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Differentiation of human adipocytes at physiological oxygen levels results in increased adiponectin secretion and isoproterenol-stimulated lipolysis

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Abbreviations: AT, adipose tissue; CM, conditioned medium; SkMC, skeletal muscle cell; SMC, smooth muscle cell; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase

Adipose tissue (AT) hypoxia occurs in obese humans and mice. Acute hypoxia in adipocytes causes dysregulation of adipokine secretion with an increase in inflammatory factors and diminished adiponectin release. O_2 levels in humans range between 3 and 11% revealing that conventional in vitro culturing at ambient air and acute hypoxia treatment (1% O_2) are performed under non-physiological conditions. In this study, we mimicked physiological conditions by differentiating human primary adipocytes under 10% or 5% O_2 in comparison to 21% O_2. Induction of differentiation markers was comparable between all three conditions. Adipokine release by adipocytes differentiated at lower oxygen levels was altered, with a marked upregulation of adiponectin, IL-6 and DPP4 secretion, and reduced leptin levels compared with adipocytes differentiated at 21% O_2. Isoproterenol-induced lipolysis was significantly elevated in adipocytes differentiated at 10% and 5% compared with 21% O_2. This effect was accompanied by increased protein expression of β-1 and -2 adrenergic receptor, HSL and perilipin. Conditioned medium (CM) of adipocytes differentiated at the three different conditions was generated for stimulation of human skeletal muscle cells (SkMC) or smooth muscle cells (SMC). CM-induced insulin resistance in SkMC was comparable for the different CMs. However, the SMC proliferative effect of CM from adipocytes differentiated at 10% O_2 was significantly reduced compared with 21% O_2. This study demonstrates that oxygen levels during adipogenesis are important factors altering adipocyte functionality such as adipokine release, in particular adiponectin secretion, as well as the hormone-induced lipolytic pathway.

Introduction

Adipose tissue (AT) is nowadays established to act as an important endocrine organ and not only as a fuel storage site. Factors released by AT, including fatty acids and a variety of proteins and peptides collectively named adipokines, are associated with the well described chronic low-grade inflammation of enlarged AT, as well as with obesity-related disorders such as insulin resistance, diabetes mellitus and the metabolic syndrome.[1-3]

It is assumed that local areas of hypoxia could occur in expanding AT of obese subjects, contributing to chronic low-grade inflammation of AT.[4] The association of hypoxia and inflammation was shown in diverse obese mouse models, revealing a reduction of the AT oxygen level down to 2% in obese animals.[5-7] AT oxygenation in lean and obese human subjects is currently described by two groups.[8,9] The first study from Pasarica et al. demonstrated a moderate reduction in AT oxygen level of obese compared with lean patients (6.2 ± 1.4% vs. 7.3 ± 1.2% O_2),[8] while the group of Goossens et al. described elevated oxygen levels in obese compared with lean controls (8.9 ± 0.8% vs. 5.9 ± 0.5%).[9] Both studies used different methods of oxygen measurement, thus Pasarica et al.[8] assessed AT oxygen tension by a Clark electrode, whereas Goossens et al.[9] used continuous measurement based on microdialysis. Furthermore, both studies differ in their study design regarding the subject matching for age, gender, ethnicity and health aspects such as type 2 diabetes. Due to the fact that these studies tested local oxygen concentrations, it could not be excluded that higher or lower levels of oxygen in different regions of the AT might occur. However, these studies demonstrated that AT oxygenation could range between 3% and 11% O_2.

The role of AT oxygen tension is not completely understood and subject of current investigations. In general, experiments are performed in a non-physiological model using human or murine adipocytes that were cultured and treated at ambient air, which is

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about 21% O_2. In general the experimental settings include differentiation and cultivation at 21% O_2 which is followed by acute hypoxia induced with 1% O_2.[10,11] As it is reported that oxygen tensions could range between 3–11% O_2 in human AT,[9] this reveals that conventional culture and hypoxic treatment do not reflect physiological conditions of human lean or obese AT. The aim of the preset study was to mimic more physiological conditions during differentiation of human primary adipocytes and to identify differences in adipocyte secretion and function in comparison to 'standard' conditions. Using two different oxygen tensions normally regarded as hypoxic conditions (10% and 5% vs. 21% O_2), we observed a similar differentiation pattern under all oxygen tensions. However, adipokine release by adipocytes differentiated at more physiological oxygen conditions is altered and in particular adiponectin levels were markedly increased. Furthermore, we could show that isoproterenol-stimulated lipolysis is elevated in adipocytes differentiated under 10% and 5% O_2 which might be mediated by elevated β-adrenergic receptor expression as well as increased HSL and perilipin expression levels. This study shows for the first time that the oxygen level during adipocyte differentiation is important for adipocyte functionality such as adipokine secretion and lipolytic activity.

Results

Differentiation of adipocytes at different oxygen levels affects lipid droplet size and triglyceride content. To mimic in vitro more physiological conditions during adipogenesis, we differentiated human primary adipocytes at 10% or 5% oxygen and compared these cells with control cells differentiated at 21% O_2. Oil Red O staining at day 14 of differentiation showed a similar differentiation of adipocytes differentiated at 21%, 10% and 5% of oxygen indicated by lipid droplet accumulation (**Fig. 1A**). Measurement of lipid droplet size revealed a significant reduction in lipid droplet diameter of adipocytes from 10% and 5% O_2 by about 25% (**Fig. 1B**). Adipocytes differentiated at 10% showed a significant increase in triglyceride content compared with cells from 21%, whereas no difference in triglyceride content from 5% differentiated adipocytes could be observed (**Fig. 1C**). We also tested protein expression of the glucose transporters (GLUT)-4 and -1 but could not detect significant changes between the three oxygen conditions (**Fig. 1D**).

We further tested the mRNA expression level of the transcription factors PPARγ and C/EBPα, which are important for adipogenesis. As shown in **Figure 1E and F**, mRNA expression of these transcriptions factors was not significantly altered during differentiation under the three oxygen conditions.

Adipokine secretion is altered in adipocytes differentiated at 10% and 5% O_2. As it is known that adipokine secretion is affected by acute hypoxia, we examined the release of distinct adipokines from adipocytes differentiated at 21%, 10% and 5%. The classical adipokine adiponectin showed a marked 3-fold increase in its protein abundance in adipocytes differentiated at 10% and 5% compared with 21% (**Fig. 2A**). Simultaneously, adiponectin release was increased from adipocytes at 10% and 5% O_2 with the highest amounts being secreted by cells at 10% O_2 (**Fig. 2B**). For leptin we observed a significant reduction in its secretion from adipocytes differentiated at 10% compared with the control at 21% while a tendency but no significant differences between 5% and 21% $O_2$was observed (**Fig. 2C**). We further analyzed the secretion of other hypoxia-related adipokines such as IL-6 and VEGF-A. VEGF-A release by adipocytes differentiated at 10% and 5% O_2 was not significantly altered (**Fig. 3A**). IL-6 concentration in CM of adipocytes differentiated at 10% O_2 was substantially elevated, while its release at 5% O_2 was not significantly altered compared with 21% O_2 (**Fig. 3B**). Furthermore, we tested the expression and the release of DPP4 that was recently described by our group as a novel adipokine released from adipocytes.[12] As shown in **Figure 3C**, DPP4 expression was significantly increased by about 2-fold under low oxygen tensions, which was accompanied by an increase in its release (**Fig. 3D**). Measurement of MCP-1 secretion revealed no differences between the different oxygen conditions (**Fig. 3E**).

Adipocytes differentiated at lower oxygen tension are lipolytically more active. We tested the lipolytic activity of adipocytes in the basal state as well as after isoproterenol-stimulation. In the basal state adipocytes differentiated at 10% O_2 showed a 2-fold increase in glycerol release compared with the adipocytes differentiated at 21% O_2 as well as to adipocytes differentiated at 5% O_2 (21% O_2 3.11 ± 0.34 nM vs. 10% O_2 10.18 ± 0.92 nM vs. 5% O_2 4.24 ± 0.86 nM). Stimulation with 1, 10 and 100 nM isoproterenol resulted in a higher glycerol release by the cells at 10% and 5% compared with 21% O_2. When stimulated with 1 nM isoproterenol, glycerol release was also significantly higher from adipocytes differentiated at 10% compared with 5% O_2 (**Fig. 4A**). We further measured protein expression of the two main lipases of adipocytes, namely adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), via western blotting. As demonstrated in **Figure 4B**, we observed no significant alteration in ATGL expression at 10% and 5% compared with 21% O_2, but HSL showed a more than 2-fold higher expression in both conditions compared with 21% O_2 (**Fig. 4C**). Additionally, the expression of the lipid coating protein perilipin was markedly increased in adipocytes differentiated at 10% and 5% compared with 21% O_2 (**Fig. 4D**). We further observed a prominent increase in the expression of β1-adrenergic receptor in cells at 5% O_2 while the β2-adrenergic receptor was elevated in adipocytes differentiated at 10% O_2 although this effect was not significant (**Fig. 4E and F**).

CM of adipocytes reduces insulin-signaling in SkMC irrespective of O_2 during differentiation. As previously shown by our group, adipocyte CM impairs insulin-stimulated Akt phosphorylation in SkMC.[13] To test whether CM from adipocytes differentiated at 21%, 10% or 5% O_2 may have a different impact on SkMC insulin signaling, we incubated SkMCs for 24 h with CM of the different conditions. As shown in **Figure 5A and B**, all CM significantly reduced insulin-stimulated Akt phosphorylation on both Ser472 and Thr308 sites with no differences in the potency of the different CM.

CM from adipocytes differentiated at 10% O_2 display a diminished proliferative effect on SMC compared with 21% and 5% O_2. We further tested CM-induced SMC proliferation as

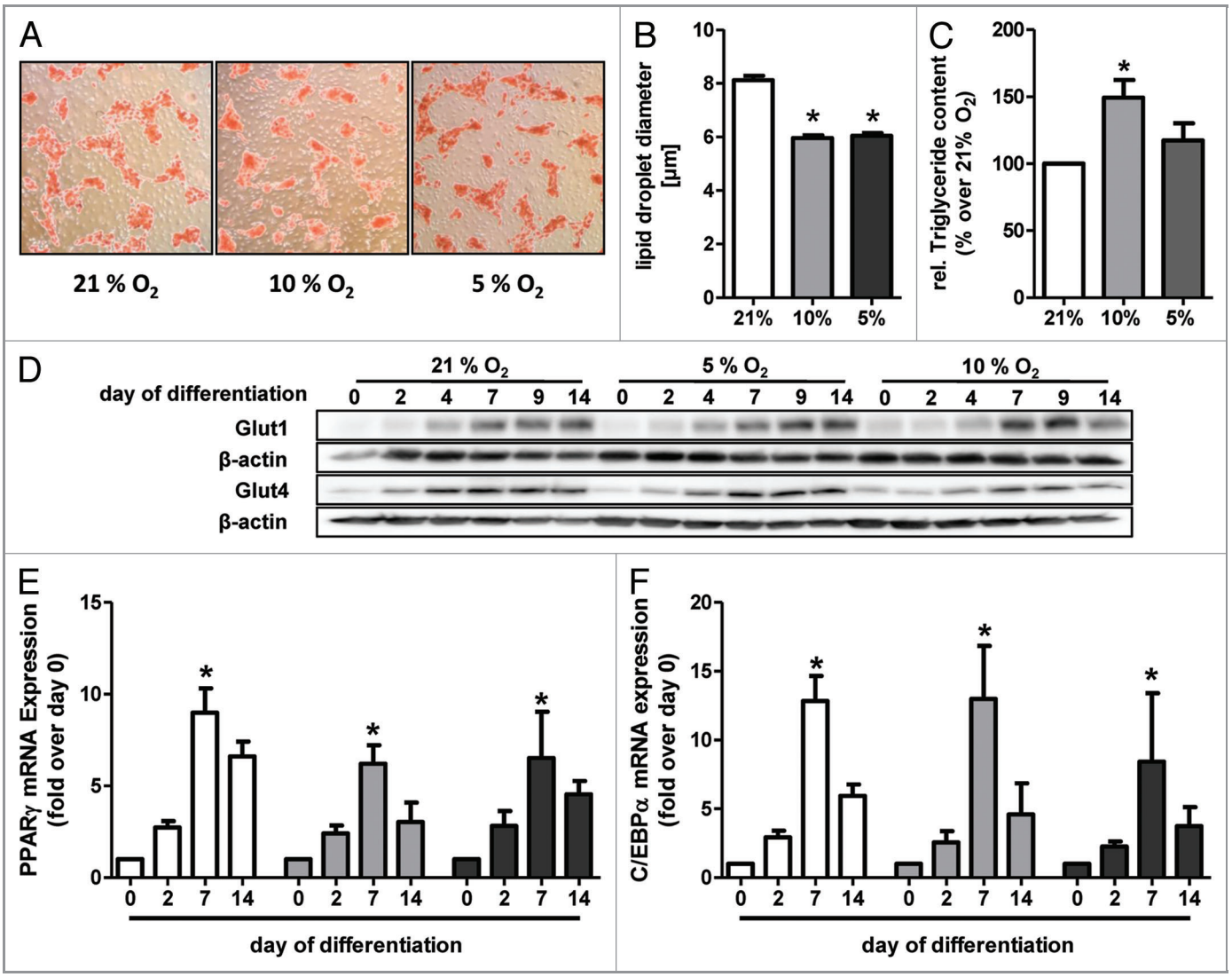

Figure 1. Differentiation of human primary adipocytes at low oxygen tensions results in a similar adipogenic profile compared with normoxic cells. Human primary preadipocytes were differentiated in vitro at 21%, 10% or 5% oxygen. (A) Lipid droplet accumulation was analyzed at day 14 of differentiation by Oil Red O staining and digitally documented. Representative pictures at 4-fold magnification are shown. (B) Lipid droplet (LD) size was analyzed using ImageJ software by measuring 200 LDs per situation and individual, using pictures of 20-fold magnification of three individuals. (C) Triglyceride content was measured using a Triglyceride Quantification kit according to the manufacturer's instructions. (D) Preadipocytes before (day 0) and adipocytes at day 2, 4, 7, 9 and 14 after induction of differentiation were harvested at the indicated oxygen concentration and directly lysed. Total lysates (10 µg) were resolved by 10% SDS-Page and analyzed via western blotting on polyvenylidenefluorid (PVDF) membranes and further processed as described in Material and Methods. Representative western blots for GLUT1 and GLUT4 are shown. (E and F) mRNA was isolated from preadipocytes before (day 0) and adipocytes at day 2, 7 and 14 after induction of differentiation. mRNA was reverse transcribed into cDNA and analyzed via quantitative real-time PCR using specific primer sets for the genes PPARγ and C/EBPα. Data were normalized to the mRNA expression level of POLR2A and expressed relative to the control at 21% O_2 of day 0. As values were not normally distributed, data was log-transformed prior to statistical analysis. Open bars, 21% O_2; light gray bars, 10% O_2; dark gray bars, 5% O_2. Data sets are mean values ± SEM of 3–5 independent experiments. *$p < 0.05$ compared with the control at 21% O_2.

described by our group in a previous study.[14] Incubation of SMC with CM generated from adipocytes differentiated at 21%, 10% or 5% O_2 significantly enhanced SMC proliferation (**Fig. 5C**). However, CM from cells of 10% O_2 showed a significantly reduced potential to induce SMC proliferation compared with the CM from cells differentiated at 21%, while CM from cells at 5% O_2 showed a tendency but no significant reduction in its proliferative effect.

Discussion

Oxygen plays a pivotal role in regulating metabolic functions such as glucose and lipid metabolism shown in diverse tissues.[15-17] Oxygen concentration in the human body is much lower than that of ambient air, ranging from 14% to 4% in the lung parenchyma and the circulation.[18] Also, the oxygen level in distinct tissues is varying, for example, the brain ranges from 0.5%

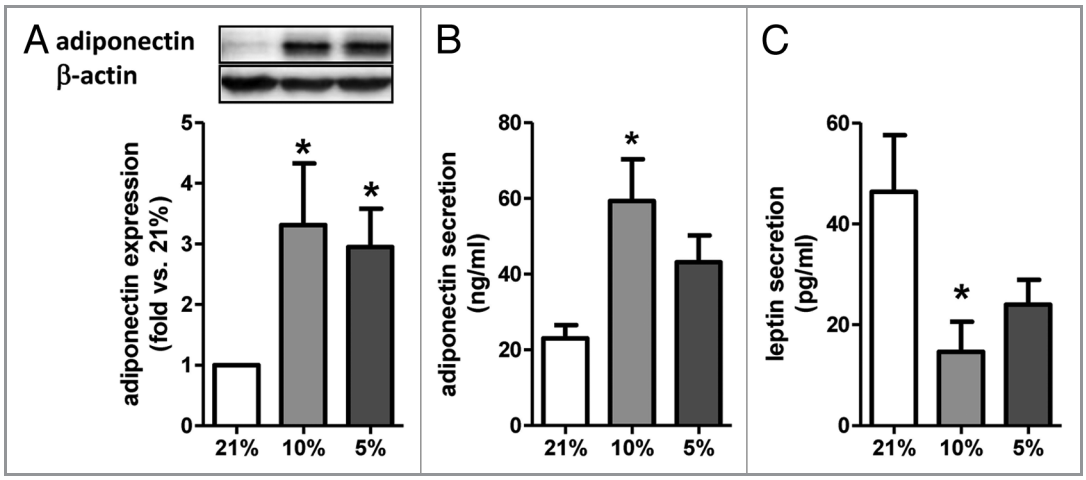

Figure 2. Differentiation of human primary adipocytes alters adiponectin and leptin release. Human primary adipocytes, differentiated at 21%, 10% or 5% O_2, were incubated for 24 h in αMEM at the same oxygen conditions. Afterwards, supernatants were collected for further ELISA measurements and cells were harvested and directly lysed. Total lysates (10 μg) were resolved by 10% SDS-Page and analyzed via western blotting on polyvenylidenefluorid (PVDF) membranes and further processed as described in Materials and Methods. (A) Protein expression of adiponectin was detected by ECL. Representative blots are shown. Supernatants were analyzed for protein concentration of (B) adiponectin and (C) leptin. Data sets are mean values ± SEM of ≥ 4 independent experiments. *p ≤ 0.05 compared with the control at 21% O_2.

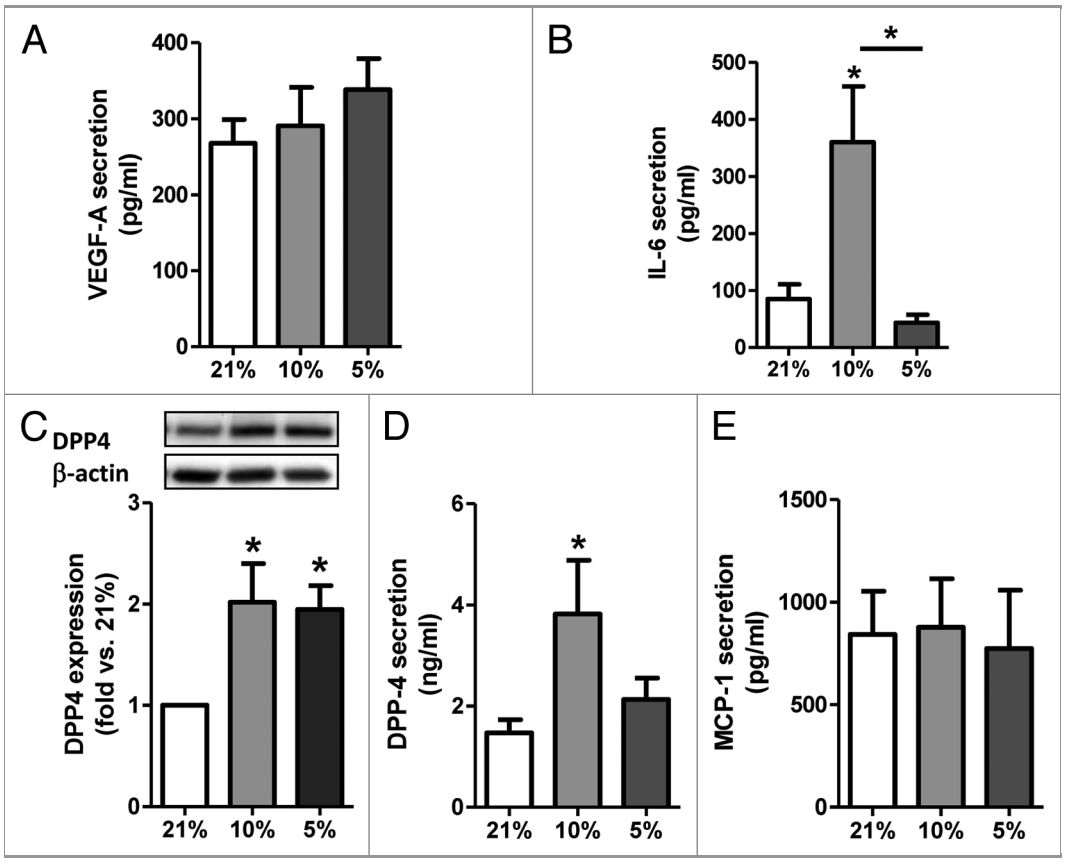

Figure 3. Regulation of IL-6, VEGF-A and DPP4 release under lower oxygen tensions. Supernatants and lysates from human primary adipocytes, differentiated at 21%, 10% or 5% O_2, were obtained and analyzed as described in **Figure 2**. Protein concentration in the supernatant was determined by ELISA measurement for (A) IL-6, (B) VEGF-A, (D) DPP4 and (E) MCP-1. (C) Protein expression of DPP4 was determined by western blotting. A representative blot is shown. Data sets are mean values ± SEM of ≥ 4 independent experiments. *p ≤ 0.05 compared with the control at 21% O_2.

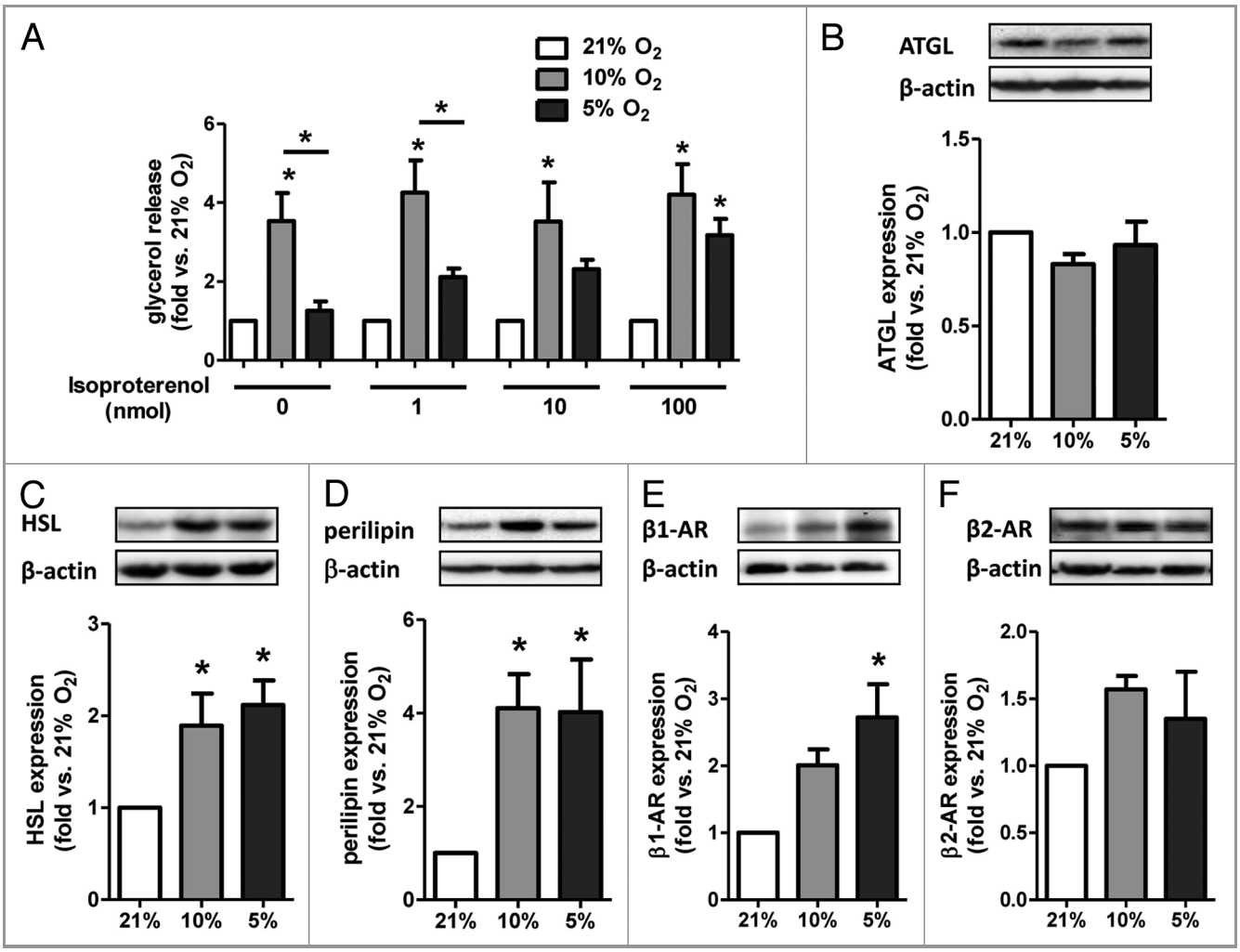

Figure 4. Increased lipolysis and expression of proteins of the hormone-sensitive lipolytic pathway at lower oxygen tensions. (A) For lipolysis experiments adipocytes at the appropriate oxygen condition were starved 24 h in αMEM. These cells were further incubated in Krebs-Ringer-buffer supplemented with 1% BSA for 4 h with or without designated concentrations of isoproterenol. Glycerol release into the supernatant was measured using the Glycerol Free reagent due to the manufacturer's instructions. Cell lysates of unstimulated adipocytes were analyzed via SDS-Page and western blotting with appropriate antibodies for (B) ATGL, (C) HSL, (D) perilipin and (E and F) β1- and β2-adrenergic receptors. Representative blots are shown. Data sets are mean values ± SEM of 3–8 independent experiments. *$p < 0.05$ compared with the control at 21% O_2.

to 7% O_2, the eye shows 1% to 5% O_2 and the bone marrow niche is nearly anoxic ranging from 0% to 4%.[18] Most cell culture models are performed at ambient air which could be defined rather a "hyperoxic" than a physiological model and it has been suggested that the physiological oxygen tension could be defined as "in situ normoxia."[18] In the present study, we investigated the impact of more physiological oxygen tensions on differentiation and cellular function of primary human adipocytes. We have chosen oxygen levels of 5% and 10% O_2, as studies investigating human AT oxygenation reported of O_2 levels ranging between 3–11%.[8,9] It is shown for 3T3-L1 as well as human bone marrow stromal cells (hMSC) that severe hypoxia of 1% O_2 inhibit adipocyte differentiation and maintain the cells in a precursor phenotype, while moderate hypoxia of 8% O_2 could stimulate adipogenesis in murine MSC.[19-21] Our data show that adipocyte differentiation at 5% and 10% oxygen is not impaired but comparable to the standard model of cell differentiation at ambient air, as we demonstrate similar lipid droplet accumulation, as well as a similar expression of adipogenesis related factors such as PPARγ, C/EBPα, GLUT1 and GLUT4 in these cells. At 10% O_2 the cells seem to exert even a higher potential of lipid accumulation, as a slight but significant increase in triglyceride content was observed. However, lipid droplets of adipocytes under more physiological conditions showed a significant lower diameter (**Fig. 1B**). This effect might directly be mediated by the observed elevated expression level of perilipin, as Sawada et al. recently showed that overexpression of perilipin in 3T3-L1 cells resulted in reduced lipid droplet size and furthermore, mediated a more brown fat-like phenotype of these cells.[22] In our study UCP-1 mRNA expression levels were very low and rather reduced at physiological oxygen conditions (data not shown).

The most intriguing observation in this study is the prominent increase in adiponectin expression and secretion of about 3-fold by adipocytes differentiated under more physiological conditions.

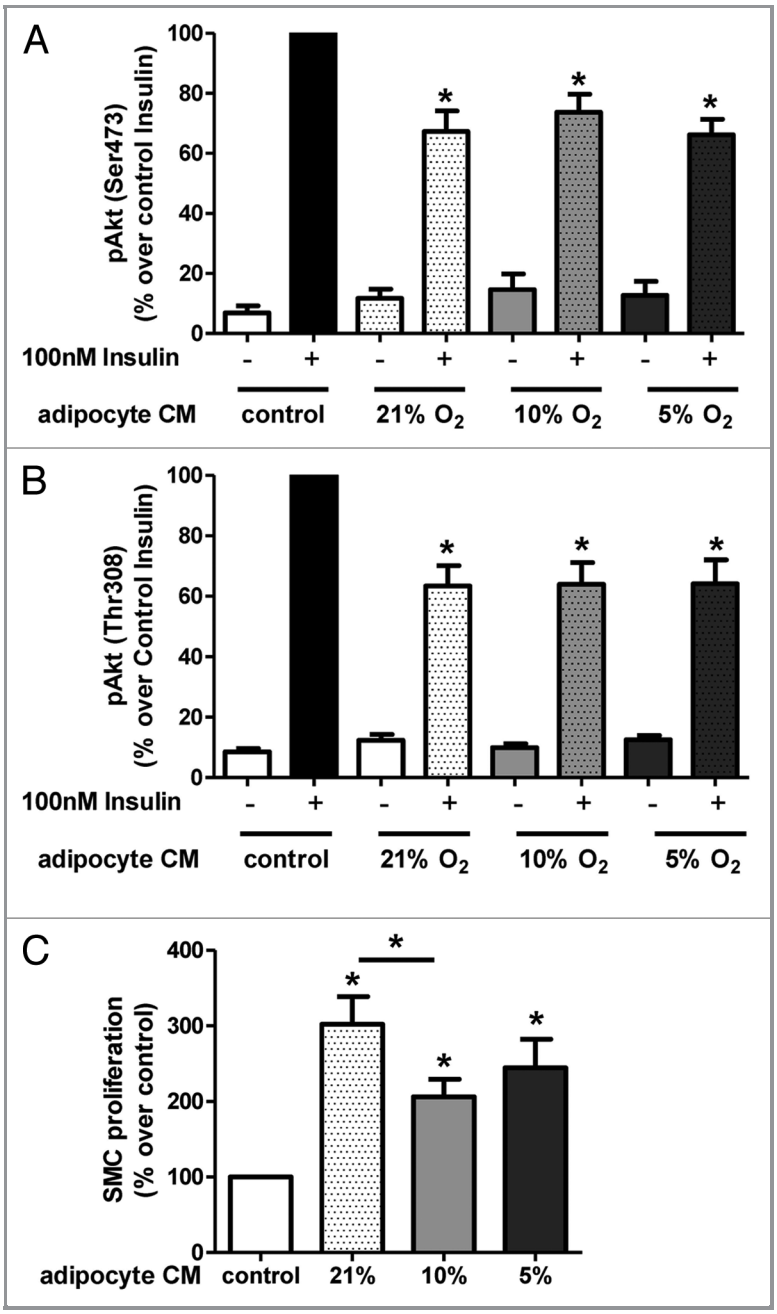

Figure 5. Effects of adipocyte-conditioned medium (CM) on human SkMC and SMC. CMs from adipocytes differentiated at 21%, 10% or 5% O_2 were generated over 48 h. (A and B) Human SkMCs were incubated for 24 h with αMEM or CM of the different adipocytes and afterwards stimulated with 100 nM insulin for 10 min. Cells were harvested, directly lysed and analyzed via SDS-Page and western blotting for the phosphorylation level of Akt (Ser473) and Akt (Thr308). (C) Human SMCs were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of CM from the differently differentiated adipocytes for further 24 h. Proliferation of SMC was measured as described in Materials and Methods. Data sets are mean values ± SEM of ≥ 6 independent experiments. *$p < 0.05$ compared with the basal value.

Adiponectin is a classical adipokine, mainly released by adipocytes, that exerts anti-inflammatory, anti-diabetic and anti-atherogenic effects and its serum concentration is negatively associated with obesity and the metabolic syndrome.[23,24] Adiponectin expression and secretion is highly regulated and the underlying mechanisms are intensively investigated but are yet not fully determined.[23] Acute treatment of adipocytes with low oxygen tensions was shown to diminish adiponectin expression and secretion and this effect correlated positively with the oxygen level.[10,25,26] Our finding of elevated adiponectin release is divergent from the described effects under acute hypoxia and this effect reflects the different cell culture model of our study. Previous studies solely investigated the impact of acute hypoxia treatment on cells differentiated at 21% O_2, while our study investigated more physiological oxygen conditions throughout differentiation. We suggest that chronic exposure of human primary adipocytes to lower oxygen tensions might result in a kind of reprogramming, altering the secretory behavior of adipocytes. It could be assumed that adiponectin expression and release depend on the oxygen level during adipocyte development. As shown in **Figure 2B**, there is a tendency of reduced adiponectin release at 5% compared with 10% oxygen. It is conceivable that optimal adiponectin release might be highest from adipocytes developed in an environment of about 10% O_2 while oxygen level above or below might reduce it.

Other adipokines related to hypoxia such as leptin or IL-6, whose release was shown to be induced after acute hypoxia treatment even at 10% O_2, are altered in a different way in adipocytes differentiated at 10% and 5% compared with 21% O_2. Leptin release is reduced in our cell culture model of adipocyte differentiation under more physiological conditions. IL-6 release shows a different picture under chronic hypoxia. Its release is markedly elevated by about 4-fold at 10%, but decreased by about 2-fold at 5% O_2, although this effect is statistical not significant. As Wood et al. demonstrated a straight dose-response correlation of IL-6 release and oxygen tension, the bell-shaped response observed in our study might also underlie the different protocol of adipocyte culture. Regarding the study of Goossens et al., who demonstrated increased oxygen tensions and inflammation in human obese AT, it is likely that physiologically rather higher than lower oxygen concentrations might be the underlying mechanism for the bell-shaped response of IL-6 release. This assumption might also be true for other inflammatory adipokines such as DPP4. Here, we demonstrate an increase in the expression and secretion level of this novel adipokine under more physiological oxygen levels. DPP4 was first identified by our group as a novel adipokine, expressed and released from human primary adipocytes.[12] Moreover, we demonstrated DPP4 to be upregulated by inflammatory TNFα, and that its release and serum levels in human subjects correlates positively with the BMI and obesity. However, in the latter study we could not demonstrate a regulatory effect on DPP4 release due

to an acute treatment of adipocytes with 1% O_2. Not much is known about the effect of hypoxia on DPP4 expression and secretion, but it is reported that DPP4 expression could be induced in endothelial cells by acute hypoxia-treatment of about 2.6% O_2 and also in a HIF-1α-dependent manner in nearly anoxic tumor xenografts.[27,28] As demonstrated in this study, physiologic oxygen tensions do affect DPP4 expression and release, resulting in higher levels of this inflammatory novel adipokine. The role of DPP4 in AT inflammation is underlined by recent reports demonstrating that DPP4 inhibition in obese mice models reduces the expression of inflammatory markers and AT infiltration with inflammatory immune cells.[29,30] As suggested by Goossens et al. physiologically rather increased than decreased AT oxygen levels might trigger an inflammatory response of human adipocytes. The increases in inflammatory IL-6 and DPP4 levels, as well as in triglyceride content and basal lipolysis, events all observable in obesity,[1,12] would support this hypothesis. However, in contrast to this hypothesis is the marked increase in anti-inflammatory adiponectin release observed in this study. Moreover, the level of MCP-1 release by adipocytes at physiologic oxygen tensions was not altered, demonstrating that not all inflammatory markers are altered and follow the pathologic situation in the obese state. If the situation of 10% O_2 in AT would reflect the more inflammatory environment, higher release of MCP-1 and less adiponectin would be expected. It is as well likely that distinct adipokines might play other roles than involvement in inflammation in this context. For example, current studies demonstrated in vivo and in vitro that IL-6 is a potent inducer of AT lipolysis and increases fatty acid oxidation.[31,32] IL-6 is assumed possibly to play an important role in lipid metabolism[33] and therefore might function as a regulator of adipocyte lipid metabolism. It is conceivable that lipid turnover induced by IL-6 is an oxygen-dependent effect that might be increased at oxygen concentrations around 10% O_2 while under lower oxygen levels IL-6-induced lipid turnover might be reduced. However, further studies are necessary to evaluate the underlying mechanisms of adipokine regulation, and to elucidate their specific role in this context.

Our cell culture model revealed an altered lipolytic activity of adipocytes under more physiological oxygen tensions. AT lipolysis is regulated by diverse effectors such as cathecholamines, glucocorticoids and insulin, but also in an autocrine manner by adipokines such as TNF-α and IL-6.[34-36] The lipolytic machinery involves a number of different lipases but it is assumed that TAG hydrolysis is mainly mediated by ATGL and HSL, as a mice model demonstrated that these two lipases account for more than 90% of lipolytic activity.[37] Lipid droplet coating proteins are as well important regulators of lipolysis as they limit or provide access of lipases to the lipid droplet.[38] In adipocytes the lipid droplet coating protein perilipin plays a pivotal role, as its protein kinase A (PKA)-mediated activation is essential for full HSL activity and indirectly also for ATGL activity.[38] Here we demonstrated a marked upregulation of isoproterenol-stimulated lipolysis in adipocytes differentiated at lower oxygen tensions compared with cells at ambient air. Our data suggests that this effect on lipolysis is caused by an upregulation of the hormone sensitive pathway, as we observed increased expression of β-adrenergic receptors, in particular of the β1-adrenergic receptor, HSL and perilipin. Basal lipolysis is elevated in cells differentiated at 10% oxygen compared with cells from 21% and 5% O_2. This increase might be mediated by an autocrine mechanism, as adipocytes at 10% oxygen released substantially more IL-6 (**Fig. 2D**), that is known to stimulate lipolysis.[35] These alterations in hormone- and adipokine-mediated lipolysis seems to be highly regulated by oxygen supply of the environment. There are only a few studies in humans and a mouse model regarding the effect of hypoxia on AT lipolysis. These studies demonstrated, that reduced oxygen supply in the air or in AT could increase adipocyte lipolysis in vivo and in vitro.[8,39-41] It was suggested by the authors that downregulation of β-adrenergic stimulation as well as reduced anti-lipolytic activity of insulin on adipocytes was involved in this effect. To our knowledge, the present study is the first to demonstrate a regulatory effect of low oxygen concentrations on the expression level of the lipase HSL, as well as the lipid coating protein perilipin in human primary adipocytes.

It is known that basal lipolysis in the obese state is elevated while catecholamine induced lipolysis is reduced.[42,43] As we observed alterations in basal as well as isoproterenol-stimulated lipolysis under physiological conditions, it could be assumed that lipolysis in obese individuals might be affected by oxygen tension. In particular, the reduced lipolytic activity after stimulation with a low concentration of isoproterenol (1 nM) in cells differentiated at 5% compared with 10% O_2 might reflect a mechanism of hypoxia-mediated impairment of cathecholamine-induced lipolysis in the obese state. However, as the current studies on AT oxygenation are conflicting and could not clearly describe if oxygen is reduced or elevated in AT of obese subjects,[8,9] we could not classify if the effects of basal and isoproterenol-induced lipolysis might contribute to the dysregulation described for the obese state. Future studies are necessary to elucidate this aspect of AT oxygenation in relationship to defects in lipolysis.

As oxygen tension significantly affects adipokine release, it might be speculated that adipokine signaling to other organs might be altered. Previous studies of our group reported that adipocyte generated CM affect other cell types like SkMC and SMC. We demonstrated via cross-talk experiments that CM induces insulin-resistance in SkMC as well as proliferation of SMC, whereas SMC proliferation negatively correlates with adiponectin amounts in the CM.[13,14] We therefore assumed that the elevated levels of adiponectin, released into CM of adipocytes differentiated under more physiological conditions, would alter the CM effect on SkMC and SMC. The alterations in CM composition and in particular the increased adiponectin amounts had no positive effect on insulin-sensitivity of CM-treated SkMC. On the other hand, we could show that SMC proliferation is reduced when treated with CM of adipocytes differentiated at 10% O_2 compared with CM from adipocytes at ambient air. Regarding that adipocytes at 10% O_2 released the highest amounts of adiponectin, this finding is in accordance to our previous study where we demonstrated that SMC proliferation is dependent on adiponectin concentration in the CM.[14] These cross-talk experiments demonstrate that oxygen dependent

alterations in CM composition and in particular elevated adiponectin amounts could not prevent the CM-induced alteration of SkMC insulin-signaling and SMC proliferation.

In conclusion, this study shows that oxygen tension is an important factor affecting adipocyte development. Adipokine expression and secretion is a highly oxygen sensitive mechanism. However, this study revealed that adipocyte culture at 21% O_2, which could be defined as hyperoxia, seems to impair adipocyte function. This is demonstrated by the reduced lipolytic activity of adipocytes at ambient air. As this study was intended to investigate the role of oxygen during adipogenesis, the specific underlying mechanisms to the observed alterations of adipocyte function have to be revealed in further studies. Future experiments should take into account that the effects in experiments of the standard culture model might show adverse effects or might be even more pronounced when performed under physiological conditions.

Materials and Methods

Materials. Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech and by Sigma. Human specific anti-phospho-Akt (Ser473) (9271), anti-phospho-Akt (Thr308) (9275), anti-Akt (9272) and anti-HSL (4107) antibodies were supplied by Cell Signaling Technology. Human anti-Glut4 (MAB1262) was purchased from R&D Systems. Human anti-actin (ab6276), anti-adiponectin (ab22554), anti-Glut1 (ab32551), anti-β 1 (ab77189) and anti-β 2 adrenergic receptor (ab69598) antibodies came from Abcam. Human anti-ATGL (sc-365278) and HRP-conjugated donkey anti-goat (sc-2020) antibodies were supplied by Santa Cruz Biotechnology. Human anti-perilipin antibody (IMG-30288) came from Imgenex and HRP-conjugated goat anti-rabbit (W4011) and goat anti-mouse (W4021) IgG antibodies from Promega. Collagenase NB4 (17465.02) was obtained from Serva. FCS (10270-106), Dulbecco's modified Eagles/HAM F12 (DMEM/F12) medium (42400-010), α-modified Eagle's (αMEM) medium (11900-016) and Ham's F-12 medium (21700-026) was supplied by Gibco (Invitrogen). Troglitazone and Oil Red O (O0625) were obtained from Sigma Aldrich. Complete protease inhibitor cocktail tablets (11697498001) were from Roche. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma Aldrich or Applichem Biochemica.

Adipocyte isolation and culture. Subcutaneous AT was obtained from healthy lean or moderately overweight women (n = 39, body mass index 26.8 ± 3.8, and aged 41.0 ± 12.3 y) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University. Preadipocytes were isolated by collagenase digestion of AT as previously described by us.[44] Isolated cell pellets were resuspended in DMEM/F12 medium supplemented with 10% FCS, seeded in 75 cm^2 culture flasks, six-well or 12-well culture dishes and maintained at 37°C with 5% CO_2. After cells were grown until confluence, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 0.25 µg/ml amphoterecin B, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 14 d under normoxia (21% O_2 supplemented with 5% CO_2) or in an Xvivo hypoxia chamber system (Biospherix) under 10% or 5% O_2 supplemented with 5% CO_2 and respective concentrations of nitrogen. Medium was changed at the appropriate oxygen tension every 2–3 d with addition of 5 µM troglitazone for the first 3 d. Oil Red O staining was performed at day 14 of differentiation. Therefore, cultures were washed with ice-cold PBS and fixed with a solution containing 71% picric acid, 24% acetic acid and 5% formaldehyde for 2 h. Afterwards, cells were washed with PBS, further incubated with 0.3% Oil Red O solution for 10 min and washed again with PBS. Subsequently, samples were photographed with a Canon EOS digital camera. To determine lipid droplet size the imaging software ImageJ was used analyzing pictures of 20-fold magnification. Triglyceride content of differentiated adipocytes was determined using a Triglyceride Quantification Kit from Biovision (K622-100) according to the manufacturer's instructions. To generate adipocyte conditioned-medium (CM) from adipocytes differentiated at 21%, 10% and 5% O_2, adipocytes were incubated with α-modified Eagle's medium (αMEM) at the distinct oxygen levels for 48 h as described in a previous study.[13]

Immunoblotting. All cells were treated as indicated and lysed in a buffer containing 20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate, pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 1% Triton X100 and complete protease inhibitor cocktail. Lysates were sonificated at 4°C and the suspension was centrifuged at 10,000 × g for 20 min. Protein concentration was determined by using a Bradford protein assay. Thereafter, 10 µg of protein were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvenylidene-fluorid (PVDF) membrane in a semidry blotting apparatus. Membranes were blocked with Tris-buffered saline containing 0.1% Tween and 5% nonfat dry milk or BSA and subsequently incubated overnight with the appropriate antibodies. After washing, membranes were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, WBKLS0500). Signals were visualized and evaluated on a BioRad VersaDoc 4000 MP work station.

ELISA analysis. Secretory products from normoxic and hypoxic adipocytes were collected over 24 h and analyzed by ELISA measurements. Concentration of adiponectin (RD191023100; detection range 1–150 ng/ml) and VEGF-A (RBMS277/2R; detection range 15.6–1000 pg/ml) was determined with ELISA kits from Biovendor, IL-6 (950.030.192; detection range 6.25–200 pg/ml) and MCP-1 (650.110.192; detection range 16–1000 pg/ml) was determined using ELISA kits purchased from Gen-Probe and leptin (DLP00; detection range 15.6–1000 pg/ml) and DDP-4 (DC260; detection range 0.31–20 ng/ml) concentrations were analyzed by ELISA kits purchased from R&D Systems. If necessary, samples were diluted with a provided dilution buffer of the manufacturer prior to the

assay, which was performed in duplicates according to the manufacturer's instructions.

RNA isolation and quantitative real-time PCR. Total RNA was isolated and reverse transcribed using the RNeasy Lipid Tissue (Qiagen, 74804) and Omniscript Reverse Transcription kit (Qiagen, 205113) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using QuantiTect primer assays and SYBR green reagents (Qiagen, POLR2A, QT00033264, CEBPA, QT00203357, PPARG, QT00029841, SYBR Green #204145) with 2.5–25 ng of generated cDNA on a Step One Plus Cycler (Applied Biosystems). POLR2A was used as a housekeeping gene as done in a previous study by us.[25] Expression levels of investigated genes were normalized to POLR2A. Gene expression was analyzed via the ΔΔCt method and compared with the designated control.

Measurement of lipolysis. Adipocytes were cultured in 12-well culture dishes and differentiated at 21%, 10% and 5% of oxygen. Lipolysis experiments were performed under the designated oxygen levels. Therefore, cells were starved 24 h in αMEM, washed with PBS and incubated for 4 h in Krebs-Ringer-buffer containing 1% BSA. Lipolytic stimulation was induced with designated concentrations of isoproterenol. Glycerol release was measured using Glycerol Free reagent (F6428) from Sigma.

Culture of skeletal muscle cells and smooth muscle cells. Primary human skeletal muscle cells (SkMC) isolated from rectus abdominis muscle of two healthy Caucasian donors (2 females, 33 and 37 of age) were supplied as proliferating myoblasts from PromoCell and cultured as described in our earlier study.[44] For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 10^5 cells/well and were grown in αMEM/Ham's F-12 medium (PromoCell) containing skeletal muscle cell growth medium supplement pack up to near-confluence. The cells were then differentiated in αMEM for 6 d. Differentiated cells were incubated for 24 h with the indicated adipocyte conditioned-medium (CM). Afterwards, cells were stimulated with 100 nmol/l insulin for 10 min.

Primary human smooth muscle cells (SMC) from two different donors (Caucasian, male, 58 y old; female, 56 y old) were supplied as proliferating cells from Lonza and PromoCell and kept in culture according to the manufacturer's protocol. SMC were characterized by morphologic criteria and by immunostaining with smooth muscle α-actin.

To analyze SMC proliferation DNA synthesis of proliferating cells was measured by incorporation of 5-bromo-2'-deoxyuridine (BrdU). Therefore, SMC were seeded in 96 well culture dishes and allowed to attach for 24 h, cultured in Smooth Muscle Cell Growth Medium 2 (C-22062) supplemented with 5% FCS and an appropriate supplemental mix (PromoCell). Following serum starvation for an additional 24 h period, cells were stimulated for 24 h with different CMs in the presence of BrdU (10 μM). To determine proliferation the BrdU ELISA Kit (11669915) from Roche was used according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer).

Presentation of data and statistics. Data are expressed as mean ± SEM. One-way ANOVA (post-hoc test: Bonferroni's multiple comparison test) was used to determine statistical significance. All statistical analyses were done using Prism (GraphPad) considering a p value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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