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Up-regulation of Mitochondrial Peripheral Benzodiazepine Receptor Expression by Tumor Necrosis Factor alpha in Testicular Leydig Cells

Possible Involvement in Cell Survival

Catherine Rey, Claire Mauduit,† Olivier Naureils,* Mohamed Benahmed,†*
Pierre Louisot and Françoise Gasnier*‡*

*INSERM U. 189 and †INSERM U. 407, Faculté de Médecine Lyon-Sud, BP12, 69921 Oullins cedex, France

ABSTRACT. Porcine Leydig cells in primary cultures are resistant to tumor necrosis factor alpha (TNFα) cytotoxicity. Here we report that these cells can be rendered sensitive to TNFα killing by treatment with the translational inhibitor cycloheximide, suggesting the existence of proteins that can suppress the death stimulus induced by the cytokine. In search of these cytoprotective proteins, we focused on the constituents of the mitochondrial permeability transition pore (PT pore), whose opening has been shown to play a critical role in the TNFα-mediated death pathway. We found that TNFα up-regulated mRNA and protein expression of the mitochondrial peripheral benzodiazepine receptor (PBR), an outer membrane-derived constituent of the pore. A strong correlation was established between the resistance of the cells to TNFα killing and the density of PBR-binding sites. Concomitantly, TNFα down-regulated Bcl-2 mRNA and protein expression. As Bcl-2 has been shown to be an endogenous inhibitor of the PT pore, we hypothesize that the TNFα-induced up-regulation of PBR expression may compensate for the decrease in Bcl-2 levels to prevent the opening of the PT pore. biochem pharmacol **60**;11:1639–1646, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. testis; Leydig cells; mitochondria; peripheral benzodiazepine receptor; Bcl-2; TNF

TNFα§ is a multifunctional cytokine that elicits numerous cellular reactions depending on the cellular context. Beside its tumoricidal activity by which it was originally defined [1], TNFα has been shown to play a major role in inflammation, septic shock, and viral replication. The biochemical basis by which TNFα mediates such a wide array of effects is not fully understood [2].

Considerable attention has recently been paid to the TNFα-mediated death pathway where production of reactive oxygen intermediates [3], caspase activation [4], loss of mitochondrial membrane potential [5, 6], and cytochrome *c* release from mitochondria [6] seem to play a critical role in activation of the signaling. All these biochemical perturbations that converge on mitochondria may be mediated by opening of the mitochondrial PT pore, a Bcl-2-regulated, large, non-specific pore also called megachannel or multiple conductance channel [7–10]. Although the molecular constituents of the pore have not been definitively established, it appears to consist of several proteins located at the mitochondrial contact sites, membrane microdomains where the outer and inner membranes come into close apposition [11, 12]. Thus, the outer membrane porin (also called voltage-dependent anion channel or VDAC) and PBR, as well as the inner membrane adenine nucleotide translocator (ANT, also called ATP/ADP carrier), have been implicated in PT pore formation [13].

While TNFα induces death of tumor cells and virally infected cells, normal cells are generally insensitive to its toxic effects. However, as many normal cells can be rendered sensitive to TNFα by treatment with RNA or protein synthesis inhibitors, a postulate has emerged that TNFα also activates a cell survival pathway that protects against its cytotoxic effects. Several cytoprotective TNF-inducible genes have been described, including the Bcl-2 family member A1 [14], manganous superoxide dismutase [15, 16], the A20 zinc-finger protein [17], plasminogen activator inhibitor type-2 [18], and γ-glutamylcysteine synthetase [19]. Nuclear factor-κB activation has been shown to play a key role in the regulation of these genes [20].

Porcine Leydig cells in primary cultures are resistant to TNFα cytotoxicity [21]. However, TNFα strongly affects their metabolism. Indeed, we have previously shown that TNFα exerts an inhibitory action on Leydig cell steroido-

‡ Corresponding author: Dr. Françoise Gasnier, Physiopathologie Subcellulaire, INSERM U 189, Faculté de Médecine Lyon-Sud, B.P. 12, F-69921 Oullins cedex, France. Tel. +33 478 86 31 58; FAX +33 478 50 71 52; E-mail: fgasnier@lyon-sud.univ-lyon.fr

§ *Abbreviations:* TNFα, tumor necrosis factor alpha; PT pore, permeability transition pore; PBR, peripheral benzodiazepine receptor; and RT–PCR, reverse transcription–polymerase chain reaction.

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genesis through a decrease in steroidogenic acute regulatory protein expression and that such an inhibitory action is probably mediated by TNFα receptor p55 expressed in the testicular cells [22]. We report here that these cells can be rendered sensitive to TNFα killing by treatment with the translational inhibitor cycloheximide. In search of the mechanism by which Leydig cells resist TNFα challenge, we focused on the PT pore constituents. We show that TNFα up-regulates PBR expression and concomitantly down-regulates Bcl-2 expression in these cells. As the resistance of the cells to TNFα killing was correlated with the density of PBR-binding sites, it is suggested that the increase in PBR expression may compensate for the decrease in Bcl-2 levels to prevent the opening of the PT pore and rescue cells from TNFα-induced death.

MATERIALS AND METHODS

Materials

[^{3}H]PK11195 (1-(2-chlorophenyl))-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline-carboxamide), specific activity 75 Ci/mmol, was purchased from NEN. Unlabeled PK11195 was a gift from Dr C. Caillard of Rhône-Poulenc Rorer Co. Human recombinant TNFα was obtained from Prepro Tech. Dulbecco's modified Eagle's/Ham's F-12 medium, Moloney murine leukemia virus reverse transcriptase, and TRIzol were obtained from Life Technologies. Collagenase/dispase was obtained from Boehringer. Insulin, transferrin, vitamin E, HEPES, and deoxyribonuclease type I (DNase) were purchased from Sigma Chemical Co. [α-^{33}P]dCTP was purchased from Amersham. Taq polymerase was obtained from Appligene-Oncor and oligonucleotide primers from Genset.

Leydig Cell Preparation and Culture

Leydig cells were prepared from immature porcine testes (2–3 weeks old) by collagenase treatment as described in [23]. They were cultured in 10-cm Petri dishes (20×10^6 cells/dish) at 32° in a humidified atmosphere of 5% CO_2, 95% air in Dulbecco's modified Eagle's/Ham's F-12 medium (1:1) containing sodium bicarbonate (1.2 mg/mL), 15 mM HEPES and gentamicin (20 μg/mL). This medium was supplemented with insulin (2 μg/mL), transferrin (5 μg/mL), and α-tocopherol (10 μg/mL).

Preparation of Purified Mitochondria

Mitochondria were isolated by differential centrifugation as described in [22].

Viability Assay

Cell viability was determined by the release of lactate dehydrogenase into the culture medium, using the detection kit from Boehringer. Cells were seeded at 10^6 cells/well in triplicate in 6-well plates. They were pretreated with TNFα (20 ng/mL) for 50 hr and then challenged for an additional 15 hr with TNFα (20 ng/mL) and different doses of cycloheximide (0–5 μg/mL). Control cells were treated with cycloheximide alone (0–5 μg/mL) for 15 hr. Cycloheximide was not cytotoxic at the concentrations used within the time frame of the experiments.

Radioligand-Binding Assays

Cultures were washed with medium, scraped from the dishes, and collected by centrifugation at $180 \times g$ for 10 min. [^{3}H]PK11195 binding studies on 20 μg protein from cell suspensions in Tris-buffered saline (TBS) or on 5 μg protein from mitochondria were performed at 0° in a final incubation volume of 0.25 mL, using 0.9 nM of the radiolabeled ligand. Non-specific binding was determined in the presence of 10^{-5} M unlabeled ligand. After a 30-min incubation, the assays were stopped by filtration through Whatman GF/C filters pretreated with 10 μM unlabeled ligand and washed with 15 mL ice-cold TBS. Radioactivity trapped on the filters was determined by liquid scintillation counting. Total binding was approximately 10% of the total free radioligand included in the assay and specific binding was 90% of the total binding.

Western Blot Analysis

Proteins from whole Leydig cells were resolved on 12% SDS/polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes using 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) pH 11, containing 10% methanol. The transfer was performed at a constant voltage of 100 V for 30 min. Following transfer, the membrane was incubated in a blocking buffer (TBS buffer containing 5% non-fat dry milk) for 2 hr at room temperature. The membrane was rinsed three times with TBS/Tween 0.1% (3×10 min), then incubated with an anti-Bcl-2 monoclonal antibody from Santa Cruz Biotechnology (1/1000 dilution in TBS containing 2% non-fat dry milk) for 2 hr at room temperature. The membrane was rinsed with TBS/Tween 0.1% (3×10 min) and then incubated with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin G. Bound antibodies were detected by chemiluminescence using a Pierce detection kit and Biomax MR film from Kodak. Band intensities were estimated by densitometric scanning using the BioImage scanner. Protein concentration was determined by the Bradford assay [24].

RNA Extraction

Total RNAs were extracted from porcine Leydig cells with TRIzol reagent. The amount of RNA was estimated by spectrophotometry at 260 nm.

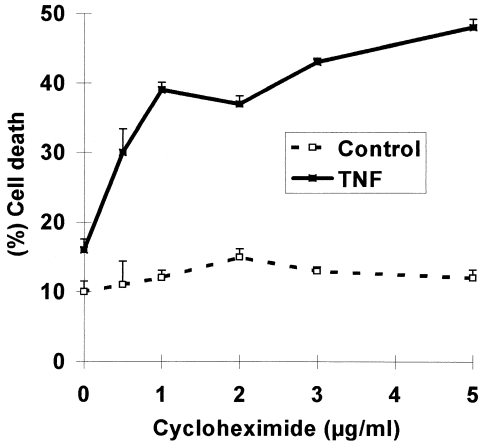

FIG. 1. Sensitization of Leydig cells to the cytotoxicity of TNFα by cycloheximide. Cells were pretreated with TNFα (20 ng/mL) for 50 hr and then challenged for an additional 15 hr with TNFα (20 ng/mL) in combination with increasing concentrations of cycloheximide (0–5 µg/mL) for 15 hr. Viability was determined by the release of lactate dehydrogenase into the culture medium. The results represent the means ± SD of three experiments.

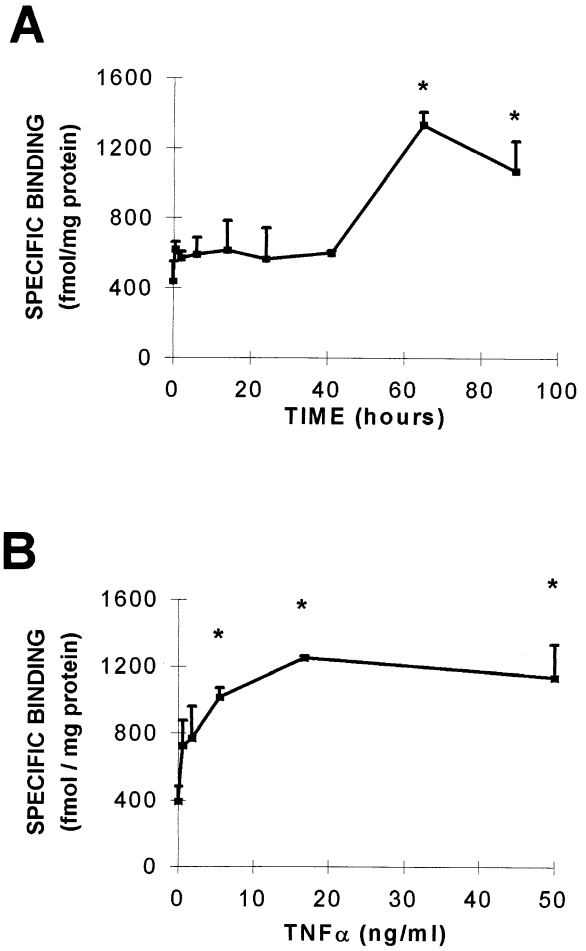

FIG. 2. (A) Effect of the duration of TNFα treatment on [^{3}H]PK11195 binding in Leydig cells. Leydig cells were treated with 20 ng/mL of TNFα for various time periods. [^{3}H]PK11195 binding studies were performed on 20 µg protein from cell suspensions as described in Materials and Methods. Data shown represent mean values ± SD from three different experiments. * indicates that the values were significantly different from control values (time 0) as determined by one-way analysis of variance followed by Tukey–Kramer's *post hoc* test ($P < 0.001$). (B) Dose–response relationship of TNFα on [^{3}H]PK11195 binding in Leydig cells. Porcine Leydig cells were exposed for 65 hr to various concentrations of TNFα. Cultures were then processed for binding as described in Fig. 2A. Data shown represent mean values ± SD from three independent experiments. * indicates that the values were significantly different from control values (no TNFα treatment) as determined by one-way analysis of variance followed by Tukey–Kramer's *post hoc* test ($P < 0.01$).

RT–PCR Analysis

Single-stranded complementary DNAs (cDNAs) were obtained from reverse transcription of 2 µg of total RNAs using random hexanucleotides as primers (5 µM) in the presence of dNTP (0.2 mM), dithiothreitol (10 µM), and Moloney murine leukemia virus reverse transcriptase (10 U/µL), 1 hr at 37°. cDNAs (1 µL of RT mixture) were amplified by PCR with Taq polymerase (0.01 U/µL), dNTP (100 µM), [α-^{33}P]dATP (0.045 µCi), and specific primers (2 µM). The mixture was first heated at 92° for 5 min. Amplification was carried out for 28 cycles (PBR and Bcl-2) or 18 cycles (β-actin) at 92° for 30 sec, 50° (PBR), 57° (Bcl-2), and 61° (β-actin) for 30 sec, and 70° for 30 sec using a Perkin Elmer 9700 thermocycler. At the end of the cycles, the reaction mixture was heated at 70° for 10 min. PCR products were analyzed on 8% polyacrylamide gels and visualized by autoradiography. The oligonucleotide primers for PBR were: 5′TGGAAAGAGCTGGGAGGCTTC 3′ (forward), 5′CGCCATACGCAGTAGTTGAG 3′ (reverse). PBR-amplified products were 266 bp. The oligonucleotide primers for Bcl-2 were: 5′AGCGTCAACGGGAGATGTC 3′ (forward), 5′GTGATGCAAGCTCCCACCAG 3′ (reverse). Bcl-2-amplified products were 212 bp. The oligonucleotide primers for β-actin were: 5′TTGCTGATCCACATCTGCTG 3′ (forward), 5′GACAGGATGCAGAAGGAGAT 3′ (reverse). β-Actin-amplified products were 146 bp. PCR analysis for PBR, Bcl-2, and β-actin were carried out from the logarithmic phase of amplification. PCR-amplified products were checked by restriction enzymes. RT–PCR primers were designed inside separate exons to avoid any bias due to residual genomic contamination. Moreover, for all primers, no amplification was observed when PCR was performed on RNA preparations.

Data Analysis

All experiments reported here were repeated three times with independent cell preparations. Significant differences among groups were examined by one-way analysis of variance followed by Tukey–Kramer's *post hoc* test. Statistical analysis was performed using the GraphPad Instat tm program.

TABLE 1. Binding characteristics of PBR in control and TNFα-treated Leydig cells

	Cell homogenate		Mitochondria	
	K_d (nM)	B_{max} (pmol/mg prot)	K_d (nM)	B_{max} (pmol/mg prot)
Control	7.2 ± 1.4	4.1 ± 0.3	4.1 ± 1.7	18.1 ± 0.5
TNFα	5.0 ± 0.6	8.5 ± 1.0*	7.6 ± 4.2	40.0 ± 7.8†

Leydig cells were treated with 20 ng/mL of TNFα for 65 hr or kept untreated. [^{3}H]PK11195 (0.9 nM)-binding studies were performed on 20 μg of protein from the cell homogenate in the presence of increasing concentrations of unlabeled ligand (1 nM to 0.1 μM). Binding characteristics of PBR were also measured on isolated mitochondria. Curve fitting was performed by computer-assisted non-linear regression analysis (CurveExpert 1.3 program). Values of dissociation constant (K_d) and maximal number of binding sites (B_{max}) are expressed as the means ± SEM of three independent experiments.

*$P < 0.03$ versus homogenate from control cells (paired *t*-test).

†$P < 0.05$ versus mitochondria from control cells (paired *t*-test).

RESULTS

Sensitization of Leydig Cells to the Cytotoxicity of TNFα by Cycloheximide

Porcine Leydig cells in primary cultures are resistant to TNFα killing [21]. However, these cells can be rendered sensitive to TNFα cytotoxicity by treatment with the translational inhibitor cycloheximide. As shown in Fig. 1, as little as 0.5 μg/mL of cycloheximide was effective in eliciting cellular death in TNFα-treated cells (30% dead cells versus 15% dead cells in TNFα-alone-treated cells). With 5 μg/mL of cycloheximide, almost 50% of the TNFα-treated cells died within 15 hr. Data from cycloheximide-alone-treated cells indicated that cycloheximide was not cytotoxic at the concentrations used in the experiments. It appears likely, from these results, that Leydig cells are protected from TNFα-mediated cytolysis by proteins that need to be continuously synthesized.

Effect of TNFα on [^{3}H]PK11195 Binding

In search of the cytoprotective proteins, we focused on one of the putative constituents of the mitochondrial PT pore, namely PBR. PK11195 was used as a prototypic ligand of this receptor. Primary cultures of porcine Leydig cells were treated with TNFα for various time periods. The cells were then harvested and assayed for [^{3}H]PK11195 binding. As shown in Fig. 2A, the addition of 20 ng/mL of TNFα to Leydig cells resulted in a significant increase in [^{3}H]PK11195 binding to these cells. A maximal effect to 2.5- to 3-fold of control occurred after long-term treatments ≥65 hr. When cells were exposed to various concentrations of TNFα for 65 hr, a significant dose-dependent increase in [^{3}H]PK11195 binding was observed (Fig. 2B). The maximal response was achieved with concentrations close to 20 ng/mL, after which a plateau was reached. In the concentration of 16–50 ng/mL, an increase of ~175–200% in ligand binding above the level in untreated cells was observed.

In non-treated cells, non-linear regression analysis revealed binding sites for PK11195 having K_d and B_{max} values of 7.2 ± 1.4 nM and 4.1 ± 0.3 pmol/mg, respectively (Table 1). Treatment of Leydig cells with 20 ng/mL of TNFα for 65 hr resulted in a 2-fold increase in the density of the binding sites (B_{max} = 8.5 ± 1.0 pmol/mg), whereas the equilibrium dissociation constant was unaffected (K_d = 5.0 ± 0.6 nM).

As a subset of PBR at the plasma membrane has also been demonstrated in some cells [25], studies were performed to verify that TNFα elicited an increase in the density of the mitochondrial PBR sites. As seen in Table 1, purified mitochondria from TNFα-treated cells exhibited a 2-fold increase in the maximal number of PK11195-binding sites when compared with mitochondria from control cells (40.0 ± 7.8 pmol/mg of protein versus 18.1 ± 0.5 pmol/mg). No significant alteration in the equilibrium dissociation constant was observed in the mitochondria from TNFα-treated cells.

Effect of TNFα on PBR mRNA

As TNFα up-regulated the number of PBR-binding sites in Leydig cells, we investigated whether the cytokine also affected PBR mRNA levels through the RT–PCR approach. The expression of β-actin was also determined for each sample and used as an internal control for the efficiency of each RT–PCR reaction. Although this method is only semiquantitative, Fig. 3A indicates that short-term treatments of Leydig cells with TNFα (20 ng/mL) did not significantly affect PBR mRNA levels. However, periods of treatment longer than 40 hr elicited an increase in PBR mRNA levels, a maximal response being achieved with 65 hr of treatment. TNFα increased PBR mRNA levels in a dose-dependent manner as shown in Fig. 3B. The concentration of TNFα required to achieve a maximal response was in the range of 16–50 ng/mL.

Correlation between the Density of PBR-Binding Sites and Cell Viability

Because Leydig cells can be rendered sensitive to TNFα cytotoxicity by treatment with cycloheximide, studies were done to determine whether PBR levels were correlated with cell survival. Leydig cells were treated with different doses of cycloheximide in combination with 20 ng/mL of TNFα. PBR levels were evaluated by measuring the binding of [^{3}H]PK11195 in whole Leydig cells, while cell death was

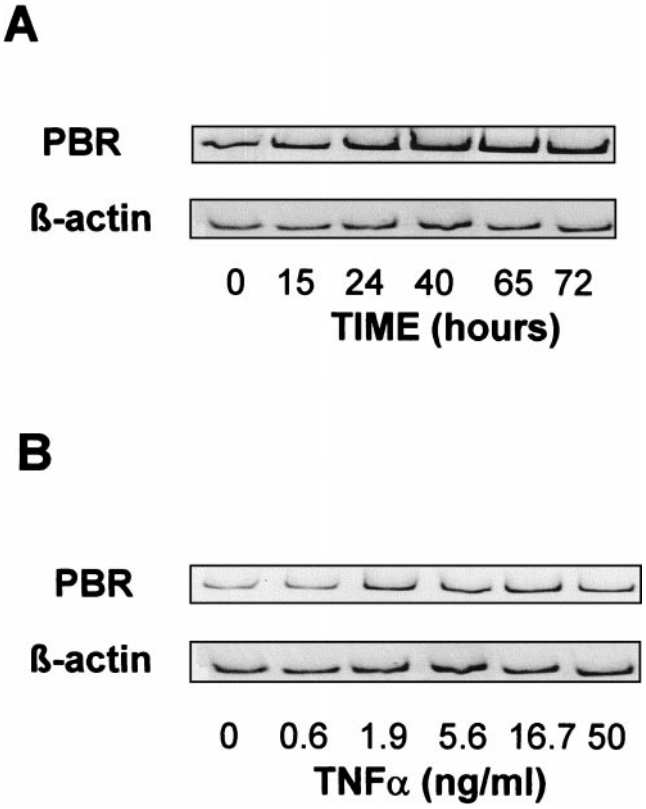

FIG. 3. (A) Effect of the duration of TNFα treatment on PBR mRNA levels. Leydig cells were treated with 20 ng/mL of TNFα for various time periods (0–72 hr). RT–PCR experiments were conducted as described in Materials and Methods. A representative PCR is shown. (B) Dose effect of TNFα on PBR mRNA levels. Leydig cells were cultured for 65 hr in the presence of various concentrations of TNFα (0–50 ng/mL). RT–PCR experiments were conducted as described in Materials and Methods. A representative PCR is shown.

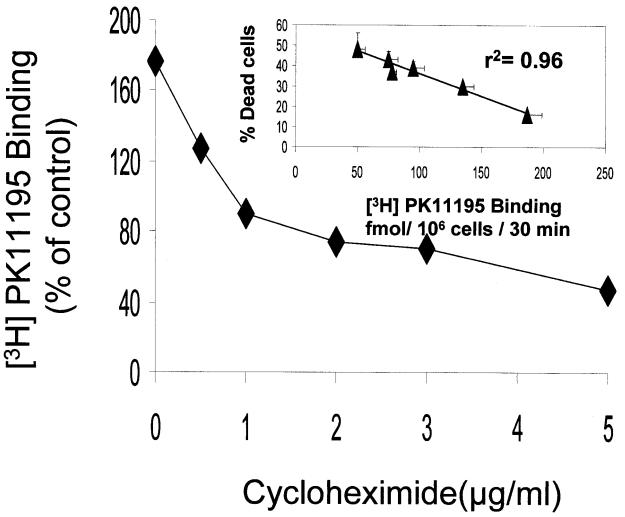

FIG. 4. Effect of cycloheximide on [^{3}H]PK11195 binding in TNFα-treated cells. Cells were pretreated with TNFα (20 ng/mL) for 50 hr and then challenged for an additional 15 hr with TNFα (20 ng/mL) and different doses of cycloheximide (0–5 µg/mL). Cells were then assayed for [^{3}H]PK11195 binding as described in Materials and Methods. The results represent the means ± SD of three experiments. The average [^{3}H]PK11195 binding from untreated cells (100%) was 106 ± 12 fmol/10^6 cells/30 min. Inset shows the correlation between the density of PBR sites and the resistance to TNFα cytotoxicity. Cell viability, determined by the release of lactate dehydrogenase into the culture medium, was plotted against PBR levels evaluated by measuring the binding of [^{3}H]PK11195 in Leydig cells. The line represents the best fit to the data points with a correlation coefficient r^2 = 0.96.

determined by the release of lactate dehydrogenase into the culture medium. As stated in Fig. 4, increasing concentrations of cycloheximide strongly antagonized the increase in PBR ligation induced by TNFα. While TNFα alone increased [^{3}H]PK11195 binding to 176% of the binding observed in untreated control cells, the combination of TNFα with 3 µg/mL of cycloheximide decreased [^{3}H]PK11195 binding to 70% of the control. Under these conditions, the B_{max} value was decreased to 3.1 pmol/mg (B_{max} value with 3 µg/mL of cycloheximide in the absence of TNFα treatment was 3.3 pmol/mg), while the K_d value was unchanged. When cell viability was concomitantly analyzed, a correlation was found between the decrease in PBR levels and enhanced cell death (Fig. 4, inset).

Effect of TNFα on Bcl-2 Protein and mRNA Expression

As PBR is known to be associated with the voltage-dependent anion channel and the adenine nucleotide translocator in a complex that participates in the mitochondrial permeability transition, we were interested in studying the effect of TNFα on the expression of Bcl-2, a protein known to regulate the opening of the PT pore. As shown by anti-Bcl-2 Western blotting (Fig. 5A), long-term treatments of Leydig cells with TNFα (20 ng/mL) down-regulated Bcl-2 expression, a 65-hr treatment resulting in a 3-fold decrease in the expression of the protein. RT–PCR for Bcl-2 demonstrated that TNFα also affected Bcl-2 mRNA expression. Indeed, while short-term treatments did not significantly reduce Bcl-2 mRNA levels, a decrease in the levels of Bcl-2 mRNA was observed for periods of treatment longer than 65 hr (Fig. 5B).

DISCUSSION

Porcine Leydig cells in primary cultures are resistant to TNFα killing, although this cytokine dramatically but reversibly disturbs the steroidogenic function of the cells [21, 22]. Yet, we have shown that those cells that express the TNFα receptor 55 [22] can be rendered sensitive to the cytotoxic effects of the cytokine by treatment with the protein synthesis inhibitor cycloheximide. These results are consistent with the existence of proteins that can suppress the death stimulus generated by TNFα in Leydig cells.

In search of the mechanism by which Leydig cells resist TNFα challenge, we focused on the mitochondrial permeability transition pore. Indeed, the opening of this pore,

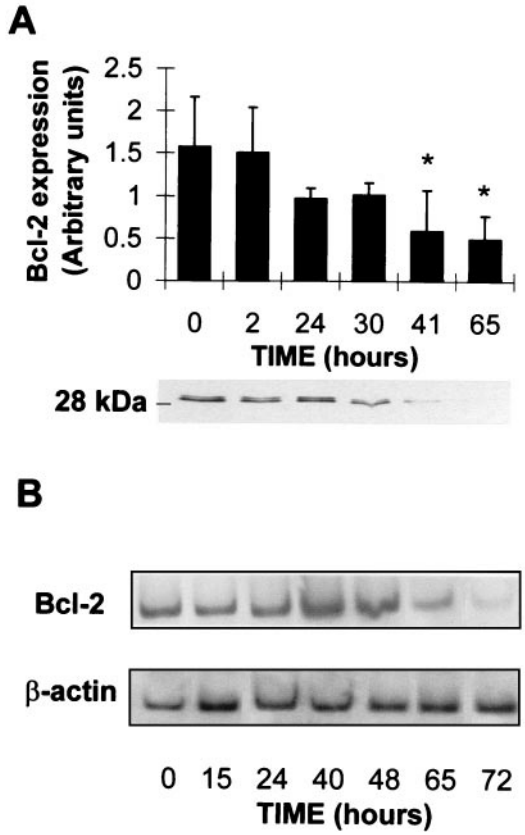

FIG. 5. (A) Long-term time–course study of TNFα on Bcl-2 protein expression. Leydig cells were treated with 20 ng/mL of TNFα for various time periods (0–65 hr). Western blot analyses were performed as described in Materials and Methods. *Upper panel,* Integrated intensities of Bcl-2 protein (from whole Leydig cells, 30 μg in each sample) Western blotted from three independent experiments. * indicates that the values were significantly different from control values (time 0) as determined by one-way analysis of variance followed by Tukey–Kramer's *post hoc* test ($P < 0.01$). *Lower panel,* a representative autoradiograph showing the immunodetected protein. (B) Effect of duration of TNFα treatment on Bcl-2 mRNA level. Porcine Leydig cells were incubated with TNFα (20 ng/mL) for various time periods (between 0–72 hr). Total cellular mRNAs were then extracted and RT–PCR was performed as described in Materials and Methods. A representative PCR is shown.

which is regulated by Bcl-2, has been shown to be a critical event in the process of lethal cell injury induced by TNFα [7]. While the molecular constituents of the pore remain only partially defined, a speculative model includes the inner membrane adenine nucleotide translocator and outer membrane proteins such as the voltage-dependent anion channel and the mitochondrial benzodiazepine receptor. Although PBR are found in virtually all mammalian tissues, they are particularly abundant in steroid-producing tissues such as adrenal, testis, and ovary [26]. Various functions have been ascribed to the PBR, including control of cell proliferation and differentiation [27, 28], modulation of monocyte functions [29], intracellular transport of anion [30] porphyrin and heme [31], as well as intramitochondrial cholesterol translocation [26]. However, the failure to generate PBR-negative gene knockout mouse because of the early death of animals during embryogenesis [32] suggests that PBR are also involved in critical functions. Of particular interest when considering this context are experiments investigating PBR ligation in various cultured cells that have shown either a facilitation of the induction of the mitochondrial permeability transition by a number of stimuli including chemotherapeutic drugs and irradiation [33, 34] or a protection against apoptosis induced by TNFα [35].

Recently, PBR have been reported to participate in the protection of hematopoietic cells against oxygen radical damage [36]. Indeed, Carayon *et al.* established a strong correlation between the ability of hematopoietic cell lines to resist H_2O_2 cytotoxicity and the level of PBR expression. Moreover, the transfection of Jurkat cells that do not express PBR with the human PBR cDNA increased their resistance to oxygen radical damage. Noteworthy are the findings of Yeliseev *et al.*, who showed numerous parallelisms between PBR and a bacterial "oxygen" sensor, the tryptophan-rich sensory protein (TspO) of *Rhodobacter sphaeroides* [37]. The striking similarity between the proteins even led the authors to propose TspO as a model for the structure and function of the mammalian PBR [38].

In the present work, we found that TNFα up-regulated PBR expression in porcine Leydig cells. As an overproduction of intracellular reactive oxygen intermediates (ROI) generated from mitochondria seems to be a critical event in mediating the cytotoxic effects of TNFα [3, 16], it appears likely that this up-regulation could be involved in the resistance of Leydig cells to the oxidative stress induced by the cytokine.

Of particular interest was the finding that the increase in PBR sites occurred concomitantly with a down-regulation of Bcl-2 expression. The Bcl-2 oncoprotein resides mainly on the cytoplasmic face of the mitochondrial outer membrane, where it is located at the contact sites between the outer and inner membranes [39]. As PBR shares properties with Bcl-2, it has been suggested that the proteins could have some common functions [36]. Indeed, recent findings of Schendel *et al.* [40] provided biophysical evidence that Bcl-2 forms channels in lipid membranes, while three-dimensional modeling of PBR revealed that the receptor could function as a channel [41]. As Bcl-2 has been shown to be an endogenous inhibitor of mitochondrial PT, our data support the hypothesis that the up-regulation of PBR expression observed in TNFα-treated Leydig cells could compensate for the decrease in Bcl-2 levels to prevent the opening of the PT pore. Moreover, this hypothesis is consistent with the observation that a large number of PBR sites associate with low Bcl-2 expression in phagocytic cells known to produce high levels of reactive oxygen species [36].

The fact that TNFα induced similar effects (up-regulation of PBR expression and down-regulation of Bcl-2 expression) in Sertoli cells (data not shown) may indicate that gonadal cells have developed common mechanisms to

protect against TNFα killing. Indeed, the long half-lives of these terminally differentiated cells suggest that they are very efficiently protected from cell death. It appears, therefore, that PBR could act as an antidote to TNFα cytotoxicity in cells that require an extended life span, such as Leydig and Sertoli cells. Worth mentioning in this context are the findings that higher PBR levels are present in various tumor tissues when compared to normal ones [42–44] and that patients with tumors expressing high PBR levels have a shorter life expectancy than patients with tumors having lower PBR contents [44].

An increase in PBR sites has also been observed in brain after experimental injuries as well as in certain neuropathological states [45–47]. *In vitro* studies suggest that this up-regulation of PBR expression may be mediated by cytokines such as interleukin-1 and TNFα, which are released by cells of the monocytic lineage found in the injured brain [48]. In our models of gonadal cells, the TNFα could originate from the interstitial macrophages that might be activated during an immune challenge or chronic inflammatory diseases.

In conclusion, PBR was known to mediate the intramitochondrial cholesterol transport in steroidogenic cells [26]. However, considering the recent work of Papadopoulos *et al.*, which provides evidence that PBR is a component of the mitochondrial apoptosis cascade in corpus luteum cells [49], as well as the data presented here, it appears likely that PBR may also fulfill other functions in these cells related to the protection from cell death.

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