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Hypoxia induces apelin expression in human adipocytes

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Abstract

Adipokines play a central role in the development of diseases associated with insulin resistance and obesity. Hypoxia in adipose tissue leads to a dysregulation of the expression of adipokines. The effect of hypoxia on the more recently identified adipokine apelin in human adipocytes is unclear. Therefore, we aimed at investigating the role of hypoxia on the expression of the adipokine apelin.

Differentiated human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes were cultured under hypoxic conditions for varying time periods. To create a hypoxic tissue culture environment (defined as 1% O_2, 94% N_2 and 5% CO_2) we used a modular incubator chamber. In addition, we mimicked hypoxic conditions by using $CoCl_2$. The effect of hypoxia on the expression of the investigated adipokines was measured by real-time PCR and the secretion of apelin was quantified by ELISA.

Induction of hypoxia significantly induced mRNA expression of leptin and apelin in differentiated SGBS adipocytes compared with the normoxic control condition. Expression of adiponectin was significantly decreased by hypoxia. In addition, the amount of secreted apelin protein in response to hypoxia was elevated compared to untreated cells. Furthermore, we could demonstrate that the observed hypoxia-induced induction of apelin mRNA expression is in the first phase dependent on HIF-1α.

In our study we could demonstrate for the first time that apelin expression and secretion by human adipocytes are strongly induced under hypoxic conditions and that the early response on hypoxia with apelin induction is dependent on HIF-1α.

Keywords

adipokines; white adipocytes; oxygen-regulated gene expression

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Introduction

White adipose tissue (WAT) is a major endocrine organ, which regulates energy homeostasis and other physiological processes by releasing several factors called adipokines. A variety of adipokines has been discovered over the last years that regulate different systemic processes in an autocrine, paracrine or endocrine manner [1]. In obesity, the production of pro-inflammatory adipokines increases, whereas the expression of anti-inflammatory factors, such as adiponectin is decreased. This dysregulation results in a chronic mild inflammatory state within the adipose tissue and plays a central role in the development of diseases associated with insulin resistance and obesity, in particular of type 2 diabetes and of the metabolic syndrome [2-4].

Obesity is characterized by tissue mass expansion [5] and direct evidence for formation of hypoxic areas within adipose tissue has been reported in different obese mouse models [6-8]. The hypoxic response is mediated through the activation of several hypoxia-sensitive transcription factors including hypoxia-inducible transcription factors (HIF), nuclear factor-kappa B (NF-κB) and cyclic AMP response element binding protein (CREB) [9-11]. Obesity-induced hypoxia leads to a change in the expression of several adipokines such as adiponectin and leptin, which was demonstrated in rodent and also in human adipocytes [6, 8, 12-16]. This dysregulation further contributes to the development of the chronic inflammatory state observed in obese individuals.

A more recently identified adipokine is apelin, a 77 amino-acid prepropeptide which is cleaved to shorter bioactive peptides that bind to the endogenous ligand for the orphan G-protein coupled receptor APLNR (apelin receptor) [17]. Expression of apelin has been demonstrated in several tissues [18, 19] and this peptide seems to have different regulatory functions, depending on the expressing tissue [20]. Apelin has been reported to have an effect on the cardiovascular system [21, 22], appetite, drinking behaviour [23], angiogenesis [24], and it is regulated by insulin [25]. Apelin is also expressed in adipose tissue by the adipocyte itself. Increased plasma concentrations were observed in obese and insulin-resistant mice and humans [25, 26]. Furthermore, apelin expression is induced under hypoxic conditions in enteric rat cells [27] and in mouse adipocytes [28] in a HIF-1α-dependent manner. However, the effect of hypoxia on apelin regulation in human adipocytes is currently unknown.

Therefore, this study was performed to determine the effect of hypoxia on the expression of apelin in human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes.

Materials and methods

Cell culture and reagents

SGBS preadipocytes [29] were maintained in DMEM/Ham's F12 (1:1) medium (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 33 μM biotin and 17 μM pantothenate. To differentiate SGBS cells into adipocytes, near confluent cells were washed three times with PBS and cultured in differentiation medium: DMEM/Ham's F12 (1:1) medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 33 μM biotin, 17 μM pantothenate, 10 μg/ml human transferrin, 10 nM insulin, 100 nM hydrocortisone, 0.2 nM triiodothyronine, 25 nM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 2 μM rosiglitazone (Cayman Chemical, MI, USA). After 4 days, this medium was replaced by a differentiation medium excluding dexamethasone, IBMX and rosiglitazone, and cells were further cultured for 10 days. Cells were incubated at 5% CO_2 and 37°C in a humidified atmosphere and medium was changed every 3-4 days.

At day 15 after induction of differentiation, fully differentiated SGBS cells were exposed to hypoxia and cultured with the HIF-1α-stabilizer cobalt chloride ($CoCl_2$; 150 μM), respectively. To create a hypoxic environment, cells were placed in a MIC-101 modular incubator chamber (Billups-Rothenberg, Inc., CA, USA), flushed with a mixture of 1% O_2, 5% CO_2 and 94% N_2 and incubated at 37°C. Cells were harvested and culture media was collected after treatment for different time periods (3, 6 and 16 hours). For suppressing HIF-1α accumulation we used the HIF-1α-inhibitor CAY10585 in a concentration of 90 μM (Cayman Chemical). Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

RNA extraction and real-time PCR

Total RNA was prepared from SGBS cells with peqGOLD TriFast™ according to the manufacturer's instructions (peQLab, Erlangen, Germany). RNA (1μg) was reverse transcribed using the SuperScript III First-Strand Synthesis Kit (Invitrogen). Expression levels of the analyzed genes were assessed by real-time PCR using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and fluorescence was detected with the LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany).

The primers were designed using Gene Runner software (Hastings Software, Inc.; version 3.05) and synthesized by Microsynth (Balgach, Switzerland) and the following sequences for the primers were used: leptin (forward 5′-GGATTCTTGTGGCTTTGGC-3′ and reverse 5′- CTTTCTGTTTGGAGGAGACTGACT-3′), adiponectin (forward 5′-GTGATGGCAGAGATGGCAC-3′ and reverse 5′-CGATGTCTCCCTTAGGACCAA-3′), apelin (forward 5′-CAGGGAGGTCGGAGGAAAT-3′ and reverse 5′-ACCAATCTATGGAGGAGACATAACC-3′), GAPDH (forward 5′-TGTTCGTCATGGGTGTGAACC-3′ and reverse 5′-GCAGTGATGGCATGGACTGTG-3′) and TATA box-binding protein (TBP; forward 5′-GGGAGCTGTGATGTGAAGTTT-3′ and reverse 5′-AAGGAGAACAATTCTGGGTTTG-3′). The thermal profile of an initial 10 min melting step at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C was used. A melting curve profile was processed after each run to confirm specific transcripts. All reactions were performed in triplicates and the samples were normalized to the endogenous reference values. The results are expressed as fold changes of cycle threshold value relative to controls using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

For preparation of nuclear SGBS extracts, NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, IL, USA) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail were used. Total protein concentration was determined using the protein assay reagent (Bio-Rad Laboratories, Munich, Germany). Extracts were dissolved in 4× SSB loading buffer containing 20% β-mercaptoethanol and boiled. Fifteen micrograms of nuclear extracts were separated by SDS-PAGE electrophoresis and then transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked and incubated with primary antibodies specific for HIF-1α (R&D Systems, MN, USA) and GAPDH (Cell Signaling, Frankfurt, Germany), washed and then incubated with horseradish-peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare, Buckinghamshire, UK), respectively. Specific bands were visualized by enhanced chemiluminescence reagent (ECL Plus; GE Healthcare) and analyzed in an AutoChemi detection system (UVP, Cambridge, UK).

Apelin release measured by ELISA

Apelin secretion (pg/ml) was measured with the RayBio® human apelin-C terminus enzyme immunoassay kit (RayBiotech, Inc., GA, USA) following the manufacturer's protocol. The used kit is designed to detect the C-terminus of the active forms of apelin including apelin-36 and apelin-13 (sensitivity: 29.1 pg/ml). The assay was conducted on a DTX 880 multimode detector (Beckman Coulter, Inc., CA, USA).

Statistical analysis

The results are expressed as mean values ± SEM. Statistical differences between groups were analyzed using unpaired two-tailed Student's t tests. All analyses were performed using the statistical software package SPSS 11.0 for Windows, SPSS Inc., Chicago, USA.

Results

HIF-1α expression during induction of hypoxia in human SGBS adipocytes

To test whether HIF-1α can be induced with both hypoxia-inducing methods applied in our study, we determined HIF-1α protein levels in human differentiated SGBS adipocytes. Hypoxia was induced for different time periods by cultivation of mature SGBS adipocytes (day 15 post-induction) under an environment containing 1% O_2 and hypoxia was mimicked by treatment with 150 μM $CoCl_2$, respectively. Incubation of the cells under hypoxia resulted in an increase of HIF-1α protein, which remained increased for up to 16 hours compared to the control cells cultured under normoxic conditions (21% O_2) (Fig. 1A). Furthermore, $CoCl_2$ treatment also led to a HIF-1α accumulation in differentiated SGBS adipocytes already after 3 hours (Fig. 1B).

Adiponectin and leptin expression is regulated by hypoxia in human SGBS adipocytes

To determine hypoxia-induced regulation of the major adipokines adiponectin and leptin in SGBS adipocytes, we measured mRNA expression after treatment of the cells with hypoxia (1% O_2, 94% N_2 and 5% CO_2) for different time periods. In addition, we treated the adipocytes with 150 μM $CoCl_2$ to mimic hypoxic conditions by stabilization of HIF-1α. Adiponectin mRNA expression measured by real-time PCR was significantly decreased after 6 hours of hypoxia and resulted in a 3.5-fold repression after 16 hours (Fig. 2A). $CoCl_2$ treatment resulted in a decrease of adiponectin mRNA, which achieved a 1.8-repression after 16 hours (Fig. 2B). Furthermore, leptin mRNA expression was increased 2-fold after 3 hours of hypoxia and reached a 7.4-fold induction after 16 hours compared to the control cells cultured in a normoxic environment (Fig. 2C). Treatment of the mature adipocytes with $CoCl_2$ also led to an induction of leptin mRNA (Fig. 2D), but the levels of a 2.6-fold induction after 6 hours and a 1.9-fold induction after 16 hours, respectively, were lower than those obtained in the experiments with 1% O_2.

Induction of apelin mRNA expression under hypoxia in human adipocytes

Real-time PCR analysis revealed a significant increase of apelin mRNA already after 3 hours of hypoxia. After 16 hours a 14.2-fold induction compared to the control could be observed (Fig. 3A). Longer incubation of the cells under hypoxic conditions for up to 48 hours did not result in a further increase of apelin gene expression (data not shown). Again this effect could be confirmed by treatment of the cells with $CoCl_2$. Treatment of the cells with $CoCl_2$ resulted in a 4.8-fold induction of apelin mRNA expression after 16 hours (Fig. 3B). However, compared to 1% O_2, 94% N_2 and 5% CO_2 atmosphere the impact of $CoCl_2$ was significantly weaker ($p < 0.05$) at all time points investigated.

Hypoxia induces apelin secretion

To investigate the effect of hypoxia on apelin expression on the protein level, we measured the secretion of apelin into the cell culture medium after treatment of the mature adipocytes with hypoxia (1% O_2) for different time periods. Hypoxia induced the secretion of apelin after 3 and 6 hours and the regulation reached in a 2.6-fold induction after 16 hours compared to the apelin secretion of differentiated SGBS control cells cultured under normoxic conditions (Fig. 3C).

Early hypoxia-induced apelin mRNA expression is HIF-1α-dependent

Despite our observation that HIF-1α accumulates in the nucleus of hypoxia-treated SGBS adipocytes, the involvement of this transcription factor in the hypoxia-induced apelin expression is not clear. Therefore, we used a HIF-1α-inhibitor to prevent accumulation and transcriptional activation of HIF-1α in the nucleus of differentiated SGBS adipocytes (Fig. 4A). Hypoxia-induced HIF-1α accumulation could be completely abrogated by the inhibitor. To investigate the consequence of HIF-1α-inhibition, we measured the expression of apelin after treatment of the SGBS adipocytes with hypoxia and the HIF-1α-inhibitor together (Fig. 4B). Apelin mRNA expression was not induced after 3 hours and only slightly induced after 6 hours of hypoxia in combination with the inhibitor with a fold-increase of 2.2 compared to the hypoxia-induced fold-increase of 17.3. Of note, after 16 hours inhibition of HIF-1α could not longer abrogate the hypoxia-induced expression of apelin in SGBS cells.

Discussion

The present study is the first to demonstrate hypoxia-induced expression and secretion of apelin by human adipocytes. Because adipose tissue hypoxia is one of the functionally most important consequences of obesity, this novel finding contributes to the understanding of obesity-related disorders such as insulin resistance and type 2 diabetes. Hypoxia-induced regulation of apelin up to now has been investigated only in the cardiovascular system and in rat and mouse cell cultures of the gastrointestinal tract [27] and studies in the adipose tissue have been exclusively conducted in murine adipocytes [28]. We now provide the first data on human adipocytes.

It has been assumed that apelin is regulated directly by HIF, based on the presence of putative hypoxia responsive elements (HREs) identified in the human, mouse and rat apelin promoter sequence [24, 27, 30]. Further, it was shown that insulin-induced apelin expression is mediated via HIF-1 [28]. Therefore, it is hypothesized that the induction of apelin under hypoxic conditions is mediated by direct binding of HIF-1 to the apelin gene. This hypothesis has been supported by a study of Eyries et al. [31], who recently demonstrated that hypoxia-induced apelin expression was mediated via HIF binding to an HRE sequence located in the first intron of the human apelin gene. In our study, we could also observe that hypoxia-induced expression of apelin is HIF-1α dependent. However, apelin expression was only dependent on HIF-1α up to 6 hours of hypoxic treatment. After 16 hours of cultivating the adipocytes under hypoxic conditions, inhibition of HIF-1α did not further prevent hypoxia-induced apelin expression. Therefore, we suppose that HIF-1α is directly responsible for the early transcription of apelin mRNA under hypoxia in SGBS adipocytes, but under longer exposures to a hypoxic environment further transcription factors are additionally involved in the regulation of apelin expression. HIF is the central transcription factor activated in response to cellular hypoxia, but various other transcription factors have also been reported to be hypoxia-responsive [9]. Furthermore, sequence analysis identified different putative binding sites for the transcription factors activator protein 1 (AP1), upstream stimulatory factor (USF), and replication initiator 1 (AP4) in the core promoter

region of the human apelin gene [32]. Therefore further expression regulation mechanisms of the apelin gene are supposable.

Of note, we observed that the effect of the HIF-1α-stabilizer $CoCl_2$ on adipokine regulation was weaker than the effect induced by 1% O_2 predominantly after 16 hours of hypoxia. These findings support our theory of the involvement of further regulatory mechanisms beside HIF-1α accumulation and activation. Alternatively, this effect could also be explained by an insufficient stabilization of HIF-1α by $CoCl_2$. However, previous experiments in our laboratory conducted with different $CoCl_2$ concentrations from 50 μM up to 250 μM showed that the effect on adipokine expression could not be increased by using concentrations higher than 150 μM (data not shown). Therefore, we can suggest that the weaker effect of $CoCl_2$ compared to the hypoxic environment was obtained due to a suboptimal $CoCl_2$ concentration.

The elucidation of the hypoxia-induced apelin regulation is of great importance because the protein was not only expressed but also secreted from SGBS cells, which was also reported for differentiated mouse adipocytes *in vitro* [25]. Apelin has been described to promote proliferation, migration, and tube formation of endothelial cells [33, 34]. As angiogenesis is essential for the maintenance and expansion of adipose tissue, Glassford et al. [28] speculated that apelin may contribute as a proangiogenic factor to the development of new vasculature which is necessary for an expanding fat depot. Similarly, leptin was also reported to have angiogenic activity, as it induced neovascularization in corneas from rats [35].

We observed a strong repression of adiponectin and an induction of leptin expression under hypoxic conditions as well as under the treatment of the HIF-1α-stabilizer $CoCl_2$. These findings observed in human SGBS cells are in accordance to previously published data indicating that adiponectin is repressed under hypoxic conditions in murine adipocytes [6, 12] and human adipocytes [15, 36]. Similarly, leptin has been shown to be regulated in response to low oxygen in 3T3-F442A murine adipocytes [13] as well as human adipocytes [15, 36].

With regard to our results observed in this study, we suggest the SGBS cell strain as an excellent human cell culture model to study adipokine regulation. Further, we observed that the key transcription factor in hypoxic gene regulation HIF-1α is accumulated after the induction of hypoxia (induced in the modular chamber and by treatment with $CoCl_2$, respectively). Thus, we can ensure that with the methods used in this study to induce hypoxia, the hypoxic signaling is mediated in SGBS cells.

In conclusion, our study shows that hypoxia has an important impact on the expression of apelin in human SGBS adipocytes. We could identify HIF-1α as the major regulation factor involved in the hypoxia-induced expression of apelin. The elucidation of the precise mechanisms resulting in the dysregulation of adipokines will contribute to a better understanding of the mechanisms leading to the development of obesity-related diseases such as type 2 diabetes and the metabolic syndrome.

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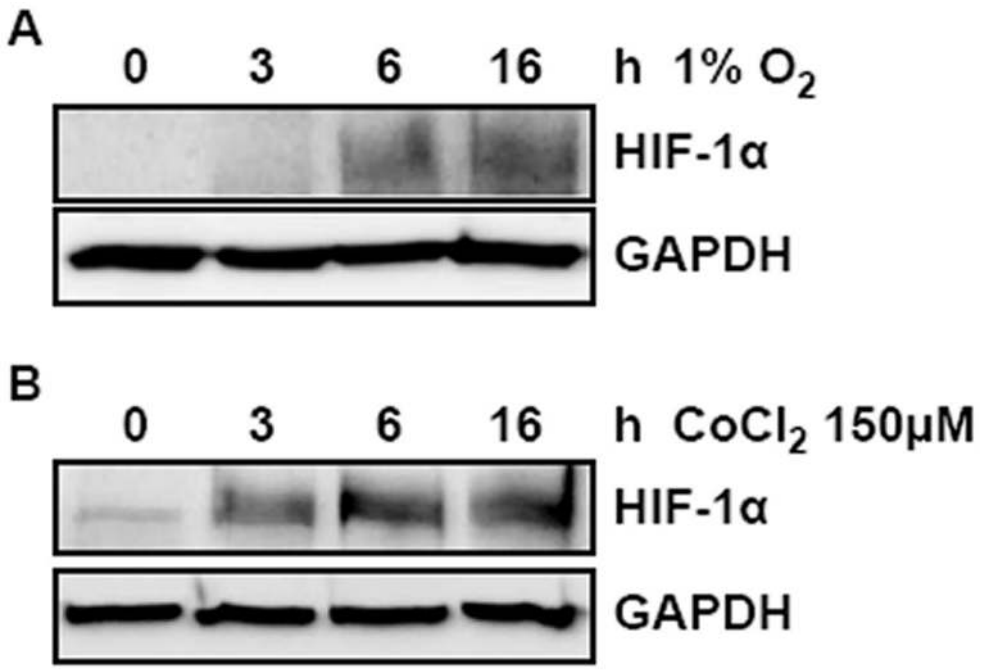

Fig. 1.

Induction of HIF-1α protein under hypoxic conditions in human differentiated SGBS adipocytes. Effect of 1% O_2 (A) and 150 μM $CoCl_2$ (B) treatment was assessed for the time periods indicated by immunoblotting, respectively. GAPDH was used as a loading control.

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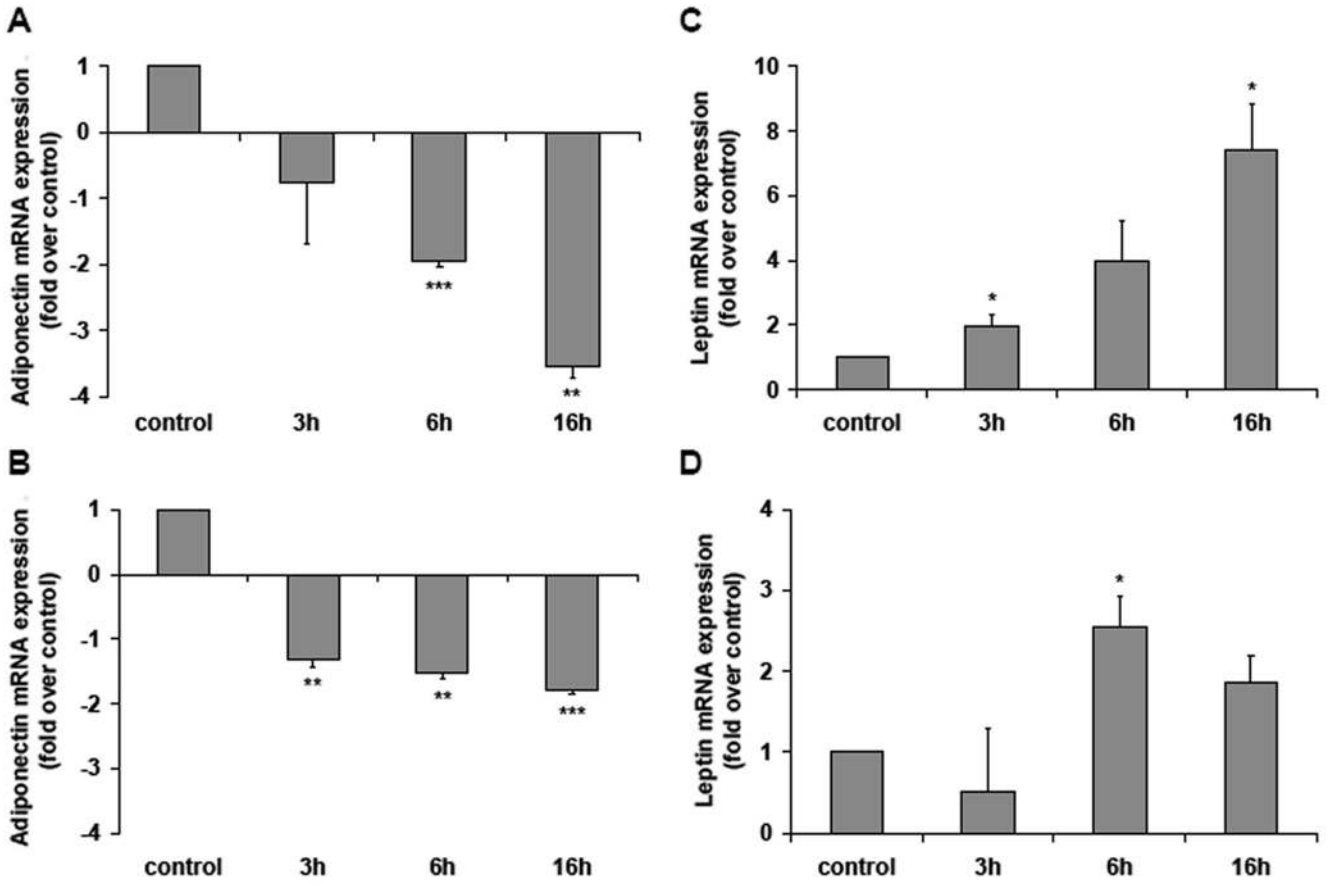

Fig. 2.

Impact of hypoxia on adiponectin and leptin mRNA expression in human SGBS adipocytes. Total RNA was prepared from differentiated SGBS cells after exposure to 1% O_2 (A and C) and treatment with $CoCl_2$ (150 μM; B and D) for the time periods indicated, respectively. mRNA expression of adiponectin (A and B) and leptin (C and D) was determined by real-time PCR. Values represent means ± SE depicted as fold-repression or fold-induction of the untreated control. Results of three independent experiments each performed in triplicate are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

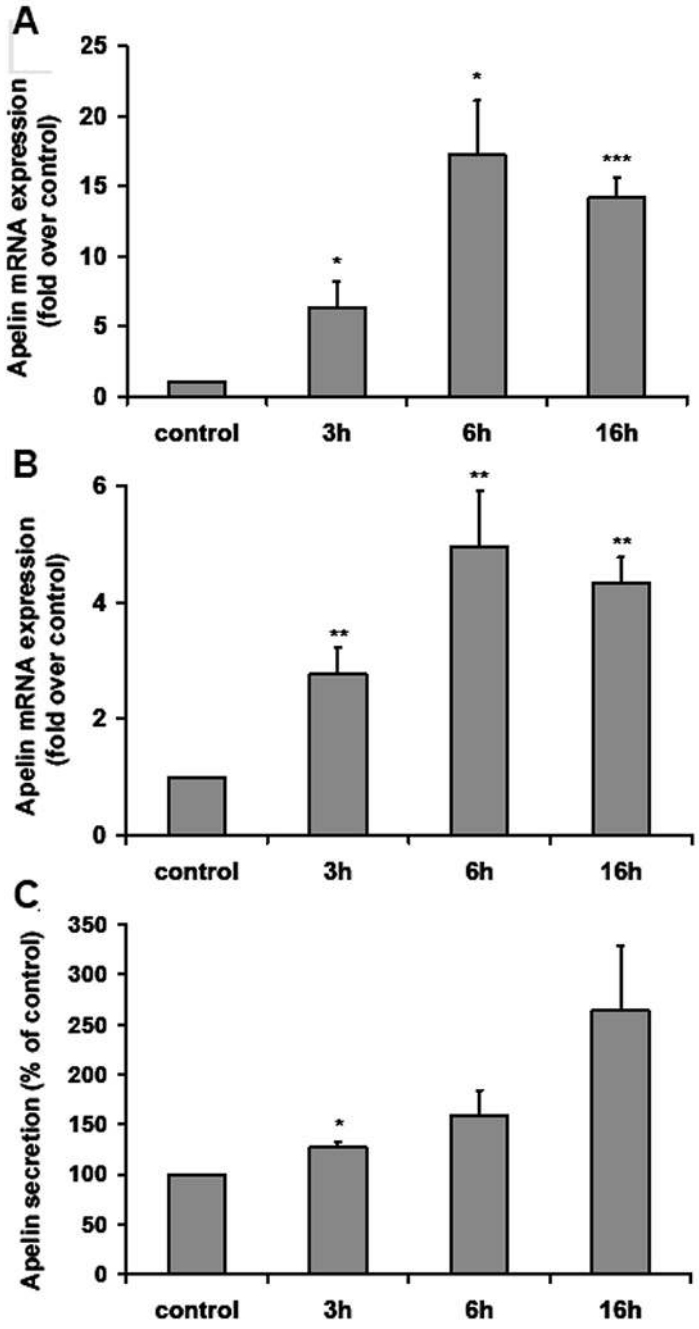

Fig. 3.

Effect of hypoxia on apelin mRNA expression and secretion in human SGBS adipocytes. Differentiated SGBS cells were cultured under hypoxia (1% O_2; A) or treated with 150 μM $CoCl_2$ (B) for the indicated time periods. Apelin mRNA expression was measured by real-time PCR. Results of three to five independent experiments each performed in triplicate are expressed as mean values ± SE. Values are depicted relative to the untreated control. (C) Secretion of apelin by differentiated SGBS adipocytes was determined after incubation of the cells under 1% O_2 for the time periods indicated. Control adipocytes were cultured under normoxic conditions. Apelin protein in the supernatant was measured by ELISA. Results of

three independent experiments each performed in triplicate are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

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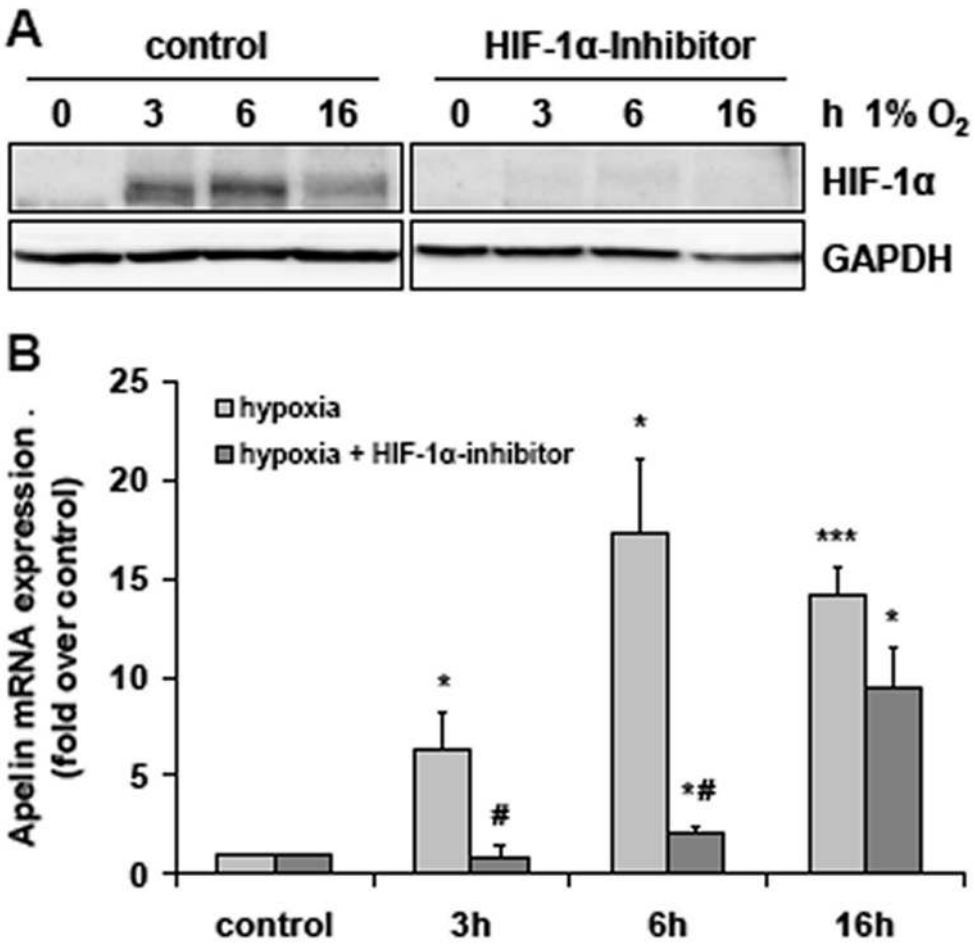

Fig. 4.

(A) Accumulation of HIF-1α protein under hypoxic conditions was examined by immunoblotting in differentiated SGBS cells after treatment of the cells with the HIF-1α-inhibitor CAY10585 (90 μM) for the time periods indicated. GAPDH was used as loading control. (B) Apelin mRNA expression in differentiated SGBS adipocytes was measured after treatment of the cells under hypoxic conditions alone or in combination with the HIF-1α-inhibitor CAY10585 (90 μM) for different time periods by real-time PCR. Four to five experiments were performed in triplicate and are expressed as mean values ± SE. * $p < 0.05$; # $p < 0.05$ compared to matched treatment period without HIF-1α-inhibitor treatment.