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Changes in ghrelin, CCK, GLP-1, and peroxisome proliferator-activated receptors in a hypoxia-induced anorexia rat model

Zmiany w stężeniu greliny, CCK, GLP-1 i ekspresji receptorów aktywowanych przez proliferatory peroksysomów w szczurzym modelu anoreksji wywołanej przez hipoksję

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Abstract

Introduction: A high-altitude environment causes appetite loss in unacclimatised humans, leading to weight reduction. Ghrelin, cholecystokinin (CCK), and glucagon like peptide-1 (GLP-1), are gut hormones involved in the regulation of food intake and energy metabolism. The liver is an important site of metabolic regulation, and together with the gut it plays a role in food intake regulation. This study intends to study the time-dependent changes occurring in plasma gut hormones, PPARα, PPARδ, and PGC1α, in the stomach and liver during hypoxia.

Material and methods: Male Sprague Dawley rats were exposed to hypobaric hypoxia in a decompression chamber at 7620 m for different durations up to seven days.

Results: Hypoxia increased circulating ghrelin from the third day onwards while CCK and GLP-1 decreased immediately. An increase in ghrelin, ghrelin receptor protein levels, and GOAT mRNA levels in the stomach was observed. Stomach cholecystokinin receptor (CCKAR), PPARα, and PPARδ decreased. Liver CCKAR decreased during the first day of hypoxia and returned to normal levels from the third day onwards. PPARα and PGC1α expression increased while PPARδ protein levels reduced in the liver on third day.

Conclusion: Hypoxia alters the expression of ghrelin and ghrelin receptor in the stomach, CCKAR in the liver, and PPAR and its cofactors, which might be possible role players in the contribution of gut and liver to anorexia at high altitude. **(Endokrynol Pol 2015; 66 (4): 334–341)**

Key words: *ghrelin; CCK; GLP-1; anorexia; hypoxia*

Streszczenie

Wstęp: Przebywanie na dużej wysokości powoduje utratę apetytu u osób do tego nieprzystosowanych i prowadzi do redukcji masy ciała. Grelina, cholecystokinina (CCK) i peptyd glukagonopodobny 1 (GLP-1) są hormonami przewodu pokarmowego biorącymi udział w regulacji przyjmowania pokarmu i metabolizmu energii. Wątroba stanowi ważne miejsce regulacji metabolicznej i razem z jelitem spełnia istotną funkcję w regulacji przyjmowania pokarmu. Niniejsze badanie miało na celu ocenę zmian zależnych od czasu w stężeniu hormonów przewodu pokarmowego oraz ekspresji PPARα, PPARδ oraz PGC1α w żołądku i wątrobie występujących podczas hipoksji.

Materiał i metody: Szczury Sprague Dawley płci męskiej poddano działaniu hipoksji hipobarycznej w komorze dekompresyjnej na wysokości 7620 m. Pobyty w komorze różniły się czasem trwania, do maksymalnie 7 dni.

Wyniki: Hipoksja podwyższała stężenie krążącej greliny od trzeciego dnia pobytu z komorze do końca jego trwania, podczas gdy stężenia CCK i GLP-1 uległy natychmiastowemu obniżeniu. Zaobserwowano podwyższenie stężenie greliny, białka receptora greliny i GOAT mRNA w żołądku. W żołądku ekspresja receptorów cholecystokininy (CCKAR), PPARα i PPARδ uległy obniżeniu. stężenie wątrobowego CCKAR uległ obniżeniu pierwszego dnia wystąpienia hipoksji, powrócił do prawidłowego stężenia trzeciego dnia i pozostał prawidłowy do końca pobytu w komorze. Ekspresje PPARα i PGC1α wzrosły, podczas gdy stężenie białka PPARδ uległo obniżeniu w wątrobie.

Wnioski: Hipoksja zmienia ekspresję greliny oraz receptora greliny w żołądku, CCKAR w wątrobie i PPAR oraz jego kofaktory, które mogą odgrywać rolę w przyczynianiu się jelit i wątroby do anoreksji przy przebywaniu na dużej wysokości. **(Endokrynol Pol 2015; 66 (4): 334–341)**

Słowa kluczowe: *grelina; CCK; GLP-1; anoreksja; hipoksja*

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Introduction

Anorexia, one of the complications experienced at high altitude, is due to hypoxia-induced disturbances in the peripheral signals that indicate energy status and control appetite. Hormones secreted from the gastrointestinal tract play an important role as an endocrine signal by sensing the nutrient composition and thus

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influencing the control of hunger and satiation by the hypothalamus through specific receptors. The gut plays an important role in energy balance through the release of appetite regulatory hormones like ghrelin, cholecystokinin (CCK), and glucagon-like peptide-1 (GLP1) [1]. In humans as well as animals ghrelin is known to stimulate food intake, increase in fat mass, and decrease in fat utilisation [2–4]. Ghrelin, the only known peripheral orexigenic hormone, is mainly secreted from the fundus region of the stomach, exists in unacylated and active acylated forms, and is a ligand for the growth hormone secretagogue (GHS) receptor [5]. Ghrelin exerts its orexigenic action through GHS receptor in the hypothalamus and also by inhibiting vagal afferent neurons in the periphery [2, 6]. Ghrelin O-acyltransferase (GOAT) facilitates the acylation of ghrelin at serine-3 residue, which is essential for its binding to the growth hormone secretagogue receptor to exert its action on appetite [7]. A decrease in plasma ghrelin level [8] and both increase and decrease in plasma CCK have been reported in humans exposed to high altitude [9, 10]. CCK inhibits feeding by acting on the cholecystokinin A receptor (CCKAR) in the gastrointestinal tract [11]. The importance of peripheral CCKAR receptor in regulating food intake has been demonstrated in a study in rats by Reidelberger et al. [12]. GLP-1 is a secretory peptide derived from proglucagon mainly from the gut and also from the brain [13]. It is an anorectic peptide that suppresses food intake upon peripheral administration [14]. It exerts its action via a widely expressed G-protein coupled receptor, GLP-1R [15]. CCK and GLP-1 excite vagal afferent neurons to inhibit food intake [6]. CCK and GLP-1 are secreted into circulation upon food ingestion from the gut [1]. Insulin increases in circulation to decrease plasma glucose before a meal, and a delay in food intake has been observed when there is no decrease in glucose [11]. Hence, it can be assumed that hypobaric hypoxia may alter these circulating gut hormones, which may in part contribute to anorexia.

Peroxisome proliferator-activated receptors (PPAR) are a family of nuclear receptors involved in various cellular processes including stress response and regulation of genes involved in energy homeostasis. Fatty acids and other lipid metabolites are their activating ligands. There are three isoforms of PPAR (α, δ, and γ), which are differentially expressed in various tissues. PPARα is expressed in liver, heart, and muscle while PPARδ is expressed ubiquitously [16]. PGC1α and PGC1β are cofactors of PPARγ and they play an important role in many cellular pathways. The liver is a vital organ in maintaining blood glucose at the optimal level during fasting as well as in the fed state. Since glucose is one of the satiety factors it is important to maintain glucose homeostasis during hypoxia [17]. PGC1α and PGC1β both play a role in regulating glucose uptake and release from liver. PGC-1α expression is rapidly induced in the liver during fasting, a process that is mediated by cAMP and glucocorticoid signalling pathways [18]. Similarly, a fasting-induced increase in PGC1β in liver has also been reported, but their targets seem to be gluconeogenesis for PGC1α and fatty acid oxidation for PGC1β [19]. Hence, the present study was aimed at identifying the effect of hypobaric hypoxia on circulating ghrelin, CCK, and GLP-1, and the molecular changes in the stomach and liver during different durations of hypoxia in relation to anorexia as an attempt to explore potential targets for improving food intake of lowlanders at high altitude.

Material and methods

Material

Enzyme-linked immunosorbent assay (ELISA) kits for ghrelin (CSB-E13167r), CCK (CSB-E08114r), and GLP-1 (CSB-E08117r) were purchased from Cusabio, China. A glucose estimation kit was purchased from AutoSpan diagnostics Ltd, India. Anti-ghrelin (ab15861), anti-ghrelin receptor (ab85104), anti-CCKAR (ab75153), anti-PPARα (ab2779), anti-PPARδ (ab23673) primary antibodies, and anti-rabbit/mouse horseradish peroxidase (HRP) conjugated secondary antibodies (ab97051, ab97023) were purchased from Abcam, Inc. First strand cDNA synthesis kit and other PCR reagents were purchased from Fermentas, USA. Primers were synthesised by Eurofins Genomics, India. β-actin primary antibody (A1978), Trizol, Bicinchoninic acid, and all other chemicals were purchased from Sigma Aldrich Co, LLC.

Animals

Male Sprague Dawley rats weighing 150–200 g were obtained from the animal breeding facility of the institute in which the study was undertaken. They were fed ad libitum with standard laboratory rodent's chow and allowed free access to drinking water. Animals were maintained under laboratory conditions in a controlled environment of temperature 28 ± 2°C and 12-hour light/dark cycle as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, and the study protocol was approved by the institutional Animal Ethics Committee.

Grouping of animals and hypoxia exposure

The experimental rats were divided into six groups with six animals in each group. Groups were named according to the time of hypoxic exposure: Control, 6 h, 12 h, 24 h, 3 day, and 7 day. Except for the control group all of the other groups were exposed to hypobaric hypoxia at a simulated altitude of 7620 m, pressure equivalent to

Table I. *PCR primer sequences and annealing temperature*
Tabela I. *Sekwencje starterów PCR i temperatura annealingu*

Gene	Upper sequence (5´-3´)	Lower sequence (5´-3´)	Cycles	Product (bps)	Temperature (°C)	Reference
GOAT	CCCCCTGTGAGGTTCTACATC	TTGGTCTTTGAACGCAAGCC	35	247	60.0	NM001107317.2
PPARα	GGTCAAGGCCCGGGTCATACTCG	AAGCATTGCCGTACGCGATCAG	40	350	55.0	[20]
PGC1α	GTGCAGCCAAGACTCTGTATGG	GTCCAGGTCATTCACATCAAGTTC	35	124	58.0	[21]
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	35	496	58.0	Fermentas, USA

282 mm Hg, with an air flow of 0.8 L/min/rat into the hypobaric chamber. The temperature of the chamber was maintained 28 ± 2°C with relative humidity 55 ± 2%. Every day at 10:00 A.M. the chamber was opened to replenish food and water. All animals were fasted overnight and sacrificed by administering a lethal dose of xylazine (10 mg/kg body wt.) and ketamine (100 mg/kg body wt.) intraperitoneally. Blood was drawn by cardiac puncture, collected into heparinised tubes, and centrifuged at 1000 g for 15 minutes to separate plasma. Plasma samples were stored at –80°C until analysis. Whole stomach tissues and liver tissues collected for western blot analysis were stored at –80°C until use. Around 100 mg of the liver tissues were stored in RNA Later solution at –20°C for RNA isolation.

Food intake and body weight measurements

Two groups with 12 animals each were kept for body weight and food intake measurements. One group was a normoxia control and the other group was exposed to hypoxia for seven days. Both groups of animals were monitored for their food intake and body weight every 24 hours for seven days. Food intake and body weight of the hypoxia-exposed group were monitored every day at 10 A.M. while the chamber was open for food and water replenishment. Data was analysed by calculating the percentage change.

Quantification of plasma ghrelin, CCK, GLP-1, and glucose

Plasma ghrelin, CCK, and GLP-1 were quantified using ELISA kits as per the manufacturer's instructions. 100 μl of plasma was used for the test and quantified using Gen5 software by four-parameter logistic analysis. Plasma glucose was estimated by glucose oxidase method following the manufacturer's instructions.

Western blotting

Stomach and liver tissue homogenates were made in RIPA buffer and centrifuged at 16000 g for 20 minutes, after which the supernatant was estimated for protein content by bicinchoninic acid assay. The stomach and liver tissue protein extracts (40 μg) of all groups were resolved in polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane for blotting. The transferred blots of stomach tissues were incubated with anti-preproghrelin, anti-ghrelin receptor, anti-CCKAR, anti-PPARα, anti-PPARδ, and anti-β-actin primary antibodies, respectively, overnight at 4°C. The blots of liver tissues were incubated with anti-CCKAR, anti-PPARδ, and anti-β-actin primary antibodies, respectively, overnight at 4°C. Following incubation with primary antibodies, blots were incubated with anti-rabbit/mouse horseradish peroxidase (HRP) conjugated secondary antibody for two hours at room temperature for respective blots. The blots were developed using diaminobenzidine as a substrate, and densitometry analysis was done using ImageJ software.

Semiquantitative reverse transcriptase polymerase chain reaction (PCR)

Total Ribonucleic Acid (RNA) was isolated from stomach and liver tissue using TRIzol reagent according to the manufacturer's protocol. RNA quality was checked by resolving in 1.0% agarose gel and visualised for 28S and 18S rRNA bands under UV light with ethidium bromide staining. RNA concentrations were quantified using a nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary deoxyribonucleic acid (cDNA) was prepared from 1 μg of total RNA of each sample as per kit protocol using a first strand cDNA synthesis kit. Specific primers for GOAT were designed using Primer Pick tool of NCBI, primers for PPARα and PGC1α were taken from a literature source [20, 21], and primers for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Fermentas, USA (Table I). Amplification of specific cDNAs were carried out in 20 μl reaction mixture containing PCR buffer (750 mM Tris-HCL pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 20 mM $MgCl_2$), 0.2 mM dNTP mix, 0.05 U/μL Taq polymerase enzyme, 1 μM of each forward and reverse primer and 1 μl of respective cDNA. The annealing temperatures used for different

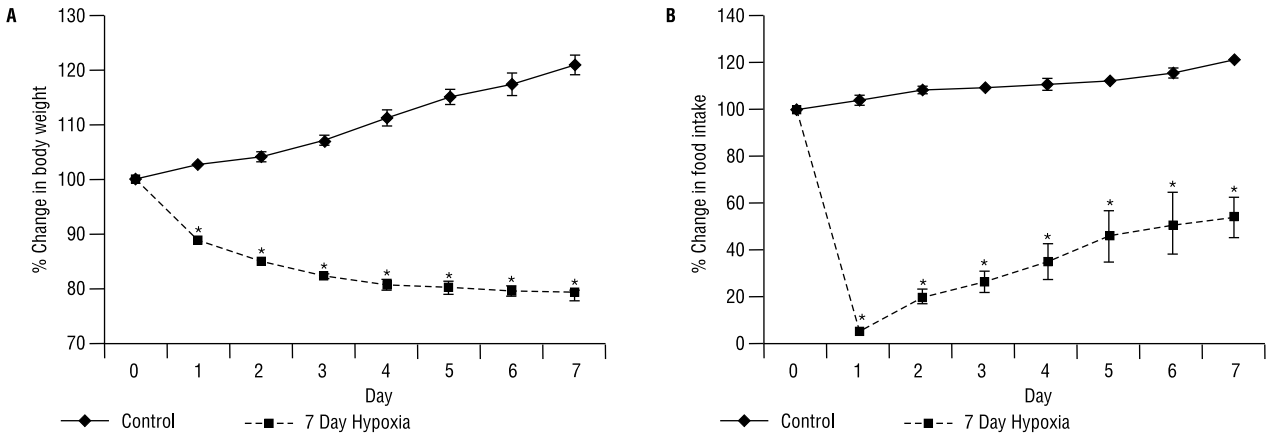

Figure 1. *Changes in food intake and body weight of rats during hypoxia exposure.* **A.** *Percentage change in body weight graph shows that hypoxia exposed rats lost around 23% of their initial weight by the end of exposure.* **B.** *Percentage change in food intake shows maximum reduction in food intake on the first day of exposure. *P < 0.05 versus control group at each time point*

Rycina 1. *Zmiany w przyjmowaniu pokarmu i masie ciała szczurów podczas poddania ich działaniu hipoksji.* **A.** *Wykres procentowej zmiany w masie ciała wskazuje, że szczury poddane działaniu hipoksji straciły około 23% początkowej masy ciała po ustaniu hipoksji.* **B.** *Procentowa zmiana w ilości przyjmowanego pokarmu ilustruje maksymalną redukcję przyjmowania pokarmu pierwszego dnia działania hipoksji. *P < 0,05 wobec grupy kontrolnej w każdym punkcie czasowym*

sets of primers are listed in Table I. All PCR products were resolved by electrophoresis using 1.5% agarose gel and stained with ethidium bromide; the images were captured under UV illumination, and densitometry analysis was done using ImageJ software.

Statistical analysis

Data are expressed as mean ± SEM. Significant differences were calculated for hypoxia-exposed groups against the control group using one-way ANOVA followed by Dunnets's *t* post hoc analysis using SPSS version 22 (IBM Corporation). Statistical significance was set at $P \leq 0.05$. The levels of significance are denoted in the figures as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Anorexia and weight loss during hypoxia

The body weight of hypoxia-exposed rats started decreasing from the first day onwards, and they continued to lose weight until the end of exposure (Fig. 1A). Animals when exposed to hypobaric hypoxia experienced anorexia with a reduction in food intake to 4.3% on the first day. By the end of exposure food intake improved to 53% of pre-exposure food intake (Fig. 1B).

Hypoxia alters gut hormones of energy metabolism and glucose in circulation

Exposure to hypoxia increased ghrelin levels in plasma on the third and seventh day compared to the control group (Fig. 2A). CCK and GLP-1 levels sharply decreased soon after exposing the rats to hypoxia at 6 hours and remained low until the end of exposure (Fig. 2B, 2C). Hypoxia exposure lead to an increase in circulating glucose levels with maximum at 24-hour time point (Fig. 2D).

Changes in stomach tissue during hypoxia

Pre-proghrelin increased to about 50% on exposure to hypoxia, and a 30% increase in ghrelin receptor was observed. GOAT mRNA levels rose from the third day of exposure. CCKAR protein was decreased from 24 hours until the end of hypoxia exposure. PPARα and PPARδ protein levels were decreased during hypoxia in the stomach with maximum reduction at 24 hours (Fig. 3).

Changes in liver tissue during hypoxia

On exposure to hypoxia CCKAR initially decreased to 41% at 24 hours, which then increased to 113% by day seven. PPARδ increased at 6 hours and 12 hours but decreased below control at 24 hours and on the third day but on day seven increased to 140% (Fig. 4A). PPARα mRNA increased to 167% at 6 hours and remained high throughout the exposure. There was an increase in PGC1α mRNA on exposure to hypoxia (Fig. 4B).

Discussion

The present study reports that exposure to high-altitude hypoxia causes anorexia, and CCKAR, PPARs, and PGC1s in liver and stomach might be potential targets in alleviating anorexia. On exposure to hypoxia, circulating levels of ghrelin increased, but the increase was from the third day only. During the first day of

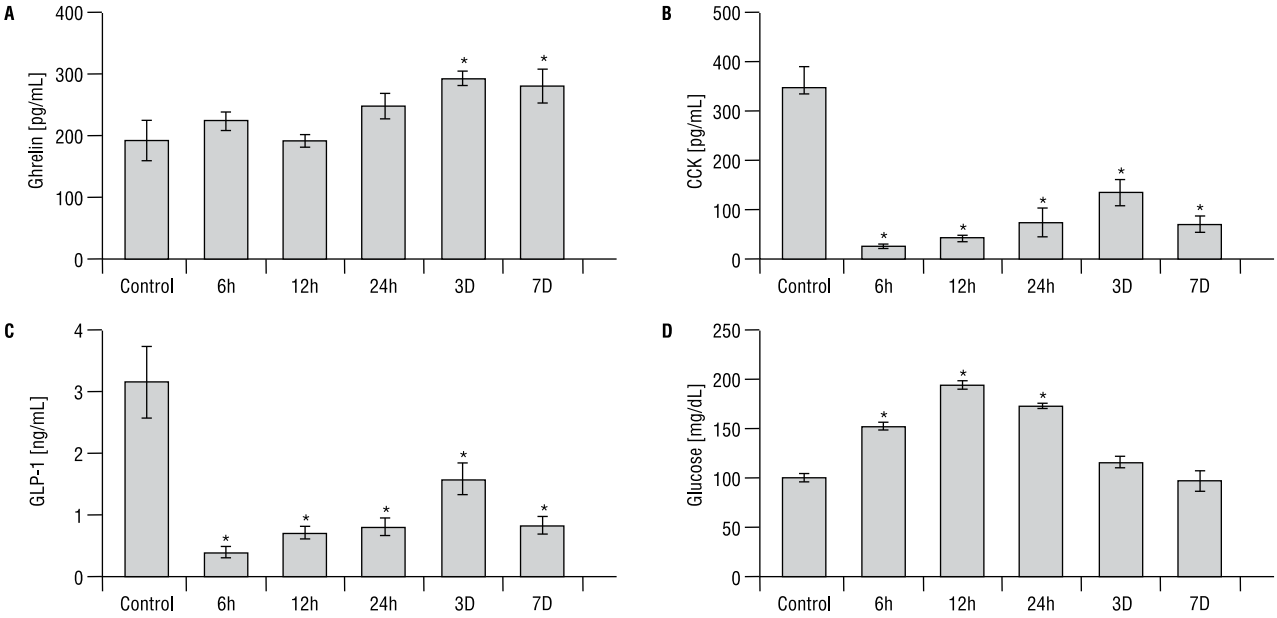

Figure 2. *Plasma levels of hormones involved in appetite regulation.* **A.** *Plasma ghrelin levels increased during hypoxia.* **B.** *Plasma cholecystokinin (CCK).* **C.** *Plasma glucagon-like peptide-1 (GLP-1). Hypoxia exposure caused reduction in both CCK and GLP-1 levels in circulation.* **D.** *Plasma glucose levels increased during hypoxia exposure. *P < 0.05 versus unexposed group*

Rycina 2. *Stężenie hormonów w osoczu biorący udział w regulacji apetytu.* **A.** *Podwyższony poziom greliny w osoczu podczas hipoksji.* **B.** *Stężenie cholecystokininy (CCK) w osoczu.* **C.** *Stężenie glukagonopodobnego peptydu-1 (GLP-1) w osoczu. Hipoksja wywołała redukcję zarówno stężenia CCK, jak i GLP-1.* **D.** *Zwiększone stężenie glukozy w osoczu wywołane hipoksją. *P < 0,05 wobec grupy niewystawionej na działanie hipoksji*

exposure circulating ghrelin remained the same as in controls even though the highest reduction in food intake was observed on day 1, indicating that fasting did not immediately induce ghrelin secretion, but from the third day ghrelin levels increased in response to decreased food intake. A study by Pardo et al. on an activity-based anorexia (ABA) model showed similar results, with increased ghrelin secretion and decreased insulin sensitivity. In the present study also there was an increase in circulating total ghrelin from the third day of anorexia, but a loss of insulin sensitivity was observed during the first day, evident from high glucose levels [22]. Plasma CCK and GLP-1 decreased immediately after hypoxia exposure, which may be due to the decrease in food intake as they are secreted upon ingestion of food [23]. Ghrelin expression is reported to increase upon fasting to induce hunger by acting on the GHS receptor in the hypothalamus [1]. On exposure to hypoxia, a slight increase in ghrelin and ghrelin receptor was observed in the stomach. Our results show that reduced food intake failed to sufficiently increase ghrelin and its receptor to prevent anorexia during hypoxia. The GOAT expression was low in stomach tissue during the first day of hypoxia but it tripled on day 3 and day 7. This result may explain why the initial increase in total ghrelin and ghrelin receptor in the stomach was not sufficient to improve appetite. The rise in plasma ghrelin from day 3 onwards might have taken effect due to its acylation by increased GOAT expression and hence might have been reflected as an improvement in food intake towards the end of hypoxic exposure. During anorexia ghrelin levels are supposed to increase in the circulation due to low nutritional availability, but in the present study anorexia induced pre-proghrelin at 6 hours of hypoxia in stomach tissue while circulating levels raised only after the third day. The reason behind this delayed increase in plasma total ghrelin might also be attributed to the high plasma glucose observed during the first day of hypoxia as it is shown that elevated plasma glucose suppresses ghrelin secretion [24]. Other gut hormones GLP-1 and CCK are known to inhibit ghrelin secretion [25], but in this study it is unlikely that these hormones could have had an effect on ghrelin levels because both of these hormones decreased in response to low nutrient intake. Increasing ghrelin expression and activating GOAT on the first day might prevent loss of appetite in a hypoxic environment. CCKAR receptor reduced in the stomach as well as liver, which might be due to reduced circulating CCK levels during hypoxia.

PPARα is known to promote fatty acid oxidation in the liver and it is upregulated during fasting. Free fatty acid, which arises from lipolysis in the adipose

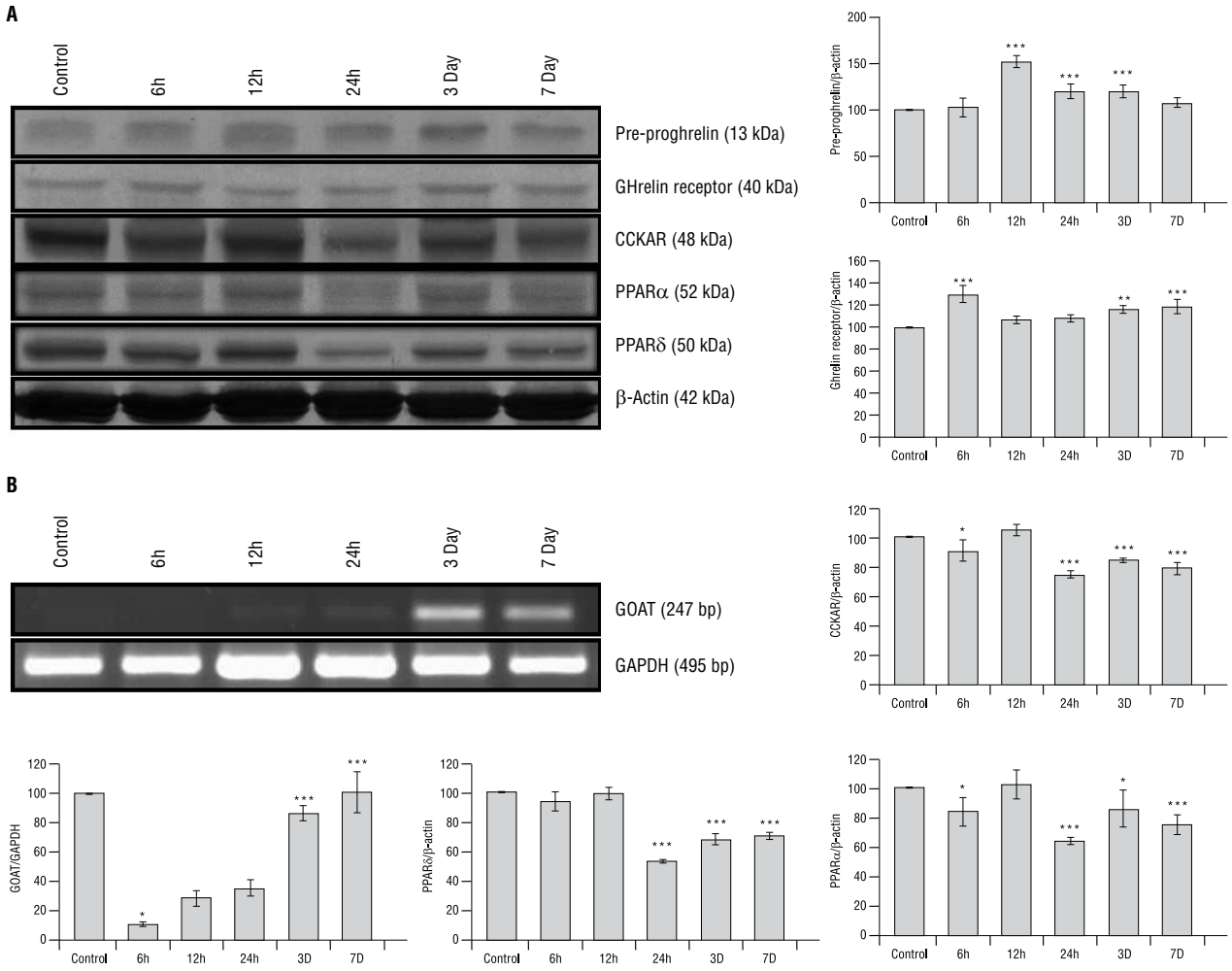

Figure 3. *Western blot and PCR in stomach tissue of hypoxia exposed and control rats.* **A.** *There was an increase in pre-proghrelin and ghrelin receptor and a decrease in CCKAR, PPARα, and PPARδ protein levels, and* **B.** *An increase in GOAT mRNA levels during hypoxia exposure. *P < 0.05, **P < 0.01, ***P < 0.001*

Rycina 3. *Western blot i PCR w tkance żołądka u szczurów poddanych i niepoddanych działaniu hipoksji.* **A.** *Odnotowano wzrost pre-progreliny, receptora greliny i obniżenie CCKAR, stężenie białek PPARα i PPARδ oraz* **B.** *wzrost stężenia GOAT mRNA podczas poddania działaniu hipoksji. *P < 0,05; **P < 0,01; ***P < 0,001*

tissue, acts as an activating ligand for PPARα [26]. The absence of PPARα during fasting lead to hypoglycaemia and higher FFA [27, 28]. Like PPARα, PPARδ also promotes fatty acid oxidation in the liver but it decreases glucose production, while PPARα stimulates gluconeogenesis [26]. Activation of PPARδ using ligands prevented weight gain [29]. Our results show a reduction in PPARα and PPARδ protein levels in the stomach upon hypoxic exposure, which might have an impact in the energy balance. An increase in PPARα and PGC1α expression in the liver was as expected because earlier reports stated that fasting induces its expression [18] and its increase might have activated the gluconeogenic pathway leading to an increase in blood glucose levels during hypoxia. Increased PPARδ in the liver from the seventh day of exposure might explain the continued weight loss even though food intake improved on the first day of hypoxia. The increase in PPARδ might also be attributed to the normal blood glucose from the seventh day onwards, which was high on the first day.

Hence, from the present study it can be concluded that ghrelin secretion is not sufficiently stimulated by reduced food intake during hypoxia and might be an important reason for the loss of appetite. CCK and GLP-1 were secreted in response to the amount of food consumed. The metabolic function of the liver in the maintenance of blood glucose and fatty acid might have been impaired due to the altered expression of PPARs and PGC1s. The study revealed that targeting PPARα and PGC1α during initial days and PPARδ in later days of hypoxia exposure might alleviate the disturbances in the energy homeostasis and prevent anorexia at high altitude.

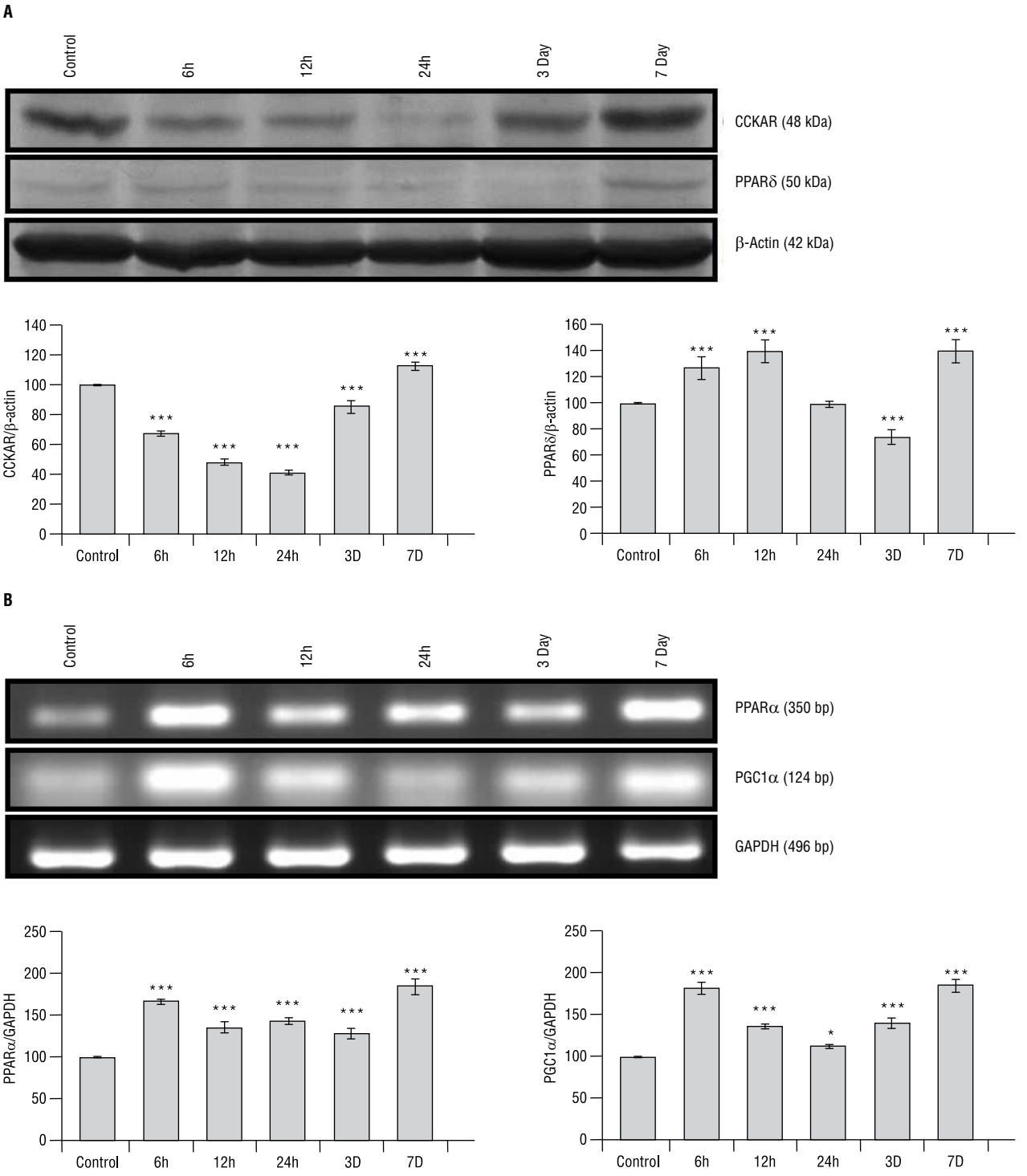

Figure 4. *Western blot and PCR in liver tissue of hypoxia-exposed and control rats.* **A.** *Western blot results showed decreased CCKAR and initial increase in PPARδ during hypoxia exposure.* **B.** *Semiquantitative PCR results showed an increase in PPARα and PGC1α mRNA on exposure to hypoxia. *P < 0.05, ***P < 0.001*

Rycina 4 *Western blot i PCR w tkance wątroby u szczurów poddanych i niepoddanych działaniu hipoksji.* **A.** *Wyniki western blot wykazały obniżony poziom CCKAR i początkowy wzrost PPARδ podczas trwania hipoksji.* **B.** *Wyniki półilościowej PCR wykazały wzrost PPARα i PGC1α mRNA podczas poddania działaniu hipoksji. *P < 0,05; ***P < 0,001*

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