THE ENDOGENOUS CANNABINOID AND THE NITRICOXIDERGIC SYSTEMS DIFFERENTLY INFLUENCE HEAT AND COLD STRESS-INDUCED ANALGESIA

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Abstract. *Stress-induced analgesia (SIA) is a well-known phenomenon, in which mechanisms of development opioid and non-opioid components take part. The endogenous cannabinoid system (ECS) takes part in the non-opioid pathways and modulates nociception. Nitric oxide (NO) is also proverbial to interfere with pain perception. The present study was performed to investigate the effects from interaction between the ECS and NO after heat (heat stress) or cold (cold stress) exposure. Male Wistar rats subjected to one hour of heat or cold stress were injected with different combinations of cannabinoid receptor type 1 (CB1) agonist anandamide (AEA) or antagonist (AM251) along with NO-donor, NO-precursor or inhibitor of the NO-synthase (NOS). Nociception was evaluated using Paw pressure (Randall-Selitto) test. The results showed that AEA-administration immediately after the end of stress let to a tendency to increase cold-SIA, but decreased heat-SIA. AEA along with NO-donor increased both cold- and heat-SIA but to a different degree. AM251 and NOS-inhibitor decreased SIA. Our experiments confirmed that the endogenous cannabinoid and the nitricoxidergic systems interact between them in the modulation of SIA. The ECS exerts a more prominent influence on cold rather than heat SIA. Differences in modulation probably depend on the type of stress, due to the different participation of ECS in the mechanisms of SIA development.*

Key words: *endogenous cannabinoid system (ECS), nitricoxidergic system, cold stress, heat stress, stress-induced analgesia (SIA)*

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INTRODUCTION

Stress induces functional and structural changes in the organism triggering interactions between the central nervous system, the endocrine and the immune systems. The possible outcome of such activation could be the development of pathological conditions and even diseases of social

significance, named stress-induced (e.g. coronary heart disease, arterial hypertension, diabetes mellitus, peptic ulcer disease, Graves' disease, malignancies, depression, reproductive disorders) [1].

During stress, different physiological parameters change, as for example pain perception is known to decrease. Such a phenomenon is noted as stress-induced analgesia (SIA) [2].

Two components take part in SIA development – an opioid and a non-opioid one [3]. Non-opioid SIA includes several neurotransmitter systems. Both the endogenous cannabinoid-ergic system (ECS) and the nitric oxide (NO)-ergic system have been documented to take part in modulation of non-opioid SIA – ECS being involved in pain perception and modulation of behavioral responses after stress, while NO-ergic system participates in stress reactions and analgesia [2, 4, 5].

The ECS is a neuroactive lipid signaling system. It includes two Gi/o-protein coupled receptors, CB1 and CB2 [6, 7, 8], their endogenous ligands (endocannabinoids, eCBs), and the enzymes engaged in their metabolism. The main cannabinoid receptor in the brain – CB1, is highly expressed throughout the rodent and the human brain [9, 10, 11]. The two best-characterized eCBs are N-arachidonylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol [12, 13]. eCBs are synthesized “*on demand*” by the postsynaptic neuron, but their effect is presynaptically evident – retrograde signaling. Activation of cannabinoid receptors causes decrease of different neurotransmitters release from the presynaptic endings. Recent studies have shown that CB1- activation reduces nociceptive processing in acute and chronic animal pain models [14, 15]. The ECS regulates brain function and the synaptic activity also during stress by modulating the neurotransmitter release and thus, through different anatomical circuits, the stress-response [16, 17]. eCBs decrease nociception by suppression of cAMP synthesis and activation of Gi-proteins [18]. The ECS has a well-established role in modulation of pain perception at both central and peripheral level, and thus becomes a candidate for therapeutic implication [19, 20, 21].

NO is synthesized from L-arginine by specific neuronal and non-neuronal forms of NO-synthase (nNOS and endothelial/eNOS or inducible/iNOS, respectively). NO and its associated enzymes are involved in many physiological and pathophysiological processes [22].

The NO-ergic system modulates pain perception at both central and peripheral levels. Implication of NO in mediation of the analgesic effects of some drugs has also been documented [5, 23, 24, 25]. Still the exact significance of NO-ergic system in pain mediation at central and peripheral levels is not completely understood. Complex and controversial effects have been documented, and the mechanisms underlying such effects have yet to be elucidated. Interactions with other mediatory systems are to be taken in consideration: activation of potassium and calcium channels or implication of the same second messengers [5, 26].

It's known that both endocannabinoid and NO-ergic systems take part in the descending antinociceptive system [4, 17, 23, 24]. Some of the eCBs' effects have been documented to be mediated by NO [27].

Our study focused on the outcomes from interaction between eCBs and NO, and the resulting effect from such interaction on cold and heat stress-induced analgesia.

MATERIALS AND METHODS

Drugs

All substances applied were purchased by Tocris Bioscience (Bristol, UK). The cannabinoid receptor type 1 (CB1-receptor) agonist N-arachidonylethanolamide (AEA, 1 mg/kg); the CB1-receptors antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251, 1,25 mg/kg); the NO-precursor L-arginine (L-arg, 1 mg/kg); the NO-synthase inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME, 10 mg/kg), and the NO-donor 3-morpholinylsyringonimine (SIN-1, 0,2 mg/kg) were administered intraperitoneally dissolved in DMSO.

Animal care

The experiments included 96 male Wistar rats (180-200 g). The animals were housed individually in polypropylene boxes at constant temperature environment ($22 \pm 2^\circ\text{C}$). 12 h light/dark cycle (lights on at 6.00 a.m.) was observed. Free access to food (standard rodent chow) and water was provided. All experiments were carried out according to the „Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the „Guide for the Care and Use of Laboratory Animals” Washington DC, National Academy Press.

Acute models of stress:

- 1) 1-hour-cold stress (1h CS) was induced by placing the animals in a refrigerating chamber at 4°C for 1 hour;
- 2) 1-hour-heat stress (1h HS) was induced by placing the animals at high environmental temperature (38°C) for 1 hour.

Free movements were allowed, but no food or water was at disposition during the time of cold/heat exposure.

Nociception evaluation:

The changes in the mechanical nociceptive threshold of the rats were measured by the use of an analgesiometer (Randall-Selitto) – Paw-pressure test

(PP). Increasing pressure was applied to the hind-paw and the value (in arbitrary units, AU) required to elicit a nociceptive response (squeak or struggle) was taken as the mechanical nociceptive (PP-) threshold. A cut-off value (24 AU = 480 g/cm²) was observed in order to prevent paw damage.

The experimental studies were performed following several protocols:

- 1) In first place the impact of stress (1h CS/1h HS) on nociception was estimated;
- 2) The effect of AEA-administration immediately after the end of stress (1h CS/1h HS) was evaluated and compared with the combination AEA+L-arg;
- 3) The participation of the NO-ergic system in the effects described was confirmed by L-NAME-pretreatment before AEA+L-arg – combination;
- 4) The relevancy of NO for the effects described was once again confirmed by NO-donor (SIN-1) administration after L-NAME in animals with cold/heat stress;
- 5) The participation of the ECS in the effects described was confirmed by AM-251-pretreatment before AEA+L-arg – combination.

Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by t-test comparison, and presented as the mean $\pm$ S.E.M. A p-value ≤ 0.05 was considered as statistically significant.

RESULTS

Both 1h CS and 1h HS increased pain thresholds of experimental animals compared with controls (Fig. 1A and 1B).

AEA alone administration after the end of 1h CS was followed by a tendency to increase in pain-thresholds of the experimental animals compared to controls on the 10th min (Fig. 1A). Instead, 1 h HS-induced analgesia was decreased after AEA-administration after heat stress during the whole estimated time compared to control values (Fig. 1B).

Administration of NO-precursor L-arg along with AEA increased both cold (on the 10th and 20th min, Fig. 1A) and heat SIA (on the 10th min, Fig. 1B)

compared to controls and animals with AEA alone administration.

Pain thresholds comparable to the controls were evaluated after NOS-inhibitor L-NAME administration in animals with both cold- (Fig. 1A) and heat (Fig. 1B) stress.

After NO-donor (SIN-1) administration along with L-NAME in animals with cold/heat stress PP-thresholds in both experimental groups (1h CS+AEA+L-NAME+SIN-1, Fig. 1A, and 1h HS+AEA+L-NAME+SIN-1, Fig. 1B) exceeded the controls on the 10th min, while on the 20th min they were higher also than animals after 1h CS+AEA- and 1h CS+AEA+L-arg (Fig. 1A), 1h HS+AEA and 1h HS+AEA+L-arg (Fig. 1B), respectively.

AM251-pretreatment abolished SIA in all experimental groups (with AEA+L-Arg-combination and with SIN-1) after both cold and heat stress. PP-thresholds of animals from 1h HS+AM+AEA+L-NAME+SIN-1- and 1h HS+AM+AEA+L-Arg-groups were comparable to the controls on the 20th and 30th min, while for the same time a tendency toward hyperalgesia was detected in 1h CS+AM+AEA+L-NAME+SIN-1 and 1h CS+AM+AEA+L-Arg experimental groups (Fig. 2A and 2B).

Fig. 1. Modulation of (A) cold and (B) heat SIA by anandamide (AEA) and the nitric oxide-ergic system after 1 hour of cold (1h CS) or heat (1h HS) stress. Pain thresholds are presented as mean values $\pm$ S.E.M. in arbitrary units (AU). ***p < 0.001 vs. controls. A. xxxp < 0.001, xp < 0.05 vs. 1h CS; +++p < 0.001 vs. 1h CS+AEA; &&&p < 0.001 vs. 1h CS+AEA+L-arg. B. xxxp < 0.001 vs. 1h HS; +++p < 0.001, ++p < 0.01, +p < 0.05 vs. 1h HS+AEA; &&&p < 0.001 vs. 1h HS+AEA+L-arg

Fig. 2. Modulation of (A) cold and (B) heat SIA by AEA and L-arg after AM251 (AM) pretreatment. Pain thresholds are presented as mean values $\pm$ S.E.M. in arbitrary units (AU). *** $p < 0.001$, ** $p < 0.01$ vs. controls. A. xxxp < 0.001 , xxp < 0.01 vs. 1h CS+AEA+L-arg. B. xxxp < 0.001 vs. 1h HS+AEA+L-arg.

DISCUSSION

Cannabis sativa shares its origins with the inception of the first agricultural human societies in Asia. It contains a number of chemically active compounds, such as cannabinoids, terpenoids, flavonoids, and alkaloids [28].

The cannabinoid receptors CB1 and CB2, located extensively in the brain and peripheral tissues, are G-protein coupled receptors, linked to the Gi/o system in the same manner as opioid receptors and their activation results in a reduction in afferent neuronal transmission. Due to the diffuse presence of the ECS in the brain and periphery, its activation or inhibition regulates several pathophysiological phenomena. ECS is involved in various disorders, including metabolic and neuroinflammatory pathologies. The use of different preparations of *C. sativa* (both natural and synthetic) is associated with therapeutic strategies for many diseases. Especially, in the brain, cannabinoids modulate hunger/satiety, neuroinflammation, analgesia, and in the periphery,

they are involved in the peripheral metabolic reactions of liver, fat, muscles, immunomodulation – anti-inflammatory response in the blood cells and cancer [21, 29, 30].

Cannabinoids have been officially admitted among non-opioid analgesics. The current public opinion is moving toward more widespread acceptance of cannabinoids and physicians will increasingly be encountering the use of cannabinoids in their practice [31].

The gaseous retrograde messenger NO has been shown to participate in both nociception and analgesia [5]. Colocalization of CB1 with nNOS in rat brain has also been recently demonstrated [32].

The results described confirmed our expectations about the role of the endogenous

cannabinoid and the nitricoxidergic systems in modulation of SIA.

Activation of CB1-receptors by exogenous AEA sustains cold-SIA, but decreases heat-SIA. L-arg-administration after AEA increased both cold and heat SIA, and yet some differences are worth to be mentioned: cold SIA-increase was more pronounced and long lasting than heat SIA one. The differences described can be explained by the different relevance of each of the two components – opioid and non-opioid, in SIA development. The non-opioid one seems to be the most expressed in cold-SIA, while the opioid one prevails in heat-SIA mechanisms. The ECS takes part in the non-opioid component [2] hence it differently affects cold and heat SIA modulation.

The importance of both systems for the effects described was demonstrated. Inhibition of CB1-receptors by AM251 abolished SIA after both cold and heat stress. Yet, AM-pretreatment in animals with 1h HS decreased pain thresholds to the control level, while in animals with 1h CS a tendency to hyperalgesia was even detected, confirming that the ECS contributes more for the proper development of cold rather than heat SIA.

The importance of NO for the effects described was also evident – inhibition of NO-synthesis led to pain thresholds comparable to control levels in both

animals after cold and heat stress. Administration of NO-donor SIN-1 increased PP-thresholds, but only in animals with AEA, and not with AM251, confirming the importance of both systems for the analgesic effect.

A possible explanation of the interaction between the ECS and NO-ergic system could be that both systems influence the same ion channels directly or by adopting the same secondary messengers. NO itself could even mediate some of AEA effects [27]. Literature data support cyclooxygenase's participation in eCBs' degradation [33]. Indications about interactions between eCBs, cyclooxygenase-1, and the NO-ergic system in modulation of pain perception and analgesia also exist [33, 34]. Colocalization of CB1 with nNOS [32] should also be taken in consideration, as well as the possible implication of additional mediatory systems [35]. Recent investigations also suggest a possible modulation of CB1 by allosteric ligands or indirect regulators [36, 37].

Future investigations of interrelations between cannabinoids and nitricoxidergic mechanisms in nociception will be relevant for at least one reason: cannabinoids have been acknowledged as painkillers; interactions with other participants in pain perception could point out therapeutic protocols in order to decrease their dosage or avoid unwanted effects.

CONCLUSION

The endogenous cannabinoid and the nitricoxidergic systems interact between each other in the modulation of SIA and both of them contribute for the analgesic effect. The ECS exerts a more prominent influence on cold rather than heat SIA.

Although SIA is a promising and systemically explored scientific field many of the underlying mechanisms are not yet fully elucidated, and still represent a challenge to us. Participation of other mediatory systems and their interactions with eCBs and NO should also be taken in consideration.

Compliance with ethical standards

This study was performed in accordance with the „Principles of Laboratory Animal Care“ formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” Washington DC, National Academy Press.

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