contribution of the different investigated mediators to inflammation. To better understand the pathophysiological background of the inflammatory processes, in our study we wanted to investigate the role(s) of endogenous substances (which are physiologically produced in the body) and receptorial mechanisms related to the development of systemic inflammation.

Therefore, because of their established roles in inflammatory processes, in different animal models:

- we focused a part of our study on the role of bilirubin in systemic inflammation;
- we attempted to characterize the role of the TRPV1 channel in thermoregulation under physiological conditions;

- we wanted to elucidate the importance of the TRPV1 channel in aseptic and microbial sepsis;
- we studied the influence of PACAP on thermoregulatory processes;
- and we analyzed the role of the NK1 receptor in inflammatory mechanisms.

Here we provide a summary of the most important aims and findings, while the detailed description of the original studies can be found in the papers that served as the basis of the present work (for list of references, please see page 2). For additional references to the statements in the present excerpt, we refer the Reader to the full text of the Doctoral Thesis.

3. Materials and methods

In our experiments we used adult male Wistar rats, as well as normobilirubinemic (heterozygous, asymptomatic; J/+) and hyperbilirubinemic (homozygous, jaundiced; J/J) Gunn rats. Other studies were conducted in mice of both sexes with the *Trpv1* gene either present (*Trpv1*$^{+/+}$) or missing (*Trpv1*$^{-/-}$); or with the *Tacr1* gene (i.e., the gene encoding the NK1 receptor) homozygously either present (*Tacr1*$^{+/+}$) or absent (*Tacr1*$^{-/-}$); or with the *Pacap* gene homozygously either present (*Pacap*$^{+/+}$) or absent (*Pacap*$^{-/-}$). The animals were housed under standard conditions. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committees.

Surgeries were performed under ketamine-xylazine [55.6 and 5.5 mg/kg intraperitoneally (i.p.), respectively] anesthesia on rats, and under ketamine-xylazine (81.7 and 9.3 mg/kg i.p., respectively) anesthesia on mice. In order to administer substances to rats and mice in a non-stressful manner, the animals were preimplanted with an intravenous (i.v.) or i.p. catheter or with an intracerebroventricular (i.c.v.) cannula. The experiments were performed 4-7 days after the surgeries.

To record abdominal temperature (T_{ab}, a measure of deep T_b) and gross locomotor activity in freely-moving mice, a miniature telemetry transmitter (G2 E-Mitter series; Mini Mitter) was implanted. Mice were then placed on top of the receivers in the telemetry setup (see below) in their home cages.

For cecal ligation and puncture (CLP), the cecal wall was punctured through at the antimesenteric side with a needle. The survival rate and T_b were monitored for 108 h, at the end of experiments, any survivors were euthanized.

We used three different experimental setups to measure the various thermophysiological parameters of rats and mice under physiological conditions and in forms of systemic inflammation. In the thermocouple setup, the animals were placed in a confiner and equipped with two copper-constantan thermocouple (Omega Engineering) to measure colonic temperature (T_c) and tail skin temperature (T_{sk}). The animal in its confiner was then placed in an environmental chamber (Forma Scientific) set to a neutral ambient temperature (T_a).

In the the respirometry setup, each animal in its confiner was transferred to a Plexiglas chamber of the four-chamber open-circuit calorimeter integrated system (Oxymax Equal Flow, Columbus Instruments, Columbus, OH, USA). In another setup, animals in their confiners were placed inside a sealed cylindrical Plexiglas chamber (Sable Systems). The Plexiglas chambers were continuously ventilated. The rate of oxygen consumption (VO_2) was calculated by comparing the oxygen fraction in the air exiting the chamber occupied by an animal to the oxygen fraction in the air exiting an empty chamber.

In the telemetry setup, mice were studied inside their home cages. The home cages of mice preimplanted with a telemetry transmitter were placed on top of the telemetry receivers (model ER-4000; Mini Mitter) in order to measure T_{ab} and locomotor activity in freely-moving animals.

To induce SIRS in experimental animals, LPS was injected in a bolus (10 or 1,000 µg/kg i.v. for rats; 0.12 or 40 mg/kg i.p. for mice) through the extension of the i.v. or i.p. catheter. AMG 517, a highly potent and selective TRPV1 antagonist, (210 µg/kg subcutaneously) or its vehicle was administered as a bolus, 1 h before the administration of the pyrogen agent or saline. PACAP38 was delivered at final doses of 100 µg/kg i.v. and at 10 and 100 µg/kg i.c.v. for rats. SP was infused into the lateral ventricle at a total dose of 100 µg/kg.

Levels of total bilirubin and markers of renal dysfunction and hepatocyte damage were determined in blood samples collected by cardiac puncture from rats 24 h after LPS injection. Cell counts positive for c-Fos were determined by immunohistochemistry in the median preoptic nucleus (MnPO) and medial preoptic area (MPO) of the *Pacap*$^{-/-}$ and *Pacap*$^{+/+}$ mice.

In order to determine LPS-induced changes in COX-2, lung, liver, and brain tissue samples were collected, and then snap frozen in liquid nitrogen for RT-qPCR and Western blot. All tissue samples were stored at -80°C.

For statistical analyses, the following tests were used, as appropriate: two- or three-way ANOVA followed by Fisher's LSD post hoc tests, Student's t test, the $\chi 2$ test, the logrank test, and the Cox proportional hazard survival regression model. The effects were considered significant when $P<0.05$. All data are reported as mean ± standard error.

4. Results

4.1. The role of bilirubin in systemic inflammation

To the low dose (10 μg/kg) of LPS, both J/J and J/+ rats responded with a triphasic fever with peaks at ~50, 130, and 280 min post-injection, however there were no significant differences in the T_b dynamics between the genotypes. To the high dose (1,000 μg/kg) of LPS, J/+ rats responded with early hypothermia followed by fever. A different T_b response of J/J rats was observed to the high dose of LPS, the early hypothermic response was remarkably exaggerated, and the subsequent fever response was significantly attenuated.

The total plasma bilirubin level in saline-treated J/J rats was significantly higher (by 2 orders of magnitude) than that in J/+ rats, moreover the low dose of LPS did not affect the total bilirubin level in either genotype. However, in response to the injection of the high dose of LPS, the total bilirubin level elevated in both J/+ and J/J rats, while in J/J rats it has still remained higher than in J/+ controls.

As markers of renal function, neither blood urea nitrogen nor creatinine levels differed significantly between saline-treated J/J and J/+ rats, or after administration of the low dose and the high dose of LPS. Activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) were within the normal range in J/J and J/+ rats after the injection of saline or the low dose of LPS. When LPS was administered at the high dose, rats showed marked rises in ALT, AST, and GGT. In J/J rats, the surge was blunted for GGT and tended to be reduced for ALT and AST, as compared to J/+ rats.

4.2. The role of TRPV1 channels in thermoregulation under physiological conditions and in systemic inflammation

In order to determine the role of TRPV1 channel in thermoregulation we studied circadian fluctuations in deep T_b and thermoeffector activities of a large number of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice in our telemetry and respirometry experimental setups. In both setups, the light-phase mean deep T_b of *Trpv1*$^{-/-}$ mice was slightly, but significantly ($P<0.05$) lower than that of the controls. During the dark (active) phase, however, the intergenotype T_b difference was attenuated in the respirometry setup and completely disappeared in the telemetry setup. It has been revealed, that *Trpv1*$^{-/-}$ mice maintained a lower metabolic rate and showed a more pronounced cutaneous vasoconstriction than their *Trpv1*$^{+/+}$counterparts, regardless of the phase. In summary, *Trpv1*$^{-/-}$ mice have a thermoregulatory phenotype that shows a specific thermoeffector pattern: these mice are hypometabolic and vasoconstricted while maintain lower T_b as compared to control mice, therefore this animal model is suitable to investigate the role of TRPV1 in systemic inflammation.

Young adult mice responded to LPS (40 mg/kg, i.p.) with a marked, rapidly progressing SIRS. Pretreatment with AMG517, a potent and selective TRPV1 antagonist, profoundly decreased the survival rate at multiple time points (e.g., from 50% to 5% at 26 h, $P<0.001$) and also shortened the mean time to death (from 26±2 to 19±1 h, $P=0.003$). Overall, the results of our experiment show that pharmacological blockade of TRPV1 increases mortality of young mice in LPS-induced SIRS. The outcome of LPS-induced SIRS in older mice was more severe than in young mice. Pretreatment of aged mice with AMG517 increased the survival rate, delayed the mean time to death and decreased the risk of mortality, which effects are directly the opposite of those observed in the young mice.

Then, we tested whether genetic deletion of TRPV1 would have the same effects on SIRS in middle-aged mice as a pharmacological blockade. LPS caused death in all wild-type control mice but only in 80% of *Trpv1*$^{-/-}$ mice. Genetic deletion of TRPV1 channels decreased the risk of mortality and tended to delay death. Importantly, *Trpv1*$^{-/-}$ mice exhibited a 70% suppression of the TNF-α response at 12 h post-LPS.

We also observed that CLP sepsis caused substantial mortality in aged mice of both genotypes, however, *Trpv1*$^{-/-}$ mice died significantly faster than the *Trpv1*$^{+/+}$ controls. The mean

time to death in *Trpv1*$^{-/-}$ mice was 20±2 h, compared to 52±11 h in *Trpv1*$^{+/+}$ mice ($P<0.01$), and the survival rate in *Trpv1*$^{+/+}$ mice was 3.9 times higher than in *Trpv1*$^{-/-}$ mice (86% vs. 22%, $P<0.001$).

4.3 Characterization of the thermoregulatory effects of PACAP

To identify the thermoregulatory effect of PACAP38 in details, we infused 10 or 100 μg/kg of the peptide (or saline) into the lateral cerebral ventricle of rats and recorded their T_c, T_{sk}, and VO_2 in our respirometry setup. Both of the applied doses of PACAP38 caused a marked rise in the T_c starting already at 10 min after the injection. The magnitude of the PACAP38-induced hyperthermia was dose-dependent. In the case of the higher dose, it was accompanied by significant tail skin vasoconstriction [as indicated by a decreased heat loss index (HLI); $P<0.05$] and elevated VO_2, reaching a maximal rise of 21±6 and 14±6 ml/kg/min at 100 and 10 μg/kg, respectively ($P<0.001$ for both). Our results demonstrate that simultaneous immediate activation of both autonomic cold-defense thermoeffectors (cutaneous vasoconstriction and brown adipose tissue thermogenesis) contribute to the development of hyperthermia in response to PACAP38. When PACAP38 at 100 μg/kg was infused i.v., both the maximum T_c elevation and the duration of the hyperthermia were substantially less than what i.c.v. administration of the same dose evoked ($P<0.05$), indicating that the site of action for the thermoregulatory response to PACAP38 is situated within the CNS.

Pacap$^{-/-}$ mice were more active than their wild-type littermates during both phases of the day ($P<0.001$). During most of the light phase, the increased locomotor activity resulted in a slightly higher T_{ab} in the KO mice compared to controls ($P<0.05$); but in the night, there was no significant difference in T_{ab} between the groups. The basal VO_2 appeared to be significantly lower in *Pacap*$^{-/-}$ mice as compared to controls in the respirometry setup ($P<0.001$). With immunohistochemistry, we have not found statistical difference in the number of c-Fos-positive cells between genotypes in the MnPO, however, c-Fos expression was nearly three times higher ($p<0.05$) in the MPO of the *Pacap*$^{-/-}$ mice as compared to controls.

4.4. The role of the NK1 receptor in systemic inflammatory processes

When treated with LPS (120 μg/kg, i.p.), the mice of both genotypes developed fever as compared to saline-treated groups. In *Tacr1*$^{-/-}$ mice, the LPS-induced fever response was less pronounced, reaching the level of significance at 40-140 min compared to controls ($P<0.05$).

The LPS-induced increase in deep T_b was generated by an elevation of VO_2, changing with parallel dynamics as T_b in both genotypes. In *Tacr1*$^{-/-}$ mice the LPS-induced elevation of the T_b and VO_2 were markedly suppressed compared to their *Tacr1*$^{+/+}$ littermates starting from 40 min post-LPS infusion until the end of the experiment; both parameters were significantly attenuated at 40-120 min ($P<0.05$ for intergenotype difference). Our results demonstrate that LPS-induced fever is attenuated in *Tacr1*$^{-/-}$ mice already in the early stage.

In response to PGE_2 administered i.c.v., in the mice of both genotypes a marked elevation in deep T_b and VO_2 rapidly developed, but there was no attenuation in either the T_b or VO_2 rise in the *Tacr1*$^{-/-}$ mice compared to their *Tacr1*$^{+/+}$ littermates. The administration of LPS resulted in a massive rise of TNF-α ($P<0.001$ vs. saline for both genotypes) and IL-6 ($P<0.01$ for *Tacr1*$^{+/+}$ mice and $P<0.001$ for *Tacr1*$^{-/-}$ mice), but we did not find any significant intergenotype difference in the TNF-α and IL-6 levels. LPS caused transcriptional upregulation of COX-2 mRNA in the lungs, in the liver, and in the brain of both genotypes ($P<0.001$ vs. saline for all three tissues) without any significant intergenotype difference. In *Tacr1*$^{+/+}$ mice, the administration of LPS resulted in a marked increase in COX-2 protein expression in the lungs ($P<0.01$ vs. saline) and in the liver ($P<0.05$), but not in the brain ($P=0.264$). In LPS-treated *Tacr1*$^{-/-}$ mice, the COX-2 protein expression did not change significantly in either the lungs or the liver compared to saline treatment. In LPS-treated mice, the COX-2 protein expression was attenuated in the lungs of *Tacr1*$^{-/-}$ mice compared with their controls presenting the gene ($P<0.01$), while in the liver only a tendency could be seen for reduced COX-2 expression in *Tacr1*$^{-/-}$ mice ($P=0.101$). These findings indicate that the absence of the NK1 receptor interferes with the increase of COX-2 expression at the protein level.

5. Discussion

We identified novel mechanisms which contribute to the modulation of systemic inflammatory processes.

We showed that hyperbilirubinemia in J/J Gunn rats was associated with a marked exaggeration of the early hypothermic response to the high dose of LPS, supposedly through a direct inhibition of nonshivering thermogenesis by bilirubin and possibly also through a direct vasodilatory action of bilirubin in the skin. This novel, hypothermia-exaggerating effect might

be responsible, at least in part, for the observed tendency of J/J rats to respond to the high dose of LPS with attenuated hepatic damage. Hyperbilirubinemia in Gunn rats was also associated with a deep attenuation of the late febrile response to the high dose of LPS, but did not attenuate the fever response to the low dose. The attenuation of the fever response to high dose of LPS could be due to either direct actions of bilirubin on thermoeffectors (inhibition of nonshivering thermogenesis and induction of skin vasodilation) or the ROS-scavenging action of bilirubin attenuating pyrogenic signaling. However, the experiments with the low dose strongly suggest that ROS signaling is not involved in the fever response to low doses of LPS. For details, please see the original study by Pakai et al. 2015 (reference II. in the list of publications related to the subject of the thesis on page 2).

We described that *Trpv1* KO mice possess a distinct thermoregulatory phenotype, which includes hypometabolism, enhanced skin vasoconstriction, preference for a lower T_a, and an increased locomotor activity. The main thermoregulatory abnormality of *Trpv1*$^{-/-}$ mice appears to be not an altered level of T_b, but a different pattern of thermoeffectors used to regulate T_b under various conditions. Having excluded profound deficiencies in the two major autonomic effectors directly and knowing that a decrease in the thermopreferendum often occurs secondarily to an increase in locomotor activity, we hypothesized that the increased locomotion may be one of the "primary" symptoms of the *Trpv1*$^{-/-}$ phenotype. For details, please see the original study by Garami et al. 2011 (reference V. in the list of publications related to the subject of the thesis on page 2).

We showed that the anti-inflammatory role firmly established for TRPV1 channels in young rodents is reversed with aging. Whereas pharmacological or genetic TRPV1 antagonism decreases the survival rate in aseptic SIRS and in antibiotic-treated sepsis in the young, both types of TRPV1 antagonism have the opposite effect on aseptic SIRS in middle-aged mice. The age-dependent reversal of the anti-inflammatory role of TRPV1 to proinflammatory is likely due, at least in part, to a reversal of the suppressive control of TRPV1 on TNF-α production. These pathobiological changes are highly important, as evident from the decreased ability of aged *Trpv1*$^{-/-}$ mice to resist polymicrobial sepsis. For details, please see the original study by Wanner et al. 2012 (reference IV. in the list of publications related to the subject of the thesis on page 2). These findings may influence the development of TRPV1 antagonists, widely viewed as new-generation painkillers. If what we found for murine models applies to human

sepsis, anti-TRPV1 therapy may suppress the systemic inflammatory response in the previously uninfected (untreated with antibiotics) elderly and, hence, decrease their resistance to bacterial infection and sepsis. This potential side effect is especially serious, because recognition of infection is often complicated in older patients by a variety of factors, including the absence of fever, which often delays treatment.

We showed that PACAP38 causes hyperthermia by acting on targets within the CNS. The PACAP38-induced hyperthermia is brought about through the simultaneous activation of both autonomic cold-defense effectors: elevation of non-shivering thermogenesis and cutaneous vasoconstriction. We hypothesize that gamma-aminobutyric acidergic neurons within the MnPO are involved in mediation of thermoregulatory response to PACAP38. In freely-moving *Pacap*$^{-/-}$ mice, the absence of PACAP results in hyperkinesis and daytime hyperthermia through mechanisms which need to be clarified, but an involvement of TRPV1 and altered central biochemical processes can be suspected. The increased locomotor activity is presumably a compensatory mechanism for the hypometabolism and hypothermia, which is present under resting conditions in the absence of PACAP. The similar thermoregulatory consequences of the absence of PACAP and TRPV1 can be explained with the alteration of the same neural pathways as PACAP38 is released from activated capsaicin-sensitive (i.e., TRPV1-expressing) neural afferents into the systemic circulation. For details, please see the original study by Banki et al. 2014 (reference III. in the list of publications related to the subject of the thesis on page 2).

We showed that in *Tacr1*$^{-/-}$ mice, the febrile response was attenuated to LPS already in the early phase of fever. When we looked at the molecular mechanism, we did not find a difference in the PGE_2-induced febrile response between *Tacr1*$^{+/+}$ and *Tacr1*$^{-/-}$ mice. The LPS-induced serum cytokine production and COX-2 mRNA expression in the lungs, liver, and brain of the mice were also statistically indistinguishable between the genotypes. In contrast with mRNA, when we measured COX-2 expression at the protein level, we found that the LPS-induced surge was significantly attenuated in the lungs and tended to be suppressed in the liver of *Tacr1*$^{-/-}$ mice as compared with their *Tacr1*$^{+/+}$ littermates. These results indicate that the NK1 receptor contributes to the early phase of LPS-induced fever by enhancing COX-2 protein expression in the periphery. These findings about the role of the NK1 receptor in LPS-induced fever further advance our understanding about the interactions between SP signaling and the

"cytokine-COX-2-PGE_2" axis in experimental fever. As a perspective, these results can help to identify the NK1 receptor as a drug target, as recently proposed against the novel coronavirus (COVID-19) by Vanda Pharmaceuticals.

6. Summary and conclusions

In summary, we showed that 1) hyperbilirubinemia exaggerates the hypothermic response to bacterial endotoxin; 2) genetic ablation of the TRPV1 channel results in minor changes of thermoregulatory phenotype of mice as they use different pattern of thermoeffectors to regulate T_b; 3) in systemic inflammation, TRPV1 has an anti-inflammatory role in the young, which is reversed to pro-inflammatory in older age; 4) the thermoregulatory effects of PACAP38 make the peptide a potential contributor to the febrile response in systemic inflammation; and 5) SP signaling modulates LPS fever through peripheral COX-2 protein expression. Taken together, these findings can help the better understanding of the pathophysiological mechanisms involved in inflammatory processes and, as a perspective, can serve as the bases of new prognostic and therapeutical approaches in patients with systemic inflammation, including sepsis. We explored ligand, receptor, and neurotransmitter mechanisms which play a part in the processes of systemic inflammation. By further extending the plethora of inflammatory mediators, these results can advance our knowledge about the pathophysiology of systemic inflammation, and, from a translational research point of view, they can open new ways for the development of diagnostic and prognostic tools as well as for prevention and perhaps for drug development.

Acknowledgement

I would like to express my thanks to my supervisor, Dr. András Garami for tutoring me through all these years. I am most grateful to Professors Miklós Székely and Zoltán Szelényi, for their scientific advice and knowledge, and continuous support and encouragement in my research, as well as to Prof. Ákos Koller for his helpful discussions and suggestions. I especially thank Prof. Andrej A. Romanovsky for offering me the opportunity to work in FeverLab at St. Joseph's Hospital and Medical Center (Phoenix, Arizona, USA) and for his help and support. I am also very grateful to Dr. Samuel Wanner from FeverLab for his explanations and stimulating discussions during the cooperative work. I would like to extend my gratitude to Prof. Péter Hegyi, the Director of the Translational Medicine Program and Head of the Institute for providing the inspiring scientific environment. I would like to also thank the members of our research group for helping me through all these years regarding both the theoretical and technical parts of the research. I especially thank Anikó Várnagyné Rózsafi for her valuable technical assistance in my experiments. I would like to thank Prof. Dóra Reglődi at the Department of Anatomy, Medical School, University of Pécs for giving me the opportunity to join the PACAP research projects. Many thanks to Drs. Andrea Tamás, Balázs Gaszner, and Eszter Bánki for their expert help with the immunohistochemistry experiments in the PACAP project. I would like to thank Prof. Erika Pintér at the Department of Pharmacology and Pharmacotherapy, Medical School, University of Pécs for her kind and professional help in the NK1 receptor project and I am also grateful to Drs. Valéria Tékus, Ágnes Kemény, Krisztina Pohóczky for their expert help in conducting molecular biology experiments in the project. I would like to thank Katalin Ördög and Drs. László Deres and Róbert Mátics for their expert help with regard to the Western blotting. Last but not least, I wish to express my warmest thanks to my family for their infinite support and patience during my PhD studies.

The author's research summarized in this work has been supported in part by the Hungarian National Research, Development and Innovation Office (grant FK 124483), GINOP "Stay alive - translational medicine for better healthcare" (2.3.2-15-2016-00048), EFOP "Live longer" (3.6.2-16-2017-00006), and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.