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Research Article

A novel interplay between membrane and nuclear melatonin receptors in human lymphocytes: significance in IL-2 production

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Abstract. Human lymphocyte melatonin, through membrane and nuclear receptors binding, acts as an activator in IL-2 production. Antagonism of membrane melatonin receptors using luzindole exacerbates the drop of the IL-2 production induced by PGE_2 in peripheral blood mononuclear and Jurkat cells. This paper studies the melatonin membrane and nuclear receptors interplay in PGE_2-diminished IL-2 production. The decrease in IL-2 production after PGE_2 and/or luzindole administration correlated with downregulation in the nuclear receptor RORα. We also highlighted a role of cAMP in the pathway, because forskolin mimicked the effects of luzindole and/or PGE_2 in the RORα expression. Finally, a significant RORα downregulation was observed in T cells permanently transfected with inducible MT_1 antisense. In conclusion, we show a novel connection between melatonin membrane receptor signalling and RORα expression, opening a new way to understand melatonin regulation in lymphocyte physiology.

Keywords. Melatonin receptors, RORalpha, cAMP, melatonin, MT_1.

Introduction

Melatonin (MLT, N-acetyl-5-methoxy-tryptamine), the first methoxyindole identified in mammalian tissue, is a major human chronobiotic agent with a remarkable role in synchronising physiological processes such as reproduction, metabolism, seasonality, thermoregulation, and immunity [1]. Furthermore, anti-oxidant [2], oncostatic [3] and immunomodulatory [4] effects attributed to MLT have been extensively reported. This functional versatility is linked with an array of molecular mechanisms, i.e., binding to high-affinity G-protein coupled membrane receptors, interaction with cytosolic and nuclear proteins, and direct radical scavenging [5]. Two distinct classes of putative MLT receptors have been reported: 1) cell surface G-protein-coupled receptors (GPCR), termed MT_1 and MT_2 [6]; 2) nuclear retinoid orphan receptors (RZR/ROR) which are members of the superfamily of steroid receptors [7].

In relation to the human immune system, MLT administration stimulates the production of interleukin 2 (IL-2), interferon γ (IFN-γ), IL-6, and IL-12 in *ex vivo* cultured peripheral blood mononuclear cells (PBMCs) [8,9]. MLT also increases IL-1, IL-6, IL-12, TNFα and ROS production as well as decreasing IL-10 levels in human monocytes and macrophages. Furthermore, repression of 5-lipoxygenase gene has been described in B cells after MLT administration [4].

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Some of these immunomodulatory effects occur via a direct action of MLT on its receptors. In fact, both membrane and nuclear MLT receptors have been widely described in the immune system [10].

Although MLT was considered as an exclusive product of the pineal gland for a long time, its identification in a large number of extra-pineal sites has now been confirmed [11, 12]. MLT has been localized in thymus and immune cells such as mast, natural killer, eosinophilic leukocytes, platelets, and endothelial cells [13]. This suggests that the immune system might be affected by this endogenous MLT. Additionally, human lymphocytes have been reported to be another source of naturally synthesized MLT [14]. Blockage of the enzymes required for the MLT synthesis caused a decrease in IL-2 production, which was restored by adding exogenous MLT. These findings support the role of the MLT as an intra, auto-, and/or paracrine factor involved in the IL-2 regulation [15].

Jurkat T cells have also been described as a source of physiologically active MLT, which takes part in IL-2 production through an action mechanism mediated by both membrane and nuclear receptors [16]. Although the MLT/IL-2 connection suggests a relevant role of MLT in immune control, signalling mechanisms are still poorly understood.

Early evidence indicated nuclear receptors as being mainly responsible for MLT effects on IL-2 production [17, 18], but recent observations have also implicated the MT_1 MLT membrane receptors. Thus, exogenous MLT is able to counteract the inhibitory effect of PGE_2 on IL-2 production via its MT_1 receptor through a cAMP-dependent signal transduction pathway [19]. Additionally, after blocking MLT membrane receptors with the specific antagonist luzindole, the inhibitory effect of PGE_2 on IL-2 production was strengthened [15, 16]. Whether membrane and nuclear pathways are independent or coupled, remains elusive. Therefore, the current study was designed to evaluate a potential relationship between membrane-triggered signalling on nuclear MLT receptors and the relation with IL-2 production both in human PBMCs and Jurkat cells.

Materials and methods

Compounds. PHA (phytohemagglutinin), PGE_2 and forskolin were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Luzindole was purchased from Tocris (Ellisville, MO, USA). All compounds were dissolved in DMSO (dimethylsulfoxide) and added to the cultures at a final concentration of 1 μM, excepting PGE_2 which was also used at nanomolar concentration. PHA was dissolved in PBS. Control cultures were tested with the according vehicles.

Monoclonal anti-human RORα/NR1F1 (nuclear receptor subfamily 1, group F, member 1) antibody was from R&D Systems (R&D Systems, Minneapolis, MN), and monoclonal anti-GAPDH antibody was from CHEMICON (Chemicon, Germany). Secondary mouse antibody linked to horseradish peroxidase was from Promega (Promega, Madison, WI).

Plasmid Constructions and Transfection. To generate the antisense expression vector, the human MT_1 receptor cDNA was inversely cloned into the vector pcDNA4/TO (Invitrogen, Carlsbad, CA, USA) containing the zeocin-resistance gene and two tetracycline operator sites within the human cytomegalovirus immediate-early promoter to allow for tetracycline-regulated expression of the antisense sequence in transfected cells.

Cell Culture and Inducible Expression System Establishment. T-REx-*Jurkat* cells (Invitrogen) were transfected with pcDNA4/TO-MT1 by the use of DMRIE-C reagent (Invitrogen) in accordance with the manufacturer's protocol and then selected with 100 μg/ml zeocin. The selected cells were grown in RPMI 1640 medium supplemented with 25 mM HEPES, 10 % FBS, 2mM L-glutamine, 10 μg/ml blasticidin and 100 μg/ml zeocin. Induction of antisense expression in transfected cells was accomplished by exposing cells (1×10^6 cells/ml) to 1 μg/ml of tetracycline (Invitrogen) for 24 h.

Cell culture. Human peripheral venous blood was obtained from healthy volunteers (aged 25–55 years). PBMCs were then obtained by centrifugation over 1.077 g/ml Ficoll-Hypaque gradient (Seromed Biochrom KG, Berlin, Germany) [20]. Cells were washed in saline and finally cultured at 0.25×10^6 cells/ml in RPMI 1640 medium supplemented with 25 mM HEPES, 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, Dorset, UK). After incubation at 37 °C in 5 % CO_2 humidified atmosphere, the cells were collected and stored at -80 °C until RNA or protein extraction. The supernatants were stored at –20 °C for IL-2 determinations. Jurkat T cells were cultured under the same conditions as PBMCs.

RNA extraction and reverse transcription. Total RNA was extracted from the samples by a modification of Chomczynski and Sacchi's method using TriPure Isolation Reagent (Roche, Mannheim, Germany) as a denaturing solution and the appropriate chloroform volume. After cell lysis and RNA extraction, RNA was precipitated with isopropanol, and the pellet was washed in 75 % ethanol. The RNA samples were recovered by centrifugation at 14 000 g for 5 min and

then dried. Each RNA pellet was dissolved in 50 or 100 µl RNase-free water and then quantified spectrophotometrically at 260 nm.

Single-strand cDNA was then synthesized using the following method: 2 µg of RNA were denatured in 19 µl RNase-free water at 85 °C for 10 min, and then rapidly chilled on ice. Then, 21 µl of a mixture formed by 1X reverse transcription (RT) buffer, 20 mM dithiothreitol (DTT), 2′-deoxyribonucleoside-5′-triphosphates (0.5 mM of each), 40 U recombinant RNasin ribonuclease inhibitor, 0.5 µg oligo $(dT)_{15}$ primer and 200 U Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) were added to give a final volume of 40 µl and incubated for 60 min at 42° C (all reagents were purchased from Promega, Madison, WI, USA). The RT reaction was terminated by placing it on ice after deactivation at 95° C for 5 min.

Real-time PCR. Real-time quantitative PCR was performed by the MiniOpticon cycler system (Bio-Rad®). Reactions were performed in a 25 µl volume containing 5 µl of RT product as template DNA (200 ng), 2X FastStart SYBR Green Master (Roche Molecular Biochemicals) and 200 nM for each primer set (Table 1). The following experimental protocol was used: after an initial denaturation step (95 °C for 10 min.), denaturation, amplification and extension steps were repeated 45 times (95 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s with fluorescent data acquisition after extension step), melting curve program (60 °C – 95 °C with a heating rate of 0.5 °C/s with continuous fluorescence measurement) and finally a cooling step at 30 °C.

The cycle number at which the fluorescent signal of a given reaction well crossed the threshold value was denoted as the C_T. C_T data for multiplex targets were normalized to the internal standard, β-actin C_T, by use of the formula: ΔC_T = target C_T-internal standard C_T. Calculations for relative quantitation were performed as outlined in User Bulletin #2: ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The relative expression of each mRNA was calculated with the comparative $\Delta\Delta C_T$ method. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This is a subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. The relative amount of each mRNA was determined by evaluating the expression of $2^{-\Delta\Delta CT}$.

Table 1. Primers used in the real-time quantitative PCR assay

Primer	Sequence (5'→ 3')
β-actin	
Forward	GCCATGTACGTTGCTATCCA
Reverse	CATGAGGTAGTCAGTCAGGT
RORα	
Forward	CGCACCGCGCTTAAATGATG
Reverse	CATACAAGCTGTCTCTCTGC
IL-2	
Forward	ATGTACAGGATGCAACTCCTGTCTT
Reverse	GTCAGTGTTGAGATGATGCTTTGAC

Western blot analysis. Cells were solubilized for 30 min at 4 °C in lysis buffer containing 50 mM HEPES; pH 7,5; 150 mM NaCl; 1,5 mM $MgCl_2$; 1 mM EGTA; 10 % glycerol; 1 % Triton X-100 with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, protein concentration was determined by the Bradford method [21]. Total proteins (50 µg) were denatured at 85 °C for 5 min in Laemmli Buffer 2X (Sigma-Aldrich, St. Louis, MO, USA), subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. The membranes were blocked with Tris-buffered saline-0.05 % Tween 20 (TBST) containing 5 % non-fat dry milk for 1 h at RT. The blots were then incubated with an antihuman RORα antibody for 2 h, washed in TBST, and further incubated with a secondary antibody linked to horseradish peroxidase. Bound horseradish peroxidase was visualized using a highly-sensitive chemiluminescence system (Super Signal; Pierce, Rockford, IL, USA). The bands obtained in the blots were scanned and analyzed by the PCBAS2.0 program. The amount of protein loaded in each lane was controlled by immunoblot with anti GAPDH antibody.

IL-2 determination. IL-2 concentrations in the cell free supernatants were determined by specific BD OptEIA™ Set ELISA (Pharmingen, San Diego, CA, USA). Mouse anti-human IL-2 in 0.2 M sodium phosphate pH 9.0 was coated overnight to high-binding microtiter plates. The plates were washed twice with PBS/0.05 % Tween 20, incubated with 1 % BSA in PBS for 1 h as a blocking step, and washed again. Samples and standards (recombinant human IL-2, from 0 to 2000 pg/ml) were diluted in 1 % BSA PBS and incubated overnight. After being washed three times, biotinylated mouse anti-human IL-2 Ab was added, and bound IL-2 detected using streptavidin-horseradish peroxidase conjugate, and 3,3′, 5,5′tetramethylbenzidine and hydrogen peroxide as the substrate of the enzyme. The reaction was stopped by adding 2N HCl.

Analysis of data. Differences between two groups were evaluated with Student's *t*-test. Differences among more than two groups were evaluated by one-way ANOVA with *post-hoc* (Newman-Keuls) testing. A probability value (*P*) less than 0.05 was considered statistically significant. Statistical analyses were performed using SYSTAT 10 program (Systat Software Inc., Richmond, CA, USA).

Results

Modulation of RORα expression by PGE_2 and luzindole in Jurkat cells. Early results from our group describing that the inhibitory effect of PGE_2 on IL-2 production by Jurkat cells was significantly higher when MLT membrane receptors were blocked using luzindole [16] were confirmed. Thus, PGE_2 decreased IL-2 production in a dose-dependent manner. This inhibitory effect was even higher in presence of luzindole, reaching complete inhibition after incubating cells at micromolar concentration of PGE_2 and luzindole (Fig. 1). Two significant readouts rise from this data: 1) the lymphocyte innate proliferate response after PGE_2 treatment is modulated by endogenous MLT; 2) membrane receptors play a critical role in the autocrine/paracrine effect of the endogenous MLT. However, we wondered what was happening with nuclear MLT signalling when IL-2 production was totally inhibited. To determine whether nuclear MLT receptors were in any way altered in our culture conditions, we studied the RORα expression levels by RT-PCR and Western blot. PGE_2 significantly decreased the RORα mRNA expression, and the inhibition was even higher when the cells were incubated with luzindole. The concomitant administration of PGE_2 and luzindole led to a significant reduction in the RORα expression compared to each one individually (Fig. 2A). In accordance with the mRNA expression study, the RORα protein levels also diminished in the presence of both compounds (Fig. 2B), showing a decrease at translational level as well. Equal loading was controlled by re-probing the blot with an anti-GAPDH antibody.

Effect of PGE_2 and luzindole on IL-2 levels in PBMCs. To test that our findings were not a restricted response to the Jurkat cell line, we moved on to a more physiological system such us *ex vivo* culture of human PBMCs, where IL-2 mRNA expression was also inhibited by PGE_2 (Fig. 3A). The decrease (approximately 90 %) was not higher after adding luzindole to the cultures. At the same time we assayed IL-2 production in the supernatants of these cultures and a decrease in IL-2 levels was also observed both in the presence of PGE_2 and luzindole together with PGE_2 (Fig. 3B).

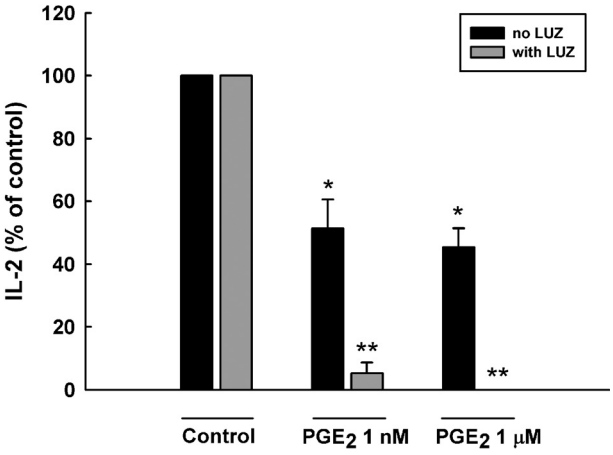

Figure 1. Effect of luzindole (LUZ) on PGE_2-inhibited IL-2 production in Jurkat cells. Cells were incubated for 24 h with PHA (2μg/ml), at the indicated concentrations of PGE_2, and when indicated 1μM LUZ, and IL-2 content was determined by ELISA in culture supernatants. Data are expressed as mean values ± SE of eight experiments performed in triplicate. Control values of IL-2 production: 654,04 ± 21,3 (no LUZ) and 615,74 ± 76,9 (with LUZ) *Significantly lower ($P<0.001$) than control group (no PGE_2). **Significantly lower ($P<0.001$) than corresponding PGE_2-treated group without LUZ.

RORα is downregulated by PGE_2 and luzindole in PBMCs. Having demonstrated the inhibition of IL-2 production by PGE_2 and luzindole, we investigated the RORα expression in stimulated PBMCs under the described conditions. Whereas luzindole inhibited approximately 60 % on mRNA levels, 80 % inhibition was obtained with 10^{-6} M PGE_2, the decline was even higher when both compounds were administered together (Fig. 4A). Furthermore, the inhibition of mRNA levels correlated with a decrease in RORα protein levels, as observed by Western blot assays (Fig. 4B).

cAMP downregulates RORα expression in PBMCs and Jurkat cells. It is well established that binding of PGE_2 to specific membrane receptors inhibits lymphocytes IL-2 production by elevating cAMP levels [22]. On the other hand, MLT inhibits forskolin-stimulated cAMP production in human lymphocytes via membrane receptors coupled to Gi proteins [23]. Moreover, we have previously shown that the MLT binding to human lymphocyte MT_1 receptors counteracts cAMP-dependent inhibitory effect on IL-2 production driven by PGE_2 [19]. Since MLT and PGE_2 trigger membrane signalling events that converge to cAMP modulation, we studied whether the interplay between both compounds in the regulation of RORα levels involved a cAMP-dependent signal transduc-

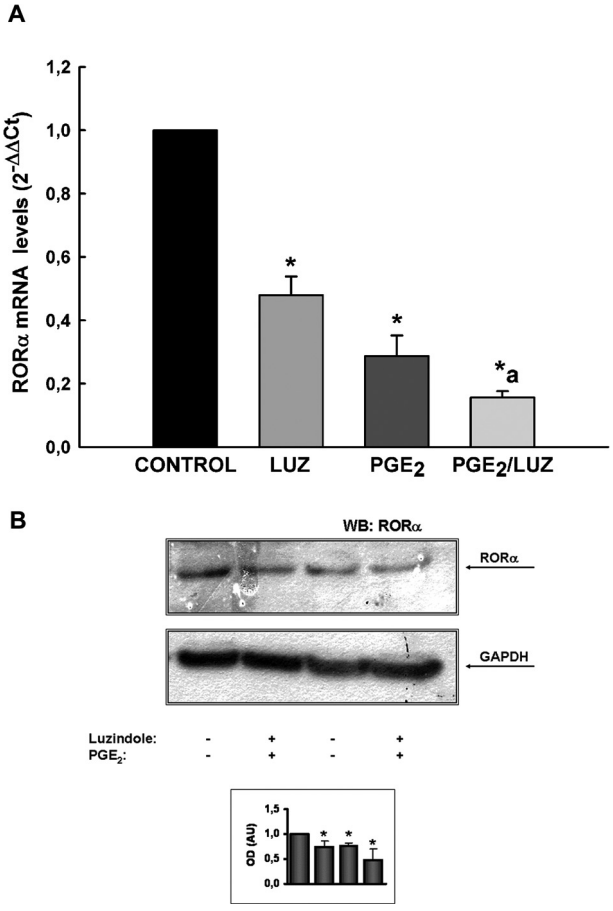

Figure 2. RORα expression is modulated by PGE_2 and luzindole in Jurkat cells. Cells were incubated for 24 h with PHA (2 μg/ml) together with 1μM LUZ or PGE_2. **2A.** Real-time PCR was performed and results of RORα mRNA expression were calculated in relation to stimulated cells without treatment after normalization against β-actin. Data were obtained from three experiments performed in triplicate. Statistically significant differences were determined (*, $P<0.05$), compared with control group, or compared with LUZ-treated cells (a, $P<0.05$). **2B.** After treatment, the cells were analyzed for RORα content by Western blot. A representative picture of RORα from three experiments is shown above in a graph representing quantification of the bands by densitometry. Anti-GAPDH immunoblot was employed to check the general amount of protein in the blots. Significant differences were observed compared with control group (*, $P<0.05$).

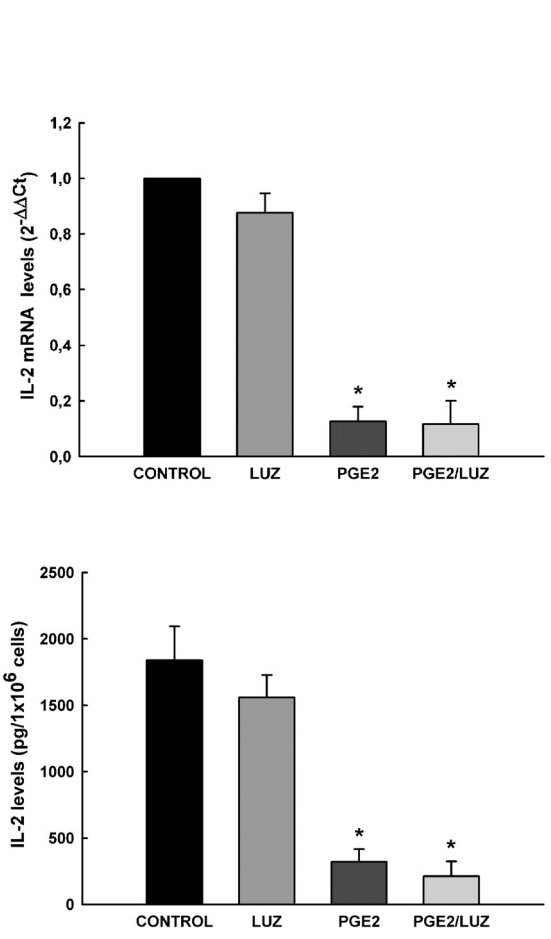

Figure 3. Effect of luzindole and PGE_2 on IL-2 levels in PBMCs. Cells were incubated for 24 h with PHA (2 μg/ml) together with 1μM LUZ or PGE_2. **3A.** Real-time PCR was performed and results of IL-2 mRNA expression were calculated in relation to stimulated cells without treatment after normalization against β-actin. Data were obtained from three experiments performed in triplicate. **3B.** IL-2 content was determined by ELISA in culture supernatants. Data are expressed as mean values ± SE of six experiments performed in duplicate. Statistically significant differences were determined (*, $P<0.05$) compared with control group and LUZ-treated cells.

tion pathway. Thus, in the presence of 10^{-6} M forskolin both human PBMCs and Jurkat cells underwent a significant decrease of RORα mRNA expression (Fig. 5A, 5C). This decrease correlated with a drop in protein levels when forskolin was added to the PBMCs cultures (Fig. 5D), while a slight decline was observed in Jurkat cells (Fig. 5B).

Transcriptional blockage of MT_1 receptors inhibits the RORα expression. The amplified effect of PGE_2 on the RORα expression after luzindole administration highlights a connection between MLT membrane receptors signalling and the modulation of MLT nuclear receptors expression. However, luzindole is a relatively specific MT_1 receptor antagonist with high affinity for both membrane receptor subtypes [24], and the MT_2 receptor activation also inhibits cAMP formation induced by forskolin [25]. Thus, we investigated the relative significance of the MT_1 receptor in the previously observed decrease of RORα levels by using Jurkat cells transfected with a tetracycline-controlled MT_1 antisense expression system. After 24 h of tetracycline incubation, the decrease in RORα mRNA levels was comparable to that shown after luzindole treatment (Fig. 6) supporting a main role of the MT_1 receptors in the process.

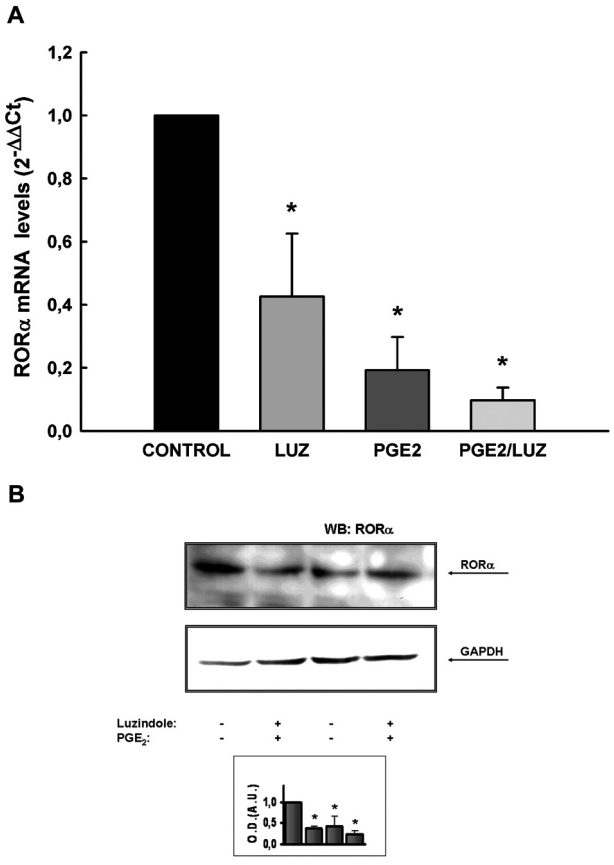

Figure 4. Effect of luzindole and PGE_2 on RORα melatonin receptor expression in PBMCs. Cells were incubated for 24 h with PHA (2 μg/ml) together with 1μM LUZ or PGE_2. **4A.** Real-time PCR was performed and results of RORα mRNA expression were calculated in relation to stimulated cells without treatment after normalization against β-actin. Data were obtained from three experiments performed in triplicate. Statistically significant differences were determined (*, $P<0.05$) compared with control. **4B.** After treatment, the cells were analyzed for RORα content by Western blot. A representative picture of RORα from three experiments is shown above in a graph representing quantification of the bands by densitometry. GAPDH loading control is also shown. Statistically significant differences were determined (*, $P<0.05$) compared with control.

Discussion

Although originally described as a potent *in vitro* T cell growth factor [26], the main non-redundant role of interleukin-2 (IL-2) *in vivo* is now known to be the maintenance of peripheral T cell tolerance. Besides promoting the proliferation and survival of recently activated effectors T cells [27], IL-2 also plays a critical role in regulatory T cell (Treg) homeostasis and is involved in thymic development, peripheral homeostasis and suppressive function of Tregs, reviewed in [28]. The description of Tregs cells as a pivotal element controlling immune-mediated diseases such as autoimmunity has emerged onto the immune scene as a revolutionary concept [29]. Therefore, a better understanding of the mechanisms underlying the natural occurrence and control of Tregs presents a new therapeutic opportunity.

The effect of prostaglandins (PG) as potent, local regulators of the immune response is well known. To this effect, the PGE_2 capacity to inhibit human T cell responses through an IL-2 decrease is well established [22]. A PGE_2-mediated increase in the inhibitory function of human Tregs as well as induction of regulatory phenotype in CD4+CD25- T cells has been recently described [30, 31]. PGE_2 exerts its effects through binding to at least four different receptors, termed E-prostanoid (EP) 1 to EP4, which activate different second messengers, accounting for the specificity and diversity of PGE_2 effects. The EP1 receptor induces a rise in intracellular calcium concentration. The EP2 and EP4 receptors activate Gs and thus increase intracellular cAMP concentration. Multiple EP3 isoforms have been described, which couple to different signalling pathways [32].

We have previously described that the inhibitory effect of PGE_2 on IL-2 production in Jurkat cells was significantly strengthened after blocking MLT membrane receptors by using luzindole, reaching complete inhibition at micromolar concentration of PGE_2 [16]. This fact indicates a critical role of endogenous MLT in the natural control of lymphocyte activation, as has been previously shown in PHA-activated human PBMCs [14]. Likewise, a significant function of membrane receptors in the process emerges. Additionally, involvement of nuclear receptors for MLT in the regulation of cytokines production, such as IL-2, has been described both in human lymphocytes [8] and Jurkat cells [33]. Due to this, we wondered why endogenous MLT was not able to overcome the decrease in IL-2 production induced by PGE_2 and/or luzindole via binding to its nuclear receptors. Firstly, we assayed the nuclear MLT receptor status in Jurkat cells. Interestingly, RORα mRNA and protein levels were downregulated by PGE_2, whereas the inhibition was more drastic after incubation with luzindole along with PGE_2. A single administration of luzindole also downregulated RORα expression, highlighting an interesting role of MLT membrane receptors activation in the nuclear receptors regulation. In addition, the downregulation of RORα could also be caused, as there were more MLT levels available for nuclear receptors. These results suggest that the decrease in RORα might be responsible for the fall in IL-2 production induced by PGE_2.

When trying to determine whether the inhibitory effect on MLT nuclear receptor expression could be reproduced in a more physiological system as normal human T cells, *ex vivo* cultured human PBMCs were used to analyze the IL-2 levels, in the first place, and

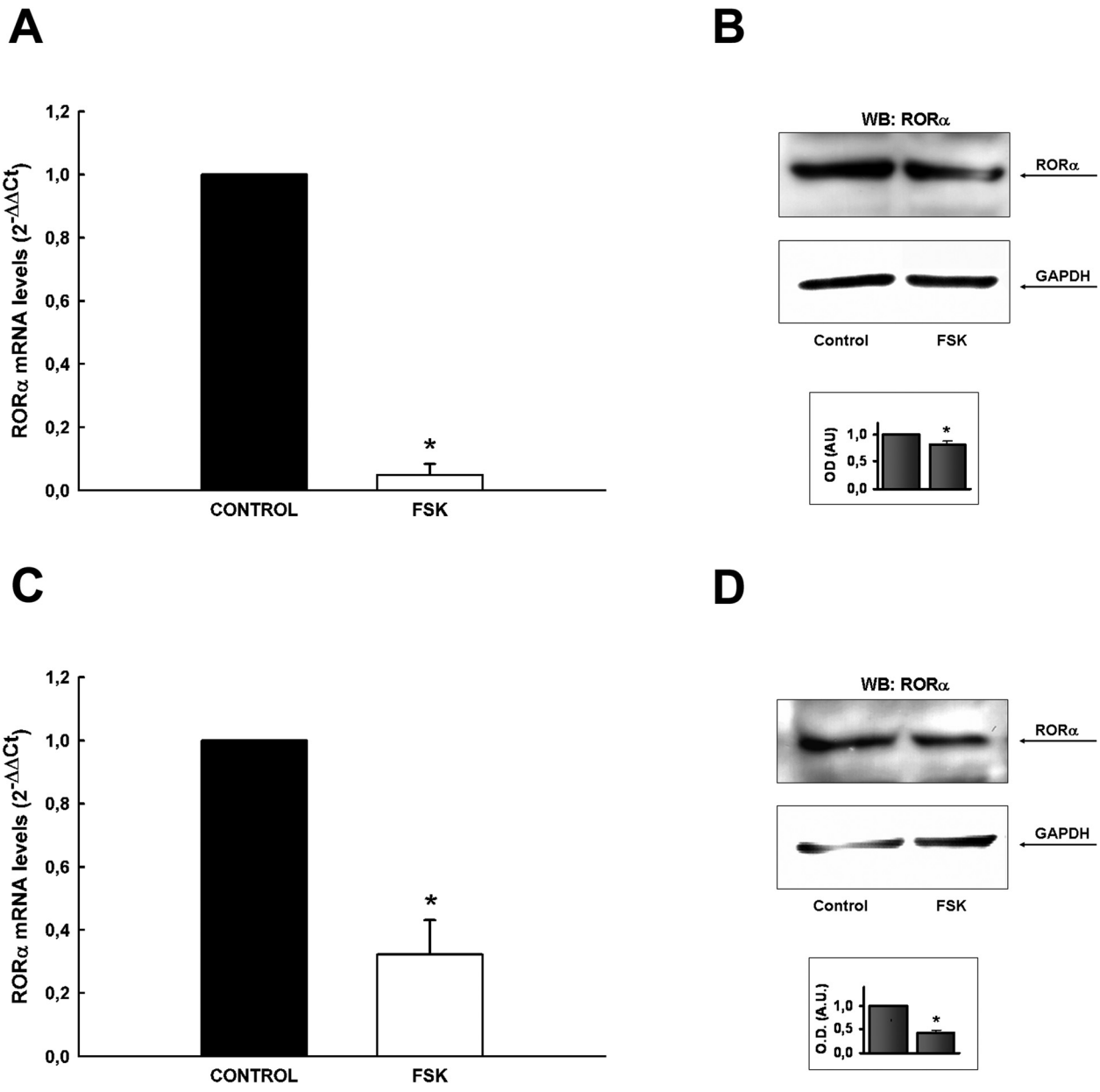

Figure 5. Effect of Forskolin (FSK) on RORα melatonin receptor expression in Jurkat (A, B) and PBMCs (C, D). Cells were incubated for 24 h with PHA (2 μg/ml) together with 1μM FSK. Real-time PCR was performed and results of RORα mRNA expression were calculated in relation to stimulated cells without treatment after normalization against β-actin. Data were obtained from three experiments performed in triplicate. Significant differences were observed between cells treated with FSK and group without FSK (*, $P<0.05$). After treatment, the cells were analyzed for RORα content by Western blot. Representative pictures of RORα and GAPDH from three experiments are shown.

RORα expression later on. Similar results to those in Jurkat cells were observed, while the slight differences can be attributed to the complexity of the PBMCs system with many possible interactions between different cell populations. The reason why the IL-2 production after luzindole administration did not show a significant statistically drop might lie in the inability of the sub-stimulated cells (PHA 2 μg/ml) to produce an adequate amount of membrane receptors-bound MLT. Supporting this matter, substantial amounts of endogenously-synthesized MLT have been described, occurring only under full activation conditions [19].

The ability of PGE_2 to inhibit IL-2 production through an increase in cAMP levels has been well established [34], while MLT binding to membrane receptor in human lymphocytes is coupled to a pertussis toxin-sensitive G protein that involves the inhibition of adenylyl cyclase [23]. Therefore, we studied whether the cAMP-dependent mechanism could be involved

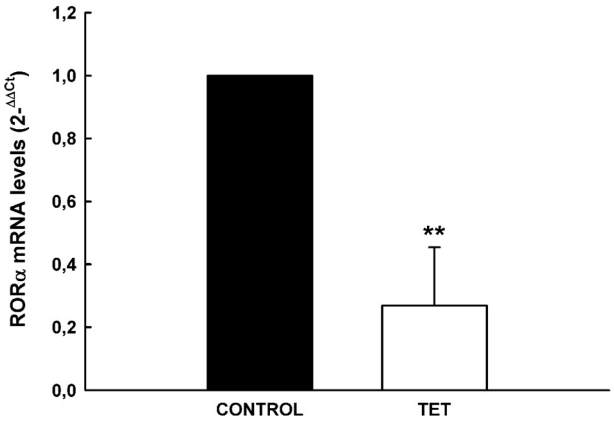

Figure 6. Effect of MT_1 antisense induction on RORα levels in Jurkat cells. Cells were incubated for 24 h with tetracycline (1 μg/ml) for maximum induction of antisense and then for 24 h with PHA (8 μg/ml). Real-time PCR was performed and results of RORα mRNA expression were calculated in relation to stimulated cells without tetracycline after normalization against β-actin. Data were obtained from three experiments performed in triplicate. Significant differences were observed between cells treated with tetracycline and group without the antibiotic (**, $P<0.001$).

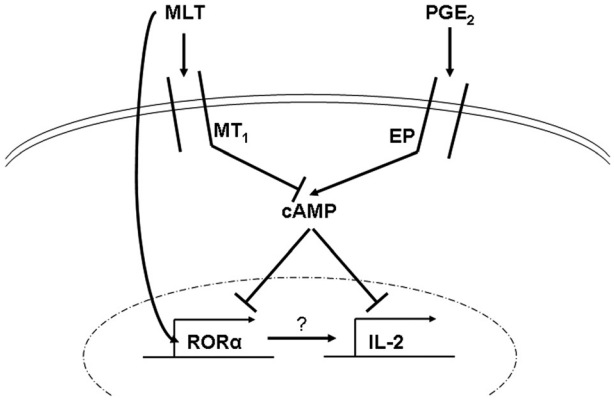

Figure 7. Schematic picture of how the MT_1-RORα interaction might control the IL-2 production in human lymphocytes. The binding of melatonin (MLT) to MT_1 receptors in the lymphocyte membrane overcomes the increase in PGE2-induced cAMP levels, giving rise to the RORα and IL-2 up-regulation. It is not known whether in the absence of exogenous MLT the RORα downstream signal modulates the IL-2 expression. PGE_2 receptors were named in a general way as EP. Arrows indicate a stimulatory effect. Back-crossed lines indicate an inhibitory effect. The question mark indicates a signalling pathway which still requires further investigation.

in the RORα inhibition induced by luzindole and PGE_2. After incubation with forskolin, Jurkat T cells and PBMCs showed lower levels of RORα, suggesting cAMP-mediated regulating. In line with this, it is generally accepted that the classical nuclear receptors function is regulated by phosphorylation. In fact, phosphorylation by upstream-activated protein kinases through signal transduction from membrane bound receptors has been described as a potential mechanism of orphan nuclear receptor activation-inhibition [35]. Thus, activation of MT_1 or MT_2 receptors by MLT would lead to inhibitory responses on the cAMP signal transduction cascade, resulting in decreased activity of PKA and the ensuing decline in CREB phosphorylation, reviewed in [36], which, in turn, would affect RORα expression levels (Fig. 7). Besides the cAMP-dependent cascade, MT_1 receptors can couple to a stimulation of PLC-dependent signal transduction cascades and can activate PKC [37]. In addition, the transcriptional activity of RORα can also be enhanced by activation of the calcium-signalling pathway, as some authors have suggested [38].

MT_1 and MT_2 receptors are G-protein coupled receptors that exhibit sub-nanomolar affinity for MLT [39]. Although luzindole has a 25-fold higher affinity for MT_2 receptor than for MT_1 receptor [24], both receptors are fully blocked with administration of luzindole at micromolar concentrations [40]. Consequently, the relative contribution of either receptor to the process required further approaches. For that reason, the modulation of RORα expression was tested in Jurkat T cells permanently transfected with an inducible MT_1 antisense. The significant down-regulation of RORα mRNA, by approximately 70 %, suggests a feasibly role of the MT_1 receptors in the event. In line with this, other authors have described that MLT and the MT_1 agonist, AMMTC (N-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole) can repress the transcriptional activity of the RORα in human breast cancer cells [41].

In conclusion, in the present study we describe for the first time a molecular mechanism controlling the expression of the MLT nuclear receptor RORα via the activation of the MT_1 membrane receptor in the immune system. Additionally, the way in which this novel interplay could regulate the IL-2 production by lymphocytes is suggested (Fig. 7), thus opening a new path to understanding MLT regulation in lymphocyte physiology.

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