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The characterization of tumor necrosis factor alpha (TNF-α), its role in cancerogenesis and cardiovascular system diseases and possibilities of using this cytokine as a molecular marker

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ABSTRACT

The inflammatory process is directly associated with secretion of cytokines, e.g. tumor necrosis factor alpha (TNF-α). This molecule is one of the 22 proteins which belong to TNF family and is secreted mainly by: macrophages, monocytes, T lymphocyte and mast cells. The biological effects of TNF-α is possible through binding this cytokine to specific receptors – TNFR1 and TNFR2. The large number of reports provides that this cytokine plays extremely important role in cancers and cardiovascular disease – two groups of inflammatory diseases. Unfortunately, these diseases are the main cause of death in spite of advances in medicine and increasing public awareness of prevention.

It is believed that better understanding both molecular potential of this cytokine and the impact in cancerogenesis and others inflammatory diseases may cause using TNF-α as a molecular marker in these diseases and will make it possible to observe the effects of anti-inflammatory therapy. It will be able to cause a drop in the incidence of these diseases and better monitoring of them.

KEY WORDS: cytokine, inflammatory process, cancerogenesis, cardiovascular diseases, marker

Introduction

Tumor necrosis factor alpha (TNF- α) as an example of cytokine

Cytokines are broad and small proteins, molecules and hormone-like proteins (5-20kDa) (Badowska-Kozakiewicz 2013) playing an extremely important role in many cellular processes, such as growth, differentiation, migration, and apoptosis. They also play an important role in immune reactions,

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inflammatory processes, maintenance of tissue homeostasis. Cytokines can cause pyrogenic effects on energy balance of the body due to the change of appetite, impact both on the structure and functioning of the cardiovascular system or the regulation of activity of the autonomic nervous system. The biological effects of cytokines are possible because they are important mediators of signaling pathways (Chechlińska 2003, Commins *et al.* 2010).

The family of cytokines includes: chemokines, interferons, interleukins, lymphokines and tumor necrosis factors. These molecules are produced by different kinds of cells, for example: active immune cells, keratinocytes, cardiomyocytes, myocytes, fibroblasts, neurons and glial cells. The characteristic feature of the cytokines is the fact that they have pleiotropic, often opposing activity depending on both the type of cell by which they are secreted and acting (Ufnał & Wołynczyk-Gmaj 2003). This fact can cause a problem with classification of cytokines but generally it is carried out on the basis of pro- and anti-inflammatory effects (Bergler-Czop & Brzezińska-Wcisło 2011).

The characterization of tumor necrosis factor alpha (TNF- α)

One of the best characterized cytokine is a tumor necrosis factor alpha (TNF-α) which is known also as a cachectin or differentiation-inducing factor (DIF) and secreted mostly by: monocytes, macrophages, T lymphocytes and mast cells. It is a pro-inflammatory cytokine and one of the 22 proteins which belong to the TNF family (Badowska-Kozakiewicz 2013). The gene encoding this compound is built with 4 exons and is relatively small (3kbp) compared to genes encoding, for example: interleukin 1β (31 kbp) or isoform 1 of cyclooxygenase (22 kbp) and isoform 2 of this enzyme (8.3 kbp) (Korobowicz 2006).

Polymorphisms in the promoter region of the TNF-α gene play an extremely important role because they regulate the transcriptional activity of this cytokine and also have an influence on the biological activity. The best known TNF-α polymorphism is biallelic polymorphism -308G / A. The allele -308A is associated with increasing transcriptional activity of the TNF-α gene in vitro and synthesis of greater amounts in vivo than the allele -308G. Other polymorphisms having the ability to modulate gene expression of TNF-α are: -238G / A, -865C / A, -859G / A, -1032T / C (Kocierz *et al.* 2007).

The role of TNF- α and its receptors in transduction of molecular signals

This proinflammatory cytokine is present in two forms. First is a membrane (precursor) form with a molecular weight of 26 kDa and second is a 17 kDa secretory form after the enzyme modification (Korobowicz 2006, Horiuchi *et al.* 2013).

The result of the translation process is 26 kDa precursor form, called the transmembrane TNF-α (tmTNF-α). It is modified involving metalloproteinase enzyme TACE (TNF-α-converting enzyme, EC3.4.24), resulting in cutting off 76 N-terminal amino acids and making biologically active form of the cytokine - sTNF-α (soluble TNF-α, 17 kDa). Both forms are homotrimers capable to interact with receptors (Juszczyński & Warzocha 2002, Horiuchi *et al.* 2013).

There are evidences that both forms of TNF-α - soluble and precursors, participate in the inflammatory response. They also indicate that tmTNF-α may act as a bipolar molecule transducing the signal, as a ligand by attaching to receptors of TNF-α or as a receptor for transmitting signals to cells producing TNF-α. Transmembrane form of TNF-α is capable of binding to both receptors - TNFR1 and TNFR2, but it seems that the

signaling pathway with the participation of TNF-α anchored in the membrane is initiated by receptor-2 (Horiuchi *et al.* 2013).

The biological changes caused by TNF-α still remain not fully explained, however it is known that these changes occur via two receptors for this cytokine – TNFR1 and TNFR2 and it will help to understand the transduction of the molecular signal. There are two types of this receptors – transmembrane (tmTNFR1 and tmTNFR2) and soluble form (sTNFR1 and sTNFR2) (Wcisło *et al.* 2002, Badowska-Kozakiewicz 2013).TNFR1 is expressed on the surface of most nucleated cells while TNFR2 only on the surface of immune cells.

Transmembrane form of TNFR1 and TNFR2 are built by three domains – ECD (extracellular domain), TMD (transmembrane domain) and ICD (intracellular domain) (Korobowicz 2006). The soluble form is built only by ECD domain and is formed by proteolytic hydrolysis of the extracellular domain of immune cells, particularly monocytes and macrophages. There are evidences that sTNFR1 and sTNFR2 are present in the cerebrospinal fluid – spinal, tissues with difficulty of draining the lymph (Serwin *et al.* 2004). Soluble receptors play an important role in the processes associated with the modulation of the immune response. In addition, they are involved in the binding and inactivation of TNF-α, contributing to the modification of the activation of programmed cell death (Mielczarek *et al.* 2011). Increased expression and elevated plasma concentrations of both forms of the soluble receptors for TNF-α are observed in obesity. However, sTNFR1 has the greatest influence on the development of obesity and responds to insulin resistance, increasing energy expenditure and in consequence reducing the body weight (Olszaniecka-Glianowicz 2005, Goral 2008, Szalecki *et al.* 2008).

The signaling pathways of TNF-α cause two different effects: cell death (mainly by TNFR1 which has death domain associated with ECD) or activating NF-κB signal transduction leading to cells survival (Krzyżowska *et al.* 2009, Szołtysek *et al.* 2011, Skórka & Giannopoulos 2012). It can be observed that signaling pathway of this cytokine is a complex process.

Cancers and cardiovascular disease as a global health problems

The cancers and cardiovascular diseases are two main death causes. The World Health Organization's (WHO) statistical analysis shows that in 2008, 30 percent of all global deaths were associated with cardiovascular disease and this number has been increasing. Data collected by WHO highlights also that „cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012" (www.who.int). According to these data cancer and cardiovascular diseases are very serious global problem despite advances in medicine and increasing public awareness of prevention. Therefore, besides better understanding of the mechanisms associated with the disease, it would be important to find a complementary diagnostic molecular marker, which may be TNF-α. TNF-α is called a „new marker of inflammatory process" (Będowska 2007, Kabłak-Ziemnicka 2010).

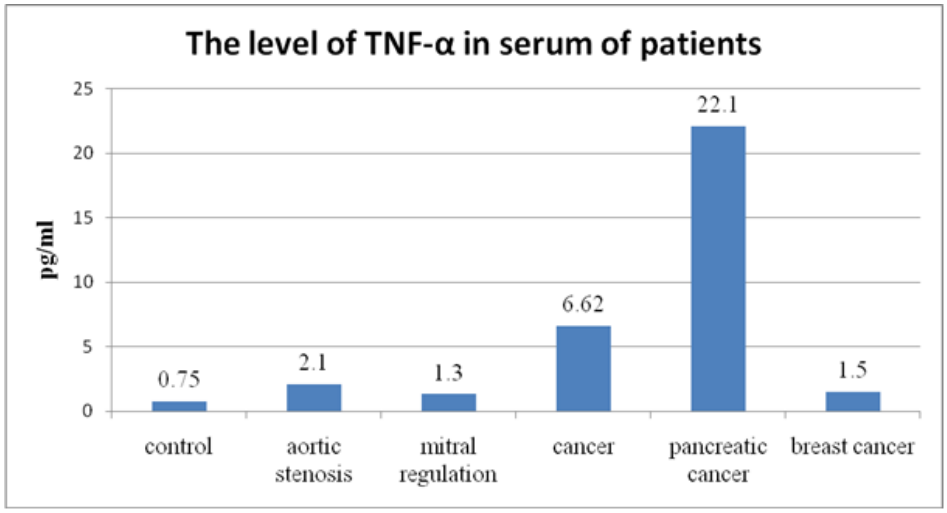
TNF-α as a molecular marker in diseases

Some reports show that TNF-α is useful as a potential marker. Kacperska *et al.* noted that changes in serum concentration of TNF-α may be a potential marker in multiple sclerosis (MS) which is an inflammatory and neurodegenerative disease (Kacperska *et al.* 2014). The possibilities of using this cytokine as a marker were observed in inflammatory bowel disease (IBD)

although some reports show the opposite information (Eder *et al.* 2007). In spite of these discrepancies, Komatsu *et al.* provided a higher concentration of TNF-α in serum of patients with IBD, recommending to analyse the changes using immuno-PCR, which is more sensitive than ELISA method (Komatsu *et al.* 2001). Olczyk-Kwiecień *et al.* observed that patients with rheumatoid arthritis treated aggressively had low levels of TNF in serum what confirmed the role of this cytokine in inflammatory process (Olczyk-Kwiecień *et al.* 2006).

The figure 1 shows the changes in level of TNF-α in serum of patients with cancers and cardiovascular diseases. It can be observed that during these processes this cytokine is secreted with higher amounts compared to control (healthy people). The graph shows the wide variation in specific tumor types (from 1.5 pg/ml in the breast cancer to 22.1 pg/ml in the pancreatic cancer). According to this results it can be observed that cardiovascular diseases and cancers are pro-inflammatory diseases of the substrate, accompanied by increased secretion of inflammatory agents including TNF-α (Scheen-Chen *et al.* 1997, Kapadia *et al.* 2000, Gasiorowska *et al.* 2016).

Figure 1. The level of TNF-α in different cancers and types of cardiovascular diseases and control. This chart was based on data from: Scheen-Chen *et al.* 1997, Kapadia *et al.* 2000, Gasiorowska *et al.* 2016.



TNF- α in cancer and cancerogenesis

TNF-α in carcinogenesis has two opposite ways of action. On the one hand it inhibits the proliferation of tumor cells and increases apoptosis of this kind of cells although it can promote metastasis by influencing the synthesis and activity of matrix metalloproteinases. TNFR1 is responsible for cytotoxicity of tumor necrosis factors against tumor cells. In contrast, TNFR2 is responsible for effects of the acute phase (Wolańska *et al.* 2010).

It was observed that the synthesis of small amounts of TNF-α in the tumor microenvironment, promoting development of tumor growth as well as the surrounding cells, induces apoptosis. However, the higher amounts initiate tumor cell death and stimulate the antitumor response (Tse *et al.* 2012). It is believed that chronic inflammation is one

of the main risk factors for the development of carcinogenesis process, in which TNF-α plays an extremely important role. It is involved in all aspects of the carcinogenesis: transformation, proliferation, angiogenesis and metastasis (Wang & Lin 2008). The antitumor effect of TNF-α is associated with modulation of immune response and leads to instant destruction of tumor stroma by cytotoxic T lymphocyte or activation of dendritic cells.

The reports show the higher level of this pro-inflammatory cytokine in the serum of patients with various types of cancers. Furthermore, the transcriptional activity of TNF-α increases in precancerous stages and is a negative prognostic indicator of tumor development (Wang & Lin 2008).

TNF-α exhibits a devastating effect on tumor vessels, including by changing the properties of endothelial cells from anticoagulant to procoagulant leads to stimulation of tissue factor expression and inhibition of thrombomodulin (Juszczyński & Warzocha 2002). NF-κB stimulated by TNF-α has the opposite effect, anti-tumorigenic in the organ that regenerates itself rapidly (liver) cells and pro- tumorigenic in colon, which regenerates slowly (Wang & Lin 2008).

The data analysis provide ambivalent effect of TNF-α on cancerogenesis, which can be a problem with the use of this cytokine as a marker. Despite this fact, some reports show possibilities of using this agent to observe and diagnose the process associated with cancerogenesis.

The role of TNF-α in cardiovascular diseases

In hypertension, which is one example of cardiovascular disease there is an increase in level of pro-inflammatory cytokines in the blood and tissues of the cardiovascular system (Ufnał & Wołynczyk-Gmaj 2011). The studies have confirmed the higher serum levels of many pro-inflammatory proteins, for example: CRP, IL-6, IL-8, TNF-α in patients with hypertension (Głuszek & Kosicak 2011). It is believed that TNF-α and other cytokines produced in large amounts are the main factors responsible for the progress and development of heart failure. Depending on the concentration, TNF-α exerts protective or harmful effect on the cell function of the heart and myocardium. Low levels of this cytokine is associated with a protective effect (Kurrelmeyer *et al.* 2000), while the higher leads to toxic effect on the myocardium, for example: dysfunction and remodeling of left ventricular, myocardial metabolism disorder, intensification of oxidative stress and endothelial dysfunction. Otherwise activating sphingomyelinase exacerbates the apoptosis of cardiomyocytes (Agnoletti *et al.* 1999).

TNF-α is involved in the production of other pro-inflammatory cytokines, i.e. IL-1, IL-6, which in turn increases metabolic disorders associated with myocardial infarction (Puszkarska & Głuszek 2010, Głuszek & Kosicak 2011). TNF-α is one of the most important cytokine responsible for the endothelial dysfunction, which is characteristic of hypertension (Pacholczyk *et al.* 2008). The mechanism occurring in this process is the activation of NF- κB, which by binding to the promoter sequence of the genes that encode cell adhesion molecules: VCAM-1, ICAM-1, MPC-1, E-selectin in endothelial cells and vascular smooth muscle cells increases their expression (Ouchi *et al.* 1999). TNF-α is also responsible for the damage to the integrity of the endothelial cells by induction of apoptosis of these cells (Hermann *et al.* 2000).

Summary

The large number of reports shows that TNF-α is directly associated with

inflammatory process, cancer and cardiovascular disease. It is extremely important to better understand the mechanisms and signaling pathways of TNF-α to use this cytokine as a molecular marker of pro-inflammatory diseases. This action will be useful in thediagnosis and treatment of cancers and cardiovascular diseases.

The studies and statistical data show how cancer and cardiovascular diseases are a serious problem and highlight the need to find new diagnostic marker. TNF-α can be used as a potential marker, which was confirmed in a large number of reports, however a better understanding of the biological role of this cytokine is absolutely essential.

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Streszczenie

Proces zapalny jest bezpośrednio związany z sekrecją cytokin, np. czynnika martwicy nowotworu alfa (ang. Tumor Necrosis Factor alpha; TNF-α). Ta cząsteczka jest 1 z 22 białek należących do rodziny TNF i wydzielana jest głównie przez: makrofagi,

monocyty, limfocyty T oraz komórki tuczne. Biologiczne efekty działania TNF-α zachodzą dzięki wiązaniu się tej cytokiny ze specyficznymi dla niej receptorami – TNFR1 i TNFR2. Duża liczba prac potwierdza kluczową rolę TNF-α w nowotworzeniu i chorobach układu sercowo-naczyniowego, będących chorobami o podłożu prozapalnym. Niestety, mimo postępu medycyny i wzrostu świadomości społeczeństwa, wymienione choroby stanowią główne przyczyny śmierci na świecie. Lepsze zrozumienie roli tej cytokiny w kancerogenezie i chorobach zapalnych może spowodować wykorzystanie TNF-α jako markera tych chorób oraz do monitorowania przeciwzapalnych efektów terapii.

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Optimization of flotation assay conditions for syndapin binding to phosphatidic acid containing liposomes

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ABSTRACT

Flotation is one of the best method for preliminary identification of protein-lipid interactions. In most widely used approach it utilizes large unilamellar vesicles, that are excellent models of freestanding membranes and do not require any additional components, like solid supports or beads that are needed in other methods commonly used for protein-lipid binding studies. Here we present results obtained during our studies on phosphatidic acid - syndapin interactions and discuss some technical aspects of this method underlying how relatively small changes in the conditions can influence the results.

KEY WORDS: protein-lipid interactions, LUVs, density gradient, ultracentrifugation

Introduction

Phosphatidic acid (PA) is the simplest phospholipid that consists of phosphate group attached to glycerol backbone in sn-3 position and two acyl chains. PA has a very small anionic head group and under physiological conditions it has a molecular shape of a cone (Kooijman *et al*. 2005). Although the amount of PA in cells is very low, (1% of total phospholipids in plants) (Guo *et al*. 2011) it is involved in many processes like regulating membrane dynamics or serving as an intermediate in lipid biosynthesis. It is also a binding partner for many proteins, being often the key point in their signaling pathways. Syndapin belongs to the group of defined PA- biding proteins. It is a member of F-BAR superfamily of membrane binding proteins (Brian 2004), that plays significant role in membrane tubulation, and by that it is identified as a regulator of synaptic vesicle endocytosis (SVE) (Quan & Robinson 2013). The presence of PA is essential for syndapin to fulfill its function. It was observed that the membrane tubulation activity of syndapin was decreased in membranes of lower concentrations of PA, what suggests that PA may be the membrane recruiting molecule. In fact in in vitro studies it appeared that syndapin binds to the model membranes by direct interactions with PA (Srinivas *et al*. 2013).

In this work we would like to present a method suitable for identification of protein-membrane interactions. There are many experimental approaches to establish the presence of protein-lipid interactions, such as lipid overlay assays (Castellana & Cremer 2006). Such methods use lipids deposited on solid supports, which are not present in

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physiological context of lipid bilayer. Moreover, it is usually difficult to quantitate the bound fraction of a protein. Further issues are connected with the fact that the deposition of various classes of lipids may not be equally efficient and some lipids may be partially removed from the substrate during the experiment. Going further with experimental procedures, interaction of lipids with proteins may also be identified using lipid monolayers. Such approach is highly homogenous but the single lipid leaflet is far different from natural membrane state (Maget-Dana 1999).

Flotation is the method where large unilamellar vesicles (LUVs) are used. Significant advantage of this approach over other lipid protein interaction identification methods, like overlay assay is the use of lipid vesicles also known as liposomes. This method is based on the effect of the flotation of vesicles in density gradient of a biochemically inert sugar, where the highest density is at the bottom of a test tube. The liposomes float during the ultracentrifugation until they reach the conditions where there is no difference between their own effective density and the density of the surrounding medium (Fig.1). Comparison of the protein concentration in the fractions collected after centrifugation allows to determine the presence of interactions with membranes, which is reflected by the presence of a protein in the upper fractions together with liposomes. If protein stays at the bottom and does not migrate together with liposomes it reveals that no interaction exists (Bigay & Antonny 2006). Sedimentation is a similar method that utilize lipid vesicles and ultracentrifugation. In this approach protein concentration in the pellet (sedimented vesicles) is compared with the supernatant. However this method has some drawbacks, namely during the centrifugation process the unbound protein can precipitate and sediment together with the vesicle-bound fractions and thus give false positive results (Bigay & Antonny 2006).

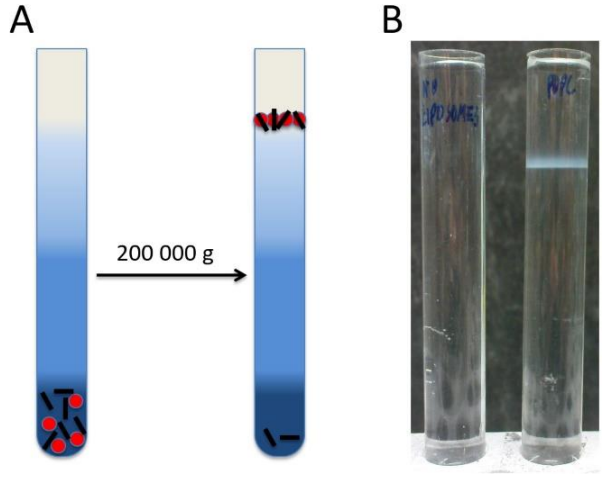

Figure 1. Flotation in the sucrose gradient. (A) Flotation scheme with sucrose gradient represented by blue shades of different intensities, protein as black sticks and liposomes as red dots. Left tube shows the situation before ultracentrifugation while right part shows the state after ultracentrifugation, directly prior to sample collection; floated liposomes with bound protein are at the interface between 10% and 0% of sucrose. (B) A representative image of test tubes after ultracentrifugation: without liposomes (left) and with POPC liposomes visible as a white cloudy fraction (right).

It is highly important that liposomes, used in the experiment are as homogenous as possible to obtain reliable results. Preparation of liposomes based on dry lipid film hydration leads to formation of a mixture heterogeneous vesicles with broad size distribution and lamellarity. This is why extrusion through polycarbonate filters with e.g. 200 nm pore size (diameter) results in obtaining vesicles smaller than 200 nm, and substantial fraction of very small liposomes passively go through the filter. Heterogeneous vesicle populations may hamper the analysis in membrane-protein interaction studies. It has been postulated that the use of heterogeneous liposome population would add the effect curvature-selective binding of amphipathic proteins, which may distort lipid-protein specificity (Czogalla *et al*. 2014). It was also shown that more homogeneous suspensions of the liposomes could be obtained by extrusion using membranes with 100 nm pore diameter (Kunding *et al*. 2008).

Materials and methods

Materials

POPA, POPC were bought from Avanti Polar Lipids (Alabaster, AL). ECL™ Prime Western Blotting Detection Reagent was bought from Amersham. Primary antibodies recognizing syndapin (pacsin) were bought from Santa Cruz and secondary antibodies Goat-Anty Rabbit were bought from Cell Signaling. Sucrose and NaCl was purchased from Chempur, HEPES from Roth, and Nitrocellulose from Amersham. The pGEX-6P-1 plasmid vector containing the sequence encoding mouse syndapin1 (Gen Bank accession number NM_011861) was a generous gift from Yvonne Groemping (Max Planck Institute for Developmental Biology, Tuebingen, Germany) and it was also deposited in Addgene repository (Plasmid #36859).

Protein purification

Protein was overexpressed in bacterial system in *E.coli* (BL21 (DE3) pLysS) (Promega). Bacteria was transformed with the plasmid vector and cultured on LB agar plates with ampicillin (100 μg/ml) overnight at 37°C. Then a single culture was transferred to test tube with LB medium with ampicillin (100 μg/ml). Overnight 37°C pre-culture was centrifuged (10 min, 10000 g, 4°C,) and the pellet was transferred to fresh LB medium with ampicillin (100 μg/ml), cultured at 37°C to reach OD_{600} = 0.6 and induced with IPTG (final concentration 1mM) and cultured overnight at 18°C with constant shaking (200 rpm). Bacteria was then centrifuged (15 min., 4°C, 10000 g) and frozen with liquid nitrogen. Bacteria were thawed on ice and treated with lysis buffer (10 mM HEPES, 300 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 8.0), sonicated (Hielscher sonicator) 10 times for 15 seconds with 15 seconds intervals on ice and centrifuged (35000 g, 30 min., 4°C). The supernatant was loaded onto a 1 mL bed volume Glutathione Sepharose 4B (GE Healthcare) column which was pre-equilibrated with the lysis buffer. The resin was then washed with 50 mL of SLB buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) and 20 mL of prescission buffer (10 mM HEPES, 150 mM NaCl, 1mM EDTA, 1 mM DTT pH 7.0) until absorbance A_{280} was lower than

0.05. 1 mL of Prescission buffer with protease 3C (Max Planck Institute for Cell Biology and Genetics, Dresden, Germany) (900 µL + 100 µL, respectively) was added to the resin and incubated overnight at 4°C. Elution was performed with prescission buffer. Concentration of Syndapin was determined using a Cary 1E spectrophotometer at λ=280 nm employing extinction coefficient parameters determined using ProtParam tool (www.expasy.com). Purity and concentration were also estimated from coomassie stained SDS-PAGE gels.

Liposome preparation

The required amounts of POPC and POPA in chloroform were dried under a stream of nitrogen and subsequently under vacuum in a glass tube and hydratated in SLB buffer (10 mM HEPES, 150 mM NaCl, pH=7.4). Liposomes were frozen/thawed five times and extruded through 0.2 µm and 0,1 µm filters (sequentially, 11 times each). Size and potential were determined using Malvern Zetasizer Nano ZS (Malvern). Liposomes were snap-frozen in liquid nitrogen and stored at -80°C.

Flotation assay

Protein concentration of 30nM and 0.4 mg/ml of liposomes were mixed and incubated at room temperature for 30 minutes. Samples were transferred to ultracentrifuge tubes and mixed with 250 µL of 60% sucrose (in SLB buffer). This fraction was overlayed with 0.8 mL of 15% sucrose. Next two layers of the gradient consisted of 1.8 mL of 10% sucrose and 1 mL of SLB buffer. Ultracentrifuge tubes were placed in 60Ti rotor (Beckman Coulter). Samples were centrifuged (200 000 g for 2 hours at 4°C) using Beckman Coulter Optima^{TM} L090K Ultracentrifuge). From each tube six fractions were gently collected. After addition of SDS (1% final concentration) fractions were analyzed by Dot Blot.

Dot Blot

Equal amounts of samples were loaded into the wells of Dot Blotter (Hoefer Scientific Instruments) and aspirated using vacuum pump. Membrane was blocked with milk for 1hour at room temperature after entire samples were filtered through the membrane. Incubation with primary antibodies (1:1000) was either for 3 hours at room temperature or overnight at 4°C. Membrane was washed three times in TBS-T (5 min. each). Secondary antibodies (1:10000) were added and incubated for 1 hour in room temperature and subsequently washed as previously. For detection commercial ECL was used. Images were made using the UVP (Biospectrum) in chemiluminescence mode.

Results

Thin layer chromatography (TLC)

TLC chromatography was performed to control lipid composition of liposomes used in the further experiments. Extracted lipids correspond to lipids used for liposomes preparation (Fig.2).

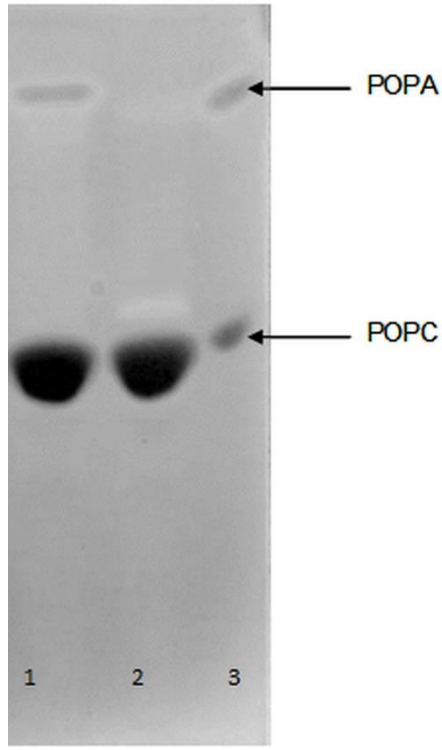

Figure 2. Liposome composition analysis using TLC. TLC analysis of: 1) liposomes with POPC/POPA in 9/1 molar ratio, 2) liposomes made of 100% POPC, 3) chloroform dissolved standards of POPC and POPA.

LUVs and the liposome-bound protein floated to the top fraction

As a result of vesicle flotation experiment, it is expected that after centrifugation liposomes will locate between two top fractions: 0% and 10% of sucrose concentration. Indeed in our experiments bound protein was detected within this liposomal fraction and any protein excess stayed at the bottom of test tube (30% sucrose). When no binding occurred, protein did not float and stayed at the bottom.

Searching for an optimal protein concentration

Protein concentration can have a big impact for study protein-lipid interactions using flotation method. In our first approach the protein concentration was overestimated and this led to some technical difficulties in the densitometry quantification of the dot blots. When too high amount of the protein was used the unbound fraction, that stayed at the bottom of the test tube largely exceeded the amount of protein present in liposomal fraction (bound to liposomes). This impeded the dot blot imaging due to fast overexposure of the parts of the image that correspond to bottom fractions and underexposure of the rest of the image, which makes the result unreliable and difficult to ascertain the presence of eventual interactions. Such situation can be avoided by changing the liposome-protein ratio in the sample. In accordance to that change of the protein concentration from 500 nM to 250 nM diametrically improved the obtained results.

Setting up the gradient and collecting the fractions

Appropriate approach to build the density gradient and collect the fractions after ultracentrifugation are the key steps for obtaining a clear results in a standard flotation assay. Even when all factors are optimized perfectly it does not guarantee legible result. Figure 3 presents result of an experiment when the two steps were conducted using inappropriate tools (i.e. automatic pipettes offering limited fluid expel precision). Such misconduction leads to the presence of a protein in all fractions although this should not be expected for the control, where only protein (without liposomes) was tested (compare Fig. 3A with Fig. 4A). This example also clearly shows how important is to conduct the control experiment with protein not mixed with liposomes in parallel to the liposome-protein samples in order to achieve necessary reliability.

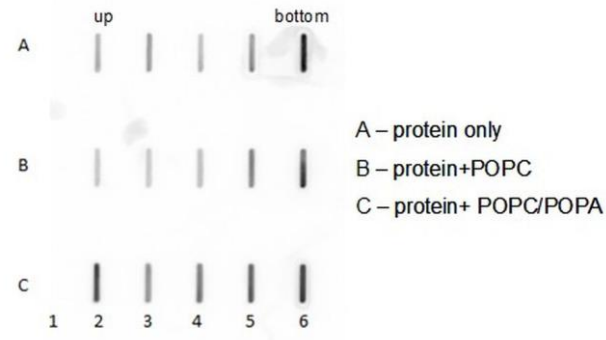

Figure 3. Dot blot analysis of fractions after flotation in non-optimized procedure. Fraction were collected from top (1) to the bottom (6) of the tube. (A) control sample containing syndapin without liposomes, (B) sample containing syndapin mixed with POPC liposomes, (C) sample containing syndapin mixed with liposomes composed of POPC/POPA 9/1 molar ratio.

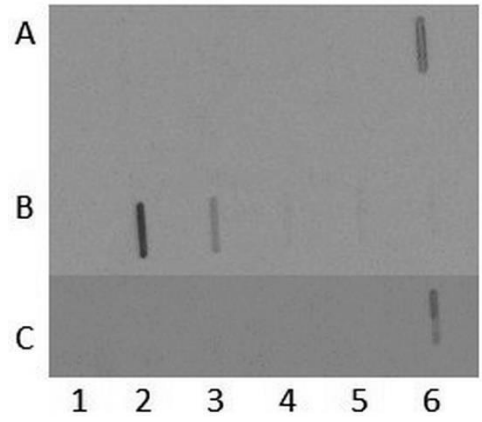

Figure 4. Dot blot analysis of fractions after flotation after optimization of the procedure. Fraction were collected from top (1) to the bottom (6) of the tube. (A) control sample containing syndapin I without liposomes, (B) sample containing syndapin I mixed with liposomes composed of POPC/POPA 9/1 molar ratio, (C) sample containing syndapin I mixed with POPC liposomes.

Figure 4 presents the experiment done properly, with optimized amount of the protein and under careful gradient building and fraction collection. First line is a control, only with protein. Protein without liposomes does not float, which means that 100% of protein stayed in the bottom fraction. As another control liposomes with POPC were used. No protein floating can be observed, which shows that syndapin I does not interact with membranes composed of pure phosphatidylcholine. On the other hand, a substantial fraction of the protein floated together with liposomes composed of POPC/POPA (90/10 mol%). Visible protein in second and third fractions provide evidence that almost all the syndapin I present in the sample was bound to the liposomes.

Optimizing sucrose concentration

The concentration-volume configuration of a sucrose gradient prior to ultracentrifugation can obviously have an impact on experiment result. It is important to choose proper gradient to let all the liposomes in the sample float. It may occur that too small differences in sucrose concentrations between neighboring gradient steps cause blurred fractionation, where liposomes are not concentrated within a certain range of the density gradient. A good practice is to estimate not only the amount of protein but also concentration of liposomes (or lipids) in each of the fractions collected after centrifugation. One of the easiest approaches consists of fluorescent labeling of liposomes coupled with spectrofluorimetric analysis of the fractions. Another issue to consider is to use an adequate gradient generator. While utilizing OptiPrep™ or other similar compounds it should be considered that buffer conditions should be equalized throughout the whole density gradient. It is because such compounds are available as water solutions of certain concentration (e.g. 60% in case of OptiPrep™) and includes neither salt nor buffering agents. Self-prepared sucrose solutions could be easily made by dissolving crystals directly with e.g. HEPES buffer. On the other hand, ready to use compounds, such as OptiPrep™ may exhibit superior properties such as relatively low viscosity and osmolarity.

Discussion

It is highly important to extend the existing knowledge about the protein-lipid interactions. Phosphatidic acid is essential for various signaling pathways. Its presence is often necessary to sustain activity of varius proteins. Thus, fully understanding the nature of its interactions with proteins may be critical for understanding regulatory mechanisms of signaling pathways, thus may play a significant role in drug development.

Among the numerous methods of protein-lipid interaction identification, flotation is unique for several important aspects. First of all, flotation method is usually performed using calibrated LUVs, which helps to achieve lipid organization analogous to what can be found in cellular membranes. Other methods used for protein-lipid interactions identification (i.e. overlay assay) are proceeded with conditions far away from physiological. This fact together with the simplicity of the flotation gives it superiority over other methods. Moreover, LUV's used in flotation assay are composed with pure lipids and the composition can be fine-tuned in a broad range. Liposomes calibration to 100 nm allows obtaining required size and membrane curvature homogeneity which is not achievable in case of membrane-assisted calibration (extrusion) with filter pore size larger or equal to 200nm. This is also true in terms of multilamellarity, as during extrusion

through larger pores it is much more difficult to eliminate all multilamellar vesicles. It should be stressed that the latter are highly undesirable in lipid-protein studies, as the inner membranes of such liposomes are usually not accessible to the protein added to liposomal suspension.

Selection of appropriate conditions for the experiment allows to get clear and unequivocal results. Ultracentrifugation in density gradient is one of the best approaches for preliminary evaluation of lipid-protein interaction. However one should be aware that there are some minor drawbacks of this method. Namely, the whole procedure is based on a relatively long centrifugation time. The kinetics of protein-lipid interactions may be an issue, as some proteins that have a faster rate of dissociation may dissociate from the floating vesicles before centrifugation is finished. Nevertheless, it also gives us the information about the reaction kinetics. In this case flotation can give us a hint about how stable in time the interaction is (in terms of the Koff value). However in some cases, when centrifugation time is extended, the protein will be on the bottom of the centrifuge tube and interaction will not be detected, even if it occurs. In such cases one should be aware that this result does not stay as a lack of interaction and the shortened centrifugation time may change the observations dramatically. Moreover, the described method does not provide fully quantitative analysis. Therefore, we would like to stress that each report on protein-lipid binding events should be underlined by results obtained using at least two fully independent experimental approaches, most preferentially based on various membrane model systems (Czogalla *et al*. 2014).

Acknowledgements

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Streszczenie

Flotacja jest jedną z najefektywniejszych metod wstępnej identyfikacji oddziaływań białko-błony lipidowe. W większości przypadków wykorzystuje się w niej małe jednowarstwowe pęcherzyki lipidowe, które służą jako modele błonowe i nie wymagają dodatkowych nośników, takich jak membrany czy nanocząstki polimerowe, które są często używane w innych metodach mających na celu identyfikację oddziaływań białko-lipid. W poniższej pracy prezentujemy wyniki uzyskane podczas badań oddziaływań kwasu fosfatydowego i syndapiny. Omawiamy także niektóre techniczne aspekty metody, kładąc nacisk na to jak małe zmiany w warunkach metody mogą wpłynąć na otrzymywane wyniki.

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Conditional probability of the daily minimum temperature in certain types of circulation on a seasonal basis in the Zywiec Valley in Southern Poland

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ABSTRACT

Air minimum temperature is very important for the natural environment and human activity. This paper presents certain aspects related to the variability of daily minimum temperature of air in the winter (XII, I, II) in the Zywiec Valley, in relation to the synoptic situation in the valley. The analysis is based on the results of research carried out at one point node (the grid) obtained from the base Carpat Clim database. The node is located at the bottom of the Zywiec Valley in the period 1961-2010. The study was complemented with a comprehensive analysis of local conditions for atmospheric circulation and temporal variability over a 50 years period. For this purpose, the classification of types of atmospheric circulation (Niedźwiedź 1981) was used for the upper Vistula river basin. Extreme temperatures included an average minimum temperature of air exceeding the 90^{th} and 95^{th} percentile. The relationship between the extremes of air temperature and atmospheric circulation types was examined by analyzing the frequency of occurrence of extreme values and their conditional occurrence in each particular type of atmospheric circulation.

KEY WORDS: Zywiec Valley, conditional probability, minimum air temperatures, extreme values, atmospheric circulation

Introduction

Air minimum temperature is very important for the natural environment and human activity. Apart from the circulation conditions needed for their existence, air minimum temperature is affected by relief and terrain type, local water balance and local vegetation (evapotranspiration). The Zywiec Valley is regarded as a geographic unit best known with respect to minimum air temperatures. The coldest winter, a so called "winter of the century" occurred here in 1929. The coldest month was February 29^{th}, and the minimum air temperature recorded that day was -40.6^{o} C (Starkel 1999).

The Zywiec Valley's environmental conditions and related impact on local climatic conditions are very adverse to the health and life of the population living there. The concave landforms give rise to stagnant cold air and frequent thermal

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inversions, which are a highly unfavorable weather phenomenon. They facilitate the formation of low stratus-type clouds, stratocumulus (St, Sc), prevent the formation of convection (vertical air movements) and horizontal air movement, which results in the weakening of local atmospheric circulation. They also contribute to the development and persistence of fog (Milata 1959).

The local terrain favors the long duration of the period of ground frost, hence agricultural problems, which are faced by the residents of the Zywiec Valley every year. Problems related to the change of the start and end dates of the period without the presence of ground frost play a key role, especially in the transitional seasons (spring and autumn). They influence the rate of plant growth, yield and labor in the fields. Any changes associated with the date of the beginning and end of the period without the occurrence of frost ground period are of immense importance (Bielec-Bąkowska & Piotrowicz 2011).

Materials and methods

The minimum air temperature based on the Meteorological Dictionary (Niedźwiedź 2003), is the lowest recorded temperature of the air during a given period of time. This definition was developed on the basis of a relatively rich collection of data obtained from the CarpatClim database the climate database of the Carpathian Region (http://www.carpatclim-eu.org). The coordinates of the grid point used in this study are 49.70ºN, 19.20ºE. It is located at the bottom of the Zywiec Valley. All analyes in this study were prepared on the basis of daily resolution data and the longest possible sequence (1961-2010).

The CarpatClim database, was established in order to improve the availability of climate data. It has very good temporal resolution (one day) and spatial resolution (0.1˚ x 0.1˚). Because of access issues and limited duration of the study, the synoptic situation was identified with the type of atmospheric circulation. Moreover, taking into account the availability of various typologies and their application to the region of southern Poland a calendar of types of circulation was used for the analysis of the synoptic circulation - TN (Niedźwiedź 1981). The basis for the separation of 21 types of atmospheric circulation in the case of this calendar is the dominant barometric system and the direction of advection. The index "a" denotes anticyclonic systems (high-pressure) and "c" cyclonic systems (low-pressure). It should be noted that this typology is subjective and non-automatic (Ustrnul & Wypych 2011). At the beginning, the data were analyzed in relation to the spatio-temporal differentiation of minimum air temperature in certain types of atmospheric circulation in each of the four seasons (winter, spring, summer, autumn). The likelihood of the stated ranges of air temperature was also analyzed during the most frequently noted advection of air masses. As a result, synoptic situations were identified that are particularly conducive to the occurrence of the minimum air temperature. The final stage of this study was to investigate and determine the frequency and likelihood of occurrence of air minimum temperature in the types of atmospheric circulation in light of the classification of types of synoptic situations made by Niedźwiedź. On the basis of synoptic maps of Europe, 21 types of synoptic situations were distinguished in this study. To make the distinction easy, universally applied lettermarks were introduced to determine the direction of advection with the letter ‘a’ for anticyclonic, and ‘c’ for cyclonic

situations: Na, Nc—situations with an advection of air masses from the north, NEa, NEc—situations with an advection of air masses from the northeast, Ea, Ec—situations with an advection of air masses from the east, SEa, SEc—situations with an advection of air masses from the southeast, Sa, Sc—situations with an advection of air masses from the south, SWa, SWc—situations with an advection of air masses from the southwest, Wa, Wc—situations with an advection of air masses from the west, NWa, NWc—situations with an advection of air masses from the northwest, Ca—centre of high pressure, Ka—anticyclonic wedge, Cc—center cyclonic situation, Bc—cyclonic trough, x—situations which cannot be classified (Twardosz & Niedźwiedź 2001).

Results

Average minimum air temperature versus atmospheric circulation type

Among the 21 types of synoptic situations commonly studied, the most frequently occurring situations is the anticyclonic situation. In the Zywiec Valley, the lowest minimum temperature during the winter is observed in an anticyclonic situation, mainly in the Ca, Ka and advection of air masses from the east, NEa, E and SEa. Average minimum temperatures oscillate between Ca (-10.7°C), Ka (-10.0ºC), while the NEa (-10.4°C), in the SEa (-10.1°C) and NEa (-9.3°C). During the examined time period, average minimum temperature in the spring was noted in the Ca (0.3ºC), NEa (1.9ºC), while in the Ka (1.8ºC). Advection of air masses from the east, particularly in situations of Ea produced a great impact on the minimum temperature in the autumn. In the summer and autumn, as in the previous seasons, the lowest values were recorded during the anticyclonic situation: Ca, Ka, and advection of air masses from the east. The average minimum air temperature in the summer ranged from Ca (10.2ºC), Ka (10.8ºC), while in the autumn (2.1ºC) and (3.8ºC) respectively (Fig. 1 and 2).

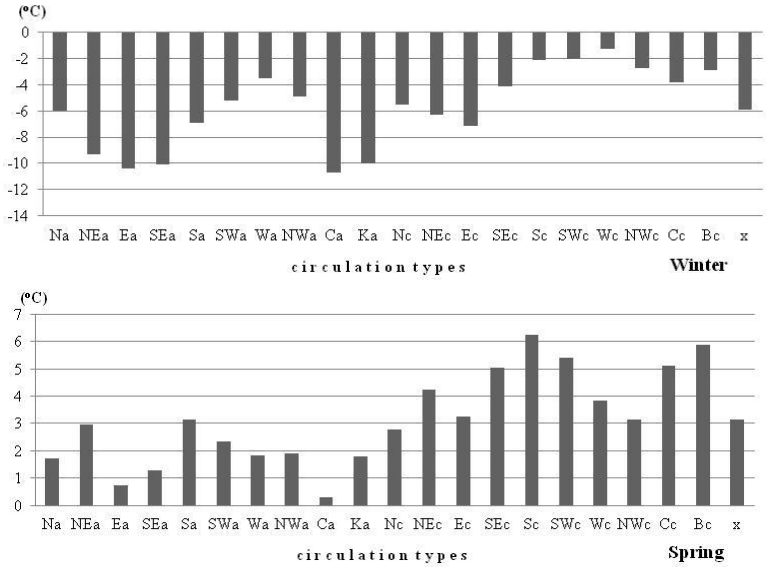

Figure 1a. Average minimum air temperature in a certain type of atmospheric circulation in the winter and spring.

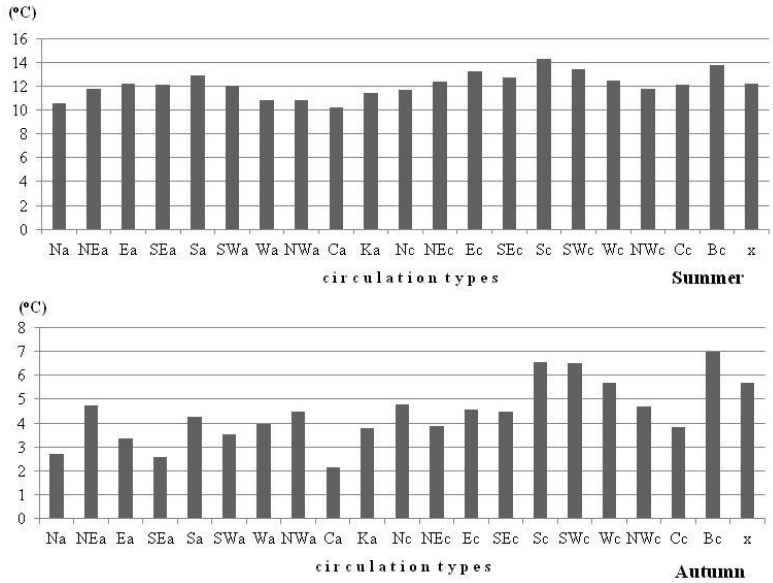

Figure 1b. Average minimum air temperature in a certain type of atmospheric circulation in the summer and autumn.

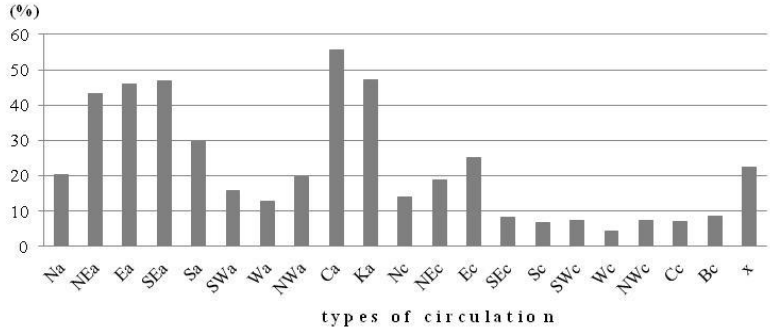

Figure 2. Conditional probability (%) of the daily minimum temperature the probability of < -10ºC in certain types of circulation in the winter.

Conditional probability of the daily minimum temperature in certain types of circulation on a seasonal basis

All four seasons (winter, spring, summer, autumn) were identified across the floor of the Zywiec Valley for the examined fifty-year period for which the probability of occurrence of minimum temperature in the range of local air temperature has been calculated. The range of air temperature for the winter was ≤ -10°C, spring and autumn ≤ 0°C, and the summer ≤ 10°C.

The research showed that anticyclonic situations occurred more often than cyclonic situations in the study area during the winter (Fig. 3). The most frequent situations were: Ca (55.6%) and Ka (47.2%). The next most frequent situations were anticyclonic and cyclonic patterns with the advection from the east: Ea (46.0%), NEa (43.3%), SEa (46.9%).

The least frequent situations were: Cc (7.0%), and NWc (7.3%).

In the spring, he highest probability of a minimum temperature of ≤ 0°C was recorded also in the Ca (45.2%), SEa (42.8%) and Ea (41.9%) (Fig. 4.). The least frequent situations were noted in: Sc (7.3%). On 23,0% of days, the daily minimum temperature ≤ 0°C occurred in X.

During the summer, the probability was greater during anticyclonic situations. The probability of ≤ 10°C minimum temperature is shown in Figure 5. The most frequent situations were: Ca (48.1%) NWa (39.5%) Na (38.8%), and Wa (36.9%). The least frequent situations were: Sc (6.3%) and Bc (8.0%).

In the autumn the probability of ≤ 0°C minimum temperature reached higher values during anticyclonic situations (Fig. 6.) The most frequent situations were: Ca (37.8%), NWc (32.8%), SWa (32.7%). The least frequent situations were: Sc and Wc (11.0%), SWc (8.5%). On 11,5% of days, the daily minimum temperature ≤ 0°C occurred in X.

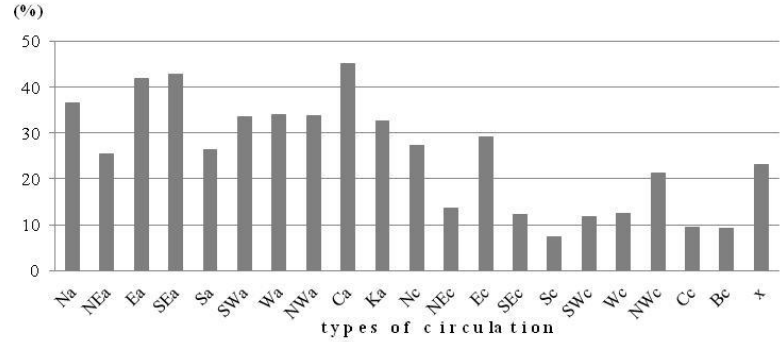

Figure 3. Conditional probability (%) of the daily minimum temperature the probability of < 0ºC in certain types of circulation in the spring.

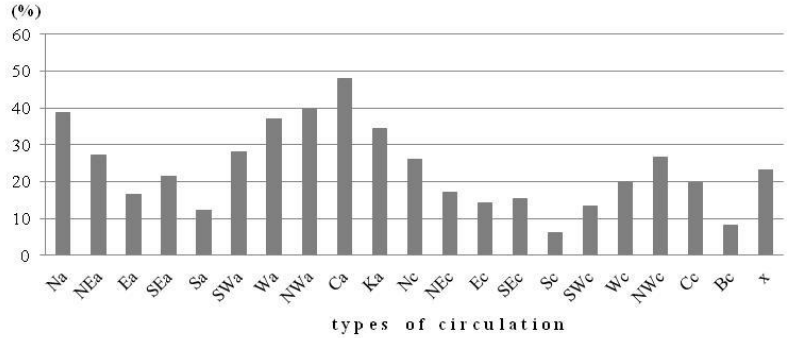

Figure 4. Conditional probability (%) of the daily minimum temperature the probability of < 10ºC in certain types of circulation in the summer.

Discussion and conclusions

This paper describes the effect of atmospheric circulation on the occurrence of the variability of daily minimum temperature of the air in the winter (DJF) in the Zywiec Valley, in relation to the

synoptic situation. Air minimum temperature is very important for the natural environment and human activity. Atmospheric circulation is crucial in shaping climat and weather conditions as well the relationships between large-scale circulation and local weather, including the occurrence of extreme phenomena and events (Ustrnul *et al*. 2014).

Research has shown that atmosphere circulation has a decisive impact on the value and occurrence of minimum temperature in the Zywiec Valley in southern Poland. There are marked differences in the formation of thermal conditions due to terrain, terrain exposure (bottom of the valley) and elevation, resulting in more contrasting effects of the conditions of circulation in winter than in summer in valleys (Niedźwiedź 1981).

The research showed that during the winter anticyclonic situations occurred more often than cyclonic situations in the study area. From among 21 synoptic situations, the most favourable for the occurrence of minimum temperature are the: center of the high pressure zone (Ca), and the anticyclonic wedge (Ka). Advection of air masses from the east is also helpful: north-eastern (NEa), eastern (Ea) and south-eastern anticyclonic situations (SEa). Minimum temperature were found to follow similar patterns in each season.

The relationship between the extremes of air temperature and atmospheric circulation types was examined by analyzing the frequency of the occurrence of extreme values and their conditional occurrence in each particular type of atmospheric circulation.

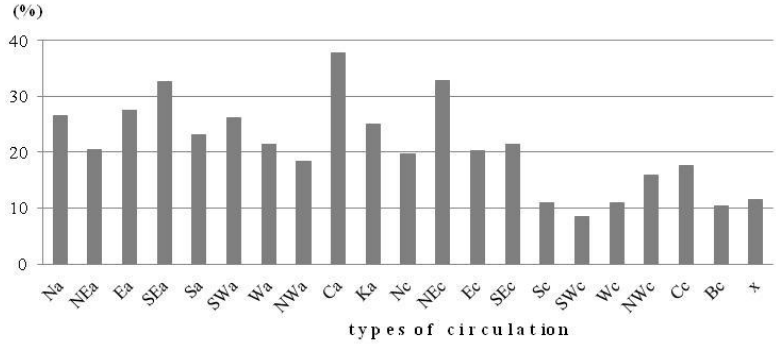

Figure 5. Conditional probability (%) of the daily minimum temperature the probability of < 0°C in certain types of circulation in the autumn.

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Streszczenie

Znaczenie temperatury minimalnej powietrza dla środowiska przyrodniczego i działalności człowieka jest niezmiernie ważne. Niniejsza praca przedstawia aspekty związane ze zmiennością dobowej temperatury minimalnej powietrza w okresie zimowym (XII-II) w Kotlinie Żywieckiej, w świetle sytuacji synoptycznych. Opracowanie bazuje na wynikach badań przeprowadzonych w jednym punkcie węzłowym pochodzącym z bazy CarpatClim, zlokalizowanym w dnie Kotliny Żywieckiej za okres 1961-2010. Studium zostało uzupełnione kompleksową analizą warunków cyrkulacyjnych i ich zmiennością czasową w ciągu 50-lecia. W tym celu zastosowano klasyfikację typów cyrkulacji atmosferycznej Niedźwiedzia (1981) dla dorzecza górnej Wisły. Za ekstremalne zjawisko termiczne uznano średnią temperaturę minimalną powietrza przekraczającą wartość progową 90 i 95 percentyla. Związki pomiędzy ekstremalnymi wartościami temperatury powietrza a typami cyrkulacji atmosferycznej rozpatrzono poprzez analizę częstości wystąpienia wartości ekstremalnych oraz ich warunkowego wystąpienia w danym typie cyrkulacji atmosferycznej.