Induction of steroidogenesis in immature rat Leydig cells by interleukin-1 α is dependent on extracellular signal-regulated kinases

N Renlund, Y Jo¹, I Svechnikova, M Holst, D M Stocco¹, O Söder and K Svechnikov

Department of Woman and Child Health, Pediatric Endocrinology Unit Q2:08, Karolinska Institute & University Hospital, Astrid Lindgren Children's Hospital, SE-17176 Stockholm, Sweden

¹Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

(Requests for offprints should be addressed to K Svechnikov; Email: konstantin.svechnikov@kbh.ki.se)

Abstract

Interleukin-1 α (IL-1 α) plays an important role in the regulation of immune responses as well as in non-inflammatory events in different types of cells. Here we have investigated the involvement of the extracellular signal-regulated kinase (ERK) cascade in IL-1 α -induced steroidogenesis by primary cultures of immature rat Leydig cells. Our findings indicate that protein kinase C functions as an upstream component of signal transduction from the IL-1 receptor type I (IL-1RI) to the ERK cascade. It was observed that IL-1 α upregulated both steroidogenic acute regulatory (StAR) protein expression and its phosphorylation when compared with controls. Selective inhibition of these mitogen-activated protein kinases (MAPKs) by UO126 enhanced both the expression and phosphorylation of the StAR protein, but suppressed androgen production by the immature Leydig cells as well as dissipating the mitochondrial electrochemical potential (Ψ_m) in these cells. The evidence that water-soluble cholesterol but not 22R-hydroxycholesterol-stimulated steroidogenesis was inhibited by UO126 suggested that an intact Ψ_m across the inner mitochondrial membrane is required for cholesterol translocation and is positively regulated by the ERK cascade. We propose that activation of ERKs by IL-1 α plays a dual role in the regulation of steroidogenesis in immature Leydig cells: these MAPKs downregulate StAR expression and phosphorylation, while at the same time they support an intact Ψ_m across the inner mitochondrial membrane, thereby promoting translocation of cholesterol into the mitochondria of the Leydig cell.

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Introduction

The multifunctional cytokine interleukin-1 α (IL-1 α) plays an important role in the regulation of immune and inflammatory responses (Dinarello 1989), and furthermore is expressed under normal physiological conditions in tissues that do not belong to the immune system (Tovey *et al.* 1988), including the Sertoli cells of the testis (Jonsson *et al.* 1999, Sultana *et al.* 2000). Two receptors for IL-1, designated, IL-1RI and IL-1 RII, have been identified, with IL-1RI being responsible for signal transduction (Dinarello *et al.* 1989). Both of these receptors are expressed by the Leydig cells, Sertoli cells and macrophages in the testis (Petersen *et al.* 2002, Svechnikov *et al.* 2003).

Recently, we have found that IL-1 α stimulates androgen production by immature, but not adult, Leydig cells (Svechnikov *et al.* 2001). Subsequent investigations in our laboratory revealed that IL-1 α is a potent inducer of the steroidogenic acute regulatory (StAR) protein in immature but not adult Leydig cells (Svechnikov *et al.* 2003). This upregulation involves activation of p38 mitogen-activated protein kinase

(MAPK) by IL-1 α , indicating that this kinase is an important component of the IL-1 α signaling pathway(s) and, moreover, may be involved in the regulation of steroidogenesis by immature Leydig cells. In addition, our most recent findings that growth hormone- and insulin-like growth factor-I (IGF-I)-stimulated steroidogenesis in immature Leydig cells is dramatically potentiated by IL-1 α (Colon *et al.* 2005) suggests that this IL may play a key role in the postnatal maturation and development of steroidogenesis in Leydig cells.

The extracellular signal-regulated kinases 1/2 (ERK1/2) belong to the family of MAPKs known to be involved in the regulation of a number of important biological functions, including cell proliferation, differentiation and apoptosis, as well as in carcinogenesis (Lewis *et al.* 1998). Although several studies have indicated that the ERK cascade participates in the regulation of steroidogenesis in steroid-producing cells (Cameron *et al.* 1996, Gyles *et al.* 2001, Seger *et al.* 2001), the conclusions drawn appear to be contradictory; i.e. both stimulatory (Cameron *et al.* 1996, Das *et al.* 1996, Gyles *et al.* 2001) and inhibitory (Seger *et al.* 2001, Tajima *et al.* 2003) effects have been reported. Recently, we have

demonstrated that ERKs upregulate *de novo* biosynthesis of StAR along with steroidogenesis in immature rat Leydig cells activated by human chorionic gonadotropin (hCG) (Martinelle *et al.* 2004). In light of previous indications that IL-1 α promotes the growth and differentiation of testicular cells, our present hypothesis was that the ERK cascade is involved in the induction of steroidogenesis in immature rat Leydig cells in response to IL-1 α .

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12, modified Eagle's medium (MEM), Hank's balanced salts solution without Ca²⁺ and Mg²⁺, BSA and antibiotics were obtained from Gibco/BRL (Life Technologies, Paisley, Scotland, UK). Trilostane, an inhibitor of 3 β -hydroxysteroid dehydrogenase, was provided by Stegram Pharmaceuticals (Billingham, Sussex, UK). Percoll, HEPES, collagenase type I, water-soluble cholesterol and 22R-hydroxycholesterol (22R-OHC) were from Sigma Chemical Co. (St Louis, MO, USA), rat recombinant IL-1 α was from RD Systems (Oxon, Oxfordshire, UK), UO126, Calphostin C and H-89 hydrochloride were from Calbiochem (La Jolla, CA, USA), and antibodies directed specifically against the phosphorylated form of p44/42 MAPK (mouse monoclonal antibody IgG, affinity-purified) or total p44/42 MAPK (rabbit polyclonal IgG, affinity-purified) were from Cell Signaling Technology (Beverly, MA, USA).

Animals

Testes from 40-day old Sprague–Dawley rats (B&K Laboratories, Sollentuna, Sweden) were used for the preparation of immature Leydig cells. These animals had access to a standard laboratory pellet diet and water *ad libitum*. These experiments were approved by the Northern Stockholm Animal Ethics Committee (registration no. N161/04).

Isolation and primary culture of Leydig cells

Leydig cells were prepared from the testes of immature rats as described previously (Svechnikov *et al.* 2001). Briefly, following decapsulation, testes were disrupted by collagenase treatment and the seminiferous tubules were separated mechanically. In order to obtain purified Leydig cells, this crude suspension was loaded on the top of a discontinuous gradient consisting of layers of 20, 40, 60 and 90% Percoll dissolved in Hank's balanced salts

solution and subsequently centrifuged at 800 *g* for 20 min. The fractions enriched in Leydig cells thus obtained were then centrifuged in a continuous, self-generating density gradient starting with 60% Percoll at 20 000 *g* for 30 min at 4 °C.

The purity of these Leydig cell preparations was shown to be 90%, as determined by histochemical staining for 3 β -hydroxysteroid dehydrogenase (Payne *et al.* 1980). The cell viability, as assessed by Trypan Blue exclusion, was greater than 90%. These purified Leydig cells were resuspended in DMEM/F12 supplemented with 15 mM HEPES (pH 7.4), 1 mg/ml BSA, 365 mg/l glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin.

For culturing, 100 μ l of a suspension containing 1.5×10^5 cells/ml was plated into each well of a 96-well Falcon plate (Falcon, Franklin Lakes, NJ, USA) and incubated for 24 h at 34 °C in 5% CO₂. At this time point, fresh culture medium was added and the cells pre-incubated for 30 min with the selective ERK inhibitor, UO126 (0.1–10 μ M), prior to incubation with IL-1 α (1 ng/ml), and/or 22R-OHC (10 μ M), and/or water-soluble cholesterol (100 μ M) for 24 h. This range of concentrations of UO126 has been earlier demonstrated to inhibit ERK activities without affecting a number of other protein kinases (Davies *et al.* 2000).

In other experiments, 0.5×10^6 Leydig cells in a total volume of 2 ml were plated into culture dishes (35 $\times$ 10 mm; Falcon) and then incubated for 24 h at 34 °C. Thereafter, fresh medium was added and the cells pre-treated with a combination of UO126 (10 μ M), H-89 (10 μ M) and Calphostin C (1 μ M), which are selective inhibitors of ERKs, protein kinase A (PKA) and protein kinase C (PKC) activities, respectively (Davies *et al.* 2000, Jarvis *et al.* 1994), followed by incubation with IL-1 α (1 ng/ml) for 15 min. This treatment was terminated by aspirating the culture medium and rinsing the cells twice with PBS. All compounds except for water-soluble cholesterol were dissolved in DMSO. The final concentration of DMSO in media did not exceed 0.1%.

Steroid assays

Culture medium samples were stored at –20 °C prior to analysis of testosterone, dehydroepiandrosterone (DHEA), pregnenolone and 5 α -androstane-3 α ,17 β -diol. The sum of testosterone and 5 α -androstane-3 α ,17 β -diol, which are the predominant steroids synthesized at this developmental age, was used as an indicator of the total capacity of Leydig cells to produce androgens in culture. Testosterone and DHEA were quantified employing the Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA), according to the manufacturer's instructions, while the concentrations of 5 α -androstane-3 α ,17 β -diol and pregnenolone were determined by RIA using specific antisera (Cosmo Bio Co., Tokyo, Japan

and Fitzgerald Industries, Concord, MA, USA, respectively). 5 α -[9,11-³H(N)]Androstane-3 α ,17 β -diol (specific activity, 40 Ci/mmol) and [7-³H(N)]pregnenolone (specific activity, 14 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, USA).

Western blot analysis

The influence of IL-1 α on the degree of phosphorylation of ERK and StAR expression was analyzed by PAGE/western blotting. For this purpose, the cells were washed twice with PBS and then lysed and sonicated in a buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol and 10% glycerol. Subsequently, the solubilized fraction was collected by centrifugation at 10 000 *g* for 6 min. The Leydig cell solubilized proteins (40 μ g from each sample) were resolved by electrophoresis on 10% SDS/polyacrylamide gels and transferred electrophoretically to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), using 25 mM Tris/HCl and 185 mM glycine, pH 8.3, containing 20% methanol.

Following this transfer, the membrane was incubated in a blocking buffer (Tris-buffered saline (TBS)/0.1% Tween (TBST) containing 5% non-fat dry milk) for 1 h, followed by washing with TBST (3 $\times$ 10 min). Subsequently, these membranes were incubated with antibodies directed towards phosphorylated or total ERK1/2, according to the manufacturer's specifications (Cell Signaling Tech., Beverly, MA, USA) and, after washing, with donkey anti-rabbit or sheep anti-mouse secondary IgG antibodies.

To assay StAR expression and phosphorylation, membranes were probed with specific antibodies recognizing either total StAR (Bose *et al.* 1999) or phospho-StAR (generated against a peptide conjugated to keyhole limpet hemocyanin corresponding to amino acid residues 190–199 of mouse StAR, with serine 194 phosphorylated, which was a kind gift from Dr Steven King, Baylor College of Medicine, Houston, TX, USA). Following incubation with the secondary antibody (anti-rabbit, conjugated with horseradish peroxidase), antibody binding was determined using enhanced chemiluminescence (ECL; PerkinElmer Life Sciences, Boston, MA, USA) and detected by exposure to X-ray film (Marsh Bio Products, Rochester, NY, USA). The ECL Hyperfilms were scanned using an HP ScanJet 5100C and HP PrecisionScan software (Hewlett-Packard Sverige AB, Kista, Sweden) and the extent of antibody binding subsequently quantified utilizing NIH Image 1.57 software.

Measurement of cytochrome P450 scc activity

In these experiments, intact Leydig cells (2 $\times$ 10⁵ cells/ml) were plated into each well of a 96-well Falcon

plate (Falcon) and cultured for 24 h at 34 °C. At this time point, fresh culture medium was added and the cells were pre-incubated for 30 min with trilostane (5 μ M), an inhibitor of 3 β -hydroxysteroid dehydrogenase (Potts *et al.* 1978), and UO126 (10 μ M) prior to incubation with or without water-soluble cholesterol and IL-1 α (1 ng/ml) for another 3 h. Since pregnenolone could be converted to DHEA by cytochrome P450c17 via the Δ^5 pathway, formation of DHEA was also measured and the sum of the above steroids was considered as an index of P450 scc activity.

To measure P450 scc activity in homogenates of Leydig cells, the cells (5 $\times$ 10⁵ cells) were plated into 35 mm culture dishes (Falcon) and cultured for 24 h at 34 °C. Then cells were pre-incubated for 30 min with UO126 (10 μ M) prior to incubation with or without IL-1 α (1 ng/ml) for 2 h. The cells were then washed twice with ice-cold PBS, scraped and sonicated (2 $\times$ 5 s) in a buffer containing 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), 1 mM EGTA and a protease-inhibitor cocktail (Roche). Debris was removed from the homogenates by centrifugation at 600 *g* for 10 min at 4 °C, followed by transfer of the supernatant to a new tube. Protein concentration was determined by the Bradford method (Bradford 1976).

To assay P450 scc enzyme activity, homogenates from all treated groups were incubated in the presence of NADPH (0.5 mM), trilostane (5 μ M) and water-soluble cholesterol (50 μ M) in a final volume of 100 μ l. After 1-h incubation at 37 °C, the reaction was stopped by centrifugation at 14 000 *g* for 10 min at 4 °C and pregnenolone and DHEA were measured by RIA.

Assay of mitochondrial membrane potential

Mitochondrial membrane potential (Ψ_m) was measured with JC-1 (Molecular Probes, Eugene, OR, USA), a fluorescent dye which detects the mitochondrial membrane potential in cells. Cells were pre-treated for 30 min with UO126 (10 μ M) prior to incubation for another 30 min with or without IL-1 α (1 ng/ml). Then cells were incubated with JC-1 (10 μ g/ml) for 15 min at 34 °C and carefully washed with medium to remove unincorporated dye and then fresh media was added back to the cells. Fluorescence was determined using 550 nm excitation and 600 nm emission filters for red J-aggregate fluorescence (Ψ_m -sensitive) and 485 nm excitation and 535 nm emission filters for green fluorescence (Ψ_m -insensitive) using a Wallac1420 microplate spectrofluorometer (Perkin Elmer). When the electrochemical potential is intact, the negative charge established by the intact mitochondrial membrane potential allows the dye to enter the mitochondrial matrix where it accumulates forming J-aggregates which become fluorescent red. If the electrochemical potential is lost, JC-1 exists in the monomeric form and stains the

cytosol green. The mitochondrial membrane potential Ψ_m was estimated as the ratio of red to green JC-1 fluorescence.

Microscopy

Cells were cultured for 24 h on cover glasses placed in 12-well plates. Then cells were pre-treated for 30 min with UO126 (10 μ M) prior to incubation with or without IL-1 α (1 ng/ml) for 24 h and fixed with 4% paraformaldehyde at room temperature for 20 min, and permeabilized with 0.3% Triton X-100 for 5 min. After washing, they were incubated in blocking buffer (0.2% BSA in TBS) for 20 min at room temperature followed by incubation with primary rabbit anti-StAR polyclonal antibody at 1:100 dilution and then with secondary Cy3-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch Labs). The cells were viewed with a Nikon fluorescence microscope (Nikon, Bergstrom Inst., Solna, Sweden).

Statistical analysis

The differences between various values were analysed for statistical significance by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keul test, using the SigmaStat (version 3.00) package (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

IL-1 α stimulates phosphorylation of ERK1/2 in a time-dependent manner

Following the overnight culturing of immature Leydig cells, activation of their ERK1/2 by IL-1 α was assessed after exposing the cells to this cytokine for different periods of time. Elevation in phosphorylation of ERK1/2 was detectable within 5 min of exposure to IL-1 α , attained a maximum level after 15 min and returned to control levels within 1 h of initiation of treatment (Fig. 1).

PKC, but not PKA, is involved in the phosphorylation of ERK1/2 caused by IL-1 α

In order to identify the upstream kinases involved in the IL-1 α -stimulated activation of ERK1/2, the cells were exposed to H-89 or Calphostin C. Under conditions where treatment of the immature Leydig cells with IL-1 α alone resulted in a 7-fold increase in the level of phosphorylated ERK1/2, Calphostin C inhibited this activation by approximately 50% whereas H-89 was without any significant effect (Fig. 2). These observations

Time (min) of treatment with IL-1 α

Figure 1 Time-course of activation of ERK1/2 in immature Leydig cells by IL-1 α . After incubation with IL-1 α (1 ng/ml) for different periods of time, the cultured cells were lysed and 40 μ g total protein were used to analyze phosphorylated (P-ERK1/2; upper panel) and total (lower panel) ERK1/2 protein levels by Western blotting. Similar results were obtained in two other experiments.

indicate the upstream involvement of PKC but not PKA in the pathway that transduces signals from IL-1 receptors to ERK1/2. As expected, the enhanced phosphorylation of these MAPKs induced by IL-1 α was completely abolished by UO126, a selective inhibitor of the ERK cascade (Fig. 3).

Figure 2 PKC is an upstream component of the pathway resulting in activation of MAPKs in Leydig cells in response to IL-1 α . Cells were pretreated with 10 μ M H-89 or 1 μ M Calphostin C (Calph), selective inhibitors of PKA and PKC respectively, or standard medium alone for 30 min and then stimulated with IL-1 α (1 ng/ml) for an additional 15 min. Whole cell lysates (40 μ g protein) were analyzed for phosphorylated and total ERK1/2 protein by western blotting (upper panel). Each band containing phosphorylated ERK1/2 (P-ERK1/2) was quantified by densitometric scanning using NIH Image software and expressed in relative densitometric units (lower panel). The means $\pm$ S.E.M. for three independent Leydig cell preparations are presented. *** $P < 0.01$ compared with untreated cells; * $P < 0.05$ and ** $P < 0.01$ compared with cells treated with IL-1 α alone.

Figure 3 UO126 inhibits the increase in phosphorylation of ERK1/2 in immature Leydig cells exposed to IL-1 α . Cultured cells were pretreated with 10 μ M UO126 or standard medium alone for 30 min and then stimulated with IL-1 α (1 ng/ml) for 15 min. The cells were subsequently lysed and 40 μ g total lysate protein used to analyze phosphorylated and total ERK1/2 protein by western blotting. Similar results were obtained in two other experiments.

Inhibition of the activation of ERK1/2 in immature Leydig cells exposed to IL-1 α suppresses steroid production

The role of ERK1/2 in IL-1 α -induced steroidogenesis by immature Leydig cells was examined using UO126, a selective inhibitor for MAPK/ERK kinase (MEK), the upstream kinase for ERK1/2. Following UO126 treatment the production of both testosterone and its 5 α -reduced form, 5 α -androstane-3 α ,17 β -diol, were inhibited in a dose-dependent fashion (Fig. 4).

Figure 4 UO126 inhibits the IL-1 α activation of steroidogenesis in immature Leydig cells. Cultured Leydig cells were pretreated with different concentrations of UO126 for 30 min and then incubated with IL-1 α (1 ng/ml) or standard medium alone for an additional 24 h. Testosterone (T) and 5 α -androstane-3 α ,17 β -diol (3 α -Diol) in the culture medium were then measured by RIA and the results expressed as ng/10⁵ cells per 24 h. Each experiment was performed four times independently obtaining similar results. * P <0.05 and ** P <0.01 compared with treatment with IL-1 α and vehicle.

Furthermore, 10 μ M UO126 also significantly inhibited basal steroidogenesis in these Leydig cells.

The site of action of UO126 on IL-1 α -stimulated steroidogenesis in immature Leydig cells

In an attempt to locate the site of action of UO126, the effect of this inhibitor on the production of steroids by immature Leydig cells from both water-soluble cholesterol and a cell-permeable form of cholesterol, 22R-OHC, was investigated. Water-soluble cholesterol was found to significantly stimulate steroidogenesis in immature Leydig cells (a 20-fold increase compared with control). Subsequently, co-treatment of the cells with UO126 and cholesterol or cholesterol and IL-1 α resulted in 4- and 3-fold decreases in androgen production, respectively (Fig. 5A). In contrast, UO126 did not inhibit steroidogenesis induced by the membrane-permeable derivative of cholesterol, 22R-OHC, alone or in combination with IL-1 α (Fig. 5B). These findings suggest that the ERK cascade may be involved in the regulation of cholesterol translocation from the outer to the inner mitochondrial membrane and/or the hydroxylation of cholesterol at the C₂₂ position by mitochondrial cytochrome P450 scc.

Influence of UO126 on the level of expression and degree of phosphorylation of StAR in IL-1 α -stimulated immature rat Leydig cells

We examined whether the inhibition of the ERK cascade by UO126 affects IL-1 α -induced synthesis and phosphorylation of the StAR protein. As expected, IL-1 α alone induced StAR expression significantly (a 25-fold increase compared with unstimulated cells) and detectably enhanced the level of phosphorylated StAR (Fig. 6A). Subsequently, treatment with both IL-1 α and UO126 resulted in a further 2-fold increase in the level of total StAR expression, as well as an 18-fold elevation in the level of its phosphorylated form. UO126 alone also markedly stimulated StAR expression but not StAR phosphorylation.

The increased expression of StAR protein induced in cultured immature Leydig cells by IL-1 α was accompanied by a 5-fold stimulation of androgen production, an effect that was dramatically attenuated by UO126, despite an even higher level of expression and phosphorylation of StAR (Fig. 6B). Similarly, this inhibitor also suppressed basal steroidogenesis by Leydig cells.

UO126 inhibits the transfer of cholesterol into mitochondria of immature Leydig cells

To further examine whether the ERK cascade is involved in the regulation of cholesterol transport across mitochondrial membranes or the hydroxylation of

Figure 5 Effect of UO126 on water-soluble cholesterol- and 22R-OHC-stimulated androgen production in cultured immature rat Leydig cells. Leydig cells were pretreated with UO126 (UO; 10 μ M) for 30 min and thereafter incubated with cholesterol (100 μ M) (A) or different concentrations of 22R-OHC (0.1–10 μ M); (B) in the presence or absence of IL-1 α (1 ng/ml) for 24 h. Androgen levels in the medium were then measured by RIA. Each experiment was performed independently three times with similar results. * P <0.05 compared with treatment with cholesterol; * P <0.05 compared with treatment with cholesterol and IL-1 α .

cholesterol at the C₂₂ position by mitochondrial cytochrome P450 scc, we measured the activity of cytochrome P450 scc directly in whole Leydig cells or in Leydig cell homogenates using water-soluble cholesterol as substrate. Co-treatment of cells with UO126 and cholesterol or cholesterol and IL-1 α resulted in a 5- and 7-fold decrease in steroid production, respectively (Fig. 7A). In contrast, when the mitochondrial membranes were destroyed and cholesterol had free access to cytochrome P450 scc in the cell homogenates, UO126 had no significant effect on the activity of this enzyme (Fig. 7B), indicating that ERKs control the

Figure 6 Effects of UO126 on the expression and phosphorylation of StAR and on androgen production by immature Leydig cells stimulated with IL-1 α . Cells were pretreated with 10 μ M UO126 or culture medium alone for 30 min, followed by incubation with IL-1 α (1 ng/ml) for 24 h. (A) The cells were lysed and whole cell lysates (40 μ g protein) employed to analyze the levels of phosphorylated and total StAR protein by western blotting as described in the Materials and methods section. The data were quantified and expressed as described in the legend to Fig. 2. The positions of total StAR (tot-StAR) and its phosphorylated forms (p-StAR) are indicated by arrows. (B) The total level of androgens secreted in the medium was assayed by RIA. In both A and B, the means $\pm$ S.E.M. for three independent Leydig cell preparations are presented. ** P <0.01 compared with untreated cells; * P <0.05 and ** P <0.01 compared with cells treated with IL-1 α alone.

transfer of cholesterol across the mitochondrial membrane without affecting activity of P450 scc.

Inhibition of the ERK1/2 by UO126 decreases the mitochondrial membrane potential in IL-1 α -activated immature Leydig cells

Since StAR-induced steroidogenesis requires maintenance of an electrochemical gradient across the inner mitochondrial membrane (King *et al.* 1999), we further examined whether UO126 could alter the mitochondrial electrochemical potential in immature Leydig cells. We observed a significant 46% decline in the red/green ratio of JC-1 fluorescence following treatment with UO126 in both control and IL-1 α -treated cells (Fig. 8).

Figure 7 Effect of UO126 on P450 scc activity in whole Leydig cells and their homogenates. (A) Leydig cells were initially incubated with 5 μ M trilostane and 10 μ M UO126 for 30 min prior to incubation with or without cholesterol (100 μ M) and IL-1 α (1 ng/ml) for 3 h. (B) Leydig cells were treated as described in the Materials and methods section and their homogenates were incubated with NADPH (0.5 mM), trilostane (5 μ M) and cholesterol (50 μ M) for 1 h. Pregnenolone and DHEA levels in the medium and supernatant of homogenates were measured by RIA. Each experiment was performed independently three times with similar results. ^{♣♣} $P < 0.01$ compared with treatment with cholesterol; ^{**} $P < 0.01$ compared with treatment with cholesterol and IL-1 α .

Figure 8 UO126 inhibits mitochondrial membrane potential (Ψ_m) in immature Leydig cells. Leydig cells were pretreated with UO126 (10 μ M) for 30 min and thereafter incubated with IL-1 α (1 ng/ml) for another 30 min. The measurement of Ψ_m in a 96-well plate was done as described in the Materials and methods section. Each experiment was performed independently three times with similar results. [♣] $P < 0.05$ compared with untreated cells; ^{**} $P < 0.01$ compared with treatment with IL-1 α .

This observation reflects the loss of the mitochondrial electrochemical potential in these cells.

Subcellular localization of StAR in immature Leydig cells treated with IL-1 α and UO126

To examine whether the UO126- and IL-1 α -induced elevation of StAR expression was located in the mitochondria, immature Leydig cells were stained with anti-StAR antibodies after treatment with IL-1 α with or without UO126. Using specific antibodies to StAR, immunocytochemistry revealed that StAR was undetectable in mitochondria in non-stimulated cells (Fig. 9A). In contrast, clear elevation in mitochondrial StAR was evident after 24 h of treatment with UO126 (Fig. 9B). IL-1 α markedly increased the mitochondrial StAR content (Fig. 9D), and co-treatment with UO126 significantly enhanced IL-1 α -stimulated mitochondrial StAR content (Fig. 9C).

Discussion

The present study demonstrates that the ERK cascade participates in the regulation of steroidogenesis and is a component of the signaling pathways initiated by IL-1 α in the immature Leydig cell. Thus, selective inhibition of the ERKs attenuates IL-1 α -induced steroidogenesis in immature rat Leydig cells despite enhanced expression and phosphorylation of the StAR protein. In contrast, steroidogenesis is not affected by UO126 when a membrane-permeable form of cholesterol (22R-OHC) is used as a substrate in these cells in both the absence and presence of IL-1 α . Further, we demonstrated that at high concentrations the water-soluble form of cholesterol is transferred across the mitochondrial membrane and can bypass StAR function and stimulate steroidogenesis, a process inhibited by UO126. This form of cholesterol is a complex of cholesterol with methyl- β -cyclodextrin, which enhance solubility of this steroid in aqueous solution (Irie *et al.* 1992). Moreover, cholesterol from such a complex was found to easily incorporate into the erythrocyte membrane (Irie *et al.* 1992), suggesting that the same process may enhance cholesterol level in the mitochondrial membrane of Leydig cells. To exclude the possible effects of UO126 on the hydroxylation of cholesterol at the C₂₂ position by mitochondrial cytochrome P450 scc, we measured the activity of cytochrome P450 scc both in intact Leydig cells and in homogenates using water-soluble cholesterol as a substrate. Inhibition of ERK activity by UO126 had no effect on the activity of P450 scc in the cell homogenates, a condition in which the integrity of the mitochondrial membranes was in all probability altered, whereas this process was inhibited in whole cells. Together, these

Figure 9 Subcellular localization of StAR on induction with IL-1 α and UO126. Leydig cells were pre-treated with UO126 (10 μ M) for 30 min prior to incubation with or without IL-1 α (1 ng/ml) for 24 h. The cells were then subjected to immunofluorescence using anti-StAR antibodies, followed by donkey anti-rabbit IgG conjugated to Cy 3. (A) Untreated control; (B) 24 h incubation with UO126; (C) 24 h incubation with IL-1 α and UO126; (D) 24 h incubation with IL-1 α .

findings indicate that the ERKs are involved in the regulation of cholesterol trafficking from the outer mitochondrial membrane to the inner mitochondrial membrane and do not influence the activities of downstream steroidogenic enzymes. In addition, our observation that UO126 dissipates the mitochondrial electrochemical potential in immature Leydig cells (quantified as the ratio of red/green JC-1 fluorescence) suggests that ERKs stimulated by IL-1 α control mitochondrial integrity and function. To the best of our knowledge, this finding provides the first example of modulation of mitochondrial function in steroidogenic cells by the ERK cascade. The observation that cholesterol- but not 22R-OHC-stimulated steroidogenesis was inhibited by UO126 also indicated that an intact Ψ_m across the inner mitochondrial membrane is

required for cholesterol translocation into the matrix as well as for StAR function and import (King *et al.* 1999). Furthermore, our findings demonstrate that PKC functions as an upstream kinase in the transduction of the signal from IL-1RI to the ERK cascade.

We observed that inhibition of ERKs enhanced the expression and phosphorylation of StAR while simultaneously causing a dramatic decrease in androgen production by Leydig cells. In an attempt to explain these contradictory observations, we speculate that after traversing the outer mitochondrial membrane, a step which does not require Ψ_m (Granot *et al.* 2003), StAR and cholesterol probably remain trapped and accumulate in the intermembrane space of mitochondria without import into the mitochondrial matrix due to dissipation of Ψ_m induced by UO126. This possibility is

in agreement with the finding that the expression of C-28 StAR, a nonfunctional StAR mutant having uncoupling activity, leads to a loss of Ψ_m and capture of the truncated protein in the intermembrane space without import into the mitochondrial matrix (Granot *et al.* 2003). In addition, this finding is in line with a recently published report demonstrating that MEK inhibition increases basal and IGF-I-mediated StAR expression, but decreases progesterone synthesis in mouse Leydig tumor cells (Manna *et al.* 2005).

Taken together, our findings are in agreement with the general notion that StAR protein functions to translocate cholesterol from the outer to the inner mitochondrial membrane (Stocco 2001), and that cholesterol requires an intact Ψ_m for its further transfer to the mitochondrial matrix. In addition, recent studies have shown that StAR acts exclusively on the outer mitochondrial membrane (Arakane *et al.* 1996, Bose *et al.* 2002) and, after being imported into the mitochondrion, presumably plays no further role in steroidogenesis (Bose *et al.* 2002). It has also been reported that accumulation of StAR can potentially damage mitochondria (Granot *et al.* 2002, 2003) but we found no changes in viability in UO126-treated cells compared with control cells (as assessed using the cell-proliferation reagent WST-1; data not shown).

Reports concerning the potential involvement of ERK1/2 in the regulation of steroidogenesis in different steroid-producing cells appear to be contradictory, some documenting stimulatory effects (Cameron *et al.* 1996, Das *et al.* 1996, Gyles *et al.* 2001, Martinelle *et al.* 2004) and others inhibitory effects (Seger *et al.* 2001, Tajima *et al.* 2003). For example, several investigations have shown that luteinizing hormone and follicle-stimulating hormone activate ERK1/2 and enhance steroid production in ovarian cells (Cameron *et al.* 1996, Das *et al.* 1996), whereas stimulation of the ERK cascade by these same gonadotropins in cell lines derived from granulosa cells was observed to suppress steroidogenesis (Seger *et al.* 2001, Tajima *et al.* 2003). In the present study, it was demonstrated that inhibition of IL-1 α -activated ERKs in immature Leydig cells enhanced the level of StAR expression, but decreased steroidogenesis, an observation in agreement with previous reports (Manna *et al.* 2005, Martinat *et al.* 2005).

In a recent study, we have demonstrated that ERKs enhanced the acute stimulatory effects of hCG on *de novo* synthesis of StAR, which were associated with increased androgen production in Leydig cells (Martinelle *et al.* 2004). In contrast, these MAPKs are involved in the downregulation of StAR expression and steroidogenesis that is observed after long-term (24–48 h) treatment of granulosa-derived cell lines with such activators (Seger *et al.* 2001). Thus the mechanisms underlying acute and long-term inhibition of ERK in steroid-producing cells may be different, involving modulation of the expression

of various transcription factors and/or of other proteins participating in the regulation of steroidogenesis. In view of the heterogeneity of signaling from hCG/luteinizing hormone and IL-1RI receptors, it is not surprising that the same MAPKs exert variable effects on the regulation of steroidogenesis in Leydig cells. Moreover, differences in the nature of the second messenger systems linked to cell-surface receptors, as well as the variety of overlapping and interacting signal pathways that are functional in various cell lines and tissues, may also help explain these seemingly contradictory findings.

The upstream events that lead to activation of the ERK cascade upon binding of IL-1 α to the IL-1RI have not yet been elucidated. The single relevant report to date demonstrated that tumor necrosis factor α and cAMP can modulate ERK activities in MA-10 mouse Leydig tumor cells (Li *et al.* 1997). Although we show here that in immature rat Leydig cells PKC is involved in the upstream machinery by which IL-1 α regulates the ERK cascade, inhibition of PKC by Calphostin C only inhibits ERK phosphorylation by 50%, suggesting that other kinases could be involved in the phosphorylation of ERK1/2 in these cells. On the basis of findings in other cell types, it can be suggested that this signaling includes activation of phospholipase C, which in turn hydrolyzes phospholipids to liberate diacylglycerol (Dinarello 1991). Diacylglycerol might then trigger PKC to phosphorylate Raf and activate the ERK cascade (Cai *et al.* 1997).

In summary, our present investigation demonstrates that activation of the ERK cascade by IL-1 α plays a dual role in the regulation of steroidogenesis in immature Leydig cells. These MAPKs downregulate the expression and phosphorylation of StAR, while at the same time positively regulating an intact Ψ_m across the inner mitochondrial membrane. As androgens promote the differentiation of Leydig cells (Buzek *et al.* 1988, Misro *et al.* 1993), we hypothesize that the ERK activities represent an important feature of the functions of IL-1 α as a growth and differentiation factor for Leydig cells.

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