

Modulation of Hypothalamic Nesfatin -1 and Insulin Resistance by CannabinoidsRatna Palit¹, Ashok Singh Chouhan², Sapan Kumar Palit³¹Associate Professor, Department of Pharmacology, HiTech Medical College & Hospital, Bhubaneswar, Odisha, India²Assistant Professor, Department of Pharmacology, HiTech Medical College & Hospital, Bhubaneswar, Odisha, India³Professor, Department of Nephrology, HiTech Medical College & Hospital, Bhubaneswar, Odisha, India

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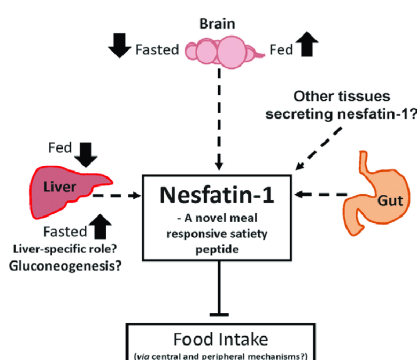
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Conflict of interest: Nil

Abstract:**Objective:** The control of sleep, metabolism, and food intake involves both the nesfatin-1 and cannabinoid systems. It is still unclear how the cannabinoid system and nesfatin-1 levels are related. This study examined the effects of cannabinoids and inhibiting cannabinoid receptors CB1R and CB2R on nesfatin-1 and insulin resistance in 70-hour REM sleep-deprived rats.**Method:** 120 mice were subjected to sleep deprivation of 70 hours. The following groups and drug administrations existed: Group 1 (the control) received a vehicle injection. Group 2 was awarded WIN. Group 3 received a WIN injection after receiving AM251 (a CB1R antagonist). Group 4 then received WIN injection after receiving SR144528 (a CB2R antagonist). Group 5 only received AM251. Group 6 only got SR144528. Two hours after administering the medication, blood samples were drawn and prepared for biochemical analysis. While insulin and nesfatin-1 levels were determined using ELISA, glucose levels were determined using a glucometer. Immunohistochemistry was also utilized to evaluate central nesfatin-1. For inter-group comparisons, one-way analysis of variance and the post hoc Tukey's test were utilized.**Results:** All study groups had similar serum nesfatin-1 levels. In the WIN group compared to controls, there were less immune-positive cells for nesfatin-1 in the brain. When CB1R or CB2R antagonists were administered, the number of nesfatin-1-positive cells did not decrease. In comparison to control and WINCB1 groups, WINCB2 and CB2 groups had higher levels of insulin resistance. Pretreatment with either a CB1R or CB2R antagonist inhibited the effect of cannabinoids on the reduction of nesfatin-1 immunoreactivity in the CNS.**Conclusion:** CB2 receptor activation, which was unrelated to central nesfatin-1 immunoreactivity, may be connected to insulin resistance.**Keywords:** Sleep deprivation, insulin resistance, nesfatin-1, and cannabinoid receptor CB2, mice.This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided original work is properly credited.**Introduction**

A powerful anorexigenic peptide known as nesfatin-1 has been demonstrated to decrease food consumption in a dose-dependent way. Both

peripheral tissues like the pancreas and portions of the central nervous system like the hypothalamus have specific nesfatin-1 binding sites [Figure 1; 1].

**Figure 1: Function and Distribution of Nesfatin-1**

Nesfatin-1 may be involved in the control of both blood glucose and sleep, according to recent investigations [2]. For example, nesfatin-1 infusion improved glucose utilization in a mouse model by increasing insulin production and activating intracellular insulin signaling [3]. Additionally, nesfatin-1 altered the excitability of hypothalamic glucose-responsive neurons, suggesting a potential function for both central glucose sensing and hypothalamic sleep regulation [4]. On the other hand, it was discovered that nesfatin-1 and melanin-concentrating hormone coexpress in the tuberal hypothalamic area, a central neuronal structure that controls rapid eye movement (REM) sleep [5]. Nesfatin-1 is a possible peptide in the hypothalamus that controls sleep [6]. In addition to its effects on cognition, sleep deprivation is a common disease that may compromise metabolic balance. In a recent study, healthy individuals who were sleep deprived for 40 hours experienced circulation insulin levels that were elevated by 3.5 times without any changes to the levels of resistin or visfatin [7]. In healthy men, regardless of when it occurs during the night, partial sleep loss or sleep reduction up to 4 hours per night decreased insulin sensitivity. [8]

A further potential modifiable risk factor for type 2 diabetes in children and adolescents is insufficient sleep [9]. The mechanisms tying poor glucose homeostasis to lack of sleep are yet unclear. Through binding to the distinct receptors CB1R and CB2R, cannabinoids are lipid signaling agents implicated in a variety of physiological processes, including pain perception, inflammatory responses, and regulation of blood glucose [10]. Insulin resistance was brought on by the overactivity of the CB1R system, whereas its inhibition had positive benefits in reducing its cardiometabolic consequences [11]. Interestingly, the cannabinoid system and nesfatin-1 may be connected and modern. Only one study looked into this connection. It has been established that the peripheral cannabinoid system controls food intake via a mechanism that suggests the creation and release of stomach nesfatin-1 [12].

In this investigation, we sought to determine if, in the context of complete sleep deprivation, the cannabinoid system might be related to both peripheral and central nesfatin-1 levels. We proposed that peripheral and central nesfatin-1 changes caused by cannabinoid receptor inhibition affect insulin resistance and blood glucose control.

Methods:

Study Design: This was a prospective study carried out at HiTech Medical College & Hospital, Bhubaneswar, within a year.

Methodology: Animals were kept in a lab environment with an 11/11 light/dark cycle, a

temperature range of 21°C–1°C, and 41% humidity. Tap water and mouse food were freely available. REM sleep deprivation lasting 70 hours was administered to all animal groups using a modified flowerpot approach. Drug injections came after sleep deprivation. Additionally, all animals were sacrificed while under general anesthesia using 5 mg/kg of xylazine and 40 mg/kg of ketamine when the medication infusions were finished. After the onset of general anesthesia, intracardiac blood samples were obtained for biochemical analysis. All animals were then severed from their necks, the skulls were taken out, and complete brains were placed in formalin solution for immunohistochemistry analysis.

There were six study groups created [n=20 each]. The only difference between each animal's 72-hour REM sleep deprivation regimen and the intraperitoneal (i.p.) medication injections was. The animals in the CONT group received a vehicle injection (77% saline, 2% ethanol, 2% tween 81, and 21% DMSO), whereas the WIN group received an injection of the cannabinoid agonist WIN 55,212,2, the WINCB1 group received an injection of the CB1R antagonist AM251, and the WINCB2 group received an injection of the CB2R antagonist SR144528. CB1R antagonist AM251 (2 mg/kg) and CB2R antagonist SR144528 (2 mg/kg) were administered to the CB1 and CB2 groups, respectively.

Drug injections were carried out once the 70-hour sleep deprivation period was complete. Following are the two injections that each group got at a 10-minute interval: WIN group, vehicle, and agonist; CB1 group, antagonist-1 and vehicle; CB2 group, antagonist-2 and vehicle; control group, vehicle and vehicle; WIN group, vehicle and agonist; WINCB1 group, antagonist-1 and antagonist-2. Blood samples were taken under general anaesthesia using a heart puncture 50 minutes following the final injection. All animals were decapitated after blood samples were taken, the craniums were surgically removed, and brain tissues were extracted and imbedded in formaldehyde solution and paraffin block.

Commercial ELISA kits were used to assess the levels of nesfatin-1 and insulin. The glucometer utilised glucose. The homeostatic model of insulin resistance (HOMA-IR = [fasting glucose, mg/dL x fasting insulin, IU/mL]/404) was used to calculate insulin resistance.

The hypothalamus tissues were sliced into 4 µm pieces after being formaldehyde-fixed and paraffin-embedded. Slides were deparaffinized, rehydrated using increasing concentrations of ethanol, and then boiled in citrate buffer for ten minutes to extract antigens. The sections were then washed in phosphate-buffered saline and submerged in

hydrogen peroxide blocking solution for 4 min. At room temperature, in a humid environment, slides were incubated with blocking solution for 11 min. Extra serum was removed, and for 2 hours at room temperature, rabbit polyclonal nesfatin-1 antibody was incubated with it at a dilution of 1:000 in antibody diluent reagent solution.

Primary antibodies were swapped out for their nonimmune isotypes as negative controls. After being exposed to a biotinylated secondary antibody that had been made in response to the primary antibody for 11 minutes, the sections were then exposed to an 11-minute HRP-streptavidin solution. Diaminobenzidine (DAB) was used to visualise staining reactions, and then hematoxylin (Merck) was used as a counterstain. A microscope with an X400 objective was used to look at the sections. By counting the number of immunopositive cells in a 0.2 mm² area of the hypothalamic paraventricular nucleus, the nesfatin-1 immunoreactivity score was determined.

Sample Size: 120 mice were included in this research study. 20 mice were divided into 6 groups.

Statistical Analysis: For descriptive analysis, means and standard deviations were utilized. The Kolmogorov-Smirnov test was used to examine the normal distribution. One-way analysis of variance was used to evaluate HOMA-IR, peripheral nesfatin, insulin and glucose levels, and nesfatin-1-positive cell counts between the six groups. Tukey's test was used to do post hoc pairwise comparisons using GraphPad Prism. The value for statistical significance was changed to $P < 0.04$.

Results

Model of insulin resistance based on serum glucose, insulin, and homeostasis: While serum insulin levels were significantly higher in CB2 than in CONT group (0.54 ± 0.14 vs. 0.28 ± 0.08 ng/mL, respectively, $P < 0.04$, [Table 1]), serum glucose levels were significantly higher in WINCB2 than in WINCB1 group (124.5 ± 30.13 vs. 79.31 ± 11.32 mg/dL, respectively, [$P < 0.04$]). The study groups' calculated HOMA-IR levels were contrasted. WINCB2 and CB2 groups had higher levels of insulin resistance than the control and WINCB1 groups did.

Table 1: Insulin resistance based on serum glucose, insulin, and homeostasis.

Glucose Level		
Group	Glucose Level	P-value
WINCB2	124.5 ± 30.13	$P < 0.04$
WINCB1	79.31 ± 11.32	
Insulin level		
Control	0.54 ± 0.14	$P < 0.04$
CB2	0.28 ± 0.08	

Serum nesfatin-1 levels and immunohistochemistry of the hypothalamus: Nesfatin-1 immune-positive cell count in the WIN group was lower than in the CONT group, according to an immunohistochemistry analysis of

hypothalamic paraventricular nucleus sections (54.74 ± 7.66 vs. 78.32 ± 6.51 , respectively, $P < 0.04$, [Table 2]). Nesfatin-1 immune-positive cell counts were greater in the WINCB1 and WINCB2 groups when compared to the WIN group [Table 2].

Table 2: Nesfatin Immune Positive cell count seen in Control and WIN group

Nesfatin-1 immune-positive cell count		
Group	Immune-positive cell	P-value
WIN	54.74 ± 7.66	$P < 0.04$
Control	78.32 ± 6.51	

Nesfatin-1 immunoreactivity was comparable between CB1 and CB2 groups. As demonstrated, the combination of WIN 55,212,2 and CB1 or CB2 receptor antagonists prevented a decrease in the number of nesfatin-1-positive cells. In all study groups, serum nesfatin-1 levels were comparable.

Discussion

The primary and original finding of this study is that pretreatment with either a CB1R or CB2R antagonist blocked the effect of cannabis treatment on the reduction of nesfatin-1 immunoreactivity in the hypothalamus of the central nervous system. In addition, regardless of whether research groups received cannabinoid agonist or antagonist

therapies, serum nesfatin-1 levels were comparable across all groups. To our understanding, this is the first study that investigated the connection between central nesfatin-1 and cannabinoid receptor blocking in REM-sleep deprivation animals.

The largest nesfatin-1 neuron individuals is found in the dorsolateral hypothalamus and zona incerta, which are closely linked to sleep-wake regulation [8]. Nesfatin-1 was initially found in brain regions involved in the regulation of food intake, such as the arcuate nucleus, paraventricular nucleus, and nucleus of the solitary tract. These results might point to a possible regulating function for nesfatin-1 in the control of both sleep and metabolism. The

expression of hypothalamic nesfatin-1 was downregulated by REM sleep deprivation before returning to normal levels during recovery sleep [8].

The fact that injection of the exogenous agonist cannabis (WIN55) had no effect on insulin resistance when CB2Rs were blocked by an antagonist is another significant finding of our investigation. Numerous investigations looked into the connection between CB2R activation and blood glucose homeostasis [13,14]. In rats, CB2R activation increased glucose tolerance following intraperitoneal glucose administration; this improvement was reversed by CB2R inhibition [14]. Another study found that the CB2R agonist SER601 increased the systemic sensitivity of diabetic mice with high-fat diet/streptozotocin induction to insulin [14]. These results show that CB2R agonism lowers blood glucose levels whereas antagonism damages glucose homeostasis. Rimonabant, a particular CB1R antagonist, reduced the amount of pancreatic insulin secreted in response to glucose stimulation [15].

Cannabinoids' coordinated activities on both CB1R and CB2R have been shown to be able to regulate glucose homeostasis in studies with selective antagonism or agonism of each cannabinoid receptor subtype. This regulation, though, could vary by species. CB2Rs are expressed on the mouse pancreatic beta-cell membrane while CB1Rs are not, according to immunohistochemistry studies [14]. The study confirmed those earlier findings and demonstrated that circulating nesfatin-1 was not involved in glucose homeostasis caused by CB2R activation. Both decreased intracellular signalling downstream of the insulin receptor and altered production of appetite-regulating peptides like asleptin, ghrelin, and neuropeptide Y may contribute to insulin resistance. It is widely known that ghrelin suppression causes insulin resistance and a corresponding rise in insulin secretion [16].

Hepatic and peripheral insulin sensitivity might be induced with just the activation of central nesfatin-1 [17]. On the other hand, Anwar et al. found a positive association between the levels of nesfatin-1, HOMA-IR, and serum insulin [18]. Additionally, Ravussin et al. demonstrated that nesfatin-1 did not affect the hypothalamic satiety pathways; rather, it reduced metabolic inflammation caused by macrophages to reduce insulin resistance [19]. To put it another way, obesity-related nesfatin-1 overexpression in macrophages may detect energy surplus that connects inflammation and insulin resistance. The significance of cannabinoid receptors in the process connecting nesfatin-1 to insulin resistance was further explored in the current work.

It's interesting to note that in this paradigm, selective CB2R blockage increased insulin resistance without the help of exogenous cannabis treatment. This result showed that insulin resistance and CB1R activation may be associated to insulin resistance and insulin sensitivity, respectively. On the other hand, nesfatin-1 down-regulation caused by cannabis was not receptor specific as seen by increased central nesfatin-1 immune reactivity in CB1R and CB2R blockage. These findings together suggested that nesfatin-1 and cannabis do not interact to control insulin sensitivity at the central level.

Conclusion

Nesfatin-1 immunoreactivity was decreased in the hypothalamus after cannabinoid therapy, and either CB1R or CB2R antagonist pretreatment blocked this effect. This is the first study that we are aware of that investigated the connection between cannabinoid receptor blockade and hypothalamic nesfatin-1. Further research revealed that central nesfatin-1 immunoreactivity was not necessary for the relationship between CB2R activation and insulin sensitivity.

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