Impact of Stimulatory Pathways on Adipogenesis and HIV-Therapy Associated Lipoatrophy

METODI V. STANKOV, REINHOLD E. SCHMIDT, AND GEORG M. N. BEHRENS[1]

Clinic for Immunology and Rheumatology, Hannover Medical School, 30625 Hannover, Germany

Objective: **Current understanding of adipogenesis derives mainly from studies with *in vitro* cell culture systems with divergent experimental requirements. We aimed to investigate the discrepancy between the anti-adipogenic effects of the HIV protease-inhibitor indinavir (IDV) *in vitro* and the lack of evidence that IDV inhibits adipogenesis in humans. *Design and Methods:* We studied cell viability and adipogenesis in murine 3T3-F442A, 3T3-L1 and primary human subcutaneous preadipocytes (phsPA). Differentiation was studied after activation of the established four signalling pathways in different combinations. We analyzed CCAAT/enhancer-binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR) γ expression and triacylglyceride accumulation. Cells were exposed to IDV at concentrations around therapeutic C_{max} levels and higher (10 μM and 20 μM) for up to 30 days. *Results:* Under insulin and fetal calf serum (FCS) input, IDV inhibited 3T3-F442A differentiation, an effect that was partially rescued by the addition of 3-isobutyl-1-methylxanthine (IBMX) stimulation. Combined stimulation with FCS, insulin, dexamethasone (DEX) and IBMX led to normal 3T3-L1 differentiation even in the presence of IDV. However, omission of IBMX rendered this cell line sensitive to IDV's anti-adipogenic effects. Differentiation of phsPA requiring complete adipogenic stimulation was not affected by IDV presence. *Conclusions:* Our data suggest that the potency of IDV to impair differentiation under partial stimulation disappears when all of the differentiation pathways are activated. Such compensatory mechanisms might be responsible for the inability of the drug to affect adipogenesis *in vivo*.** Exp Biol Med 234:1484–1492, 2009

Key words: protease inhibitors; IDV; adipocyte differentiation; antiretroviral therapy

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[1] To whom correspondence should be addressed at Clinic for Immunology and Rheumatology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. E-mail: behrens.georg@mh-hannover.de

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Introduction

Most of the current understanding of adipogenesis derives from studies with *in vitro* cell culture systems such as the 3T3-L1 and 3T3-F442A cell lines (1–3) and suggests four main adipogenic signalling pathways: the IGF-l-activated tyrosine kinase pathway, the glucocorticoid pathway, the cAMP-dependent protein kinase (PKA) pathway and the fatty acid activated receptor pathway (Fig. 1) (4). *In vitro* these pathways are activated by analogous external inducers of adipocyte differentiation such as IGF-1 (or high insulin levels), glucocorticoid, cAMP (or agents increasing cAMP) and fatty acids (Fig. 1) (4). Although it may be expected that all four signalling pathways participate in the complex maintenance of adipocyte homeostasis *in vivo,* only part of them may be sufficient to induce complete differentiation depending on the cell model used *in vitro* (1–3, 5). This becomes more relevant when choosing cell models to study the effect of certain substances on adipocyte differentiation. Certain cell line models, such as the 3T3-F442A cell line, require only partial stimulation in order to produce optimal differentiation (6). Although such models are indispensable in the identification of affected pathways and establishment of mechanisms, interference with the particular signalling pathways used for the induction of adipogenesis might affect the process differently as compared to conditions when complete stimulation is applied. Thus, the effect of the inhibited pathways may be compensated in 3T3-L1 cells and primary human subcutaneous preadipocytes (phsPA), which require complete stimulation of differentiation.

Several researchers have investigated the contribution of antiretroviral drugs to the HIV-therapy-related lipodystrophy syndrome by employing experimental *in vitro* systems. It was hypothesised that protease inhibitors (PI) and in particular indinavir (IDV) play a major role in the development of lipoatrophy in HIV patients (7, 8). This hypothesis was confirmed *in vitro* using the 3T3-F442A cell line. Caron *et al*. demonstrated that IDV affects mainly the IGF-l-activated tyrosine kinase pathway (8). However, detailed clinical observations later were not consistent with the idea that the use of PI facilitates the development of

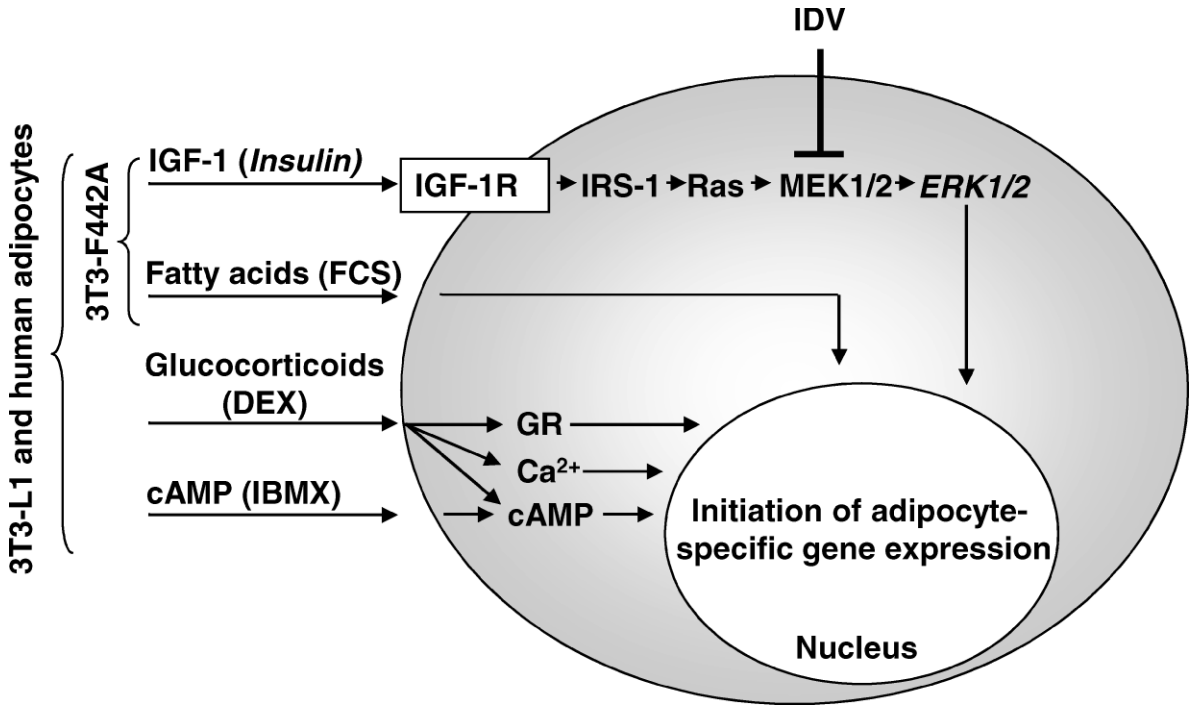


Figure 1. Induction of preadipocyte differentiation. Preadipocytes differentiation requires a number of external inducers. Depending on the cell model (3T3-L1, 3T3-F442A, primary preadipocytes), there are four main inducers that are considered necessary for the induction of adipogenesis: IGF-1 (or high insulin levels), glucocorticoids (DEX), cAMP (or agents increasing cAMP such as IBMX) and fatty acids (FCS) (4). The requirements for these four different types of inducers implicate four second-messenger signalling pathways: the IGF-I-activated tyrosine kinase pathway; the fatty acid activated receptor pathway; the glucocorticoid pathway; the cAMP-dependent protein kinase (PKA) pathway (4). Also indicated is the proposed inhibitory effect of IDV.

lipoatrophy but rather that thymidine analogues were strongly associated with the occurrence of peripheral fat loss (9). Reiss *et al*. even reported improvement in lipoatrophy in patients switched from failed NRTI-therapy to ritonavir-boosted IDV and efavirenz (10).

We hypothesised that the proper stimulation of some of the other pathways may compensate the drug effect on adipogenesis in human cells. Such compensation, if demonstrated, may also provide an explanation for the inability of the drug to induce the expected pathology (10, 11). We aimed to demonstrate that the anti-adipogenic effect of IDV observed in 3T3-F442A under dual stimulation with insulin and fetal calf serum (FCS) (8) may be reduced by the activation of the additional cAMP-dependent protein kinase (PKA) pathway. Consequently, we hypothesised that IDV will have minor effects on the process of adipogenesis of a cell line such as 3T3-L1 and phsPA, when the differentiation induction protocol provides stimulation of the complete set of transduction pathways. Finally, we speculated that the removal of cAMP-dependent protein kinase (PKA) pathway stimulation during 3T3-L1 differentiation protocol would make the cell line susceptible to IDV induced inhibition of differentiation.

Materials and Methods

Cell Culture. Murine 3T3-L1 (12) and 3T3-F442A (13) preadipocytes were maintained and differentiated as previously described (14, 15). 3T3-L1 preadipocytes were maintained in DMEM containing 10% FCS (DMEM-FCS) and 3T3-F442A preadipocytes in DMEM containing 5% newborn calf serum (NCS). The two cell lines differ in their requirements for optimal initiation of differentiation. 3T3-L1 differentiation is triggered by 48 h incubation with DMEM-FCS supplemented with a mixture of hormones including 520 μM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone (DEX) and 1 μM insulin (all Sigma-Aldrich, St. Louis, USA). 3T3-F442A differentiation is triggered by 48 h incubation with DMEM-FCS supplemented with only 1 μM insulin. After the completion of the initial 48 h both cell lines were incubated with DMEM-FCS and 1 μM insulin until day +9, with a medium change every 72 h. Cell culture media were supplemented with 100 U/100 μg/ml penicillin/streptomycin, 4 μg/ml panthotenic acid and 8 μg/ml biotin.

Isolation and maintenance of phsPA were carried out essentially as previously described (15). The phsPA were maintained in DMEM/F12 (1:1) containing 10% FBS (DMEM-FBS) and 172 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A-2-P) (Preadipocyte medium). Cell culture media were supplemented with 100 U/100 μg/ml penicillin/streptomycin. For differentiation, preadipocyte medium was supplemented with a mixture of hormones composed of 520 μM 3-isobutyl-1-methylxanthine (IBMX; for the first 7 days only), 1 μM dexamethasone (DEX), 167 nM insulin and 100 μM indometacin. At

least 70% of the cells differentiated under these conditions as estimated by the acquisition of a mature phenotype and cytoplasms filled with multiple lipid droplets (Oil Red O stained). Primary human subcutaneous fibroblasts were isolated and maintained as previously described (16) and under similar differentiation induction conditions, serving as a negative control for differentiation.

IDV was dissolved in DMSO, and used at concentrations around the therapeutic C_{max} levels and higher (10 μM and 20 μM) (17). The highest concentration of the solvent used in the incubation experiments (DMSO 0.1%) did not affect cellular viability and preadipocyte differentiation. The degree of preadipocyte differentiation was evaluated every second day by Oil Red O staining and microscopic examination for acquisition of a spherical shape and lipid droplet accumulation.

RNA Preparation and Real-Time Quantitative PCR. Total RNA extraction was carried out using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA in 20 μl, using random nonamers, oligo dT primers and the Omniscript RT Kit (Qiagen, Hilden, Germany). The design of primers was performed using Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Real-Time quantitative-PCR analyses for the genes encoding CCAAT/enhancer-binding protein (C/EBP) α, and peroxisome proliferator-activated receptor (PPAR) γ, were performed in a final volume of 25 μl using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) in an iCycler. The murine specific primers for C/EBPα, PPARγ and transferrin receptor (housekeeping gene) were as previously published (15).

The following human specific primers were used: C/EBPα forward primer 5′-GACCTAGAGATCTGGCTGTG-3′; reverse primer 5′-GAGCAAAACCAAAACAAAAC-3′; PPARγ forward primer 5′-AAGCGATTCCTTCACTGATA-3′; reverse primer 5′-GTGGAGTAGAAATGCTGGAG-3′. Amplifications were performed in specifically designed optical 96-well plates using a spectrofluorometric thermal cycler (iCycler, Bio-Rad, Munich, Germany). Genes of interest and housekeeping gene products were amplified separately, using identical cycling conditions. An initial cycle at 95°C for 15 min incubation was performed for activation of HotStarTaq DNA Polymerase, then 42 cycles of one step at 94°C (15 sec) for denaturation, one step at 55°C (30 sec) for annealing, and one step at 72°C (30 sec) for extension. Products were analyzed by melting curve analysis and on ethidium bromide-stained agarose gels to ensure that a single amplicon of the expected size was indeed obtained. The absence of residual DNA in our RNA isolation was controlled by using PCR reactions without reverse transcription. To measure PCR efficiency, serial dilutions of reverse transcribed RNA (1, 1/5 and 1/25) were amplified, and a standard curve was obtained by plotting cycle threshold values as a function of starting reverse transcribed RNA, the slope of which was used for efficiency calculations using the iCycler software. The relative quantification for any given gene was calculated after dividing the standard curve value of the given gene A by that of the calibrator gene B (Transferrin receptor or β actin) in the treated and control cells. The effect of IDV was calculated by comparing mean values obtained from three independent experiments. In each of the three experiments, RNA from three independent culture plates was isolated and pooled. The pooled RNA was reverse transcribed and cDNA obtained by three separate reactions. This cDNA was again pooled and measured in a Real-Time PCR in triplicates. The triplicate Real-Time PCR results of three independent experiments were used for statistical evaluation. These were compared to the mean value of the control cells subjected to the same procedure.

Adipocyte Staining with Oil Red O. For Oil Red O (Sigma-Aldrich, Munich, Germany) staining adherent cells were fixed in 10% formalin, washed and stained with a 0.21% (wt/vol) Oil Red O solution (60% isopropanol, 40% water). At this level Oil Red O staining was studied by conventional fluorescence microscopy. For objective quantification of triacylglyceride content, cells were dried and Oil Red O was extracted with 100% isopropanol and photometrically measured at 495 nm.

Cell Viability. The number of viable cells was measured microscopically using trypan blue exclusion. Every time cell culture medium was changed, the percentage of dead cells was calculated and the final calculation of viable cells was made by exclusion of the cumulative percentage of dead cells for the whole differentiation period.

Statistics. The statistical analyses were done by unpaired Student's *t* test. Comparisons of more than two groups were performed by ANOVA with Bonferroni's post-hoc analysis. The level of significance was set at $P < 0.05$. All data are presented as mean ± SEM. All calculations were performed using GraphPad Prism 4 (Version 4.03).

Results

IDV Affects the Differentiation-Dependent Triacylglyceride Accumulation and Adipogenic Marker Expression in Differentiating 3T3-F442A. Extending earlier observations (8, 18), the 3T3-F442A cell line was induced to differentiate in the presence or absence of therapeutic C_{max} concentrations of IDV (10 μM) (17) added on day −5 and maintained until day +9 (*i.e.* 9 days after the induction of differentiation). Adipocytes exposed to IDV presented significant decreases in the amount of triacylglycerol droplets compared to vehicle-treated cells at day +9; confirmed by objective quantification of Oil Red O staining (Fig. 2A). The 40% decrease in Oil Red O staining was accompanied by an only 10% decrease in the number of viable cells for the entire 9-day period of differentiation (Fig. 2B). To further analyse the effect of IDV on the adipogenesis, 3T3-F442A cells were treated as above and mRNA was isolated at days +4 and +6 of the differentiation protocol, reverse transcribed, and Real-Time PCR analysis was

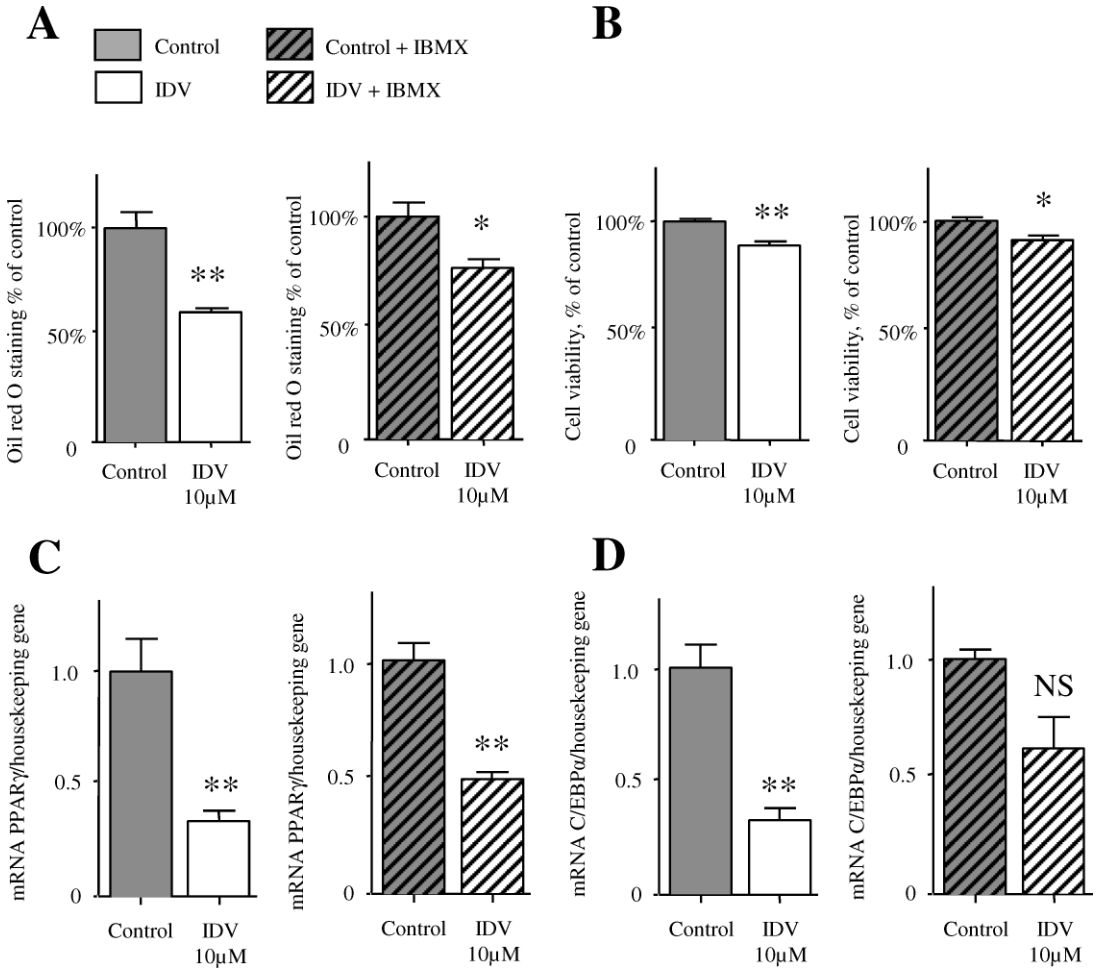
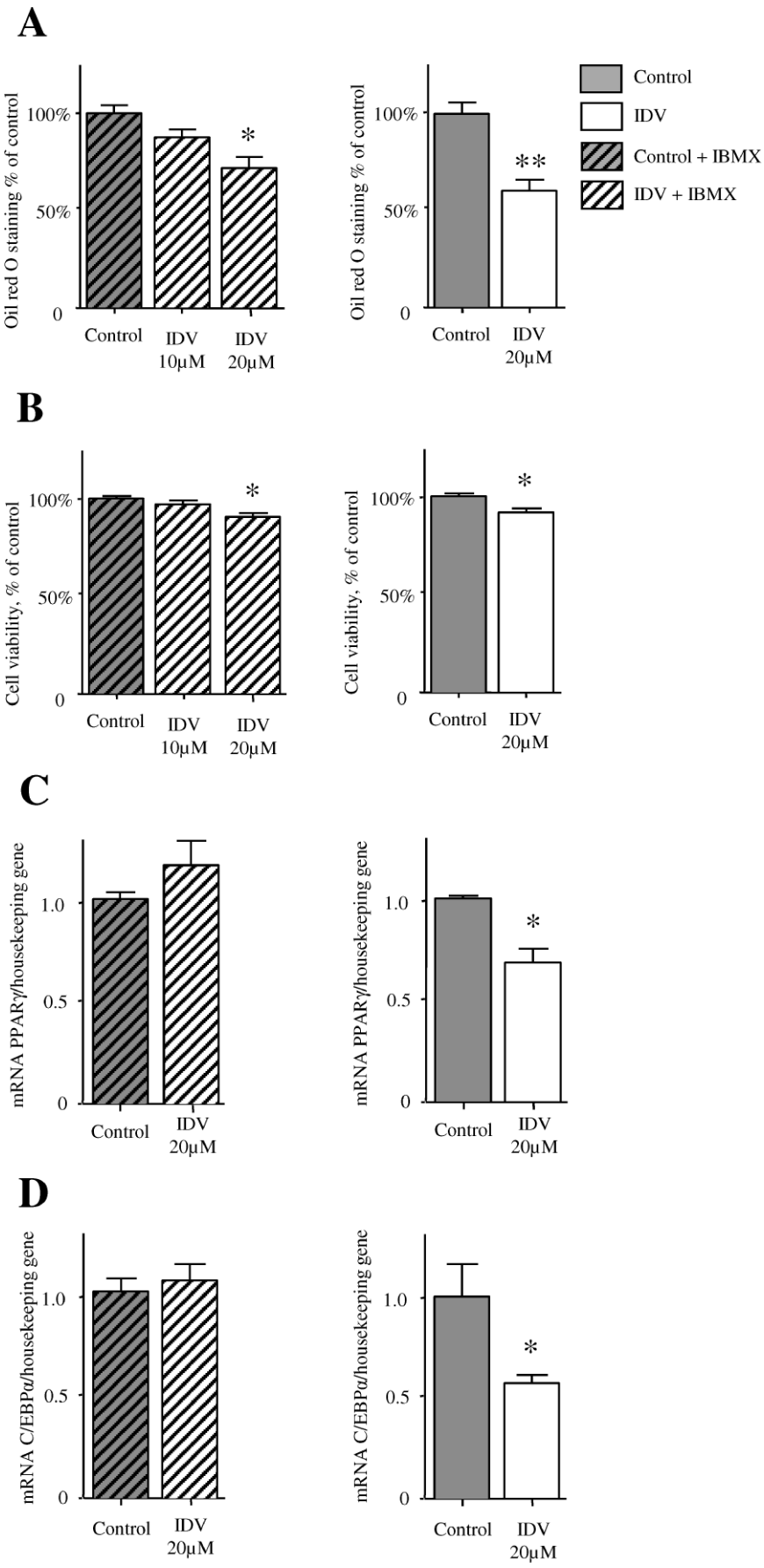


Figure 2. Effect of IDV on 3T3-F442A differentiation in the presence and absence of IBMX stimulation. (A) 3T3-F442A adipocytes were stained with Oil Red O and staining was quantified at 495 nm on day +9 after initiation of differentiation in the presence or absence of IDV with or without IBMX stimulation. (B) Cell death was measured as a percentage by trypan blue dye exclusion every third day during the 9-day differentiation protocol. Cumulative cell death for the entire culture period was used in order to calculate cell viability at the end of differentiation. Effects of IDV (10 μM) on PPARγ (C) and C/EBPα (D) expression in differentiating 3T3-F442A cells. Preadipocytes were cultured in the presence or absence of IDV from day −5, induced to differentiate on day 0 with or without IBMX stimulation and differentiated until day +9. Total RNA was isolated at day +4 (for PPARγ) and +6 (for C/EBPα). Graph A depicts data from three independent experiments. Graph B depicts data from six independent experiments. Graphs C and D show data from three independent experiments with triplicate samples per experiment. Values are the mean ± SEM. Student's *t* test was performed for comparison. * $P < 0.05$, ** $P < 0.01$, versus control.

performed to assess expression levels of two transcription factors involved in preadipocyte differentiation. Analysis of the expression levels of PPARγ and C/EBPα at their peak expression levels during adipogenesis demonstrated reductions in the IDV-treated cultures when compared to those of vehicle-treated cells (Fig. 2C and D).

IDV Induced Inhibition of Differentiation-Dependent Triacylglyceride Accumulation and Adipogenic Marker Expression in Differentiating 3T3-F442A Is Improved by the Addition of IBMX. The 3T3-F442A cell line was induced to differentiate in the presence or absence of therapeutic C_{max} concentrations of IDV added on day −5 and maintained to day +9 of differentiation. In these cultures the adipogenic stimulation was supplemented with IBMX. In the presence of IBMX, adipocytes exposed to IDV demonstrated only a 20% decrease in Oil Red O staining. After normalisation for the amount of dead cells, around 10% irrespectively of the presence or absence of IBMX (Fig. 2B), the results indicated a more than 100% increase in the reduction of Oil Red O staining in the absence of IBMX stimulation. Analysis of the expression levels of PPARγ and C/EBPα at day +4 and +6, respectively, confirmed the beneficial effect of IBMX as its presence slightly improved PPARγ expression and led to a non-significant reduction in C/EBPα expression in the IDV-treated cultures as compared to vehicle-treated cells (Fig. 2C and D).

Complete Adipogenic Stimulation Prevents IDV Effects on the Differentiation-Dependent Triacylglyceride Accumulation and Adipogenic Marker Expression in the Differentiating 3T3-L1 Cells. Next, 3T3-L1 cells were induced to differentiate in the



presence or absence of IDV (10 μM and 20 μM) from day −5 until day +9. Adipocytes exposed to IDV at C_{max} concentrations did not differ from vehicle-treated cells. Adipocytes cultures exposed to IDV at concentrations higher than C_{max} retained less Oil Red O than the vehicle-treated cells (Fig. 3A). At these concentrations there was a similar decrease in the number of viable cells (Fig. 3B), suggesting that the decreased Oil Red O staining could have resulted from an effect of decreased cell numbers leading to decreased levels of total cytoplasmic triacylglyceride per culture flask. Analysis of the expression levels of the adipogenic transcription factors PPARγ and C/EBPα at day +4 and +6, respectively, in the cultures presenting decreased amounts of Oil Red O staining (IDV 20 μM), demonstrated no significant impact on expression levels in the IDV treated cultures, when compared to those of vehicle-treated cells (Fig. 3C, *left panel* and D, *left panel*). Taken together, the unaffected expression of PPARγ and C/EBPα is consistent with the notion that decreased Oil Red O staining at higher IDV concentrations reflects mainly an increase in cell death (Fig. 3B).

IDV Affects the Differentiation-Dependent Triacylglyceride Accumulation and Adipogenic Marker Expression in Differentiating 3T3-L1 Cells in the Absence of IBMX Stimulation. To further examine the potential compensatory role of cAMP-dependent protein kinase (PKA) pathway, the 3T3-L1 cells were induced to differentiate in the presence or absence of IDV (20 μM) from day −5 until day +9 and IBMX was excluded from the differentiation stimuli. In the absence of IBMX, 20 μM IDV incubation led to a 40% decrease in Oil Red O staining (Fig. 3A) as compared to vehicle-treated cells stimulated similarly, without changes in the rate of cell death (Fig. 3B). The omission of IBMX from the differentiation cocktail resulted in slower and partially incomplete differentiation (data not shown) (19); nevertheless, the additional inhibitory effect of IDV on adipogenesis was clearly detectable. Statistically significant reductions in the expression of PPARγ and C/EBPα in IDV-treated cultures as compared to vehicle-treated cells (Fig. 3C and D, *right panels*) confirmed the inhibitory effect.

In the Presence of IBMX, IDV Does Not Affect the Differentiation-Dependent Triacylglyceride Accumulation and Adipogenic Marker Expression in Differentiating phsPA. Next, phsPA were induced to differentiate in the presence or absence of IDV (10 μM and 20 μM) from day −5 until day +30 (from start of differentiation). A trend towards reduced Oil Red O staining was observed, but this did not reach statistical significance even at an IDV concentration as high as 20 μM (Fig. 4A). At this concentration, however, we detected a cumulative 18% decrease in cell viability (Fig. 4B) in comparison to vehicle-treated cells for the entire 30-day period of differentiation. Therefore, we conclude that the reduction in Oil Red O staining is mainly the result of decreased cell numbers leading to a decrease in total cytoplasmic triacylglyceride level per culture flask. Cells incubated with a low dose IDV (10 μM) did not appear to differ from vehicle-treated cells (data not shown). Analysis of the expression levels of the adipogenic transcription factors PPARγ and C/EBPα was performed on day +18, a time point corresponding to the complete maturation (20–22), when a plateau in the acquisition of mature phenotype is reached. This time point has been used in similar *in vitro* studies (21) and represents a suitable time point for the analysis of adipogenic transcription factors. Under our experimental conditions, PPARγ and C/EBPα expression were similar in IDV (20 μM) and vehicle-treated cultures (Fig. 4C and D), confirming the lack of an anti-adipogenic effect of this drug even at a concentration above C_{max} (Fig. 4A).

Discussion

Obesity and the HIV-therapy-associated lipodystrophy syndrome constitute a serious health problem by enhancing the risk for cardiovascular disease and metabolic disorders (23–25). The high dynamics of adipose tissue turnover in adults (26) highlight the importance of better understanding of the mechanisms involved and the potential strategies to intervene in the process of adipogenesis. Accordingly, *in vitro* studies remain indispensable tools in the identification of pathological mechanisms and the establishment of new therapeutic targets for pharmacological intervention. Our analyses focused on the two most commonly used adipocyte cell line models, 3T3-L1 and 3T3-F442A, from which the major part of our current understanding of adipogenesis is derived (1–3).

The HIV protease inhibitor IDV has been reported to disturb the adipogenic conversion of 3T3-F442A cell line. This *in vitro* effect was used to explain the subcutaneous fat loss in HIV patients receiving IDV (8). However, subsequent data dissociated the link between protease inhibitor therapy and the development of lipoatrophy (9). The use of IDV containing regimens was even associated

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Figure 3. IDV effect on 3T3-L1 differentiation in the presence and absence of IBMX. (A) 3T3-L1 cells were stained with Oil Red O and staining was quantified by 495 nm absorbance on day +9 after initiation of differentiation, in the presence or absence of IDV as indicated, with or without IBMX stimulation. (B) Cumulative cell death for the whole period was used to calculate cell viability at the end of differentiation. Effect of IDV (20 μM) on the expression of the differentiation factors PPARγ (C) and C/EBPα (D) in differentiating 3T3-L1 cells. Preadipocytes were cultured in the presence or absence of IDV from day −5, followed by the induction of differentiation on day 0, with or without IBMX stimulation until day +9. Total RNA was isolated at day +4 (for PPARγ) and +6 for (C/EBPα). Graph A depicts data from three independent experiments. Graph B shows data from six independent experiments. Graphs C and D depict data from three independent experiments with triplicate samples per experiment. Values are the mean ± SEM. Student's *t* test and analysis of variance (ANOVA) with Bonferroni post-hoc analysis were performed for comparison. * $P < 0.05$, ** $P < 0.01$, versus control.

with improvement in lipoatrophy (10), suggesting significant discrepancy between *in vitro* and *in vivo* studies. Our data provide evidence for specific characteristics of the 3T3-F442A model, which we regard as relevant for the understanding of the divergent findings among the studies mentioned above.

The most commonly used external inducers of the signalling pathways that lead to adipogenesis are: IGF-1 (or high insulin levels), cAMP (or agents increasing cAMP), glucocorticoids and fatty acids (Fig. 1) (4). Important for initiation of differentiation are not insulin receptors, which are scarce in preadipocytes, but IGF-1 receptors (4). Insulin stimulation *in vitro* requires very high insulin concentrations in order to mimic the effect of IGF-1, which is also a component of the FCS (5). *In vivo* one may expect also growth hormone (GH) to affect this pathway given its ability to activate the expression and secretion of IGF-1 by preadipocytes (4).

An increase in the cAMP levels, which modulate the activity of nuclear transcription factors through protein kinase A (PKA) (27), activates the transcription factor CREB, which then induces adipogenesis (5). Increased cAMP levels may be achieved both by glucocorticoids and by IBMX, which is a cAMP phosphodiesterase (PDE) inhibitor. IBMX and cAMP decrease the levels of the transcription factor Sp1, which represses C/EBPβ promoter (5). Therefore it is possible that IBMX modulates the expression pattern of genes involved in the control of differentiation pathway such as C/EBPβ (28).

Glucocorticoids (DEX) are established inducers of preadipocyte differentiation (29) and act through the glucocorticoid receptor (GR), a nuclear hormone receptor from the superfamily to which also PPARs belong (Fig. 1). Part of the adipogenic activity of DEX may be mediated through its ability to induce C/EBPδ and to reduce the expression of preadipocyte factor-1 (pref-1) (5). In addition, it increases the intracellular levels of cAMP and Ca^{2+} (Fig. 1) (4). Fatty acids, supplemented by the addition of FCS, are supposed to stimulate clonal expansion and differentiation (4). Some fatty acids activate the PPARs among which PPARγ is a transcription factor regulating 422 (aP2), which confers adipose-specific gene expression (4). The exact role and mechanisms by which fatty acids affect differentiation require further investigation.

Our first set of experimental data is consistent with the report by Caron *et al*. (8, 18). Extensive studies by these authors support IDV's ability to inhibit insulin-induced mitogen-activated protein kinase (MAPK) activation at the level of ERK1/2 *phosphorylation* (8) (Fig. 1). This effect is in line with the fact that the expression levels of the adipogenic transcription factors PPARγ and C/EBPα have been shown to be highly increased by the activation of MEK/ERK (ERK1/2 *phosphorylation*) signalling pathway throughout the first 1–2 h of adipogenesis (30). However, such activation can be ascribed not only to the presence of insulin, but also to the presence of IBMX (4) and to a lesser

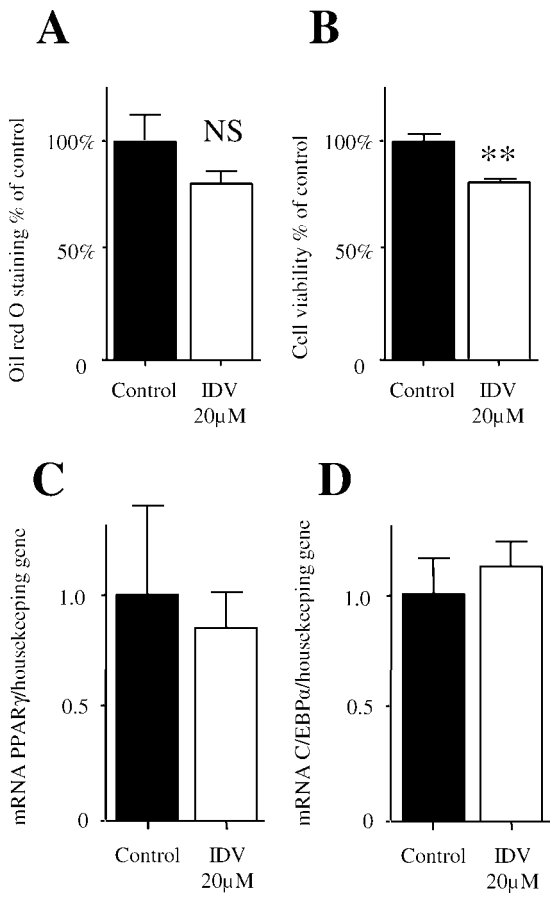

Figure 4. IDV has no effect on phsPA differentiation under complete adipogenic induction. (A) PhsPA were stained with Oil Red O and staining was quantified at 495 nm on day +30 after initiation of differentiation in the presence or absence of IDV. (B) Cell death was measured by trypan blue dye exclusion every fifth day during the 30-day differentiation protocol and cumulative cell death was calculated. Effect of IDV (20 μM) on the expression of the differentiation factors PPARγ (C) and C/EBPα (D) in differentiating phsPA. Preadipocytes were cultured in the presence of vehicle *(black column)* or with the addition of IDV from day −5, followed by the induction of differentiation on day 0 through day +30. Total RNA was isolated at day +18 (for PPARγ and C/EBPα). Graph A depicts data from three independent experiments. Graph B shows data from six independent experiments. Graphs C and D show data from three independent experiments with triplicate samples per experiment. Values are the mean ± SEM. Student's *t* test was performed for comparison. * $P < 0.05$, ** $P < 0.01$, versus control; NS, not significant.

extent to DEX in the differentiation cocktail (30). In accordance with our hypothesis that IDV-mediated disturbance in insulin signalling and MAPK activation may only contribute to altered adipogenesis in the absence of parallel stimulation, we demonstrated a partial rescue effect by IBMX during the first 2 days of 3T3-F442A differentiation protocol. We speculated that activation of the complete set of adipogenic pathways as in 3T3-L1 differentiation protocol would confer proper adipogenic conversion in the presence of IDV. Indeed, our results revealed that IDV is unable to inhibit the differentiation of 3T3-L1 cell line under complete stimulation, even at doses above the therapeutic C_{max}. To further demonstrate the compensatory

role of the cAMP-dependent protein kinase (PKA) pathway, we examined the incomplete induction of 3T3-L1 differentiation by omitting IBMX stimulation from the differentiation cocktail during the first 2 days of conversion. Unfortunately, we were unable to test the effect of DEX omission, as under such conditions only minor differentiation was observed (19), which prevented the detection of further impairment.

Insulin stimulated ERK1/2 *phosphorylation* during the first few hours of adipogenesis leads to increased expression of PPARγ and C/EBPα and ultimately results in acquisition of a mature fat cell phenotype (30). On the other hand IDV inhibits MAPK activation at the level of ERK1/2 *phosphorylation* (8). As IBMX and DEX exert insulin independent effects on the *phosphorylation* of ERKs (30), it is tempting to speculate that such a mechanism might be responsible for the observed compensatory effect. In line with such a mechanism, IBMX and DEX presence in the differentiation cocktail almost completely abrogate the effect of MEK inhibitor PD98059 on the differentiation process and lead to only slight delay, but no significant inhibition of adipogenesis (31). Furthermore, in the absence of IBMX, the same inhibitor induces strong inhibition of adipocyte formation from embryonic stem (ES) cells (32).

The established protocols for the *in vitro* differentiation of phsPA recommend stimulation of all four adipogenic pathways. Under these conditions, IDV was unable to inhibit the differentiation of phsPA even when the dose was increased to higher than the therapeutic C_{max} level. Similar results were reported by others employing phsPA derived from a large number of donors with normal BMI (20, 21). The requirement for all four differentiation pathways for the *in vitro* conversion of phsPA did not allow us to study the effect of IBMX omission. It is important to mention that *in vitro* differentiation of phsPA requires additionally either indomethacin or rosiglitazone, factors which may also play a compensatory role. Our data about compensatory effects are consistent with data from a recent clinical study demonstrating that four weeks of IDV treatment had no effect on the expression of adipogenic transcription factors in the subcutaneous adipose tissue of healthy HIV-negative volunteers (11).

Taken together, our experimental data suggest that partial interference with a specific pathway may not be sufficient to modulate the adipogenic conversion in conditions where adipogenesis per se involves multiple regulatory pathways. In addition to helping the better understanding of the pathogenesis of the HIV-associated lipodystrophy syndrome, our findings should also be of interest to scientists attempting to establish new therapeutic targets for pharmacological interventions in adipogenesis.

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