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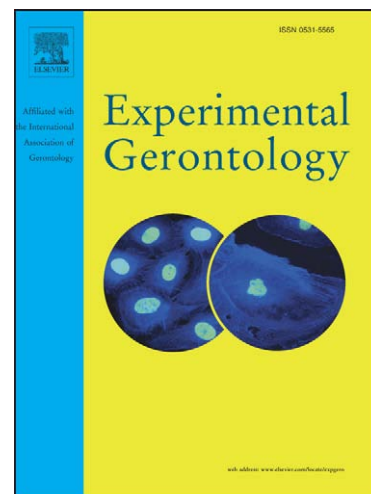
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Interplay of IGF-I and 17 β -estradiol at age-specific levels in human sebocytes and fibroblasts in vitro

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Running title: IGF-I & 17 β -estradiol signaling pathway

Abbreviations: DHEA: dehydroepiandrosterone, E2: 17 β -estradiol, ER α : estrogen receptor α , GH: growth hormone, IGF-I: insulin-like growth factor-I, IGF-IR: insulin-like growth factor receptor-I, f20: 20-year-old female levels, f60: 60-year-old female levels, MUH: 4-methylumbelliferyl heptanoate

ABSTRACT

In order to obtain greater insights into the molecular mechanisms accompanying hormonal aging the effects of growth hormone (GH), insulin-like growth factor-I (IGF-I), 17 β -estradiol, progesterone and dehydroepiandrosterone were tested as single agents in concentrations corresponding to 20- and 60-year-old females on human SZ95 sebocytes and fibroblasts. Cell proliferation and viability were measured by 4-methylumbelliferyl heptanoate and lactate dehydrogenase microassays, respectively, whereas lipid accumulation was documented via Nile red microassay and fluorescence microscopy. mRNA and protein expression was evaluated via real-time RT-PCR and western blotting or ELISA, accordingly. Our results depict the importance of IGF-I for the lipid synthesis in SZ95 sebocytes and demonstrate the lack of 17 β -estradiol, dehydroepiandrosterone and progesterone activity on lipid synthesis and SZ95 sebocytes proliferation suggesting that the action of these hormones in vivo may be implemented through indirect pathways. Fibroblasts showed to be more susceptible to 17 β -estradiol treatment, while IGF-I could significantly stimulate fibroblasts proliferation in a dose-dependent manner. Furthermore, an interplay between the 17 β -estradiol and IGF-I signaling pathway was documented in both cell types. In conclusion, IGF-I is a key regulator of human skin aging and declining IGF-I levels with age may play a significant role in the reduction of skin surface lipids and thickness.

INTRODUCTION

Aging is characterized by a gradual decline in several body functions, among them a functional decline of endocrine gland function and a reduction in the levels of circulating hormones (Roshan et al., 1999). Skin being a target for a plethora of hormones and also a peripheral endocrine organ in itself (Zouboulis, 2000) can be affected to a large extent by hormonal changes (Makrantonaki and Zouboulis, 2007). Recent data suggest that incubation of human SZ95 sebocytes with a hormone mixture consisting of growth factors and sex steroids at age-specific levels may alter the biological activity of the cells by regulating their transcriptome (Makrantonaki et al., 2006) and thus illustrate the importance of the hormone environment for the aging process. (Zouboulis and Makrantonaki, 2006).

The growth hormone (GH)/ insulin-like growth factor-I (IGF-I) axis is considered to be one of the most important signaling pathways involved in aging. Serum levels of IGF-I have been reported to increase from birth to puberty, followed by a slow decline through adulthood. This reduction has been correlated with the progressive decline of GH with advancing age (Bennett et al., 1984). Patients with isolated GH deficiency (IGHD), multiple pituitary hormone deficiency (MPHD) including GH, as well as primary IGF-I deficiency (GH resistance, Laron syndrome) present signs of early skin aging such as dry, thin and wrinkled skin. Other resulting characteristics of GH/IGF-I deficiency are obesity, hyperglucemia, reduced body lean mass, osteopenia, lowered venous access, hypercholesterolemia, cardiovascular diseases and, subsequently, premature mortality (Carroll et al., 1998; Laron, 2005; Tomlinson et al., 2001; Zouboulis et al., 2007). Treatment of normal elderly males with GH resulted in amelioration and reverse of the aging signs and symptoms (Rudman et al., 1991; Rudman et al., 1990; Veldhuis et al., 2004). However, recent reports of an association of GH substitution and increased risk of prostate, lung, colon, breast cancer as well as a possible decrease of

insulin insensitivity all make further investigations necessary regarding safety and efficacy of GH substitution in the aging population (Riedl et al., 2001).

On the other hand, menopause, which is characterized by a rapid decline of sex-steroids, has been associated with a worsening of skin structure and functions, which can be at least partially repaired by hormone replacement therapy or local estrogen treatment (Brincat, 2000). Improvement of epidermal skin moisture, elasticity and skin thickness (Fuchs et al., 2003), enhanced production of surface lipids (Sator et al., 2001), reduction of wrinkle depth, restoration of collagen fibers (Schmidt et al., 1996) and increase of the collagen III/I ratio (Affinito et al., 1999) have all been reported under hormone replacement therapy.

Recently, clinical studies have documented that sex-steroid hormones can regulate GH and IGF-I secretion. Supplementation of middle-aged and older men with high-dose testosterone attenuates IGF-I feedback-dependent inhibition of GH secretion (Veldhuis et al., 2004), whereas acute administration of estradiol accentuated IGF-I-induced inhibition of GH secretion (Veldhuis et al., 2004). The reduction of circulating sex steroids, which occurs with advancing age, can therefore lead to a defective regulation of both growth factors.

In order to decipher which hormones are the most effective in human skin cells, the effects of GH, IGF-I, 17 β -estradiol, progesterone and dehydroepiandrosterone (DHEA) were tested as single agents in concentrations corresponding to 20 (f20) and 60 (f60) year old females on human SZ95 sebocytes and fibroblasts. Cell proliferation and lipid synthesis were measured after treatment with GH, IGF-I, 17 β -estradiol, DHEA, and progesterone. In addition, an interaction between IGF-I and 17 β -estradiol signaling pathways was documented.

MATERIALS AND METHODS

Active Compounds, Reagents and Antibodies

Unless otherwise stated, hormones, chemicals (analytical grade) and assay reagents were all from Sigma (Deisenhofen, Germany) and culture media and supplements were all from Biochrom (Berlin, Germany). Experiments were performed in 75 cm² flasks and in 24- or 96-well tissue culture plates (Nunc, Wiesbaden, Germany). All antibodies were purchased from Santa Cruz Biotechnologies (Heidelberg, Germany).

Cell Cultures

Immortalized human SZ95 sebocytes (Zouboulis et al., 1999) and breast area skin fibroblasts from a 60-year-old woman undergoing surgery were maintained in Sebomed[®] medium supplemented with 10% steroid-free fetal calf serum (cc pro GmbH, Neustadt, Germany), 5 ng/ml human recombinant epidermal growth factor, 1 µM retinol, 0.1 µM linoleic acid, 1 mg/ml human protease-free albumin, 1 mM Ca²⁺ and 50 µg/ml gentamicin (Invitrogen, Karlsruhe, Germany) in culture flasks in a humidified atmosphere of 5% CO₂ at 37°C. The cells were incubated with hormones at levels corresponding to the average serum levels for females from 20 (f20) to 60 (f60) years of age, namely IGF-I, GH, 17β-estradiol, progesterone and DHEA (Table 1). The skin fibroblasts used for the experiments were between passages 1-4 and the human SZ95 sebocytes between passages 26-28, accordingly. The end concentration of the DMSO diluent did not exceed 0.1% in all treatments.

Cell proliferation

Cell proliferation was assessed by the 4-methylumbelliferyl heptanoate fluorescence assay and measured automatically, after 48 and 96 h, as described previously (Zouboulis et al.,

1991). Experiments were performed in triplicate, with 10 wells evaluated for each data point in each experiment.

Detection of cell viability

SZ95 sebocytes were incubated at a density of 20,000 cells per 96-plate well for 48 h. The cells were then washed with phosphate buffered saline (PBS) and were treated with hormones at the concentrations mentioned above. After 24 h the supernatants were collected and centrifuged to remove cell debris. Finally, 100 µl of the supernatant was used to measure lactate dehydrogenase release employing the Boehringer Lactate Dehydrogenase Assay kit (Mannheim, Germany) and adhering to the manufacturer's instructions. Experiments were performed in triplicate, with 10 wells evaluated for each data point in each experiment. No compounds used at the above-mentioned concentrations were found to show any cytotoxicity.

Lipid detection

After 48 h treatment of SZ95 sebocytes with the hormones at the concentrations mentioned above, the Nile red microassay for neutral and polar lipids was performed as previously described (Zouboulis et al., 2002). The results are presented as percentages of the control, using 485 nm excitation and 565 nm emission filters for neutral lipids and 540 nm excitation and 620 nm emission filters for polar lipids. Experiments were performed in triplicate, with 10 wells evaluated for each data point in each experiment.

Fluorescence microscopy

SZ95 sebocytes were seeded on 18 mm coverslips in culture dishes at approximately 40% confluence. After 24 h incubation with or without treatment the medium was harvested, the

cells were washed twice with PBS, and 100 μ l of a 100 μ g/ml Nile red solution in PBS was added. The plates were then incubated at 37°C for 10 min, and the released fluorescence was documented by a Zeiss, Axiophot SIP 31182 fluorescence microscope, using 485 nm excitation and 565 nm emission filters for neutral lipids and 540 nm excitation and 620 nm emission filters for polar lipids.

RNA isolation/ cDNA synthesis

The cells were washed with PBS and were then collected in extraction buffer. RNA was isolated using the RNeasy mini kit (Qiagen) with additional DNA digestion using the RNase-free DNase kit (Qiagen). RNA quantity and quality was assessed using RNA 6000 Nano Chip analysed with the Agilent 2100 bioanalyzer (Agilent, Palo Alto, USA). cDNA synthesis was performed as already described (Makrantonaki et al., 2006).

Real-time PCR

Analysis of gene expression was employed using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturers' instructions. The primers used were for IGF-I: F: ACACCATGTCCTCCTCGCATCT; R: CACACGAA-CTGAAGAGCATCCA and for PBGD (house-keeping gene): F: AATCATTGCTATGTCCACCACAGG; R: CAAGGAGTGAA-CAACCAGGTCC. The measurements were done using an ABI PRISM 7000 instrument (Applied Biosystems), following standard procedures with 40 cycles, and using 60°C as annealing temperature. The assessed data were evaluated using the "Sequence detection software" (v1.2.3. Applied Biosystems) using untreated cells as reference for gene expression.

Western blotting

Cells were washed in PBS, incubated with cold lysis buffer for 5 min and collected on ice by scraping. Lysates were centrifuged at maximum speed for 15 min at 4°C, and 40 µg of total protein from each sample was incubated for 10 min at 70°C. The protein concentration in cell lysates was determined by the BCA-200 Protein Assay (Pierce, Bonn, Germany). Proteins were separated using NuPage Tris acetate gels 3-8% (Novex; Invitrogen, Carlsbad, CA) and then transferred onto a PVDF membrane (Schleicher & Schuell). Membranes were blocked by 3% non-fat dry milk in 1% PBS/ Tween-20 over night at 4°C. After 3 washes in PBS/ Tween-20 the membranes were incubated for 1 h at room temperature with the rabbit antibody against IGF-IR (1:100) or the mouse antibodies against ER α (1:100) and β -actin (1:500) – used as control – washed again and subsequently incubated for 30 min at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies, respectively, diluted 1:5000 in PBS/ Tween-20. Protein bands were detected by an enhanced chemiluminescence system (ECL; Amersham Pharmacia). The protein band intensities were quantified densitometrically using the Fuji TINA 2.04 image analysing software.

ELISA

Cell cultures were treated for 48 and 72 h, and supernatants were collected and centrifuged in order to remove debris. 17 β -estradiol and IGF-I concentrations were measured using the Delfia Kit (PerkinElmer, Rodgau-Jügesheim, Germany) and the human IGF-I Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany), respectively, following the manufacturers' instructions.

Statistical analysis

Values represent the mean values \pm standard deviation (SD) of at least three experiments.

Statistical significance was calculated by the two-sample independent-groups *t-test*. Mean differences were considered to be significant when $p < 0.05$. Data and graphs were produced with MS Excel 2000 (Microsoft Corporation, USA).

RESULTS

IGF-I and GH amplified lipid synthesis in SZ95 sebocytes.

After treatment of SZ95 sebocytes with IGF-I for 48 h in concentrations similar to those circulating in 20- (f20) and 60-year-old females (f60), a significant increase of neutral ($p<0.001$) and polar lipids ($p<0.001$) was observed at both concentrations tested (Fig. 1A). There was a stronger enhancement of neutral lipids than that of polar lipids. Moreover, the lipids of SZ95 sebocytes which were maintained under IGF-I in concentrations similar to those of f60 showed a slight decline in comparison to SZ95 sebocytes cultured with f20 concentrations (ns). The increase of neutral lipids was also documented by means of fluorescence microscopy (Fig. 2B). On the other hand, GH at f20 and f60 concentrations were shown to significantly enhance neutral ($p<0.01$ and $p<0.05$, respectively) and polar lipids ($p<0.05$ and $p<0.01$, respectively) in SZ95 sebocytes (Fig. 1B) but not to such an extent as IGF-I did.

17 β -estradiol exhibited an effect only on the polar lipid content of SZ95 sebocytes.

SZ95 sebocytes incubated with 17 β -estradiol at f20 and f60 concentrations showed no significant difference in neutral lipid content when compared to that of the controls (Fig.1C). On the other hand, polar lipid content under 17 β - estradiol was significantly increased in contrast to that of untreated cells ($p<0.01$) (Fig.1C). SZ95 sebocytes treated with progesterone and DHEA at f20 and f60 concentrations revealed no significant change in their lipid content when compared to untreated controls (data not shown). In addition, IGF-I, GH, 17 β -estradiol, progesterone and DHEA as single agents at f20 and f60 concentrations showed no significant effect on SZ95 sebocyte proliferation (data not shown).

IGF-I mRNA expression was reduced under IGF-I and 17 β -estradiol treatment in SZ95 sebocytes in a time-dependent manner.

Gene expression analysis via real-time RT-PCR in SZ95 sebocytes showed a time-dependent regulation of IGF-I mRNA expression, comparing cells under IGF-I or 17 β -estradiol treatment at f20 concentrations to untreated cells (Fig.3). After treatment with IGF-I at f20 serum levels, IGF-I mRNA expression showed to be decreased after 6 h (-86%) of treatment, and was slightly higher after 12 h (-56%), 24 h (-33%), 48 h (-61%) and 72 h (-12%) of treatment. During treatment with 17 β -estradiol at f20 serum levels, a decrease of IGF-I mRNA expression could be shown after 12 h (-81%) and 24 h (-70%) of treatment, respectively, which were slightly higher after 48 h (-14%) and 72 h (-14%), accordingly.

17 β -estradiol synthesis was slightly enhanced in SZ95 sebocytes under treatment with IGF-I, while IGF-I synthesis remained unchanged after treatment with 17 β -estradiol.

SZ95 sebocytes produced both 17 β -estradiol and IGF-I. The 17 β -estradiol synthesis in SZ95 sebocytes treated with IGF-I at f20-similar concentrations was slightly enhanced at levels of +37.5% (p=0.06) and +14.5% after 48 and 72 h of treatment, respectively (ns; Fig. 4). On the other hand, the IGF-I synthesis in SZ95 sebocytes treated with 17 β -estradiol at f20 concentrations showed no change after 48 and 72 h of treatment, respectively (data not shown).

IGF-I and 17 β -estradiol amplified lipid synthesis in fibroblasts, whereas only IGF-I could enhance fibroblast proliferation.

After treatment of the breast fibroblasts with IGF-I for 48 h at f20 and f60 concentrations, a significant increase of neutral and polar lipids was observed at both concentrations tested ($p<0.001$) (Fig. 5A). The lipids produced by fibroblasts cultured with IGF-I in concentrations similar to f60 showed a slight but not significant decline in comparison to the lipids produced by cells cultured with IGF-I in f20 concentrations. 17β -estradiol at f20 and f60 also amplified neutral and polar lipids ($p<0.001$) in contrast to untreated fibroblasts (Fig. 5B). Proliferation of fibroblasts only exhibited a significant dose-dependent increase after treatment with IGF-I at f20 and f60 concentrations for 96 h ($p<0.001$) (Fig. 5C). 17β -estradiol did not affect the cell proliferation (data not shown).

IGF-I and 17β -estradiol synthesis was enhanced after treatment with 17β -estradiol and IGF-I, respectively, in fibroblasts.

Fibroblasts produced both 17β -estradiol and IGF-I. IGF-I synthesis in fibroblasts treated with 17β -estradiol at f20 was enhanced (+41%, $p<0.01$) at 72 h (Fig. 6A). 17β -estradiol synthesis slightly increased (+19.6% and +14.3% after 48 and 72 h, respectively; ns) (Fig. 6B).

IGF-IR expression was influenced under IGF-I and 17β -estradiol treatment in SZ95 sebocytes.

IGF-IR was expressed in SZ95 sebocytes at protein level. The regulation of IGF-IR in SZ95 sebocytes was investigated after exposure of the cells to IGF-I and 17β -estradiol at median f20 serum concentrations by means of Western blotting (Fig. 7A, B). In comparison to untreated controls, IGF-IR protein expression was first increased in SZ95 sebocytes under IGF-I treatment after 3 h (+13%) and 6 h (+21.4%) (both $p<0.05$), but was then reduced after 12 h (-30.7%, $p<0.05$) and 24 h (-27.6%), and was followed by a further increase after 48 h

(+19.9%; $p<0.01$) and 72 h (+9.0%; $p<0.05$) (Figs. 7A). On the contrary, after treatment with 17β -estradiol, a slight decrease of IGF-IR expression was shown after 3 h (9.6%), an increase after 6 h (+24%, $p<0.05$), followed by a decrease after 12 h (-35.2%; $p<0.01$), 24 h (-3.7%), and 48 h (-20.0%; $p<0.01$) and finally by an increase again after 72 h (+42.9%, $p<0.05$) (Figs.7B).

ER α expression was modified under IGF-I and 17β -estradiol treatment in SZ95 sebocytes.

ER α was expressed in SZ95 sebocytes. ER α expression was initially increased in SZ95 sebocytes under IGF-I treatment at f20 after 3 h (+49%; $p<0.05$), 6 h (+4.4%; $p<0.001$), 12 h (+3.9%; $p<0.01$), and 24 h (+13.0%; $p<0.001$) in contrast to untreated SZ95 sebocytes, followed by normalization after 48 h (-0.5 %) (Fig. 7C). On the other hand, after treatment with 17β -estradiol at f20 concentrations, an increase of ER α expression was detected after 3 h and 6 h (+37.3 % and +11.8 %) followed by a slight decrease after 12 h (-2.5 %) and an increase after 24 h (+19.7%; $p<0.001$) and 48 h (+6.7 %). After 72 h of treatment, ER α was not detectable (Fig.7C, D).

IGF-IR expression was reduced under IGF-I treatment in human fibroblasts.

IGF-IR was expressed in human fibroblasts. IGF-IR expression was significantly reduced under IGF-I treatment at f20 concentrations after 48 h (-60.3%, $p<0.01$). This reduction remained detectable after 72 h (-60.6%, $p<0.01$) (Fig. 8A). After treatment with 17β -estradiol, no change was initially observed in comparison to the untreated control, however after 72 h IGF-IR expression declined significantly under 17β -estradiol treatment (-18%, $p<0.01$) (Fig. 8B).

DISCUSSION

GH activity is considered to be mainly attributed to IGFs but GH has also been shown to exhibit direct effects on human skin cells (Deplewski and Rosenfield, 2000). The increased serum GH levels in acromegaly are associated with enhanced sebum secretion (Burton et al., 1972), an observation that could be confirmed by GH treatment of human SZ95 sebocytes in vitro. In Mini rats, GH gene expression was suppressed by an antisense transgene, whereas thinner skin with less collagen, increase of subcutaneous adipose tissue and small-sized sebaceous glands were observed (Ikawa et al., 2002).

IGF-I has been shown to stimulate sebocyte differentiation in vitro especially in combination with GH (Deplewski and Rosenfield, 1999), while in human keratinocytes it acts as a mitogen (Tavakkol et al., 1999). In acne vulgaris, increased sebum production peaks in mid-adolescence at a time that GH and IGF-I reach their highest serum levels (Deplewski and Rosenfield, 1999). In our experiments, IGF-I amplified lipid synthesis in SZ95 sebocytes corresponding to results by Smith et al (Smith et al., 2006) and fibroblasts as well as fibroblast proliferation, all in a dose-dependent manner underlining the fact that the declining IGF-I levels with increasing age may play a significant role in the reduction of skin surface lipids and thickness.

17 β -estradiol has been shown to stimulate proliferation of human keratinocytes by promoting the expression of cyclin D2 and to suppress oxidative stress-induced keratinocyte apoptosis by promoting expression of the anti-apoptotic protein Bcl-2 after binding to the membrane GPR30 on keratinocytes (Kanda and Watanabe, 2003, , 2004). In addition, 17 β -estradiol stimulates collagen synthesis, increases ER mRNA levels and enhances IGF-IR expression in cultured fibroblasts obtained from postmenopausal women (Surazynski et al., 2003).

Our results correspond to previous *in vitro* findings which reveal that estrogens do not influence the biological activity of sebaceous glands (Guy et al., 1996). On the contrary, 17 β -estradiol as shown here was able to amplify both neutral and polar lipid syntheses in our fibroblasts, thus demonstrating that fibroblasts are more susceptible to estradiol *in vitro* than SZ95 sebocytes are. This is in accordance with *in vivo* studies which identified fibroblasts as the major target of estrogens where skin thickness and collagen content had been markedly affected by estrogen levels (Schmidt, 2005).

DHEA has been related to the process of skin aging by regulating production and degradation of the extracellular matrix (Lee et al., 2000; Shin et al., 2005). Improvement of the skin quality was observed after treatment with DHEA, particularly in women, in terms of increased hydration, epidermal thickness, sebum production, and improvement of pigmentation (Baulieu et al., 2000). Consequently, several researchers have suggested the use of DHEA as an anti-aging agent (Minghetti et al., 2001; Shin et al., 2005).

Nevertheless, substitution with DHEA in elderly persons is accompanied by a small increase of testosterone and estradiol, which may indeed yield an explanation of the clinical changes demonstrated (Baulieu et al., 2000). This could explain the lack of DHEA activity on lipid synthesis and cell proliferation of SZ95 sebocytes, suggesting that the action of DHEA may be implemented through indirect pathways.

Progesterone enhances the keratinization of epidermal cells (Tammi, 1982), increases keratinocyte proliferation (Urano et al., 1995), blocks the activity of 5 α -reductase in genital fibroblasts and inhibits the growth of fibroblasts *in vitro* (Comini Andrada et al., 1985). In addition, progesterone suppresses the expression of members in the matrix metalloproteinase super family, stimulates the production of tissue inhibitor of metalloproteinases in fibroblasts (Kanda and Watanabe, 2005) and reduces the collagenolytic activity (Huber and Gruber,

2001). Human and animal experiments which attempt to study possible effects of progesterone on the activity of the sebaceous gland remain controversial. A stimulation of sebum secretion was observed in female and castrated rats. The degree of stimulation was related to the time point of gonadectomy and this was higher when it was carried out before puberty. In contrast, in intact male rats, sebum secretion was inhibited by progesterone (Shuster et al., 1977). These results of rat studies may lead to the conclusion that the effect of progesterone is sex-dependent and correlates with the early endocrine environment. In SZ95 sebocytes, progesterone did not affect their biological activity in concentrations similar to young and elderly women.

As already mentioned above, 17β -estradiol affected lipid synthesis in human fibroblasts but not in SZ95 sebocytes. By contrast, IGF-I significantly increased the lipid content of both cell types. Estrogens are known to play a significant role in ameliorating skin quality and skin surface lipids after topical or systemical application in postmenopausal women (Schmidt et al., 1996). Since human sebocytes account for the maintenance of skin surface lipids, it was originally expected that 17β -estradiol would increase the lipid content of SZ95 sebocytes in the experiments presented here, but this was not the case. Therefore we suggested that IGF-I is the key hormone for sebaceous lipid synthesis, i.e. that estrogen activity may be mediated in aged skin cells through interaction with the IGF-I signaling pathway. In order to investigate this hypothesis, the effects of IGF-I and 17β -estradiol on the expression of IGF-IR and $ER\alpha$ as well as the synthesis of IGF-I and 17β -estradiol in SZ95 sebocytes and human fibroblasts were measured. Our findings confirm an interplay between IGF-I and 17β -estradiol signaling pathways in both cell types.

The interplay between both pathways has already been documented in other cell types. In vivo, IGF-I is probably required for estradiol-induced mitosis in the uterine epithelium

(Richards et al., 1996). Oral estradiol administration resulted in a significant fall of mean IGF-I levels and transdermal administration of 17β -estradiol resulted in a slight increase in serum IGF-I (Ho et al., 2003). In vitro, 17β -estradiol increased the number of IGF-I receptors of basal, undifferentiated epidermal keratinocytes and indirectly so the number of IGF-I/IGF-I receptor complexes, whereas IGF-I was found to be produced in fibroblasts (Rudman et al., 1997). In osteoblasts, estradiol could increase the synthesis of IGF-I, which appeared to act as an autocrine growth factor (Westley and May, 1994). In uterine tissue, estradiol was shown to increase the synthesis of IGF-I in the stroma, which could then modulate the proliferation of epithelial cells (Westley and May, 1994). In the mouse uterus, IGF-IR was activated after administration of estradiol to ovariectomized adult female mice (Richards et al., 1998). Insulin receptor substrate-I, the principal substrate for the tyrosine kinase of IGF-IR was regulated by estradiol in MCF-7 breast cancer cells (Westley et al., 1998). Finally, the induction of DNA synthesis in breast cells by estradiol could be blocked completely with the antibody alpha IR3, which binds to the IGF-IR (Thorsen et al., 1992).

In this work, 17β -estradiol showed IGF-I-like effects on human fibroblasts, such as the increase of lipid production, while it was not so effective in inducing IGF-I effects in human sebocytes (Fig. 9). This led us to the assumption that estradiol in skin may unfold its action through the enhanced IGF-I production in fibroblasts and through a resulting autocrine mechanism, as already reported for osteoblasts (Westley and May, 1994). These observations could also explain why estradiol is so effective in ameliorating skin surface lipids in vivo. Since sebocytes are in close neighborhood with dermal fibroblasts, induction of IGF-I in fibroblasts through estradiol could induce a paracrine stimulation of lipid synthesis in sebocytes. In conclusion, it is likely that not only an interaction between IGF-I and estradiol signaling pathways exists but also a paracrine interaction between sebocytes and fibroblasts.

Moreover, we detected that IGF-I and estradiol induce a negative feedback on IGF-IR and that both hormones can control IGF-IR expression. With increasing age, IGF-I and estradiol levels decrease, possibly resulting in uncontrolled expression and activation of IGF-IR. Present knowledge on the effects of hormone deficiency on aging and life span are controversial. In animal models as diverse as the nematode *Caenorhabditis elegans*, the fly *Drosophila melanogaster* and the mouse *Mus musculus*, it has been documented that suppression of insulin-like peptides, insulin-like growth factor (IGF) and sterols or their receptors can increase their lifespan significantly and delay age-dependent functional decline (Antebi, 2004; Rincon et al., 2004; Tatar et al., 2003). On the other hand, in humans hormone decline has been associated with the onset of aging. Our data raise the questions, how important the IGF-I signaling pathway is for the aging process in humans, and whether or not increased and uncontrolled IGF-IR expression itself during increasing age may play a role in aging.

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TABLES

Table 1

Median hormone concentrations in serum of healthy 20 (f20) - and 60 (f60) – year-old women, which were added to cell cultures as single agents.

FIGURE LEGENDS**Fig. 1**

Content of lipids in SZ95 sebocytes incubated with IGF-I (A), GH (B) and 17 β -estradiol (C) at age-specific levels by means of Nile red microassay.

SZ95 sebocytes were seeded at a density of 10,000 cells/ well in 96-well plates and were left 48 h to adhere. The following day cells were treated with the hormones as single agents for 48 h in levels similar to young (f20) and elderly (f60) females. Neutral lipids (light columns) and polar lipids (dark columns) were measured with the Nile red microassay. Lipid content of untreated control cells was set at 0% and the lipid content in the treated cells was calculated as percentage of the change from control. Values represent the mean of ten experiments \pm SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 2

Detection of neutral lipids in untreated SZ95 sebocytes (A), after treatment with IGF-I (B) and 17 β -estradiol (C) by means of fluorescence microscopy after Nile red staining.

SZ95 sebocytes were seeded on 18 mm coverslips in culture dishes and they were treated with IGF-I (B) and 17 β -estradiol (C) at levels corresponding to 20-year-old females (f20). After 24 h the cells were stained with Nile red and the released fluorescence was documented by fluorescence microscopy.

Fig. 3**Gene expression of IGF-I in SZ95 sebocytes incubated with IGF-I and 17 β -estradiol.**

Cells were seeded at a density of 50,000 cells/ well in a 24-well plate and were left 48 h to adhere. On day 0 cells were treated with IGF-I and 17 β -estradiol, respectively at f20 levels. After 6h, 12h, 24h, 48h and 72h of treatment, the cells were collected in RLT buffer and RNA was extracted. Gene expression was calculated as relative values compared to untreated controls. Untreated control cells were set at 0% and the IGF-I mRNA expression in the treated cells was calculated as percentage of the change from control. Values represent the mean of three experiments \pm SD.

Fig. 4**17 β -estradiol synthesis in SZ95 sebocytes incubated with IGF-I.**

SZ95 sebocytes were seeded at a density of 300,000 cells/ flask in a 75 cm² flask and were left 48 h to adhere. The following day cells were treated with IGF-I at f20 for 48 h and 72 h. The production of 17 β -estradiol was measured by means of ELISA. 17 β -estradiol content of untreated control cells was set at 0% and the 17 β -estradiol synthesis in the treated cells was calculated as percentage of the change from control. Values represent the mean of three experiments \pm SD.

Fig. 5**Lipid content in fibroblasts incubated with IGF-I (A) and 17 β -estradiol (B) and cell proliferation after treatment with IGF-I (C).**

Breast fibroblasts were seeded at a density of 10,000 cells/ well in a 96-well plate and were left 48 h to adhere. The following day cells were treated with the IGF-I or 17 β -estradiol for 48 h at f20 and f60. Neutral lipids (light columns) and polar lipids (dark columns) were measured by the Nile red microassay. Lipid content of untreated control cells was set at 0% and the lipid

content in treated cells was calculated as percentage of the change from control. For measuring cell proliferation, fibroblasts were seeded at a density of 1,500 cells/ well in 96-well plates and were left 48 h to adhere. On day 0 and 2 cells were treated with IGF-I at f20 and on day 4 proliferation was measured by means of MUH fluorescence assay. Proliferation of untreated control cells was set at 0% and proliferation of treated cells was calculated as percentage of the change from control. Values represent the mean of ten experiments \pm SD (***) $p < 0.001$).

Fig. 6

IGF-I and 17 β -estradiol synthesis in human fibroblasts incubated with 17 β -estradiol (A) and IGF-I (B).

Breast fibroblasts were seeded at a density of 100,000 cells/ flask in a 75 cm² flask and were left 48 h to adhere. The following day cells were treated with 17 β -estradiol (A) and IGF-I (B) for 48 h and 72 h in levels similar to those circulating in young female individuals (f20). The production of IGF-I and 17 β -estradiol was measured by means of ELISA. Control untreated cells were set at 0% and the production of hormones in the treated cells was calculated as percentage of the change from control. Values represent the mean of three experiments \pm SD (** $p < 0.01$).

Fig.7

Time-dependent expression pattern of IGF-IR (A, B) and ER α (C, D) in SZ95 sebocytes after treatment with IGF-I (A, C) or 17 β -estradiol [E2] (B, D).

Cells were seeded at a density of 700,000 cells/ flask in a 75 cm² flask and were left 48 h to adhere. On day 0 cells were treated with IGF-I and 17 β -estradiol, respectively at f20. After 3, 6, 12, 24, 48 and 72 h treatment, cell proteins were collected and 40 μ g of protein from each probe was used for the detection of IGF-IR, and ER α by Western blotting. The picture is also

showing the expression of β -actin of the tested probes, which was used as control of the protein quantity (co: control untreated cells). Receptor expression of control untreated cells was set at 0% and expression in the treated cells was calculated as percentage of the change from control. Diagrams show the expression of IGF-IR and ER α relating to the β -actin expression of the probes. Values represent the mean of three experiments \pm SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 8

Time-dependent expression pattern of IGF-IR in breast fibroblasts after treatment with IGF-I (A) or 17 β -estradiol [E2] (B).

Cells were seeded at a density of 100,000 cells/ flask in a 75 cm² flask and were left 48 h to adhere. On day 0 cells were treated with IGF-I and 17 β -estradiol, respectively at levels corresponding to 20-year-old females (f20). After 48 and 72 h treatment proteins were collected from each probe and 40 μ g of protein was used for the detection of IGF-IR by Western blotting. The picture is also showing the expression of β -actin of the tested probes, which was used as control of the protein quantity. Receptor expression of control untreated cells was set at 0% and expression in the treated cells was calculated as percentage of the change from control. Diagrams show the expression of IGF-IR relating to the β -actin expression of the probes. Values represent the mean of three experiments \pm SD (** $p < 0.01$).

Fig. 9

A schematic overview of the effects of IGF-I (blue arrows) and 17 β -estradiol (dotted red arrows) and the interplay of both signaling pathways in human sebocytes (right) and fibroblasts (left) [C: cytoplasm, N: nucleus, IGF-IR: insulin-like growth factor-I receptor, ER α : estrogen receptor α]

Fig.1

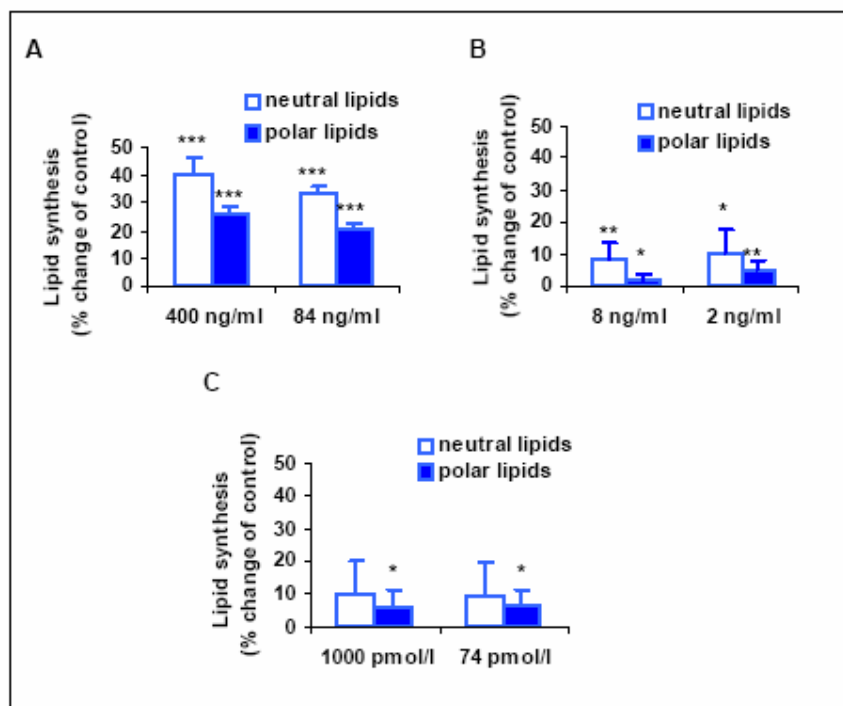


Fig.2

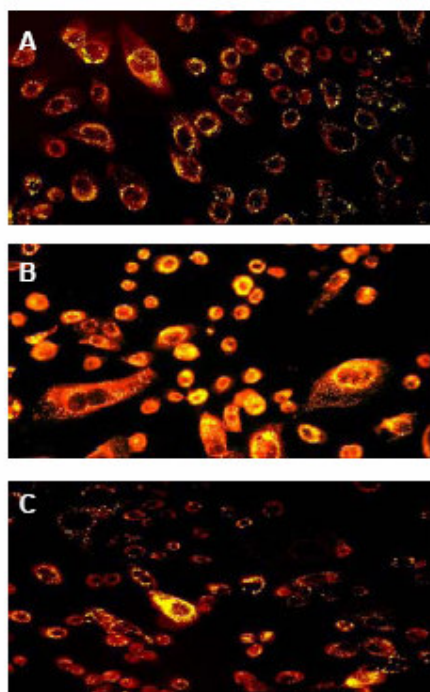


Fig.3

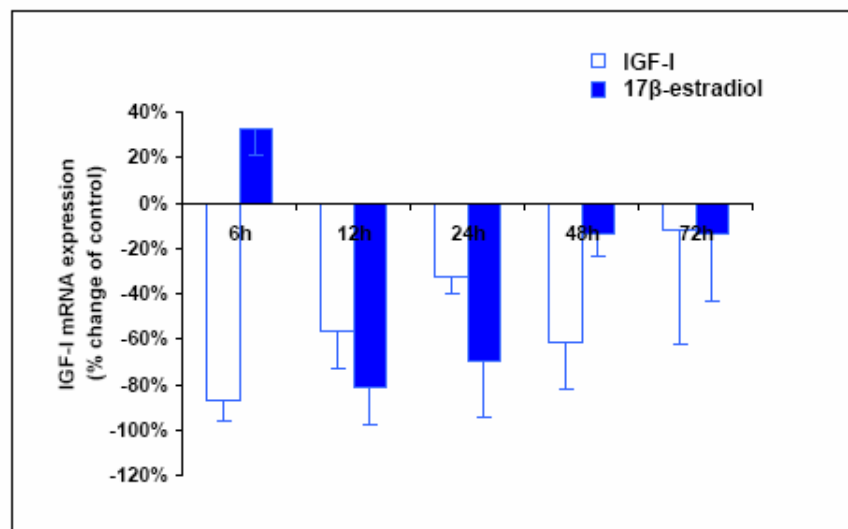


Fig.4

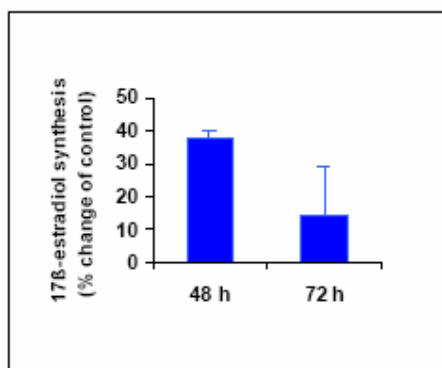


Fig.5

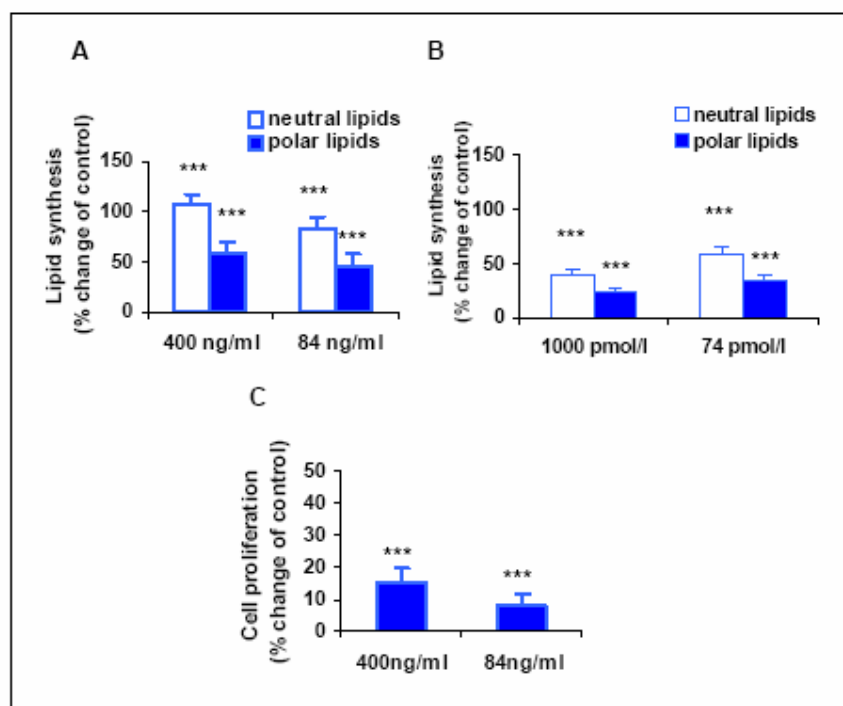


Fig.6

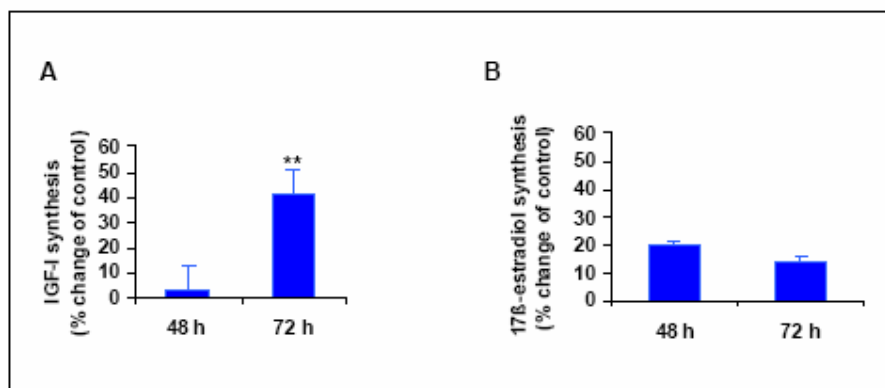


Fig.7

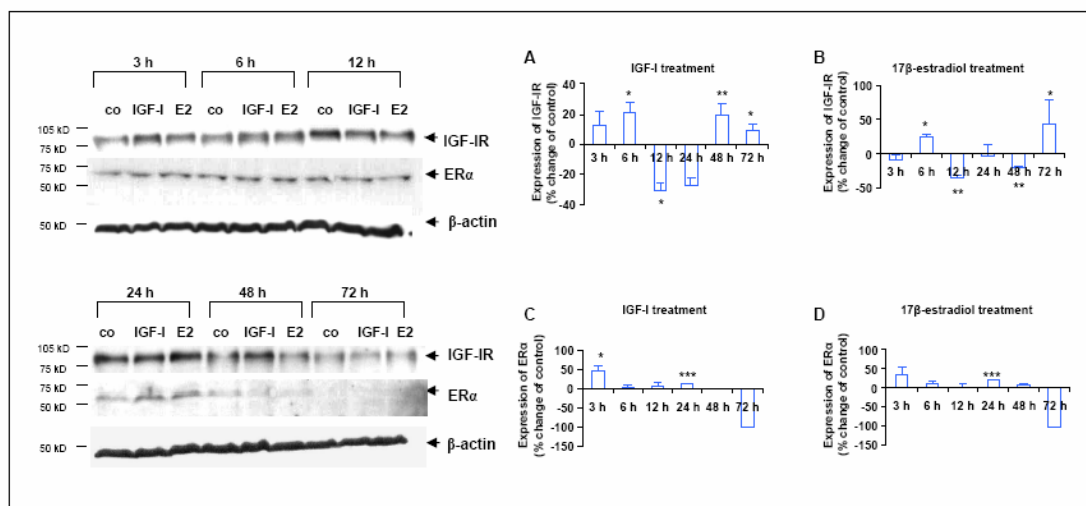


Fig.8

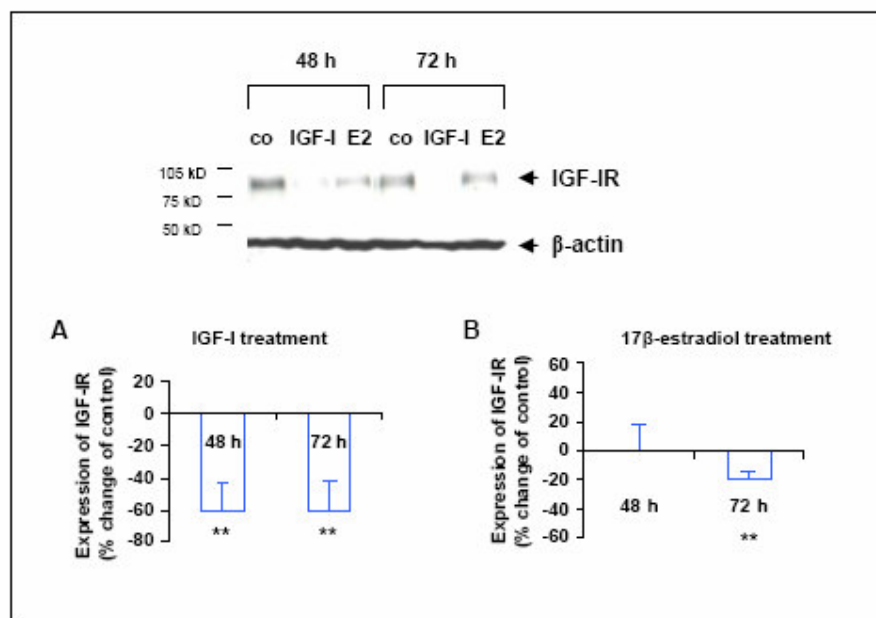
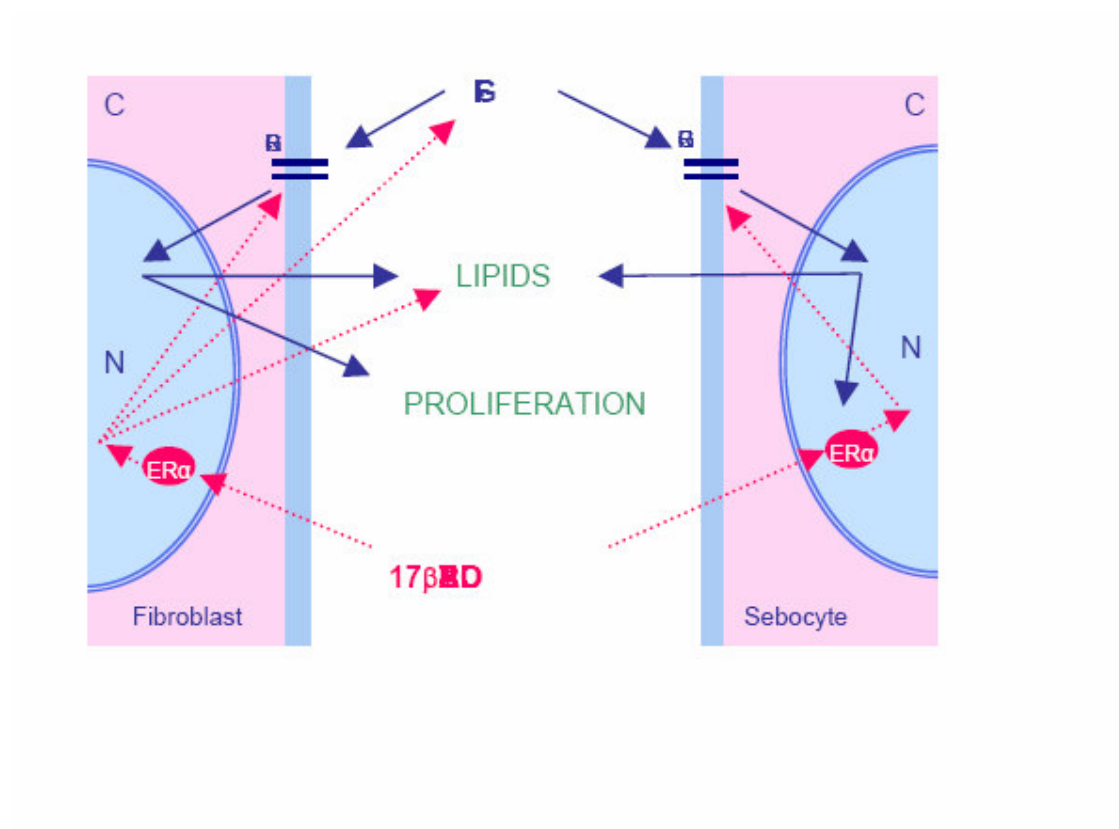


Fig.9



TABLES

Table 1

Median hormone concentrations in serum of healthy 20- and 60-y-old women, which were added to cell cultures as single agents

Hormones	20-y-old women (f20)	60-y-old women (f60)
17 β -estradiol (pmol/l)	1000	74
Progesterone (nmol/l)	50	1
Dehydroepiandrosterone (nmol/l)	20	5
Growth hormone (ng/ml)	8	2
Insulin-like growth factor I (ng/ml)	400	84