J Bioenerg Biomembr (2011) 43:739–746
DOI 10.1007/s10863-011-9388-x

In vitro mitochondrial effects of PK 11195, a synthetic translocator protein 18 kDa (TSPO) ligand, in human osteoblast-like cells

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Received: 2 August 2011 / Accepted: 1 October 2011 / Published online: 30 November 2011

Abstract The role of the TSPO in metabolism of human osteoblasts is unknown. We hypothesized that human osteoblast metabolism may be modulated by the TSPO. Therefore we evaluated the presence of TSPO in human osteoblast-like cells and the effect of its synthetic ligand PK 11195 on these cells. The presence of TSPO was determined by [^{3}H]PK 11195 binding using Scatchard analysis: Bmax 7682 fmol/mg, Kd 9.24 nM. PK 11195 did not affect significantly cell proliferation, cell death, cellular viability, maturation, [^{18}F]-FDG incorporation and *hexokinase* 2 gene expression or protein levels. PK 11195 exerted a suppressive effect on VDAC1 and caused an increase in TSPO gene expression or protein levels. In parallel there was an increase in mitochondrial mass, mitochondrial ATP content and a reduction in $\Delta\Psi$m collapse. Thus, it appears that PK11195 (10^{-5} M) stimulates mitochondrial activity in human osteoblast-like cells without affecting glycolytic activity and cell death.

Keywords Osteoblast · TSPO · VDAC · Hexokinase 2 · Mitochondria · ATP · Cell death

Introduction

The mitochondrial 18 kDa Translocator Protein (TSPO) is known to play a regulatory role in cellular apoptosis and steroid synthesis (Veenman et al. 2007). The TSPO is primarily localized on the outer mitochondrial membrane and can interact with the 32 kDa voltage dependent anion channel (VDAC) (Veenman et al. 2002). VDAC is a key factor in controlling the mitochondrial membrane potential ($\Delta\Psi$m) and ATP/ADP transfer in mitochondria, glycolysis and oxidative phosphorylation. It is also involved in the mitochondrial cellular apoptotic pathway (Casellas et al. 2002; Kroemer and Reed 2000; McEnery et al. 1993; Veenman et al. 2008). VDAC and TSPO are thought to be components of the mitochondrial permeability transition pore (MPTP), which is a complex of proteins located on and adjacent to the mitochondrial outer and inner membranes and includes several constituents, the 30 kDa adenine nucleotide translocator (ANT), hexokinase, *creatine kinase* and cyclophilin D (Casellas et al. 2002;

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Veenman et al. 2008; Papadopoulos et al. 2006). The interactions between the TSPO, VDAC and ANT, although being suggested to be important in cellular apoptosis (Casellas et al. 2002), are not completely understood (Baines et al. 2007; Galluzzi and Kroemer 2007). These three proteins, and especially TSPO and VDAC, are considered to comprise one functional entity (Veenman et al. 2002), and their role in cellular physiology of steroidogenic organs and particularly in mitochondrial cholesterol transport was extensively investigated (Avital et al. 2001; Bar-Ami et al. 1991; Fares and Gavish 1986; Krueger and Papadopoulos 1992; Papadopoulos 1993). The interaction of the hexokinase 2 and VDAC has been previously suggested to influence mitochondrial apoptosis by regulating the VDAC state of permeability (Lemasters and Holmuhamedov 2006). TSPO was also shown in various studies to regulate the $\Delta\Psi m$ (Kugler et al. 2008; Shoukrun et al. 2008; Zeno et al. 2009).

In spite of the intriguing evidence of its role in cellular metabolism, data on the role of the TSPO in the metabolism of connective tissue cells of mesenchymal origin are scarce (Kugler et al. 2008; Shoukrun et al. 2008; Zeno et al. 2009; Gavish et al. 1999). Several reports demonstrated the existence and the role of the TSPO in fibroblasts or their precursors, where TSPO were found in a density that is comparable to that of steroidogenic cells (Kroemer and Reed 2000; Weissman et al. 1990). There is no information available on the metabolic role of the TSPO in the human osteoblast, but according to its important cellular role in general and its identification in other mesenchymal cells, we hypothesized that osteoblast metabolism may be modulated by the TSPO.

In order to assess its role in human osteoblast, we evaluated the presence of TSPO in human osteoblast-like cells. We assumed that the TSPO specific synthetic ligand PK11195 might display a regulatory effect on osteoblast-like cell metabolism, based on the previous reports on the involvement of TSPO in the induction of apoptosis and modulation of cellular ATP accumulation (Veenman et al. 2010; Pastorino and Hoek 2008).

To this end we assessed in human osteoblast-like cells the *in vitro* effects of PK 11195 (10^{-5} M) on cell viability and proliferation, as well as mitochondrial mass, mitochondrial membrane potential ($\Delta\Psi m$) and ATP production.

Materials and methods

TSPO ligand

For the experiments we used a synthetic ligand to TSPO—isoquinoline carboxamide (PK 11195, supplied by Sigma-Aldrich Ltd, St. Louis, MO). The ligand was used for treatment of cells at a concentration of 10^{-5} M. The concentration of the ligand was chosen as similar to a previous study on other mesenchymal cells (fibroblasts) where the cellular role of TSPO was investigated (Weissman et al. 1990).

Cell culture

Bone chips of cancellous bone were prepared from disposable bone samples, which were collected from proximal femora of six donors (two men, four women, age range 71–85 years) during fractured hip arthroplasties. No osteoarthritic changes in the femoral head were evident under direct inspection and the samples were collected from the femoral canal—at least 4 cm distant from subchondral area. The bone samples, 2–3 g in total each, were incubated in osteogenic medium (Gundle et al. 1998; Yamanouchi et al. 2001): DMEM (Dulbecco's Modified Eagles Medium, Biological Industries Ltd, Bet Haemek, Israel) with heat-inactivated fetal calf serum (10%), 20 mM HEPES buffer, 2 mM L-glutamine, 100 μM ascorbate-2-phosphate, 10 nM dexametasone, 50 U/ml penicillin, 150 μg/ml streptomycin, at 37 °C in humidified air with 5% CO_2 (v:v) for 20–30 days. Human osteoblast-like cells grow out from the chips as a primary cell culture adherent to the plastic tissue culture plates (non-pyrogenic polysterene). The human bone cell cultures obtained by this standard method have been shown previously to express osteoblast-like characteristics, i.e. polygonal multipolar morphology, expression of the enzyme alkaline phosphatase, synthesis of a collagen-rich extracellular matrix with predominantly type I collagen and small amounts of collagen type III and V, and non-collagenous proteins such as sialoprotein (BSP) and osteocalcin (Gundle et al. 1998; Yamanouchi et al. 2001).

Additionally these cells demonstrate matrix mineralization in vitro and bone formation in vivo ((Gundle et al. 1998; Yamanouchi et al. 2001)). Furthermore, we have previously shown the osteoblast characteristics of these cells, including positive Von Kossa staining, synthesis of osteopontin, characteristic multipolar morphology and adherence to plastic surface, as well as cellular alkaline phosphatase, and osteocalcin content (Rosenberg et al. 2010). In the present study, cells were allowed to migrate from the bone chips into the medium, and proliferate in 75 cm^2 culture flasks for 21 days. Then the cells were passaged into 24 well plates, where each well was seeded with 10^4 cells. The cells were counted by direct microscopic inspection and kept at the same conditions prior to experiment commencement.

The use of these cells for the experiments was approved by the Institutional Ethical Committee.

The cultured samples were treated by PK 11195 (10^{-5} M) for 48 h prior to the biochemical assays and compared to the control cell cultures unexposed to this agent.

TSPO binding characteristics

Cells for the binding assay were cultured to a confluent layer, in 75 cm^2 culture flasks. [^{3}H]PK 11195 (New England Nuclear, Boston, MA) binding to mitochondrial membranes of the osteoblast like cell was assessed, as previously described (Weizman and Gavish 1993). Briefly, the reaction mixture contained 400 μl of the homogenized membranes in question (40 μg protein) and 25 μl of [^{3}H] PK 11195 solution in the concentration range of 0.18–6.00 nM in the absence (total binding) or presence (nonspecific binding) of 75 μl unlabeled PK11195 10 μM. Saturation curve with Scatchard analysis was used to determine the maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d) (Award and Gavish 1993). Each point was measured in duplicate and averaged.

Cell proliferation assay

Cell proliferation in culture was assayed by a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis. For this purpose we used a specially designed kit, cell proliferation ELISA BrdU (colorimetric) (Cat. No 11 647 229 001, Roche Applied Science, Basel, Switzerland). Briefly, after the passage cells were cultured with and without TSPO ligand treatment for 48 h, BrdU was added to the culture samples and they were incubated for an additional 24 h. Then the cells were fixed with 1% paraformaldehyde in PBS and the DNA was denatured by the kit reagents. Then the incorporated BrdU was labeled by the anti-BrdU-POD antibody dilution of 1:100 (Roche Applied Science, Basel, Switzerland) and the immune complexes were detected and read by a spectrophotometer ELISA reader at 450 nm wavelength. The results are expressed in absorbance units.

Apoptosis assay (TUNEL)

In apoptosis, activation of endonucleases results in fragmentation of DNA. A method of labeling DNA breaks due to fragmentation is referred to as Terminal Deoxynucleotide Transferase dUTP Nick End Labeling, or TUNEL (Bortner et al. 1995). In our study, the TUNEL assay was performed by APO-BrdU TUNEL assay kit (A-23210), (Molecular Probes, Eugene, OR). Briefly, the assay was performed as follows: the cells in well-plates (controls and after 48 h treatment by TSPO ligand) were trypsinized. Cells were fixed using 1% paraformaldehyde in PBS for 15 min and washed with PBS. Then the pellet was suspended in ice cold 70% ethanol and stored for 18 h in −20 °C prior to performing the TUNEL assay. Then cells were incubated in DNA labeling solution provided with the kit for 1 h and after that in antibody staining solution provided by the kit for 1 h. Following that procedure, cells were analyzed by flow cytometry using the FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell viability test

We used propidium iodide (PI) to perform viability tests. As described previously PI enters cells with damaged cellular membranes i.e. non-viable cells (Zeno et al. 2009). Its application is based on its fluorescent properties in damaged cells where it intercalates in the DNA (Salvioli et al. 2000). The cells in well-plates (controls and after 48 h treatment by TSPO ligand) were trypsinized, centrifuged and washed with PBS. Then the cells were treated with the solution of PI at the concentration of 5 μg/ml. Flow cytometry was performed 10 min after the exposure to the PI solution. 20,000 cells were examined per each treatment. Cells stained with PI were considered to be necrotic or late apoptotic cells (Salvioli et al. 2000). Percentage of viable, unstained cells, were compared in treated and control samples.

Cell death assay

LDH activity in culture media, due to leakage via damaged cell membranes, indicates the overall cell death. The LDH activity in the collected culture media from treated samples and controls was determined by 340 nm wavelength spectrophotometry of a reduced Nicotinamide adenine dinucleotide (NAD), which is directly proportional to the LDH activity (Gay and Bowers 1968).

[^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG) incorporation

The cells in well-plates (controls and after 48 h treatment by TSPO ligand) were washed with PBS and placed in a glucose free medium. Then 5 μCi [^{18}F]-fluorodeoxyglucose (FDG) were added to the cultures; the cells were incubated at room temperature for 45 min. The cells were then washed with PBS to eliminate free FDG, removed by trypsinization, re-suspended in PBS and counted. The suspended cells were centrifuged at 200 × g. The supernatant was discarded. The pellet and a standard solution were counted in a γ counter (Atomic Products Corporation, Atomlab, NY). The FDG incorporation was

determined as the percentage of the original concentration and normalized to 10^6 cells (Rosenberg et al. 2007).

Alkaline phosphatase activity

The maturation state of the osteoblasts was estimated by the measurement of cellular alkaline phosphatase activity. Following cell counting in each culture sample the media were collected for further assay and cells adherent to the plastic surface were washed by PBS and lysed in 10% Triton X-100 by three cycles of freezing to −20°C and thawing at 20°C. Alkaline phosphatase activity was determined in lysed cell culture samples, after incubation with P-nitrophenyl phosphate substrate, by 410 nm wavelength spectrophotometry (Bessey et al. 1946).

Western blot

Western blot analyses of TSPO, VDAC1 and *hexokinase* 2 were performed using antibodies: against human 18-kDa TSPO (produced in our laboratory (Kugler et al. 2008)), against VDAC 32-kDa component (anti-porin 31 HL(Ab-3), EMB Biosciences Itc. La Jolla, CA), and *hexokinase* 2 (goat polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz,CA). Abundance of the TSPO, VDAC and *hexokinase 2* in osteoblast-like cells exposed for 48 h to PK 11195 were compared to untreated control cells. The measurements were expressed in OD units.

Mitochondrial mass estimation

We used MitoTracker Green FM Probe (MTG) to estimate the mitochondrial mass in live, non-fixated, osteoblast like cells (Oubrahim et al. 2001). Cell culture monolayers, after 48 h of treatment with PK 11195 (10^{-5} M) and vehicle treated controls, were washed with PBS, trypsinized, collected and re-suspended in fresh culture medium. The suspended cells were incubated with 200 nM MTG at 37°C for 30 min in the dark. Then cell suspensions were subjected to flow cytometry using the FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an argon ion laser tuned at 488 nm excitation and 516–530 nm emission). From each sample 10,000 cells were analyzed. The results were expressed as mean fluorescent intensity.

ATP bioluminescence assay

Cells in cultures treated with PK 11195 for 48 h and vehicle control cultures were counted and assayed for ATP content. ATP cellular content was assayed using the CLS II kit (Roche Diagnostics, Mannheim, Germany). The method is based on light emission as part of the catalytic reaction:

$$ATP + D - luciferin + O_2 \rightarrow oxyluciferin + PP_i + AMP + CO_2 + light.$$

The light emission was measured by luminometer (TD—20/20 Luminometer, Turner Designs DLReady, Sunnyvale, CA). The light emission readings were normalized to the cell number.

Mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial membrane potential in the cellular samples was evaluated by staining with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen™), which is a lipophilic cationic probe (Cossarizza et al. 1993). JC-1 forms red-fluorescent complexes inside the mitochondria with an intact $\Delta\Psi m$ (when exited at 488 nm). JC-1 does not enter mitochondria with a collapsed $\Delta\Psi m$ and remains in its green fluorescent monomeric form in the cytosol. The fluorescence emission shift from green to red in cells stained by the JC-1 probe was indicated by a decrease in the red/green fluorescence intensity ratio. Cells treated with PK 11195 10^{-5} M and vehicle controls, were estimated by flow cytometry (at 590 nm emission for the red aggregates and 529 nm emission for the green monomers) using the FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ), as described previously (Zeno et al. 2009). From each sample 10,000 cells were analyzed. The results were expressed as a ratio between red to green fluorescent intensity in the 10,000 cells. Six samples for each condition were examined.

Real Time PCR (TaqMan)

Total RNA was isolated using the RNeasy Mini Kit (Cat 74104), which is based on Spin Technology for RNA purification (QIAGEN, Austin, TX), following the protocol provided by the manufacturer.

Reverse transcription (RT) reactions were carried out in MicroAmp reaction tubes using a high capacity cDNA reverse transcription kit (Cat 4368814, Applied Biosystems, Foster City, CA). Each reaction tube contained 1 μg of total RNA in a volume of 20 μl containing RT buffer, dNTP, RNase inhibitor, MultiScribe Reverse Transcriptase and random primers. The reverse transcription reaction was carried out at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min, 4 °C for storage.

Reaction products were stored at −20 °C for later use. PCR primers and TaqMan probes for VDAC1, TSPO and *hexokinase 2* were designed and synthesized by Applied

Biosystems (Foster City, CA). TaqMan probes were labeled with the reporter fluorescent dye, FAM(6-carboxyfluorescein), at the 5′ end and a fluorescent dye quencher, TAMRA(6-carboxymethylrodamine) at the 3′ end.

RT-PCR was performed in MicroAmp Optical tubes closed with MicroAmp caps (Applied Biosystems) and using the ABI Prism 7700 sequence detector. Each well contained a cDNA equivalent to 50 ng total RNA, 10 μl TaqMan gene expression master mix, 1 μl primers, the total volume was 20 μl. Amplification conditions were 2 min at 50°C, 10 min at 95°C and then 40 cycles of 95 °C for 30 s, 60 °C for 1 min. Samples were run in triplicate and Ct values averaged. HRPLPO was used as an endogenous reference to TSPO, VDAC and *hexokinase* 2 which were the target genes. Three samples for each treatment were used. The results are presented as mean relative quantification values (RQ), including the range of the RQ values.

Statistical analysis

At least six samples per group were used to compare the PK 11195 treated cells with the vehicle control groups. The results were analyzed by the *t* test or by the non-parametric Mann-Whitney Rank Sum test as appropriate. $P<0.05$ was considered as statistically significant. The results are expressed as mean ± SD.

Results

[^{3}H]PK 11195 binding

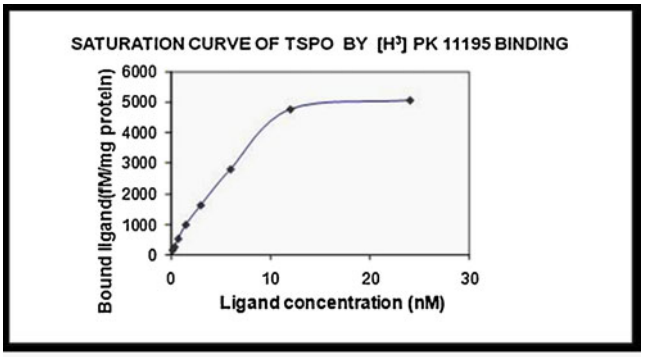
We found that the binding of the ligand [^{3}H]PK 11195 to human osteoblast-like cell TSPO is saturable with a single population of binding sites (linear regression $r=0.92$, Fig. 1). The mean equilibrium dissociation constant (K_d) of the two performed tests was 9.24 nM and mean maximal binding capacity (B_{max}) was 7,682 fmol/mg protein (Fig. 1).

Glucose incorporation

The estimation of cellular metabolism showed that glucose incorporation was not significantly affected by PK 11195 (10^{-5} M) (mean 6.0%, +/− 2.6% dose/10^6 cells in treated cells vs. mean 7.6% +/− 1.6% dose/10^6 cells in controls; $p>0.05$, $n=6$ in each group).

Osteoblast maturation state

Osteoblast maturation state was not affected by the PK 11195 (10^{-5} M), when studying the alkaline phosphatase activity in the cells (mean 0.96 +/− 0.31 U/L/cell in treated cells vs. mean 0.79 +/− 0.19 U/L/cell in controls, $p>0.05$, $n=6$ in each group).



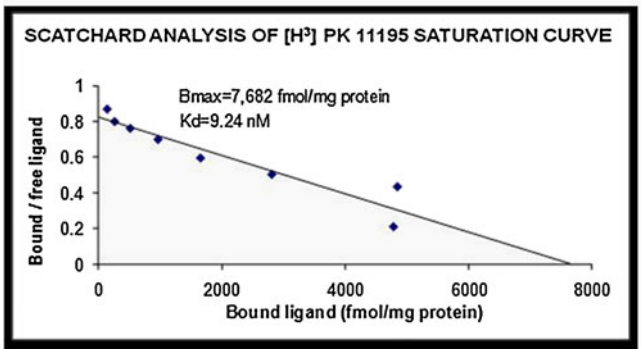


Fig. 1 Scatchard analysis of the saturation curve of [H^3]PK 11195 binding (each point is the average of two assays)

Cell number, death, apoptosis, viability and proliferation

Following treatment by PK 11195 we also did not observe any significant change in cell number in culture (by direct cell counting in low power microscopic field: mean 96 +/− 9 cells/microscopic field in treated cells vs. mean 92 +/− 10 cells/microscopic field in controls); in cell death, i.e. in cell necrosis (by estimation of LDH activity in culture media: mean 3.70 +/− 1.20 U/cell in treated cells vs. mean 3.86 +/− 1.59 U/cell, $n=6$, $p>0.05$) and in apoptosis (using a cytometric assay for the apoptosis rate by application of the TUNEL method, mean 4.4% +/− 0.4% of apoptotic cells in treated cultures vs. 4.1% +/− 0.5% of apoptotic cells in controls, $n=6$ in each group, $p>0.05$). Also, no change was observed in cellular proliferation (by cytometry assaying BrdU incorporation: mean 0.0082 +/− 0.0026 OD/cell in treated cells vs. mean 0.0075 +/− 0.0009 OD/cell in controls, $n=6$, $p>0.05$) and in the cellular viability (cytometric testing of PI incorporation into cells, mean 88% +/− 4% of treated cells, vs. 90% +/− 3% of cells in control samples, $n=6$ in each group, $p>0.05$).

TSPO, VDAC1 and hexokinase 2 proteins and gene expression

With Western blots we found a significant increase in the TSPO abundance in cells treated with PK 11195 in comparison to untreated controls (mean 228026 +/− 41564 OD in treated cells vs. 146071 +/− 22728 OD in controls, n=6, p<0.001, Fig. 2a). However, we found a significant decrease in VDAC1 abundance (mean 36749 +/− 2521 OD in treated cells vs. 42531 +/− 3761 OD in controls, n=6, p<0.001, Fig. 2b) and no significant change of *hexokinase* 2 protein abundance (mean 47260 +/− 13903 OD in treated cells vs. 47267 +/− 7162 OD in controls, n=6 in each group, p>0.05).

Similar to the Western blot analysis, the *hexokinase 2* gene expression, studied by Real Time PCR, was not changed by PK 11195 (mean RQ=1.046, range 0.753–1.451), the TSPO gene expression increased by PK 11195 (mean RQ=1.7, range 1.522–1.963) and the VDAC 1 gene expression was reduced (mean RQ=0.398, range 0.267–0.594).

Mitochondrial characteristics and functions

Treatment with PK 11195 (10^{-5} M) exerted significant effects on mitochondrial characteristics and functions. Mitochondrial mass increased significantly after treatment with PK 11195 (cytometric analysis stained by MTG: mean 257 +/− 2 counts in treated cells vs. 225 +/− 3 counts in controls, n=6 p<0.05, Fig. 3). ATP content in cells was significantly higher following treatment by PK 11195 (mean 55.6 +/− 5.6 bioluminescence/10^6 cells in treated cells vs. mean 24.9 +/− 2.3 bioluminescence/10^6 cells in controls, n=6, p<0.001, Fig. 4). The treatment with PK 11195 also appeared to prevent normal occurrence of $\Delta\Psi$m collapse, according to the JC1 assay i.e. $\Delta\Psi$m collapse occurred more in vehicle treated cells than in PK 11195 treated cells (cytometric analysis of red/green staining/cell: mean 10.94 +/− 3.22 in treated cells vs. mean 7.53 +/− 1.8 in controls, n=6 in each group, p<0.001, Fig. 5).

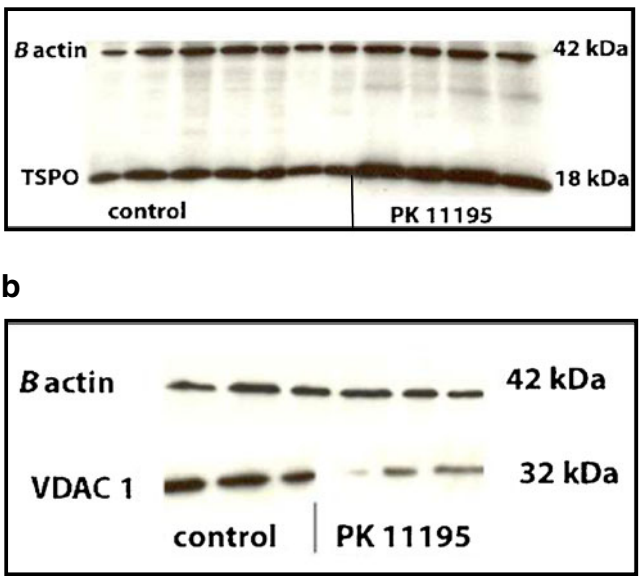


Fig. 2 Western blot analyses of TSPO and VDAC following exposure of osteoblast-like cells to PK 11195 (10^{-5} M). Representative examples of SDS-PAGE of assayed A: TSPO, B: VDAC are given, showing decrease in VDAC and increase in TSPO following treatment with PK 11195

Discussion

The TSPO was identified in the human osteoblast-like cells by the binding of [^{3}H]PK 11195 with nanomolar affinity. TSPO density in the human osteoblast-like cells was found to be similar to or higher than in mammalian tissues with typically high levels of TSPO density, e.g. kidney, placenta, ovary and uterus (Avital et al. 2001; Fares and Gavish 1986; Gavish et al. 1999; Bar-Ami et al. 2006). In addition, the TSPO density in the human osteoblast-like cells also appeared to be higher than in rat skeletal fibroblasts, which are, as osteoblasts, also from mesenchymal origin (Weissman et al. 1990). Western blot and RT-PCR confirmed the abundant expression of TSPO in these cells.

We investigated the potential involvement of the TSPO in the cellular pathways of proliferation, cell death and energy generation by using the specific TSPO synthetic ligand PK 11195 on the human osteoblasts.

The data show that PK 11195 has no effects on overall cell death, as presented by the lack of effect on LDH activity in the media, on cellular PI incorporation and on apoptosis, as seen in the TUNEL assay. These findings are supported by the rise of mitochondrial membrane stability, seen in the treated cells by the JC1 assay, which would counteract initiation of the mitochondrial apoptosis pathways. It was previously reported that PK 11195 can cause cell death in different cell lines through activation of TSPO, possibly via increasing the permeability of the mitochondrial membrane (Veenman et al. 2007; Santidrián et al. 2007). The results of the present study on the human osteoblasts show a different effect, which is mainly anti-apoptotic, because it blocks the collapse of the $\Delta\Psi$m in the mitochondria.

Human osteoblast-like cell viability, proliferation (measured by the BrdU incorporation) and maturation (measured by the cellular *alkaline phosphatase* activity), were not affected by the PK 11195. This is in accord with several previous studies where it was shown that PK 11195's direct interactions appear to be anti-poptotic, while pro-apoptotic effects may be non-specific, i.e. not

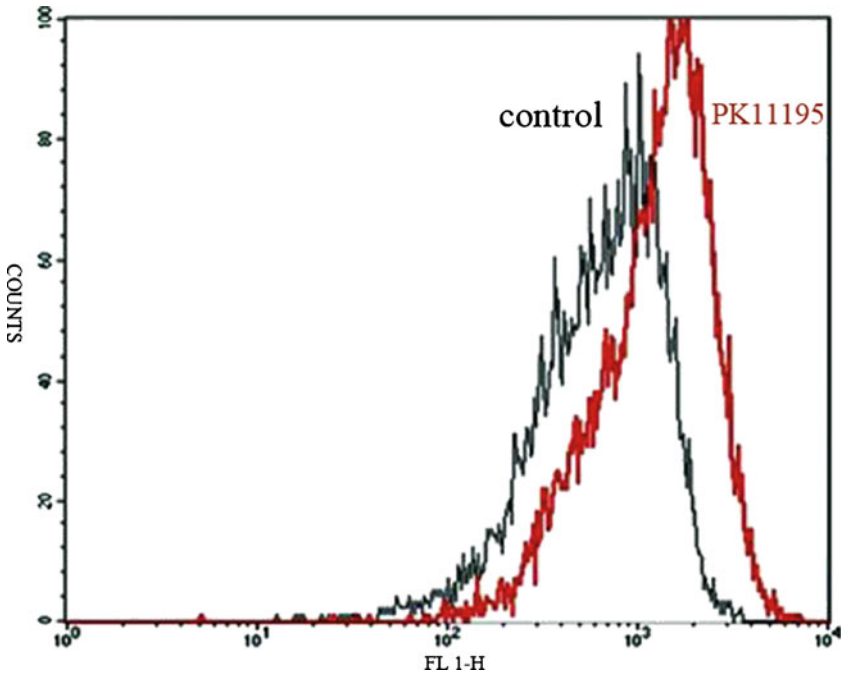


Fig. 3 Mitochondrial mass. A representible example of flow cytometry overlay histograms of cells stained by MTG. The histogram of the mitochondrial mass in osteoblast-like cells following treatment with PK 11195 (10^{-5} M) is shifted, showing an increase in the mitochondrial mass relatively to the control

via the mitochondrial TSPO (Veenman et al. 2007; Kugler et al. 2008; Zeno et al. 2009). Therefore we conclude that this TSPO ligand does not induce the overall process of the proliferation, maturation and cell death in human osteoblasts. This inert behavior of the PK 11195 in human osteoblasts might be of importance if other specific cellular effects of this compound will be found.

Indeed, in the third aspect that we investigated, i.e. cellular energy generation, PK 11195 exerted a significant effect, as demonstrated by elevation in cellular ATP content. This effect does not involve the main glycolytic pathway, since we did not find an increase in [^{18}F]-FDG incorporation into cells and also no increase in *hexokinase 2* gene expression (by RT PCR) or its cellular protein content (by Western blotting). This phenomenon was accompanied by an increase in cellular mitochondrial mass, as seen in the MTG staining assay, with a parallel increase in the TSPO gene expression (by RT PCR) and its cellular protein content (by Western blotting). These phenomena might be of compensatory nature in an attempt to provide adequate synthetic means for a higher rate of ATP generation.

Interesting is the fact that VDAC 1 gene expression and cellular protein content decreased following exposure of the cells to PK 11195. This may explain the increase in the stability of the $\Delta\Psi m$ observed in the present study. The mechanism of regulation of VDAC expression is still poorly understood and the role of TSPO in this process is also unclear (Veenman et al. 2008). At present it is unclear whether the overall suppressive effect of PK 11195 on VDAC 1 gene expression is primarily causative or compensatory. The same is true for the TSPO.

To summarize, this report indicates that TSPO may contribute to metabolic processes in human osteoblast. In

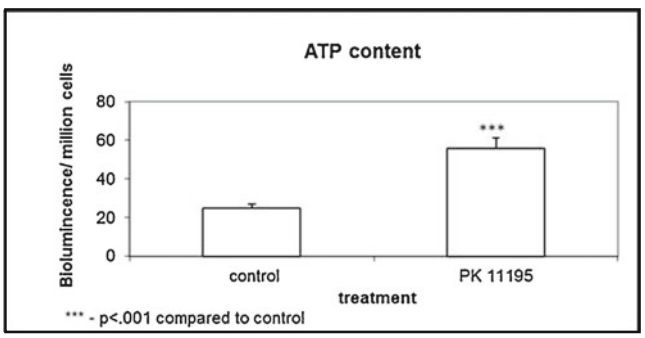


Fig. 4 ATP contents in cells with and without treatment with PK 11195 (10^{-5} M) are compared. Average values with standard deviations are presented, $n=6$ in each group, $p<0.001$

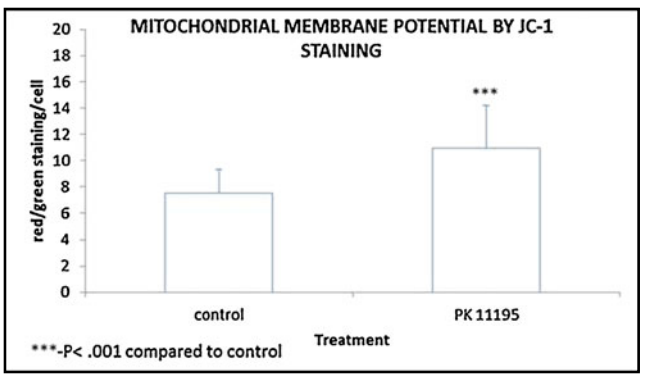


Fig. 5 Comparison of mitochondrial membrane potential ($\Delta\Psi m$) in cells treated by PK 11195 (10^{-5} M) and controls, $n=6$ in each group, $p<0.001$

particular the TSPO synthetic ligand, PK 11195, can elevate the cellular ATP content, without affecting cellular death, mitogenesis and maturation level of these cells. The cellular ATP content increase is accompanied by increases in mitochondrial mass and TSPO levels but with reduction in VDAC1 levels. These changes might indicate the existence of cell preserving compensatory pathways that involve TSPO in human osteoblast.

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