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Dynamics of changes in the concentrations of anandamide and 2-arachidonoylglycerol and their impact on the carbohydrate-lipid metabolism in patients after kidney transplantation in the follow-up study*

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ABSTRACT
Endocannabinoids are lipid metabolites that have paracrine effects. Emerging evidence indicates a role of endocannabinoids and their cognate receptors in synaptic modulation and plasticity at a wide range of synapses throughout the central nervous system. This study aimed to analyze the dynamics of changes in the concentrations of 2 endocannabinoids (anandamide – ANA, and 2-arachidonoylglycerol – 2-AG) and their impact on the carbohydrate-lipid metabolism in patients before kidney transplantation and 7 days, 1, 3, and 6 months after transplantation. Considering the pro-inflammatory action of the compound, an increase in the concentration of 2-AG causes deterioration in kidney function, which, in the case of tests performed after transplantation, is associated with a poor prognosis. In our results, the highest concentration was obtained in the group of patients before transplantation and the lowest after 6 months. In the group of patients after transplantation, ANA and 2-AG were significantly correlated with lipid metabolism parameters, adipocytokines, and inflammatory markers. As both endocannabinoids play a very important role in improving kidney function after transplantation, future studies are needed to examine the potential mechanisms for their action and clinical implication.
Keywords: endocannabinoids; transplantation; anandamide; 2-arachidonoylglycerol.

INTRODUCTION

Anandamide (ANA) is a biologically active fatty acid amide that is an agonist of the cannabinoid (CB1) and vanilloid (VR1) receptors. In addition, due to its origin, it can also be classified as an eicosanoid, because it is formed from arachidonic acid and other fatty acids. It occurs in many tissues of the body, but its concentration depends on the content of the previously mentioned precursor, i.e., arachidonic acid. It is formed by binding the above-mentioned acid to the free amine with the aid of N acetyltransferase. Anandamide is also produced by the hydrolysis of the phospholipids N of arachidophosphadiethanolamide. This reaction requires the participation of calcium ions and phospholipase D [1]. Anandamide has a short half-life due to its sensitivity to the action of fatty acid amide hydrolase, which converts it back into arachidonic acid and ethanolamine [2].

Another compound examined in this paper is 2-arachidonoylglycerol (2-AG) produced by combining omega 6 with glycerol. Rodent experiments demonstrate the neuroprotective function of 2-AG. It has been shown that in close-head injury (CHI) mice, the level of endogenous 2-AG significantly increased [3]. On the other hand, the administration of synthetic endocannabinoid resulted in a reduction in edema, a reduction in the extent of the infarction, as well reduced mortality of hippocampal cells compared to the control group. This was due to the reversal of the effects of endothelin 1 by 2-AG, which causes severe vasoconstriction. The reduced blood flow thus adversely affects brain injury causing ischemia, as well as inhibition of tumor necrosis factor alpha (TNF-α) and reactive oxygen species.

Research on 2-AG shows its influence as a mediator of inflammation. In the experiments, an increase in interleukin 8 (IL-8) and the mitochondrial pyruvate receptor was observed. This reaction is likely to be dependent on the CB2 receptor. During acute inflammation, activated macrophages release 2-AG, which stimulates the above-mentioned receptors on the remaining inflammatory cells, thereby increasing the synthesis of IL-8. Interleukin 8 has a pro-inflammatory effect, which triggers a cascade in which natural killer (NK) cells, eosinophils, and macrophages begin to migrate, creating inflammation [4]. An increase in the concentration of endogenous CB receptor

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agonists, such as ANA and 2-AG, has been observed in people with obesity and metabolic disorders manifested by increased glucose concentration, insulin resistance, high triacylglycerols (TAG) values, and low high-density lipoprotein (HDL) cholesterol. In the case of 2-AG, its higher concentration was found in visceral fat than in subcutaneous fat, in contrast to ANA. High values of 2-AG cause the activation of CB receptors in the vessel wall causing oxidative stress, which results in the migration of neutrophils and monocytes into the artery wall contributing to the development of atherosclerosis. Such results suggest a causal relationship between high endocannabinoid concentrations and the development of cardiovascular diseases [5, 6].

The study aimed to analyze the dynamics of changes in the concentrations of endocannabinoids (ANA and 2-AG) and their impact on the carbohydrate-lipid metabolism in patients before kidney transplantation and 7 days, 1, 3, and 6 months after transplantation.

MATERIALS AND METHODS

Group characteristics

The study involved 38 kidney transplant patients (19 women and 19 men) aged 25–74 years. They were the patients of the Department of Nephrology, Transplantology, and Internal Diseases of the Pomeranian Medical University in Szczecin (PMU). All patients underwent kidney transplantation without complications from rejection or inflammation. The subjects were subjected to immunosuppressive therapy, including 3 drugs: corticosteroids, calcineurin inhibitors, and mycophenolate mofetil or mammalian target of rapamycin (mTOR). The control group consisted of 50 healthy volunteers (14 women, 36 men) aged 31–61 years. Patients and volunteers from the control group gave informed consent to participate in the study. The research was approved by the Bioethics Committee at the PMU.

Sample collection

To perform the planned tests, the patients had blood taken from a peripheral vein for dipotassium edetate (K2EDTA) and a clot. The patient's blood was collected 5 times: just before kidney transplantation, 7 days, 1 month after transplantation, 3 months, and half a year after transplantation. Blood was collected from healthy volunteers once. Blood samples were centrifuged (1000 g, 10 min, 20°C). The obtained plasma and serum served as research material. Plasma and sera were stored at –80°C while awaiting the determination.

Determination of endocannabinoids by high-performance liquid chromatography HPLC MS/MS ESI-Q-TOF

After thawing the plasma samples at room temperature and thoroughly mixing, 300 µL of material was transferred to new tubes. The internal standard R-methenamine (10 ng/mL) and 900 µL acetonitrile (Sigma-Aldrich, Poznań, Poland) were added to each sample. After thorough mixing (2 min), the samples were centrifuged (10 min, 13,000 g, 4°C). The supernatant was transferred to new tubes and evaporated in a vacuum concentrator (RCV 2-25CD plus, Christ, Germany) for 3.5 h at 40°C. The resulting pellet was stored at –80°C. Samples were thawed at room temperature. To each of them, 100 µL of acetonitrile was added, followed by thorough mixing until the precipitate was completely dissolved. Four hundred µL of deionized water was added to the resulting solution and mixed again. Four hundred and fifty µL was withdrawn from the resulting solution and transferred to glass vials. To determine individual substances, a high-performance liquid chromatograph coupled with an HPLC MS/MS ESI-Q-TOF mass spectrometer (Perkin-Elmer, USA) was used. Calibration curves determined using ANA and 2-AG standards, respectively (Sigma-Aldrich, Poznań, Poland) were the basis for calculating the concentration of the tested endocannabinoids.

Statistical analysis

The mean value, standard deviation, median, and quartiles – lower and upper – were determined for all parameters. The compliance of the distribution of quantitative variables with the normal distribution was assessed using the Shapiro–Wilk test. All variables had an abnormal distribution. The significance of differences between the control and study groups at all time points (pre-transplant, week, 1 month, 3- and 6-months post-transplant) was assessed using the non-parametric Kruskal–Wallis ANOVA and Dunn's *post hoc* test. The strength of the correlation was analyzed using Spearman's rank correlation test. The threshold of statistical significance was set at $p \leq 0.05$. Statistical analysis was performed using Statistica PL13 (StatSoft, Kraków, Poland).

RESULTS AND DISCUSSION

Patients who have undergone a kidney transplant often experience severe disorders related to the metabolism of carbohydrates and lipids [7].

In this study, among healthy people, a positive correlation was found between ANA and adropin (Rs = 0.43; p = 0.0017). A positive correlation was also found with the mean albumin concentration (Rs = 0.32; p = 0.024). In the group of patients before kidney transplantation, a positive correlation was found between ANA and C-reactive protein (CRP) index (Rs = 0.51; p = 0.033). There was no correlation between the concentration of ANA and other parameters in this group. Patients on day 7 after transplantation showed a negative correlation between ANA and TAG (Rs = –0.41; p = 0.032). Among patients 1 month after kidney transplantation, a positive correlation with adiponectin was observed (Rs = 0.71; p = 0.0065), and 3 months after transplantation, a negative correlation between ANA and cholesterol was found (Rs = –0.69; p = 0.039).

Among healthy people, a positive correlation was found between 2-AG and adropin (Rs = 0.37; p = 0.01). There was no correlation between the concentration of 2-AG and other parameters in this group. On day 7 after kidney transplantation, a positive correlation was found between 2-AG and CRP (Rs = 0.65; 0.004), as well as creatinine (Rs = 0.46; p =

0.01) and resistin (Rs = 0.44; p = 0.03). In the group of patients 1 month after transplantation, a positive correlation was found between 2-AG and adiponectin (Rs = 0.71; p = 0.01). There was no correlation between the endocannabinoids ANA and 2-AG in any group.

Anandamide is one of the representatives of endocannabinoids, compounds that play more and more important roles. There is a growing interest among scientists in this compound due to its anti-inflammatory effects. It inhibits the synthesis of IL-6 and IL-8, which have a pro-inflammatory effect, as well as the chemotactic macrophage protein-1 and TNF [8]. Anandamide is a compound that is still little known, and the studies conducted so far are mainly conducted on animals, which makes it difficult to determine the correct concentrations in humans. The mean ANA value in our control group was 0.26 ±0.15 ng/mL (Fig. 1). Similar results were obtained by Quercioli et al. who indicate that the values were in line with the values of healthy people [5]. The protein discussed earlier, adiponectin, also has anti-inflammatory properties. It works by inhibiting the activity of T lymphocytes and inhibits the secretion of the same interleukin as ANA: IL-8, thus showing a negative correlation with IL-6. These facts suggest the compatibility of the above-described compounds and a common path in combating the inflammatory process inherent in organ transplantation, including kidneys. This thesis is confirmed by our results, in which both compounds show a positive correlation in the first month after surgery (Rs = 0.71; p = 0.0065) [9]. In his analysis, Tam describes the distribution of CB1 receptors throughout the human kidney, mainly in the glomeruli, tubules, or loops of Henle. Unlike the CB2 receptor, the presence of which is controversial. According to some researchers, the gene is not expressed in this organ, and according to others, it is located in the kidney cortex. Anandamide, an agonist of the CB1 receptor, plays an important role in kidney function. It has been shown that exogenous administration of this compound affects the dilation of the arteries and the production of nitric oxide (NO), which blocks the sodium and hydrogen ion transporter, suggesting its diuretic effect [10]. In our study, a relationship was found between ANA and adropin (Rs = 0.43; p = 0.0017). Adropin also affects the availability of NO in the body, and its increase causes smooth muscles to relax. Studies on the effect of this substance on the human body mainly included heart disease, where positive effects such as lowering lipogenesis were found [11]. The correlation of the 2 compounds shows a common pathway of their action and their contribution to improving kidney function after transplantation.

Li and Wang conducting studies on rats given methanandamide, showed increased urine excretion while maintaining normal sodium concentration and arterial pressure, which highlights the important role of endocannabinoids in the regulation of renal hemodynamics [12]. In the conducted studies, we found a positive correlation between ANA and albumin (Rs = 0.32; p = 0.024) [13]. Hsu et al. showed that activating the CB1 receptor, a function of ANA, increases the level of proteins through increased receptor expression and the participation of vascular endothelial growth factor [13]. Barutta et al. examined the

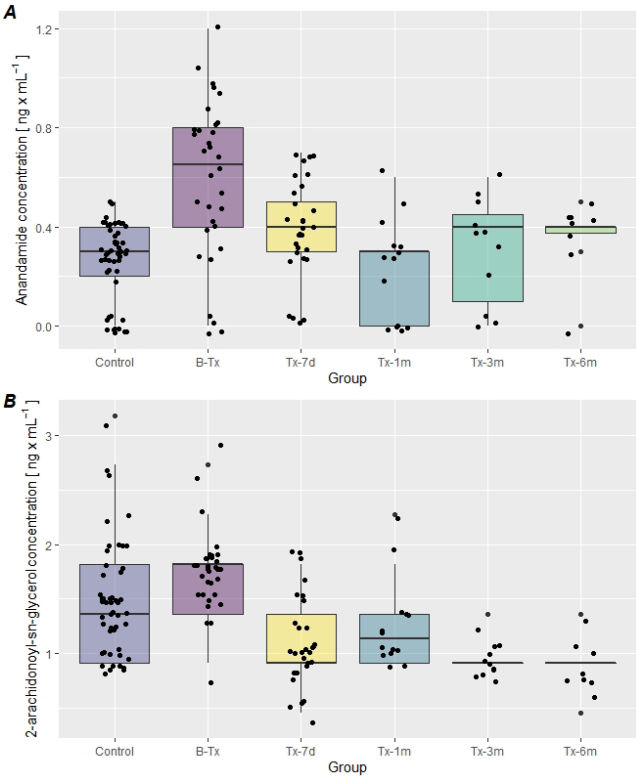

FIGURE 1. Changes in anandamide (A) and 2-arachidonyl-sn-glycerol (B) concentrations in the control and study groups (patients before and after 7 days, 1, 3, 6 months after kidney transplantation) in the Kruskal–Wallis test (A – p = 0.0001; B – p = 0.0001)

concentration of ANA in the kidney cortex of diabetic patients with normal glycemia. They showed an increase in endocannabinoids in healthy people and a low concentration in the first group. Our patients showed normal glucose levels, which were probably caused by taking hypoglycemic drugs. The highest increase in ANA was found in patients before transplantation, and the lowest in the first month after transplantation. There was no significant correlation between ANA and glucose. However, the trend was maintained, i.e., with the highest glucose concentration in the first month, and patients also had the lowest concentration of ANA, which is consistent with the findings of the above-mentioned researchers [14]. Analyzing the level of CB1 receptor expression, its decreased activity was noticed in diabetic nephropathy, which suggests the use of agonists of this receptor, i.e. ANA, as protection against further diabetes complications [15]. One of the suggested causes of high ANA levels in our patients was obesity. Engeli et al. showed a relationship between high values of this parameter and obesity in humans [16], which indicates that blocking the CB1 receptor can be used in the treatment of this disease. Rimonabant, an antagonist of this receptor, works by lowering cholesterol and TAG levels, and also increases HDL cholesterol. Anandamide, i.e. the opposite compound, by stimulating the CB1 receptor, should increase appetite, thus increasing the above-mentioned parameters. Our study did not confirm this thesis because ANA shows a negative correlation with TAG on day 746 after transplantation (Rs = –0.41; p = 0.032) and with cholesterol in the third month after surgery (Rs = –0.69; p = 0.039). The reasons

for such results can be many, among others, the serious condition of patients after transplantation or taking immunosuppressive drugs that affect the entire metabolism [17]. The study by Quercioli et al. on the relationship between endocannabinoid concentrations and coronary artery disease in obesity showed a positive correlation between ANA concentration and CRP. We obtained confirmation of these results in patients before transplantation, where the correlation coefficient was Rs = 0.510 (p = 0.033). This result may suggest a relationship between metabolic mechanisms and inflammatory processes in both cardiovascular and kidney diseases [5]. Unfortunately, no studies have been conducted to check the effect of ANA on the acceptance of transplanted organs.

2-arachidonoylglycerol is also an endocannabinoid, CB1 and CB2 receptor antagonists. The correct and desired concentration of this compound is also not known yet. In our control group, the concentration was 1.55 ±0.81 ng/mL (Fig. 1). Quercioli et al. also obtained the same results planned on a scale of approx. 2.0 ng/mL [5]. Contrary to the previously discussed compound, there are reports of its pro-inflammatory function. Our research showed a positive correlation of the described compound with the CRP (Rs = 0.65; 0.0037) which increases the inflammatory process. In animal studies, an increase in 2-AG was found during acute inflammation, which was most likely released from macrophages. In this way, stimulated CB2 receptors, located on inflammatory cells, increase the concentration of pro-inflammatory IL-8, stimulating the migration of feeding cells [4]. The described mechanism confirms the correctness of the obtained results. Moreover, the studies showed a positive correlation between 2-AG and resistin, a pro-inflammatory protein (Rs = 0.44; p = 0.034). Leśniowski et al. found an increase in this cytokine in acute pancreatitis. Both parameters show marked activity during the chronic inflammatory process stimulating the organism to produce IL-1, IL-6, IL-12, and TNF-α from macrophages [9, 18]. Blüher et al., in their research on the effect of endocannabinoids on lipid metabolism and obesity, showed an increase in the titer of 2-AG in obese and diabetic patients. However, they did not show a significant correlation between adiponectin and 2-AG (Rs = 0.71; p = 0.0065) [19]. In our research, both relationships correlate positively with each other. It is difficult to explain this phenomenon. Perhaps during the inflammation that occurs in the transplanted person, inflammatory cells are activated, and CB receptors are overstimulated. Studies have shown that chronic activation of CB receptors causes an increase in peroxisome proliferator-activated receptor gamma (PPARγ), which, as an early marker of adipocyte differentiation, causes an increase in adiponectin levels. However, the cause is not yet known [20]. Di Marzo et al. research on the role of endocannabinoids in obese people found a positive correlation between 2-AG and TAG and negative HDL [6]. Such a relationship was not observed in our results. In the obtained results, we obtained a clear correlation between the tested endocannabinoid and creatinine concentration on day 7 after transplantation (Rs = 0.46; p = 0.014). Considering the pro-inflammatory tendency of the compound, an increase in the concentration of 2-AG causes deterioration of kidney function, which in the case of tests performed after transplantation, is a poor prognosis. In our results, the highest concentration was obtained in the group of patients before transplantation and the lowest after 6 months. The results show a decrease in the concentration of 2-AG with time, which, given the pro-inflammatory nature of the compound, seems to be a positive aspect in accepting a transplanted organ.

CONCLUSIONS

In the group of patients after transplantation ANA and 2-AG are significantly correlated with lipid metabolism parameters, adipocytokines, and inflammatory markers. Both endocannabinoids play a very important role in improving kidney function after transplantation. Future studies are needed to examine the potential mechanisms for their action and clinical implication.

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