Leukemia inhibitory factor as a regulator of steroidogenesis in human NCI-H295R adrenocortical cells

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Abstract

Leukemia inhibitory factor (LIF) is a multiple function cytokine regulating the hypothalamic–pituitary–adrenal axis at the pituitary level. LIF and its receptor are expressed in the adrenal glands, suggesting their potential regulatory role also at the adrenal level. Our aim was to clarify the effects of LIF on adrenal steroidogenesis using cell culture conditions. NCI-H295R human adrenocortical cells were treated with LIF (0.01–100 ng/ml) for 3–48 h with or without 8-bromo-cAMP (8–Br-cAMP; 1 mM). LIF treatment augmented cortisol, dehydroepiandrosterone (DHEA), DHEA sulfate, androstenedione, and aldosterone production (up to 224, 149, 229, and 170% of control respectively, P<0.05 for all). It increased basal steroidogenic acute regulatory protein (STAR) and 17α-hydroxylase/17,20-lyase (CYP17A1) mRNAs (up to 142 and 170% of control respectively, P<0.05) and the respective proteins, but decreased 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2) mRNA (down to 72% of control, P<0.05), and protein. LIF also increased 8-Br-cAMP-induced cortisol and DHEA production and STAR mRNA accumulation, while it attenuated 8-Br-cAMP-induced HSD3B2 expression and androstenedione production. It had an additive effect on tumour necrosis factor-induced cortisol production. LIF had no effect on apoptosis, but it increased slightly the number of metabolically active cells (up to 120% of control, P<0.05). These findings indicate that LIF is a potential physiological and/or pathophysiological regulator of steroidogenesis at the adrenal level.


Introduction

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine produced mainly by activated T-lymphocytes in response to different stimuli (Gearing 1993, Auernhammer & Melmed 2000). It is a secreted glycoprotein with molecular mass from 38 to 67 kDa, the variation resulting from inconsistent glycosylation of the ~20 kDa protein (Hilton et al. 1988). LIF has the four α-helix cytokine structure that contains six cysteine residues; the same structure is present in many hematopoietic factors (Robinson et al. 1994, Lass et al. 2001). LIF acts through the LIF receptor (LIFR) composed of a gp130 and a specific LIFR subunit (Gearing 1993). Activation of the LIF signal transduction pathway involves heterodimerization of these subunits and subsequent rapid activation of janus kinases (JAKs; Stahl et al. 1994, Zhang et al. 1994), which in turn phosphorylate tyrosine residues of LIFR and gp130. These phosphorylated tyrosine residues form docking sites for signaling molecules including the members of the signal transducer and activator of transcription (STAT) family (Ray et al. 1996, Heinrich et al. 2003). This signal transduction pathway has been confirmed, for instance, in rat pancreatic duct cells where the specific JAK2 phosphorylation inhibitor AG490 decreased basal and LIF-induced cell proliferation (De Breuck et al. 2006).

LIF mRNA expression has been detected in different human tissues including those of the endocrine system (Ferrara et al. 1992, Patterson 1994, Paglia et al. 1997, Mylonas et al. 2005). LIF protein and receptors are expressed in bovine (Ferrara et al. 1992), mouse (Shimon et al. 1997), human fetal, and adult pituitaries (Akita et al. 1995, Ray et al. 1996, Shimon et al. 1997, Hanisch et al. 2000), and in various pituitary adenoma subtypes (Akita et al. 1995, Hanisch et al. 2000). LIF and LIFR have been detected throughout all zones of the normal human adrenal cortex and not in the medulla. LIF is located in the cytosol and LIFR in both cytosol and on the cell membrane. LIF and LIFR were also found in the NCI-H295 adrenal carcinoma cell line (Bamberger et al. 2000).

The expression of LIF and LIFR mRNA is upregulated by lipopolysaccharide (LPS), interleukin 1 alpha (IL1A), IL1B, and tumour necrosis factor (TNF) in various human cell types (Campbell et al. 1993, Hamilton et al. 1993, Arici et al. 1995, Grosset et al. 1995, Wang et al. 1996). IL1B has a synergistic effect with TNF on LIF mRNA expression in mouse AtT-20/D16v-F2 corticotroph cells (Auernhammer et al. 1998, Chesnokova et al. 1998).
LIF has a wide range of effects on diverse cell types, tissues, and organs, including the immune, hematopoietic, and nervous systems, and many of these actions can be mimicked by related cytokines, in particular IL6, oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1. However, some of the functions exhibited by LIF cannot be compensated by other cytokines. The pleiotropic effects of LIF in many physiological systems include cell proliferation, differentiation, and survival (Kamohara et al. 1997, Vogiagis & Salamonsen 1999, Savitz & Kessler 2000, Lass et al. 2001, Schere-Levy et al. 2003, Azari et al. 2006).


Adrenal steroidogenesis is under complex regulatory control involving different extra- and intra-adrenal factors. The principal regulators of adrenal steroidogenesis are ACTH and angiotensin II. However, there are dozens of additional hormones, neuropeptides, and cytokines involved in this regulation. Proinflammatory cytokines TNF (Jäättelä et al. 1990, 1991, Ilvesmäki et al. 1993, Voutilainen 1998, Barney et al. 2000, Judd et al. 2000, Chesnokova & Melmed 2002, Chesnokova et al. 2002, Mikhaylova et al. 2007), IL6 (Barney et al. 2000, Judd et al. 2000, Chesnokova & Melmed 2002), and IL1B (Chesnokova & Melmed 2002) have been shown to modulate bovine, murine, and human adrenal steroidogenesis.

The only published report on the effects of LIF at the adrenal level is by Bamberger et al. (2000). They showed that LIF increases basal and ACTH-induced cortisol and aldosterone secretion in the NCI-H295 cell line. To shed more light on the possible role of LIF as a local regulator of adrenocortical function, we examined in detail the effects of LIF on adrenal steroidogenesis, adrenocortical cell apoptosis, cell viability, and on the mRNA and protein levels of three key genes involved in steroidogenesis (steroidogenic acute regulatory protein (STAR), 17α-hydroxylase/17,20-lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2)) in the human adrenocortical cell line NCI-H295R, of which the parent cell line NCI-H295 has previously been described as an appropriate model for studying human adrenocortical steroidogenesis (Rainey et al. 1994).

Materials and Methods

Cell culture

NCI-H295R human adrenocortical cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The medium used was DMEM:F12 (Gibco BRL) supplemented with 2 mM L-glutamine (Gibco BRL), 2% Ultrosen (Biosepra, Marlborough, MA, USA), and antibiotics (125 μg/ml streptomycin and 125 IU/ml penicillin; Orion Pharmaceutical Co., Espoo, Finland). The cells were maintained at 37 °C in a 5% CO2 humidified atmosphere, and plated with a density of 1×10^6 cells/well on 35 mm plastic culture dishes (Nalge Nunc International, Rochester, NY, USA) and grown for 48 h. In dose–response experiments, the cultures were treated with or without 0-01–100 ng/ml recombinant human LIF (Chemicon International, Temecula, CA, USA) for 24–48 h. In time-course experiments, the cells were treated with or without 10 ng/ml LIF and 1 mM 8-bromo-cAMP (8-Br-cAMP; Sigma Chemical Co.) for 3–48 h. In some experiments, 1 nM TNF (R&D Systems Inc., Minneapolis, MN, USA) was used either alone or in combination with LIF. The conditioned culture media were collected, frozen immediately, and stored at −70 °C for subsequent steroid analyses.

Steroid measurements

Concentrations of cortisol, androstenedione, dehydroepiandrosterone (DHEA), and its sulfate (DHEAS) in the conditioned culture media were determined by commercial ELISA kits, according to the manufacturer’s instructions (Diagnostic System Laboratories Inc., Webster, TX, USA; catalogue numbers DSL-10-2000, DSL-10-3800, DSL-10-9000 and DSL-10-3500 respectively). Aldosterone was measured by Coat-A-Count RIA (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA; catalogue number TKAL2).

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Life Technologies, Inc.), according to the manufacturer’s protocol. Total RNA concentration was measured spectrophotometrically at 260 nm. For the RT-PCR analyses, the trace amount of genomic DNA were removed from RNA samples with DNA-free DNase treatment and removal kit (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. In short, 10 μg total RNA was incubated with 2 U DNase I and 2.5 μl tenfold DNase buffer in the total volume of 25 μl at 37 °C for 30 min. Divalent cations and DNase were removed by incubating samples with 5 μl inactivation reagent in ambient temperature for 2 min. Reverse transcription was performed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s protocol. Reactions were made in the total volume of 20 μl containing 2 μg DNase-treated total RNA, onefold reaction buffer, dNTP mixture and random primers, and 50 U MultiScribe reverse transcriptase. Reaction mixtures were incubated at 25 °C for 10 min following 2-h incubation at 37 °C. Quantitative real-time PCR was carried out in the Applied Biosystems 7500
Real-Time PCR System using TaqMan Gene Expression Assays (Applied Biosystems) for STAR (assay ID Hs00264912_m1), steroid 17α-hydroxylase (CYP17A1, assay ID Hs_00164375_m1), HSD3B2 (assay ID Hs00605123), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, assay ID Hs99999905_m1). GAPDH was chosen for endogenous control as it showed least variation after several different treatments of the cell line in TaqMan Human Endogenous Control Plate (Applied Biosystems). Standard series of five dilutions containing 96, 24, 12, 3, and 1 ng template cDNA were prepared from pooled sample cDNAs. Sample dilutions comprised 12 ng template cDNA. All standards and samples were run in the total volume of 20 μl in triplicate.

**Western immunoblotting**

Cell monolayers were washed with ice-cold Tris-buffered saline (TBS) and harvested in TBS. Cell pellets were solubilized in lysis buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol) and lysed by sonication 2X10 s. Solubilized protein was centrifuged briefly, and protein concentrations in the supernatants were quantified by bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). All samples were normalized to the same protein concentration. After β-mercaptoethanol (final concentration 350 mM) and bromophenol blue were added, the samples were incubated for 5 min at 95 °C and aliquots (25–125 μg) were separated on 12% SDS-PAGE gels. The proteins were transferred onto nitrocellulose membranes and visualized by following antibodies: STAR (FL-285, sc-25806), CYP17A1 (H-48, sc-66849), and GAPDH (FL-335, sc-25778) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); HSD3B2 (R1484) from J Ian Mason (University of Edinburgh, UK; Vani et al. 2007); and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Invitrogen) using the ECL reagents, according to the manufacturer’s instructions (Pierce Biotechnology). For protein quantification, goat anti-rabbit IgG DyLightTM 800 and goat anti-mouse IgG DyLightTM 680 fluorescent dye-conjugated secondary antibodies were used, and the membranes were analyzed with a Li-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), according to the manufacturer’s instructions.

**Apoptosis assay**

Commercial luciferase-based Caspase-Glo 3/7 Assay (Promega) was used to measure apoptosis in the NCI-H295R cell line. For all apoptosis experiments, 10 000 cells per well were subcultured in 100 μl DMEM:F12 medium on white flat-bottom 96-well plates (Nalge Nunc International) and incubated for 24 h to allow cell attachment. After that, the culture medium was removed, the cells were washed once with 100 μl PBS, and fresh medium with or without LIF (0–01–100 ng/ml) was added for 24 h. TNF (0-1 nM) was used as a positive control for the assay. The experiments were terminated by adding 100 μl Caspase-Glo 3/7 reagent. The contents of the wells were mixed on a plate shaker for 30 s, incubated at room temperature for 1 h, and the luminescence was measured by a luminometer (BioPhotometer, Eppendorf, Hamburg, Germany).

**Cell viability assay**

Commercial CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to estimate the effect of LIF on the metabolic activity of the cells, based on the amount of cellular ATP. Five thousand cells per well were seeded on a flat-bottom 96-well plate (Nalge Nunc International). After 24-h incubation, the culture medium was removed, the cells were washed with PBS, and fresh medium with or without LIF (0-01–100 ng/ml) was added. TNF (0·1 nM) was used as a control for the assay. After 24-h treatment, 100 μl prepared reagent was added to each well. The contents of the wells were mixed on a plate shaker for 2 min and incubated at room temperature for 10 min, and the luminescence was measured by a luminometer (Eppendorf).

**Statistical analyses**

Each experiment was repeated at least three times (western immunoblot experiments two times). Single experiments consisted of several treatments. The results are given as mean ± S.E.M. of at least three separate experiments each performed in triplicate, with the control mean adjusted to 100% (western immunoblot quantitation data as mean ± range of two separate experiments). The significances of the differences were assessed by Kruskal-Wallis and Mann–Whitney tests. The level of significance was chosen as P<0·05. Data were analyzed using the statistical program SPSS for Windows, release 14.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**The effect of LIF and 8-Br-cAMP on steroidogenesis**

We used the human adrenocortical cell line NCI-H295R as a model to test the effects of LIF on adrenocortical function. The steroid production data were normalized by the total RNA content of the wells at the end of the experiments (reflecting the cell number) to exclude the influence of possibly altered cell number on steroid production. In LIF-treated cultures, normalization did not essentially affect steroid production data, but due to the simultaneous use of TNF in some experiments (causing remarkable changes in cell number; Mikhaylova et al. 2007), normalization was used throughout the manuscript. LIF treatment (0·01–100 ng/ml) for 48 h increased dose dependently the basal production of cortisol (Fig. 1A) and DHEA (Fig. 1B; up to 190 and 211% of
control respectively, \( P < 0.05 \) for both). DHEAS (Fig. 1C) and androstenedione (Fig. 1D) increased also significantly in response to LIF treatment (up to 143 and 229% of control respectively; \( P < 0.05 \) for both), although their increase was not as clearly dose dependent as that of cortisol and DHEA.

The LIF concentration 10 ng/ml caused nearly maximal increase in steroid production after 48-h incubation (Fig. 1). The time-course experiments were performed with this dose. The stimulatory effects of LIF and 8-Br-cAMP (1 mM) on basal cortisol production became significant at 24 h, while those on DHEA, DHEAS, and androstenedione production could be seen earlier (Fig. 2). The effect of LIF on aldosterone production was studied only at the 48-h time point, partly due to the relatively low amount of produced aldosterone in basal conditions. LIF (10 ng/ml) increased basal production of aldosterone up to 170\% \( (P < 0.005, \text{Fig. 3}) \), but had no significant effect on 8-Br-cAMP-induced secretion of this steroid; 8-Br-cAMP (1 mM) by itself increased aldosterone production up to 110\% \( \left( \text{reflecting the cell number}\right) \). \( *P < 0.005 \) compared with the control of the same time point, \( \dagger P < 0.05 \) compared with the 8-Br-cAMP–treated wells at the same time point.

The effect of LIF and 8-Br-cAMP on STAR, CYP17A1, and HSD3B2 mRNA expression

In dose–response experiments, LIF (0.01–100 ng/ml) increased the basal accumulation of the mRNAs for STAR and CYP17A1 (up to 138 and 170\% of control after 48-h treatment respectively, \( P < 0.05 \) for both; Fig. 5A and B). By contrast, the expression of \( \text{HSD3B2} \) mRNA decreased

Figure 1 The dose-dependent effect of LIF on cortisol, DHEA, DHEAS, and androstenedione production by cultured NCI-H295R cells after 48-h treatment. One million cells per well were plated and incubated for 48 h with or without 0.01–100 ng/ml LIF. The results (steroid concentration in the conditioned medium, mean±S.E.M.) are expressed as % of control (without any treatment), and they originate from three separate experiments, each performed in triplicate wells. The steroid production data were normalized by the total RNA content of the wells at the end of the experiment (reflecting the cell number). \( *P < 0.05 \) compared with the control.

Figure 2 The time-dependent effect of LIF and 8-Br-cAMP (cAMP) on cumulative cortisol (A), DHEA (B), DHEAS (C), and androstenedione (D) production by cultured NCI-H295R cells. One million cells per well were plated and incubated for 3–48 h with or without 10 ng/ml LIF, 1 mM 8-Br-cAMP, or their combination. The results (steroid concentration in the conditioned medium, mean±S.E.M.) are expressed as % of control (without any treatment) at each time point. The data originate from three separate experiments, each performed in triplicate wells. The steroid production data were normalized by the total RNA content of the wells at each time point (reflecting the cell number). \( *P < 0.05 \) compared with the control of the same time point, \( \dagger P < 0.05 \) compared with the 8-Br-cAMP–treated wells at the same time point.

Figure 3 The effect of LIF and 8-Br-cAMP (cAMP) on cumulative aldosterone production by cultured NCI-H295R cells after 48-h treatment. One million cells per well were plated and incubated for 48 h with or without 10 ng/ml of LIF, 1 mM 8-Br-cAMP, or their combination. The results (steroid concentration in the conditioned medium, mean±S.E.M.) are expressed as % of control (without any treatment), and they originate from nine separate experiments, each performed in duplicate or triplicate wells. The steroid production data were normalized by the total RNA content of the wells at the end of the experiment (reflecting the cell number). \( *P < 0.005 \) compared with the control.
during LIF treatment, but this decrease (maximal decrease down to 71% of control with 10 ng/ml LIF, \( P < 0.05 \); Fig. 5C) required higher LIF concentrations than the simultaneous increase in STAR and CYP17A1 mRNAs (Fig. 5A and B).

In time-course experiments, LIF (10 ng/ml), 8-Br-cAMP, and their combination clearly modified the mRNA accumulation of the three key genes involved in steroid production (Fig. 6). As expected, 8-Br-cAMP (1 mM) induced time dependently the accumulation of STAR (up to 301%, \( P < 0.05 \); Fig. 6A) and CYP17A1 mRNAs (up to 320% of control, \( P < 0.05 \); Fig. 6B) after 3–48 h of treatment. The amount of HSD3B2 mRNA; however, decreased during the first hours of 8-Br-cAMP treatment (down to 51% of control at 3 h, \( P < 0.05 \); Fig. 6C). LIF (10 ng/ml) increased significantly the basal expression of STAR and CYP17A1 mRNAs (up to 140% and 143% of control respectively, \( P < 0.05 \); Fig. 6A and B). At the 3-h time point, LIF increased also the 8-Br-cAMP-induced STAR mRNA accumulation (44%, \( P < 0.05 \), but had no significant effect on 8-Br-cAMP-induced CYP17A1 mRNA accumulation. On the other hand, LIF (10 ng/ml) decreased significantly the basal and 8-Br-cAMP-induced accumulation of HSD3B2 mRNA (35% inhibition at 48 h, \( P < 0.05 \); Fig. 6C).

The effect of LIF, TNF, and 8-Br-cAMP on STAR, CYP17A1, and HSD3B2 protein expression

Western immunoblot analyses were used to estimate the effects of LIF, TNF, and 8-Br-cAMP treatments on STAR, CYP17A1, and HSD3B2 protein expression at 12 and 48 h of treatment. Quantitation of the protein levels was performed by infrared imaging of the intensity of the protein band of interest in relation to the intensity of the GAPDH band in the same sample (Fig. 7). LIF (10 ng/ml) increased STAR up to 140% and CYP17A1 up to 130% of control, but decreased HSD3B2 level down to 85% of control at the 48-h time point. 8-Br-cAMP (1 mM) increased STAR protein rapidly (up to 550 and 310% of control at the 12- and 48-h

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**Figure 4** The effect of LIF and TNF on cumulative cortisol production by cultured NCI-H295R cells after 48-h treatment. One million cells per well were plated and incubated for 48 h with 10 ng/ml of LIF, 1 nM of TNF, or their combination. The results (steroid concentration in the conditioned medium, mean±S.E.M.) are expressed as % of control (without any treatment), and they originate from three separate experiments, each performed in triplicate wells. The steroid production data were normalized by the total RNA content of the wells at the end of the experiment (reflecting the cell number). *\( P < 0.05 \) compared with the control, †\( P < 0.05 \) compared with TNF alone.

**Figure 5** The dose-dependent effect of LIF (0.01–100 ng/ml) on the accumulation of STAR (A), CYP17A1 (B), and HSD3B2 (C) mRNAs in cultured NCI-H295R cells after 48-h treatment. The cell culture experiments were the same as presented in Fig. 1 for steroid production. RNA was isolated at each time point and RT-PCR performed as described in the Materials and Methods section. The results (mean±S.E.M.) are expressed as % of control (without any treatment). The data originate from three separate experiments, each performed in triplicate wells. *\( P < 0.05 \) compared with the control.
The pre-protein and the mature form of STAR were clearly induced by 8-Br-cAMP (Fig. 7, uppermost left panel), as suggested previously (Arakane et al. 1997). The increasing effect of 8-Br-cAMP on CYP17A1 (up to 160 and 210% of control at 12 and 48 h respectively) was slower than that on STAR. HSD3B2 decreased during 8-Br-cAMP treatment down to 65% and increased up to 160% of control at the 12- and 48-h time points respectively. In combination with 8-Br-cAMP, LIF reduced HSD3B2 down to 70% of the mere 8-Br-cAMP treatment level at the 48-h time points. The effect of LIF on 8-Br-cAMP-induced STAR and CYP17A1 levels was minor and cannot be considered convincing on the basis of our western blots (Fig. 7).

TNF (1 nM) treatment increased STAR, CYP17A1, and HSD3B2 protein levels up to 125, 110, and 130% of control at the 48-h time point respectively (Fig. 7).

The effect of LIF on apoptosis and cell viability

LIF treatment (0.01–100 ng/ml, 24 h) had no significant effect on apoptotic index (measured by the luciferase-based caspase 3–7 assay), while TNF (0.1 nM) increased it significantly (Fig. 8A) as reported previously (Mikhaylova et al. 2007). LIF (0.01–100 ng/ml, 24 h) slightly increased the number of metabolically active cells (up to 120% of control, $P<0.05$; Fig. 8B), whereas TNF decreased it as shown previously (Mikhaylova et al. 2007).

Discussion

We studied the effects of LIF, 8-Br-cAMP, and their combination on adrenal steroidogenesis using the human adrenocortical NCI-H295R cell line as a model. As expected, 8-Br-cAMP increased the production of cortisol, DHEA, DHEAS, androstenedione, and aldosterone, and the accumulation of STAR, HSD3B2, and CYP17A1 mRNAs. Moreover, LIF had a significant dose- and time-dependent effect on steroid production and on the accumulation of STAR, HSD3B2, and CYP17A1 mRNAs. The LIF-induced changes in the STAR, HSD3B2, and CYP17A1 protein levels were generally in line with the steroid and mRNA data.

Our findings of the stimulatory effect of LIF on cortisol and aldosterone production are in agreement with those of Bamberger et al. (2000), who found that LIF increases basal and ACTH-induced cortisol and aldosterone production in the human adrenocortical NCI-H295 cell line, which is the parent cell line for the one we used (NCI-H295R). It is noteworthy that there are two variants (NCI-H295A and NCI-H295R) of the original NCI-H295 cell line. These variants have somewhat different steroidogenic secretory profiles (Samandari et al. 2007). We suppose that Bamberger et al. (2000) used the parent cell line NCI-H295 in their LIF studies, although the source of their cells was not clearly described. In our detailed experiments with NCI-H295R cells, LIF increased time and dose dependently the basal and 8-Br-cAMP-induced production of cortisol, DHEA, DHEAS, and androstenedione. Careful time-dependent analyses were not possible for aldosterone production due to its low production rate and relative insensitivity of the RIA.

Figure 6 The time-dependent effect of LIF (10 ng/ml), 8-Br-cAMP (1 mM), and their combination on the accumulation of STAR (A), CYP17A1 (B), and HSD3B2 (C) mRNAs in cultured NCI-H295R cells. The cell culture experiments were the same as presented in Fig. 2 for steroid production. RNA was isolated at each time point and RT-PCR performed as described in the Materials and Methods section. The results (mean ± S.E.M.) are expressed as % of control (without any treatment) at each time point. The data originate from three separate experiments, each performed in triplicate wells. *$P<0.05$ compared with the control of the same time point, †$P<0.05$ compared with the 8-Br-cAMP-treated wells at the same time point.
In any case, LIF also increased aldosterone production by 48-h treatment in our NCI-H295R cells, although this cell line produces less mineralocorticoids than the NCI-H295A variant (Samandari et al. 2007). Our time-course experiments showed that the stimulatory effects of LIF and 8-Br-cAMP on cortisol production were somewhat slower than on adrenal androgen production.

The stimulatory effect of LIF on STAR and CYP17A1 expression is a potential explanation for the LIF-induced increase of steroid production in adrenocortical cells. The results from the time-course experiments support this assumption. Although LIF increased STAR mRNA already at the 3-h time point, it took 48 h before CYP17A1 mRNA and protein levels were increased. Accordingly, LIF treatment did not increase cortisol production remarkably before the 48-h time point. It is quite possible that LIF also regulates

Figure 7 The effect of TNF-α (1 nM), LIF (10 ng/ml), and 8-Br-cAMP (cAMP) (1 mM) on STAR, CYP17A1, and HSD3B2 protein levels after 12- and 48-h treatments. Cells were treated and protein extracts were prepared as indicated in the Materials and Methods section. STAR (1:500), CYP17A1 (1:1000), and HSD3B2 (1:5000) antibodies (α-STAR, α-CYP17A1 and α-HSD3B2 respectively) were used to detect the corresponding proteins with ECL (upper panel) or with Odyssey infrared imaging system to quantify the protein bands. The bars (lower panels) represent the intensity of the band of interest in relation to the intensity of the GAPDH band in the same sample detected by the α-GAPDH antibody (1:3000). The results (mean ± S.E.M. of caspase 3/7 activity in (A) or of the luminescence reflecting the amount of cellular ATP and the number of metabolically active cells in (B)) are expressed as % of control (without LIF treatment) after 24-h incubation. The data originate from three separate experiments, each performed in triplicate wells. *P<0.05, compared with the respective control. TNF treatment was used as a positive control for the assays.

Figure 8 The dose-dependent effect of LIF on the apoptotic index (A) and cell viability (B) in cultured NCI-H295R cells. Ten thousand (for apoptosis experiments) or five thousand (for cell viability experiments) cells per well on a 96-well plate were seeded and incubated for 24 h with 0-01–100 ng/ml LIF. The results (mean ± S.E.M. of caspase 3/7 activity in (A) or of the luminescence reflecting the amount of cellular ATP and the number of metabolically active cells in (B)) are expressed as % of control (without LIF treatment) after 24-h incubation. The data originate from three separate experiments, each performed in triplicate wells. *P<0.05, compared with the respective control. TNF treatment was used as a positive control for the assays.

In any case, LIF also increased aldosterone production by 48-h treatment in our NCI-H295R cells, although this cell line produces less mineralocorticoids than the NCI-H295A variant (Samandari et al. 2007). Our time-course experiments showed that the stimulatory effects of LIF and 8-Br-cAMP on cortisol production were somewhat slower than on adrenal androgen production.
other genes and proteins involved in adrenocortical steroid synthesis, which could contribute to the steroid production pattern seen after LIF treatment.

It is of special interest that LIF decreased both basal and 8-Br-cAMP-induced HSD3B2 mRNA and protein expression, while increased STAR and CYP17A1 expression. This contrasts with the effect of another cytokine, TNF, which increases the accumulation of HSD3B2 mRNA (Mikhaylova et al. 2007) and protein (48-h time point in this study) in these cells. Thus, these two cytokines have similar (stimulatory) effects on STAR and CYP17A1 expression but opposite effects on HSD3B2 expression. Although LIF decreased HSD3B2 mRNA accumulation, cortisol and androstenedione production (requiring HSD3B2 activity) increased during LIF treatment. This indicates that HSD3B1 activity is not rate limiting for cortisol and androstenedione synthesis in these cells. Our dose–response experiments suggest that higher LIF concentrations are required for inhibiting HSD3B2 mRNA accumulation than for increasing STAR and CYP17A1 mRNA levels. This may explain the lack of straightforward dose dependency of androstenedione production in our dose–response experiments.

The effects of LIF and TNF on NCI-H295R cells differed also in another respect. TNF caused remarkable apoptosis in these cells (Mikhaylova et al. 2007; this study), whereas LIF had no effect on it when estimated by caspase activity. LIF has been reported to modify proliferation, differentiation, and cell survival of several mammary cell lines (Metcalf & Gearing 1989, Patterson 1994, Estrov et al. 1995, Kellokumpu-Lehtinen et al. 1996, Ray et al. 1996, Bousquet et al. 1997, Shimon et al. 1997, Dhingra et al. 1998, Savitz & Kessler 2000, Schere-Levy et al. 2003, Azari et al. 2006). In our experiments, LIF had no effect on apoptotic index, and it only slightly influenced the viability of NCI-H295R adrenocortical cells. Both induction (Kamohara et al. 1997, Savitz & Kessler 2000, Schere-Levy et al. 2003) and inhibition (Azari et al. 2006) of apoptosis by LIF in different cell types involve modulation of the Jak/STAT and Akt signaling pathways. Downstream-regulated genes include the anti-apoptotic molecule BIRC3, MYC, and CASP1 (Kamohara et al. 1997, Schere-Levy et al. 2003, Azari et al. 2006). In the present study, we analyzed only caspase activity and not any of the other mentioned molecules or genes.

Analysis of the LIF signal transduction mechanisms from LIFR to the regulation of target gene transcription (JAK/STAT pathway and other modifying mechanisms, reviewed in Auernhammer & Melmed (2000) and Heinrich et al. (2003)) was not within the scope of our study. However, the STAR, CYP17A1, and HSD3B2 genes and/or their upstream sequences have several possible binding sites for STAT proteins (according to TFSEARCH http://www.cbrc.jp/research/db/TFSEARCH.html), which might transmit regulation of transcription of these genes as a consequence of adrenocortical cell LIFR activation.

IL1A, IL1B, TNF, and LPS (endotoxin) have been reported to upregulate LIF or LIFR mRNA in various tissues and cell types (Campbell et al. 1993, Hamilton et al. 1993, Arici et al. 1995, Grosset et al. 1995, Wang et al. 1996, Auernhammer et al. 1998, De Breuck et al. 2006), suggesting that a complex network of different cytokines may also function at the adrenal level. Further studies are needed to clarify the significance of the whole cytokine network in the regulation of human adrenal function in vivo, but it is tempting to speculate that LIF and TNF are factors modulating the activity of the hypothalamic–pituitary–adrenal axis, possibly at several levels in septic conditions and inflammatory states.

In conclusion, our results demonstrate a significant potential of LIF to stimulate adrenal steroidogenesis. Its effects are mediated at least partly by modulation of the mRNA and protein expression levels of key steroidogenic genes. The expression of LIF and LIFR in human adrenal cortex (Bamberger et al. 2000) make this cytokine a potentially important auto- and paracrine modulator of adrenal steroidogenesis and an interesting target for further studies.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This study was financially supported by Pediatric Research Foundation, Finnish Medical Foundation, Sigrid Jusélius Foundation, Kuopio University Hospital and Academy of Finland.

Acknowledgements

We thank Ms Leila Antikainen and Minna Heinanen for their skilful technical assistance. Prof. J Ian Mason, University of Edinburgh, UK, is greatly acknowledged for supplying the human HSD3B2 antibody.

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Received in final form 12 September 2008
Accepted 16 September 2008
Made available online as an Accepted Preprint 16 September 2008