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The effect of bradykinin on the pro-inflammatory response of human adipocytes*

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The proper functioning of adipose tissue is one of the factors in maintaining energy homeostasis. Adipocytes not only store lipids but also produce active molecules such as adipokines and adipocytokines, which are involved in many functions of adipose tissue, including the secretion of hormones that regulate energy and lipid metabolism. Inflammation has been shown to underlie the deregulation of adipose tissue function. Bradykinin belongs to a family of pro-inflammatory kinin peptides that are abundant in most tissues and biological fluids. This study aimed to determine the ability to produce kinin peptides and characterize the effect of bradykinin on pro-inflammatory responses in adipocytes. The Chub-S7 human preadipocyte line was differentiated to show specific properties for adipose tissue cells. The differentiated cells expressed genes that encode proteins such as kininogen, kallikrein, and prolylcarboxypeptidase that are involved in the production of kinins and also showed the expression of kinin receptors. The response of adipocytes to bradykinin was examined in relation to kinin concentration and the presence of kininase inhibitors. The high concentration of bradykinin induced a moderate increase in lipid accumulation, increased release of pro-inflammatory cytokines, and altered gene expression of molecules involved in adipocyte function, such as adiponectin, lipoprotein lipase, and other transcription factors. This study suggests an important role for kinin peptides in inducing inflammatory responses in adipocytes, which can modify the function of adipose tissue and ultimately lead to diseases related to disturbance of energy homeostasis. The results obtained may enrich our understanding of the mechanisms underlying obesity-related disorders.

Key words: adipocyte, bradykinin, inflammation, adipogenesis, lipid metabolism

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Abbreviations: ACE, angiotensin I converting enzyme; BK, bradykinin; B1R, bradykinin receptor type 1; B2R, bradykinin receptor type 2; C/EBP α, CCAAT/enhancer binding protein alpha; CIDEC, cell death inducing DFFA-like effector c; DMEM, Dulbecco's Modified Eagle Medium; EF2, elongation factor 2; FBS, fetal bovine serum; GLUT-4, transporter glucose type 4; IRS-1, insulin receptor substrate-1; IL, interleukin; LPL, lipoprotein lipase; PBS, phosphate buffered saline; PRCP, prolylcarboxypeptidase; STAT3, signal transducer and activator of transcription 3; TCCF, total corrected cellular fluorescence; TNF-α, tumor necrosis factor α

INTRODUCTION

Bradykinin (BK), an ubiquitous peptide in human tissues and physiological fluids, regulates several processes associated with vascular functionality, including vasodilatation, vasoconstriction, and vascular permeability (Blais *et al.*, 2000). An important physiological effect of BK is related to glucose transport. A study carried out under conditions of normal glucose tolerance and poor glucose control revealed an exercise-induced increase in plasma BK concentration only in individuals with good glycemic control. Experiments with normal and hyperglycemic rats demonstrated a similar effect showing a lower glucose concentration in plasma, translocation of transporter glucose type 4 (GLUT-4) to the skeletal cell membrane, as well as increased activation of insulin-dependent pathways (Taguchi *et al.*, 2000). In fact, BK has been reported to improve the activity of insulin-stimulated receptor tyrosine kinase and the downstream insulin signaling cascade through bradykinin B2 receptor-mediated signal pathways (Motoshima *et al.*, 2000). Furthermore, in primary dog adipocytes, BK was able to increase glucose uptake through the insulin-dependent pathway and phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), which in turn increased GLUT-4 translocation (Isami *et al.*, 1996). A novel mechanism of BK mediation was also proposed in insulin-stimulated glucose transport in rat adipocytes (Beard *et al.*, 2006). These authors suggested a NO-dependent pathway that acts by modulating feedback inhibition of insulin signaling at the IRS-1 level.

However, BK can also induce pathological effects, especially when its concentration in the body and intercellular fluids is high. BK and other kinin peptide levels in blood and tissues are dynamically controlled under physiological conditions (for a review, see Blais *et al.*, 2000). Kinin concentration is regulated by various processes related to activation of the kallikrein-kinin system and degradation of kinins by specific peptidases. However, the balance between kinin production and degradation can be altered in different pathological states, including systemic or local inflammation. The specific degradation of kinins by carboxypeptidases leads to the production of peptides without Arg at the C-terminus, called des-Arg kinins, which have high pro-inflammatory activity. Kinins and des-Arg kinins are recognized by two types of receptors – the kinin receptor type 1 (B1R) and type 2 (B2R) that belong to the G-protein-coupled receptor family (Leeb-Lundberg *et al.*, 2005). The first type of receptor preferably recognizes des-Arg-kinin peptides,

while B2R primarily recognizes BK and kallidin (Lys-BK). B2R is ubiquitously present in numerous cells and is mainly responsible for the physiological functions of BK, while B1R is induced by certain stimuli associated with chronic pro-inflammatory processes. However, under certain conditions, B2R receptors can play an important role in inflammation, especially when kinin production is increased or peptide degradation is altered.

Increasing data support that inflammation of adipose tissue, with enhanced secretion of adipokines and chemokines, may be a crucial factor in diseases related to adipose tissue, such as obesity, diabetes, and cardiovascular disorders (Emanuela *et al*., 2012; Kwon & Pessin, 2013; Makki *et al*., 2013). Taking into account the above facts, in this study an attempt was made to evaluate the role of bradykinin in the processes that regulate inflammation in human adipocytes. In this study, we investigated the ability of differentiated human Chub-S7 cells to generate kinins and the activation of kinin receptors by BK in these cells. Furthermore, the effects of BK on the gene expression of proteins involved in lipid metabolism and on pro-inflammatory responses were examined depending on the concentration of peptides in the presence of kininase inhibitors and the absence of these inhibitors.

MATERIALS AND METHODS

Materials

The Chub-S7 human preadipocyte cell line was supplied by Nestle Research Center (Lausanne, Switzerland). BK was acquired from Bachem, Switzerland. Cell culture medium – Dulbecco's Modified Eagle Medium (DMEM), F12, and fetal bovine serum (FBS) were purchased from Biowest (France). 2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid was supplied by Calbiochem (USA). The fluorescent mounting medium was from DAKO (USA). Other chemicals were obtained from Merck (USA). Universal RNA/miRNA Purification Kit for total RNA isolation and M-MLV Reverse Transcriptase kit were supplied by EURX (Poland) and Promega (USA), respectively. The KAPA SYBR Green Master kit for qPCR was purchased from Kapa Biosystems (USA). Quantikine ELISA kits for adiponectin, interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) were supplied by R&D Systems (USA).

The rabbit polyclonal antibody to the bradykinin B1 receptor against a sequence that includes the third EC (Ab13305) was supplied by Abcam (UK). The rabbit polyclonal antibody to the bradykinin B2 receptor against a sequence that includes the N-terminal amino acid sequence 2-66 (NBP2-14351) was obtained from Bionovus, USA. Secondary goat anti-rabbit IgG-Alexa Fluor 488 (A-11008) was purchased from Thermo Fisher Scientific (USA).

Cell culture and differentiation

Chub-S7 cells were cultured in a medium mixture (DMEM: F12; 1:1, v/v) supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C (Darimont, 2003). For cell differentiation, cells were kept in serum-free "adipogenic medium" containing 15 mM $NaHCO_3$, 17 μM D-pantothenic acid, 15 mM Hepes, 33 μM biotin, 10 μg/ml transferrin, 1 nM triiodothyronine, 850 nM insulin, 500 μg/ml fetuin for 8 days. The medium was also supplemented with 1 μM dexamethasone and 1 μM rosiglitazone up to day 4 of differentiation. For the microscopic study, cells were seeded on glass cover slides and fixed with 3.7% paraformaldehyde before or after 8 days of differentiation. Morphological changes in differentiated cells were monitored with an ELIPSE TE300 inverse microscope (Nikon, USA) at a magnification of 20×.

Gene expression

Chub-S7 cells before and after 8-day differentiation were analyzed for the expression of genes involved in lipid metabolism (*AdipoQ*, *CIDEC*, *LPL*), in kinin production (*HK*, *KLK1, KLKB1, PRCP)* and kinin receptor genes (*BDKRB1*, *BDKRB2*). Furthermore, the effect of BK on the expression of genes involved in lipid metabolism (*AdipoQ*, *C/BEPa*, *CIDEC*, *LPL*) and the inflammatory response (*IL-6, IL-8, TNF-a*) was also studied. In this case, differentiated cells were stimulated with 1 nM or 1 μM BK for 3 or 6 hours in the presence of kininase inhibitors (500 μM bacitracin, 10 μM captopril and 20 μM 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid) or in their absence. Cells were preincubated with inhibitors for 30 minutes prior to BK stimulation. Total RNA was isolated from cells and then reverse transcribed according to the manufacturer's instructions. cDNA was used for quantitative real-time polymerase chain reaction using the C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). The primers used for gene expression analysis are listed in Table 1. Target mRNA expression was normalized to the level of the elongation factor-2 housekeeping gene (*EF-2*) and compared with samples from untreated cells. Relative gene expression was calculated using the following equation: $Q=2^{\Delta\Delta CT}$, where CT is the threshold cycle.

Cytokine determination

Differentiated Chub-S7 cells seeded in 12-well plates were stimulated for 6 and 24 hours with 1 μM BK in the presence or absence of kininase inhibitors as described above. All experiments were carried out in DMEM/F12 medium supplemented with a protease inhibitor cocktail, containing aprotinin, bestatin, leupeptin, E-64, and pepstatin (200× dilution). The levels of adiponectin, IL-6, IL-8, and TNF-α released into the medium were measured with ELISA kits according to the manufacturer's instructions. The values were normalized to 1 mg of cell lysate protein determined by the Lowry method.

Determination of lipid accumulation with Oil Red O

After 6 hours of stimulation of differentiated Chub-S7 cells with 1 nM and 1 μM bradykinin, cell lipid accumulation was measured by lipid staining with 0.5% Oil Red O (Kraus *et al*., 2016). After washing, cells were fixed in 3.7% paraformaldehyde for 30 minutes at room temperature. The solution was then removed and, after careful washing, the cells were incubated with the Oil Red O solution for 1 hour at room temperature. Subsequently, the Oil Red O solution was removed and the amount of intracellular dye was eluted with 200 μl of 100% isopropanol. The absorbance of the eluate was measured at 500 nm using the Multiskan FC microplate reader (ThermoLab Systems, USA). The total amount of Oil Red O in each sample was normalized to 1 mg of cell lysate protein.

Table 1. Sequences of primers used for qPCR analysis.

Gene	Forward primer 5′→3′	Reverse primer 5′→3′	Annealing temperature (°C)
AdipoQ	gagatggcacccctggtga	cccttaggaccaataagacctgg	60
BDKRB1	gcaactgaacgtggcagaaa	gcccaagacaaacaccagatc	58
BDKRB2	gtgcccatgccgcttgctcc	tcggcgctgaaagaggccgt	58
C/EBPα	aagaagtcggtggacaagaacag	tgcgcaccgcgatgt	60
CIDEC	tcatggcttacagtcttgagga	gggcttggaagtactcttctgtc	60
EF2	gacatcaccaagggtgtgcag	gcggtcagcacactggcata	58
KLK1	gctcattgcatcagcgacaattacc	ctgcttggcgggtgtggttct	60
KLKB1	tgggtaaccggatggggcttct	agcacagaccatccgttgggtt	58
LPL	cagcaaaaccttcatggtgat	caagttttggcacccaactc	60
PRCP	acaggtcctgggatgccattaatcg	tagtccaccattgccagattcacc	58
KNG1	gcaagagtacaggtggtggctggca	cctggaggccttttcatcagtgaga	60
IL-6	ccacaagcgccttcggtcca	ctgggggtactggggcaggg	58
IL-8	caccggaaggaaccatctcact	tcagccctcttcaaaaacttct	54
TNFα	tccttcagacaccctcaacc	aggccccagtttgaattctt	62

Abbreviations: *AdipoQ,* adiponectin*; BDKRB1,* bradykinin receptor type 1*; BDKRB2,* bradykinin receptor type 2*; C/EBPa,* CCAAT enhancer binding protein alpha*; CIDEC,* cell death-inducing DFFA-like effector protein C*; EF2,* elongation factor 2*; KLK1,* tissue kallikrein*; KLKB1,* plasma kallikrein*; LPL,* lipoprotein lipase*; PRCP,* prolylcarboxypeptidase*; KNG1,* kininogen*; IL-6,* interleukin 6*; IL-8,* interleukin 8*; TNFα,* tumor necrosis factor alpha.

Immunofluorescence detection of kinin receptors

The cell surface expression of B1R and B2R was examined by immunofluorescence microscopy using antibodies against external receptor epitopes. Briefly, Chub-S7 cells were seeded on glass cover slides and differentiated for 8 days. Then, the undifferentiated and differentiated cells were fixed with 3.7% paraformaldehyde for 30 minutes and washed with phosphate buffered saline (PBS). After 1 hour of blocking with 10% FBS, cells were incubated overnight with primary antibodies diluted in PBS (1:50 or 1:100 dilution for anti-B1R or anti-B2R, respectively) at 4°C. After washing with PBS, the samples were treated for two hours with Alexa-Fluor488 conjugated secondary antibody (1:250 dilution). Then 2 μM 4',6'-diamidino-2-phenylindole was added for 10 minutes and, after extensive washing with distilled water, the cover slides were mounted on microscope slides using fluorescent mounting medium. A negative control sample without primary antibody incubation was also prepared. The samples were visualized with an epifluorescence microscope (Leica DMI6000B, Wetzlar, Germany) at a magnification of 40× with oil immersion. Using Leica Application Suite X software, the images were normalized to a lower threshold value. The fluorescence intensity of each image was measured using ImageJ 153 with Java 1.8.1 (USA) according to a previously published method (McCloy *et al.*, 2014). Total corrected cellular fluorescence (TCCF) = integrated density – (area of the selected cell × mean fluorescence of the background reading) was calculated. For each sample, at least three different images from two separate protein immunodetection experiments were chosen for the calculation of TCCF.

Statistics

Representative data from at least three experiments were expressed as mean values ± S.D. Student's *t*-test was used for statistical comparisons of mean values with the GraphPad Prism software (GraphPad Prism v. 8.0, USA).

RESULTS AND DISCUSSION

The human Chub-S7 preadipocyte cell line, after differentiation, showed a characteristic morphology for mature adipocytes, consistent with the human adipocyte model established successfully in our previous study (Góralska *et al.*, 2017). The most notable change was the increase in intracellular lipid accumulation. An increase in the number of lipid droplets was observed after 8 days of differentiation (Fig. 1). Furthermore, cells were characterized by determining specific adipocyte gene markers by qPCR, such as adiponectin (*AdipoQ*), the cell death inducing DFFA-like effector C (*CIDEC),* and lipoprotein lipase (*LPL*). Adiponectin mRNA was significantly increased by more than 20 times in differentiated cells (Fig. 1). Adiponectin, a hormone released

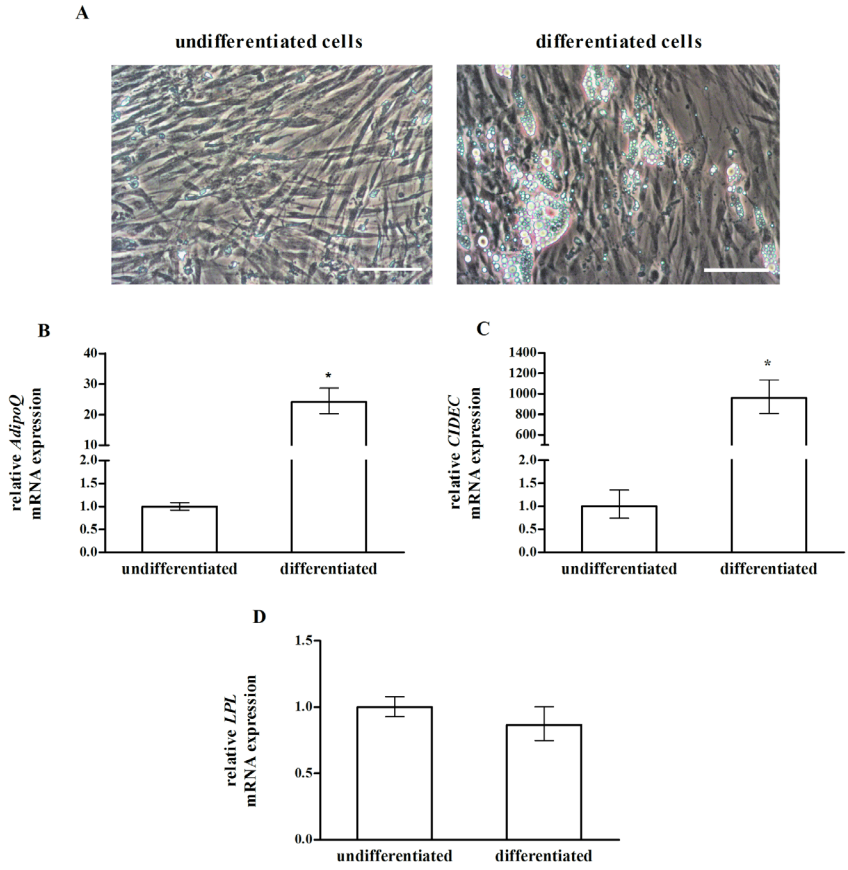


Figure 1. The effect of Chub-S7 cell line differentiation on morphological markers and adipogenesis.
Chub-S7 cells were differentiated in serum-free adipogenic medium for 8 days. Changes in cell morphology and mRNA expression of adipogenic markers were investigated. Selected images of cells before and after differentiation are presented (A). Scale bar – 50 µm. The expression of adiponectin (**B**), CIDEC (**C**), and LPL (**D**) mRNAs in cells before and after differentiation was investigated by the qPCR technique. The bars represent mean values ± S.D. of at least three experiments. *p<0.01 *vs*. undifferentiated cells.

from adipocytes, is associated with glucose and fatty acid metabolism. Therefore, the observed increase in the expression of this gene supports the achievement of a suitable adipocyte model. In fact, an earlier study reported that cells differentiated into adipocytes showed elevated expression of *AdipoQ* after just 4 days of differentiation, which lasted until the 14th day (Sheng *et al*., 2014). This assumption was corroborated by the expression of the *CIDEC* gene. This transcriptional factor, abundantly expressed in mature adipocytes, is involved in the regulation of lipid metabolism (Yin *et al*., 2014). The amount of CIDEC mRNA increased 1000 times in mature adipocytes compared to preadipocytes. Furthermore, differentiated Chub-S7 cells also expressed lipoprotein lipase (*LPL*), an enzyme secreted by adipose tissue involved in lipid metabolism. However, only a slight increase in LPL expression was observed compared to undifferentiated cells. In fact, Darimont and co-workers showed that LPL expression was unchanged in differentiated adipocytes independently of differentiation time; only specific agonists of the peroxisome proliferator-activated receptor gamma were able to enhance *LPL* expression (Darimont *et al*., 2003).

The ability of adipocytes to produce kinin peptides was tested in the developed cell model. For this purpose, the gene expression of proteins involved in the activation of the kallikrein-kinin system was investigated. Kinin peptides are generated from proteinaceous precursors, high molecular weight kininogen, and low molecular weight kininogen, through the proteolytic action of plasma or tissue kallikreins (Colman & Schmaier, 1997; Joseph & Kaplan, 2005). Other cell proteins such as prolylcarboxypeptidase (PRCP) or heat shock protein 90 are also involved in kinin generation due to their ability to activate kallikrein at the cell surface (Joseph & Kaplan, 2005). The first studies of kinin production were carried out in endothelial cells, but other cells such as smooth muscle cells, neutrophils, macrophages, microglia and neurons have been shown to also generate these peptides (Fernando *et al*., 2003; Barbasz *et al*., 2008; Guevara-Lora *et al*., 2011, 2013; Joseph & Kaplan, 2005). Although an increasing number of studies have indicated the participation of elements of the kinin production system in adipocyte functions (Peyrou *et al*., 2020; Selvarajan *et al*., 2001), the knowledge of the impact of differentiation processes on the expression of individual proteins from this system in preadipocytes is insufficient. In this study, the gene expression of essential proteins for the generation of kinins was observed in Chub-S7 cells before and after differentiation (Fig. 2). The kininogen gene (*KNG1*) was expressed in both undifferentiated and differentiated cells. No significant differences in *KNG1* expression were observed between preadipocytes and mature adipocytes, which does not exclude the activation of kinin generation systems. Stable expression of this gene suggests that adipose tissue has the ability to pro-

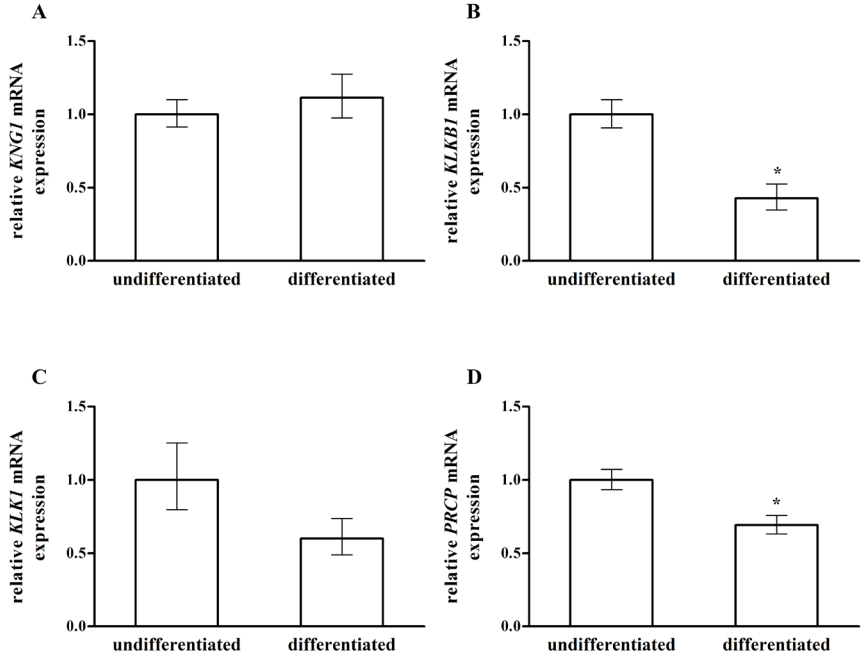


Figure 2. The effect of Chub-S7 cell line differentiation on mRNA expression of proteins involved in kinin generation.
Cells were differentiated in serum-free adipogenic medium for 8 days. The gene expression of *KNG1* (**A**), *KLKB1* (**B**), *KLK1* (**C**), and *PRCP* (**D**) was analyzed with qPCR. The bars represent the mean values ± S.D. of at least three experiments. *$p<0.05$ *vs*. undifferentiated cells.

duce kininogen that could be used for local BK production. In fact, this protein has been reported to contribute to the functions of adipose tissue. Recent studies have demonstrated that in mice adipose tissue two different kininogen genes were expressed, *KNG1* and *KNG2*. The last, which is present only in mice, showed a significant effect on the thermogenic regulation of brown adipose tissue, while *KNG1* was only slightly enhanced after the thermogenic stimulus (Rouhiainen *et al*., 2019; Peyrou *et al*., 2020). On the other hand, our study revealed that the gene expression of serine proteases, such as plasma kallikrein (*KLKB1*), tissue kallikrein (*KLK1*), and prolylcarboxypeptidase (*PRCP*), was decreased but not abolished (Fig. 2). A previous report demonstrated an important role for the plasminogen cascade in the differentiation of mouse-derived 3T3-L1 cells to adipocytes, suggesting the mediation of plasma kallikrein (Selvarajan *et al*., 2001). These authors demonstrated that the use of serine protease inhibitors during differentiation led to poor cell differentiation and low lipid accumulation. We obtained a good model of human adipocytes, suggesting that although the gene expression of kallikreins and PRCP is decreased, Chub-S7 cells were able to differentiate, showing lipid accumulation and the presence of specific markers of mature adipocytes. Although this study did not demonstrate the production of BK or its metabolites, these cells may have the ability to generate kinins due to the presence of kallikreins. Furthermore, brown rat adipose tissue showed significant amounts of BK concentration, comparable to that in blood (Campbell *et al*., 1994). Even if the amount of kinins is insufficient or these peptides are mostly degraded, it is likely that this peptide can be delivered to adipose tissue from the surrounding blood vessels.

From the point of view of our research, it was interesting to check the effect of differentiation on the expression of kinin receptors in Chub-S7 cells. The presence of B2R and B1R in adipose tissues has been widely demonstrated (Abe *et al*., 2007; Catalioto *et al*., 2013; Marketou *et al*., 2018; Mori *et al*., 2012). In this study, both kinin receptors, B1R and B2R, were expressed in Chub-S7 cells, as demonstrated by qPCR and immunofluorescence microscopy. Cell differentiation led to a strong decrease in *B1R* expression (by 80%), while a moderate increase in *B2R* expression occurred (by two times) (Fig. 3B and 3A, for B1R and B2R, respectively). Similar observations resulted from microscopy analysis of kinin receptors in differentiated cells with specific antibodies to extracellular epitopes. B2R expression in differentiated cells increased compared to undifferentiated cells (Fig. 3C and 3E). Semiquantitative analysis of immunofluorescence images showed TCCF values equal to 46.5±1.1 and 11.8±3.8 for differentiated and undifferentiated cells, respectively (Fig. 3G). In turn, the expression of B1R was reduced (Fig. 3D and 3F), achieving TCCF values of 8.3±3.2 *vs*. 23.0±5.2 for differentiated and undifferentiated cells, respectively (Fig. 3G). Strongly reduced B1R expression can be beneficial; however, this does not mean that its expression cannot be induced. Kinin receptors have been shown to be autoregulated by their agonists (Guevara-Lora *et al*., 2009, 2014), especially BK is able to regulate B1R expression through its transformation into des-Arg kinins. Since these cells express kinin receptors, the cellular model is adequate for further study. However, BK and Lys-BK can be degraded very quickly, mainly by ACE, but other membrane-linked peptidases such as neutral endopeptidase 24.11, carboxypeptidase M, and aminopeptidase are also involved in kinin degradation (Blais *et al*., 2000). In addition, enzymes present in biological fluids, such as carboxypeptidase N or released by immune system cells (e.g., elastase), are also able to hydrolyze kinin peptides (Blais *et al*., 2000; Campbell, 2000; Dulinski *et al*., 2003). Because BK degradation can result in the formation of des-Arg-BK, spe-

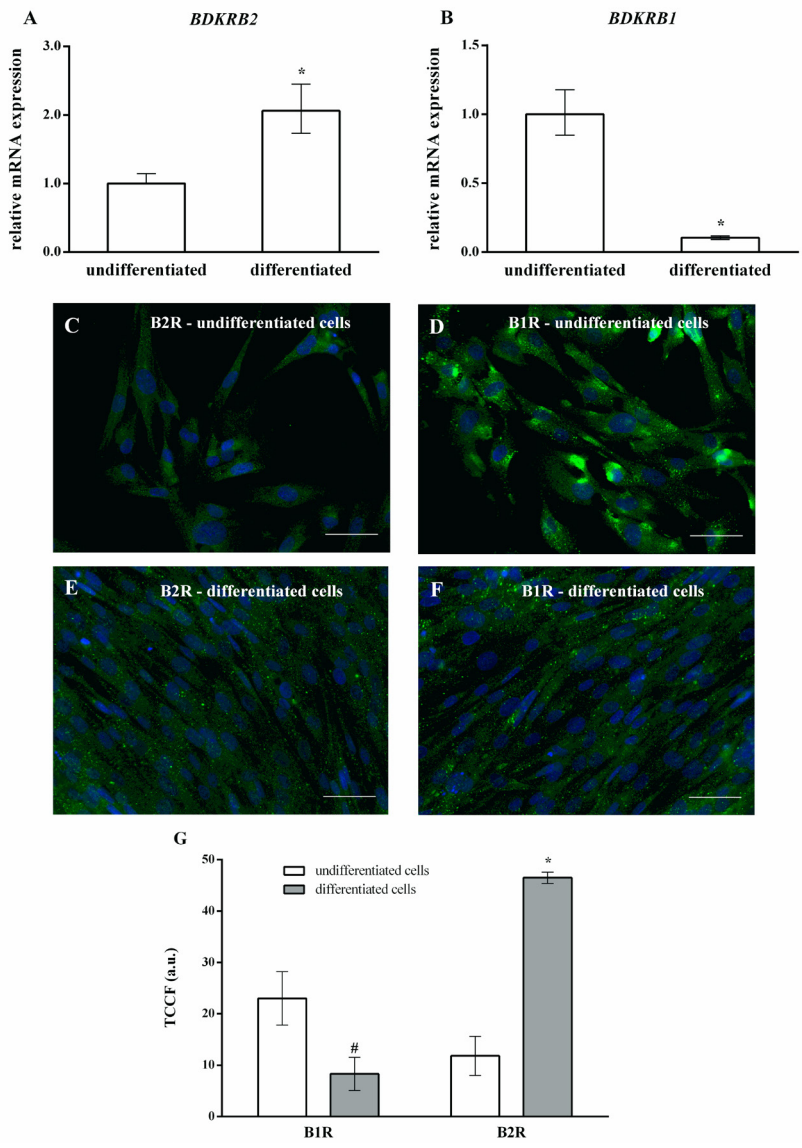


Figure 3. The effect of Chub-S7 cell line differentiation on kinin receptor expression.
Cells were differentiated in serum-free adipogenic medium for 8 days. The gene expression of *BDKRB2* (**A**) and *BDKRB1* (**B**) in cells before and after differentiation was analyzed by qPCR. The bars represent mean values ± S.D. of at least three experiments. Statistical significance vs. undifferentiated cells was *p<0.05. The detection of B2R and B1R proteins before (**C, E**) and after differentiation (**D, F**) was carried out by immunofluorescence. Representative photographs of B2R and B1R immunodetection are presented. Scale bar – 50 μm. The quantification of the images of B2R and B1R immunodetection was performed with ImageJ (**G**). The bars represent mean values ± S.D. of at least two experiments. Statistical significance *vs.* undifferentiated cells was *p<0.01 or #p<0.05.

cific kininase inhibitors were used. It is worth emphasizing here that exogenous des-Arg-BK added to serum had a half-life at least five times longer than that of BK, which was established to be approximately 30 s (Blais *et al.*, 2000). Therefore, carboxypeptidase inhibitors are especially required to avoid B1R activation, which has been reported to promote expansion of adipose tissue and contribute to metabolic and inflammatory disorders responsible for obesity (Sales *et al.*, 2019; Freitas-Lima *et al.*, 2022). The use of peptidase inhibitors allowed the appreciation of the effect of non-degraded BK in adipocytes, while incubation without such inhibitors may represent more physiologically relevant conditions.

In recent decades, an undisputed correlation has been established between obesity-induced inflammation and metabolic syndrome (Ouchi *et al.*, 2011; DeBoer, 2013; Rodríguez-Hernández *et al.*, 2013; Kwon & Pessin, 2013; Makki *et al.*, 2013; Emanuela *et al.*, 2012). Chronic low-grade systemic inflammation has been shown to contribute to the development of metabolic syndrome that leads to cardiovascular disorders and type 2 diabetes mellitus. The molecular mechanism of the expansion of inflammatory responses in obese patients is not yet fully understood. It seems that pro-inflammatory cytokines can play a central role. Therefore, in this study, the effect of exogenous bradykinin on cytokine production by differentiated Chub-S7 was analyzed (Fig. 4). As mentioned above, kinin concentration in body fluids is dynamically controlled by peptidases, the most effective being angiotensin-I-converting enzyme (ACE). The mean concentration of BK measured in blood plasma, depending on the technique used, ranges from pM to nM (Blais *et al.*, 2000). Therefore, we used two different BK concentrations – 1 nM, assumed to be in the physiological range, and 1 μM – a pathological concentration. The gene expression and protein release of interleukin 6

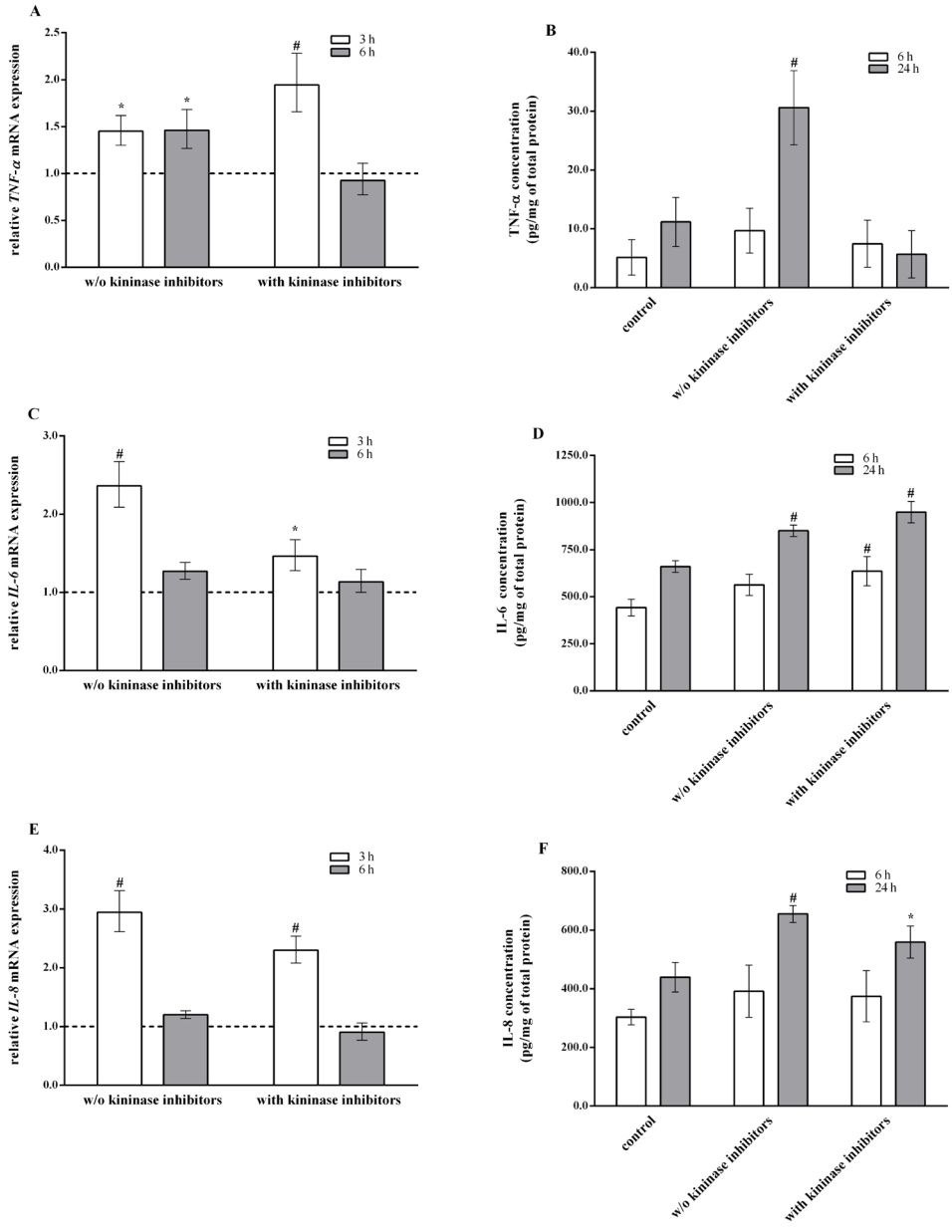


Figure 4. The effect of BK stimulation on cytokine expression in differentiated Chub-S7 cells.
Differentiated cells were incubated with 1 μM BK in the presence or absence of kininase inhibitors. mRNA expression and protein release were measured at the indicated time of incubation. Gene expression of *TNFα* (**A**), *IL-6* (**C**), and *IL-8* (**E**) was analyzed by qPCR. The protein released to medium was assessed with ELISA kits for TNF-α (**B**), IL-6 (**D**), and IL-8 (**F**). The bars represent the mean values ± S.D. of at least three experiments. Statistical significance *vs.* undifferentiated cells was $^{*}p<0.05$ or $^{\#}p<0.01$.

(IL-6), interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) were observed in treated Chubs-S7. These cytokines have been shown to be produced by adipocytes (Makki *et al.*, 2013) and play a role in inflammation of adipose tissue. TNF-α is associated with disorders of insulin and glucose metabolism, while IL-6 is mainly associated with glucose metabolism. In turn, IL-8 concentration is elevated in obese subjects, showing a closed positive correlation with fat mass (Kim *et al.*, 2006). In fact, our study showed a significant increase in TNF-α release from differentiated cells incubated with 1 μM BK and without kininase inhibitors (Fig. 4B). Interestingly, the presence of kininase inhibitors completely abolished the release of cytokines, suggesting that kininases may play an important role in the propagation of the inflammatory response in these cells. It can be assumed that adipocytes can transform BK into the strong pro-inflammatory peptide des-Arg BK. These results were correlated with the expression of the *TNFa* gene, which showed a moderate increase in mRNA levels in cells incubated without inhibitors (Fig. 4A). Furthermore, in cells pretreated with inhibitors, cytokine gene expression decreased to the control sample level, demonstrating that under these conditions the pro-inflammatory response can be inhibited. The 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid present in the inhibitor cocktail may inhibit carboxypeptidase activity. Therefore, it can be expected that in these samples there are no des-Arg peptides that are more difficult to degrade. Certainly, the importance of these enzymes in adipogenesis has been

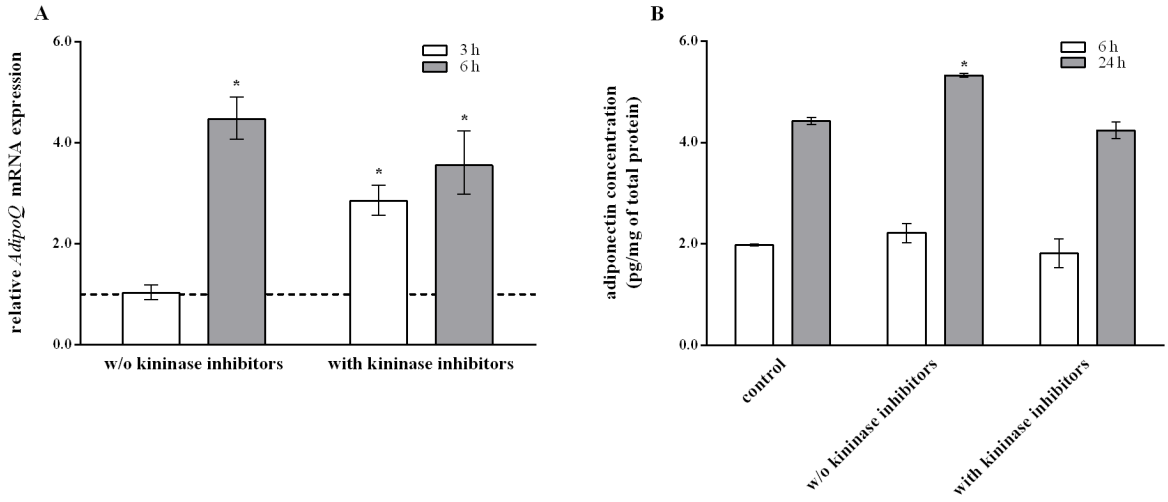
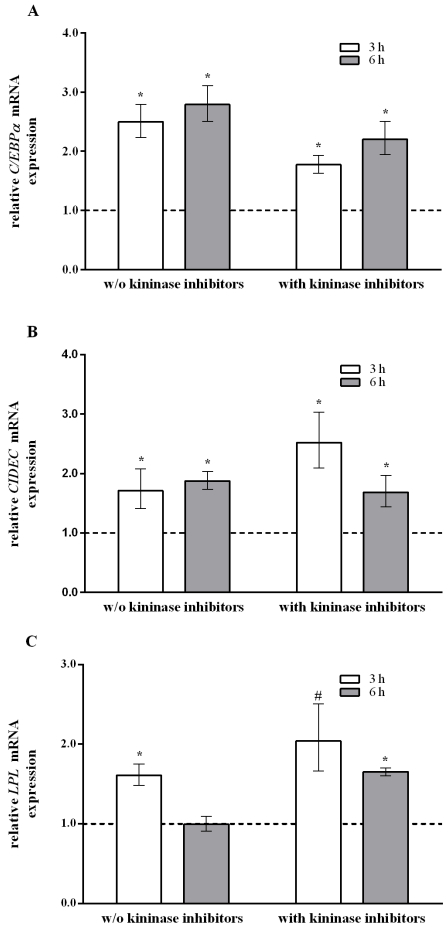


Figure 5. The effect of BK on adiponectin expression in differentiated Chub-S7 cells.
Differentiated cells were incubated with 1 μM BK in the presence or absence of kininase inhibitors. The gene expression (**A**) and the concentration of protein released to the medium (**B**) were analyzed with qPCR and ELISA, respectively. The bars represent the mean values ± S.D. of at least three experiments. Statistical significance *vs*. undifferentiated cells was **p*<0.05.



proposed. Carboxypeptidase M levels were established to be elevated in the late stage of adipogenesis, suggesting that this enzyme can cleave important protein/peptides with Lys or Arg at the C-terminus that are present in adipose tissue (Denis *et al*., 2013). On the other hand, it seems that the physiological concentration of BK does not have a significant influence on the expression of this cytokine. Although after 3 h of incubation the level of mRNA increased, after 6 hours the TNFα mRNA values returned to the level before incubation (Fig. S1A at https://ojs.ptbioch.edu.pl/index.php/abp/).

Regarding interleukins, the results were slightly different. The release of IL-6 and IL-8 from cells treated with 1 μM BK was higher compared to untreated cells, whether or not they were preincubated with kininase inhibitors (Fig. 4D and 4F). The gene expression of both cytokines was highest only in a short time of incubation with 1 μM BK, while after 6 hours it quickly decreased to the value of the control samples (Fig. 4C and 4E for *IL-6* and *IL-8* genes, respectively). It should be emphasized that high levels of BK can also induce NF-κB-mediated pro-inflammatory responses in several tissues, such as in the case of IL-8 release by smooth muscle cells under the influence of BK (Leeb-Lundberg *et al*., 2005). As noted above, it appears that the dynamics of BK metabolism and cytokine production may play an important role in shifting the expression of both types of kinin receptors, perhaps in favor of B1R, leading to the propagation of inflammatory processes. In turn, cell incubation with 1 nM BK caused a slight increase in IL-6 and IL-8 mRNA, but only without the use of kininase inhibitors. The expression of these genes was even reduced after 6 hours of BK treatment when these inhibitors were used (Fig. S1B and S1C at https://ojs.ptbioch.edu.pl/index.php/abp/).

Obesity-related inflammation is characterized by reduced adiponectin production, an anti-inflammatory

Figure 6. The effect of BK stimulation on the gene expression of lipid metabolism markers in differentiated Chub-S7 cells.
Differentiated cells were incubated with 1 μM BK in the presence or absence of kininase inhibitors. The gene expression of *C/EBPα* (**A**), *CIDEC* (**B**) and *LPL* (**C**) was measured by qPCR. The bars represent the mean values ± S.D. of at least three experiments. Statistical significance *vs*. undifferentiated cells was **p*<0.05 or #*p*<0.01.

adipokine whose secretion by adipocytes is inversely proportional to the lipid content (Ouchi *et al.*, 2011). In this study, the release of adiponectin by BK-treated adipocytes was gently increased (by 20%) only in cells incubated without kininase inhibitors (Fig. 5B). These results are correlated with the expression of the *AdipoQ* gene, showing a strong increase of more than four times in mRNA (Fig. 5A). This effect was unexpected given the increased production of pro-inflammatory cytokines discussed above. This is even more intriguing, as 1 nM bradykinin caused a similar effect in cells without pre-incubation with kininase inhibitors (Fig. 2SA at https://ojs.ptbioch.edu.pl/index.php/abp/). However, many factors are involved in the regulation of adiponectin gene expression. Cytokines such as IL-6, IL-8, and TNFα can lead to increased expression of *AdipoQ* through the signal transducer and activator of transcription 3 (STAT3). In the STAT3-initiated final stage, the signaling pathway activates FoXO1, which through the CCAAT/enhancer binding protein (C/EBP) triggers the transcription of the adiponectin gene (Shehzad *et al.*, 2012). One of the pivotal proteins involved in adipogenesis is C/EBPα, which binds to the adiponectin gene and activates the gene promoter (Park *et al.*, 2004). In this regard, our study showed a strong increase in C/EBP gene expression after 1 μM BK stimulation of differentiated Chub-S7 cells (Fig. 6A). Taking this into account, it can be assumed that increased expression and secretion of adiponectin may be related to increased expression of the *C/EBPa* gene. In turn, kininase inhibitors were able to reverse BK-induced adiponectin release (Fig. 5B). In this case, the amount of secreted adiponectin showed a value similar to that obtained in unstimulated cells. This fact is correlated with the observed low cytokine production by differentiated cells after a short incubation time with 1 μM BK (Figs 4B, 4D, and 4F). Furthermore, cell treatment with a low concentration of BK in the presence of kininase inhibitors showed a decrease in the expression of cytokine genes (Fig. S1 at https://ojs.ptbioch.edu.pl/index.php/abp/) and C/EBPα (Fig. S2B at https://ojs.ptbioch.edu.pl/index.php/abp/). These results demonstrate the complexity of the mechanism of action of BK in adipocytes. Different kinase signaling pathways, such as mitogen-activated protein kinase, Akt/PI3K and STAT3, are involved in the expression of adiponectin (Fasshauer *et al.*, 2002; Shehzad *et al.*, 2012). Furthermore, these proteins are also associated with kinin receptor signaling (Leeb-Lundberg *et al.*, 2005). Therefore, it can be concluded that the signaling pathways responsible for adiponectin production can be altered by the action of BK. The effect of 1 μM BK on *CIDEC* gene expression was also investigated (Fig. 6B). As described above, this transcriptional factor is expressed in adipose tissue and regulates lipid metabolism by promoting lipid accumulation (Kim *et al.*, 2008). A higher concentration of BK was able to induce the expression of CIDEC mRNA independently of the presence of kininase inhibitors, supporting the hypothesis that kinin peptides may promote adipogenesis. However, a lower concentration of BK caused an increase in the amount of CIDEC mRNA in cells treated without kininase inhibitors, while in the presence of such inhibitors, a lower expression of CIDEC was observed, especially after 6 h (Fig. S2D at https://ojs.ptbioch.edu.pl/index.php/abp/). Another marker of lipid metabolism evaluated in this study was LPL. Stimulation of adipocytes with 1 μM BK in the presence of kininase inhibitors resulted in poor LPL expression, while the lack of inhibitor showed the opposite effect (Fig. 6C). A study carried out with ACE knockout mice

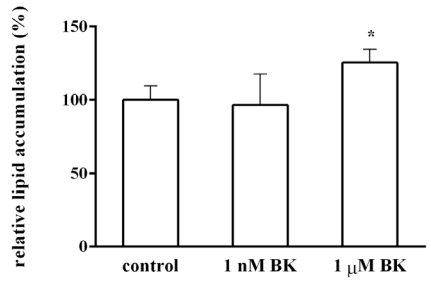


Figure 7. Effect of BK stimulation on lipid accumulation in differentiated Chub-S7 cells.
Differentiated cells were incubated with 1 nM or 1 μM BK. Intracellular lipids were stained with Oil Red O and the measured absorbance was normalized to the values of the control samples, which was assumed to be 100%. The bars represent the mean values ± S.D. of at least three experiments. Statistical significance *vs.* undifferentiated cells was *$p<0.05$.

demonstrated increased LPL production without changes in fatty acid synthase, showing reduced fat mass and improved glucose clearance (Jayasooriya *et al.*, 2008). Therefore, it can be assumed that inhibition of ACE may lead to similar effects. In fact, differentiated Chub-S7 treated with 1 μM BK without inhibitor showed an increase in lipid accumulation (by 25%) compared to untreated cells (Fig. 7). In turn, a lower concentration of BK did not cause lipid accumulation. The effects of 1 nM BK on LPL mRNA were slightly different (Fig. 2SD at https://ojs.ptbioch.edu.pl/index.php/abp/). The use of kininase inhibitors led to lower gene expression, while the lack of these inhibitors promoted the expression of the *LPL* gene. Therefore, the addition of BK to adipocytes under physiological conditions results in increased expression of LPL, which can lead to a decrease in lipid accumulation. In contrast, the opposite situation was observed when a higher concentration of BK was used, especially when BK was not degraded because of the presence of kininase inhibitors.

In properly functioning adipose tissue, a balance between pro-inflammatory and anti-inflammatory adipokines is maintained. On the contrary, during intense hypertrophy-based adipogenesis, too large fat-filled adipocytes lose their ability to control metabolic processes. The consequence of impaired adipocyte function is a change in adipokine secretion that regulate metabolism and the inflammatory process at the level of both tissue (autocrine, paracrine) and the entire organism (endocrine) (Taylor, 2021). In summary, our study demonstrated that BK can potentiate the production of adipokines, including pro-inflammatory cytokines. These processes depend on both the concentration of the peptide and the presence of kininase inhibitors that could degrade it. An important element of the involvement of BK in these processes appears to be the dynamics of degradation and/or generation of this peptide by adipocytes. The fact that adipocytes can degrade kinins is known and in this study we showed that the cell model used has the enzymatic proteins necessary for kinin generation. We propose that BK at pathological concentration can act through the B2 receptor inducing the pro-inflammatory response in adipocytes. Although strong pro-inflammatory des-Arg-BK generation can be expected, cells incubated without kininase inhibitors also led to increased cytokine release, which can autocrinnely enhance adipocyte dysfunction, such as reduced lipid degradation (decreased *LPL* expression) and enhanced adipogenesis (increased *CIDEC* expression). However, it seems that the effect of BK at

physiological concentration, especially in the presence of kininase inhibitors, does not indicate a significant impairment of adipocyte functions. It can be concluded that the influence of BK on energetic homeostasis is related not only to the modulation of glucose metabolism but also to the regulation of lipid metabolism in adipocytes.

Declarations

Conflict of interest. The authors have no conflict of interest to declare.

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