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RAPID COMMUNICATION

Mitochondrial Peripheral-Type Benzodiazepine Receptor Expression

CORRELATION WITH GONADOTROPIN-RELEASING HORMONE (GnRH) AGONIST-INDUCED APOPTOSIS IN THE CORPUS LUTEUM

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ABSTRACT. We have demonstrated that continuous administration of a gonadotropin-releasing hormone agonist (GnRH-Ag) decreases the expression of the mitochondrial peripheral-type benzodiazepine receptor (PBR) and increases the rate of DNA degradation in a time-dependent manner in the corpora lutea of pregnant rats. In the present study, we show *in situ* the GnRH-Ag-induced DNA fragmentation and correlate the increase of the rate of DNA degradation with the decrease in mitochondrial PBR ligand binding ($r = 0.89$). The GnRH-Ag-induced decrease in the 18-kDa PBR protein also correlated with the reduction in the Bcl-X_L, but not Bcl-2 (cell survival), gene product levels and the increase in the Bax (cell death) gene product expression in the luteal mitochondrial preparations. Considering the function of PBR in cholesterol uptake and intramitochondrial movement, we propose that decreased PBR expression may lead to reduced levels of mitochondrial membrane cholesterol, which, together with the ability of Bcl-X_L and Bax to form ion channels, produces breaks in the outer membranes allowing the exit of cytochrome *c*, thus triggering apoptosis. Alternatively, PBR may exert an as yet unidentified anti-apoptotic function. BIOCHEM PHARMACOL **58**;9: 1389–1393, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. corpus luteum; GnRH; peripheral benzodiazepine receptor (PBR); cholesterol

Apoptosis is a process whereby cells die in a controlled manner in response to a specific stimulus [1]. Apoptosis produces specific degradation of DNA into regularly sized small MW fragments. Recent observations support a concept that an intrinsic GnRH¶ system, complete with ligand [2], receptor [3], and biological action, exists in the ovary. In support of this hypothesis, the CL of the rat has been shown to have high-affinity, low-capacity binding sites for GnRH [4], and more recently human granulosa-lutein cells and ovary have been reported to express mRNA for the GnRH receptor [5]. Considering that the highest levels of GnRH are present in the ovary/CL around the time of parturition [6], when serum progesterone levels are declining, GnRH can be considered to be a physiological luteolysin in the pregnant rat. Thus, in an *in vivo* model system that we developed to evaluate the role of GnRH in luteolysis, we have demonstrated that continuous administration of a GnRH-Ag to a pregnant rat suppresses serum levels of progesterone [7, 8]. This is a time-dependent phenomenon starting as early as 8 hr after initiation of the treatment [7, 8]. During these studies, we made the following observations: (a) GnRH-Ag increases the degree of DNA degradation and expression of mRNA for *Bax*, a cell death gene, and, at the same time, decreases the mRNA expression of *Bcl-X*, a cell survival gene, in the CL [7]; and (b) compared with corresponding sham controls, GnRH-Ag treatment decreases the expression of CL PBR mRNA and ligand binding expression at all time periods starting at 4 hr [8]. During the analysis of these data, we noticed a close similarity between the time courses of the GnRH-Ag-induced DNA degradation and the PBR ligand binding expression. This observation led to additional experiments and the present report, where we present the specific localization of the DNA damage in the CL, the correlation between the GnRH-Ag-induced DNA degradation and the PBR ligand binding expression, and the GnRH-Ag-induced changes in the mitochondrial membrane proteins PBR, Bcl-2, Bcl-X_L, and Bax.

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¶ *Abbreviations*: GnRH, gonadotropin-releasing hormone; GnRH-Ag, GnRH agonist; PBR, peripheral-type benzodiazepine receptor; CL, corpus luteum; PT, permeability transition pore; VDAC, voltage-dependent anion channel protein; and ANT, adenine nucleotide translocator.

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MATERIALS AND METHODS

Animals and Treatment

GnRH-Ag (5 μg/day) was administered to timed-pregnant Holdzman Sprague–Dawley rats continuously, using osmotic minipumps (model 2001D, Alza Corp.), starting on the morning of Day 8 of pregnancy; sham-operated control rats received no treatment, as we described previously [7, 8]. GnRH-Ag ([Pyro]-Glu-His-Trp-Ser-Tyr-D-Trp-NMeLeu-Arg-Pro-ethylamide-luteinizing hormone-releasing hormone; Wyeth-40972) was a gift from Wyeth-Ayerst Laboratories. Rats were killed at 0, 4, 8, and 24 hr after the commencement of treatment. At autopsy, ovaries were removed; CL from the ovaries of each animal was separated, mitochondria were isolated from either controls or animals treated with GnRH-Ag for 8 hr [8], and the mitochondrial preparations were frozen. The Institutional Animal Care and Use Committee approved all procedures involving animals.

Immunoblot Analysis

Mitochondrial fractions (40 μg of protein) were resolved by SDS–PAGE, electro-transferred onto nitrocellulose membranes, and treated for immunodetection of PBR using anti-PBR (1:1000 dilution) antiserum [9]. The blots then were stripped and re-blotted for the detection of Bcl-2, Bcl-X_L, and Bax proteins using anti-Bcl-2 (1:500 dilution; monoclonal YTH-10C4), anti-Bcl-X_L (1:500 dilution; monoclonal YTH-2H12), and anti-Bax (1:500 dilution; monoclonal YTH-6A7). Goat IgG-horseradish peroxidase was used as a secondary antibody at 1:6000 followed by chemiluminescent detection (Dupont-NEN). Densitometric analysis of the immunoreactive protein bands was performed using Sigmagel software (Jandel Scientific).

Extraction and Analysis of DNA for Intranucleosomal Cleavage

Genomic DNA from CL was prepared, purified, and radiolabeled as we described previously [7]. Labeled DNA samples were resolved by electrophoresis and exposed to autoradiography. Low MW DNA fractions (<15 kb) were excised from the gels, mixed with scintillation fluid, and measured by liquid scintillation spectrometry to provide a quantitative estimate of the degree of intranucleosomal DNA cleavage as previously described [7].

In Situ *Localization of DNA Fragmentation*

The extent of DNA breakdown in fixed ovarian tissue sections was assessed using a non-isotopic, streptavidin-biotin-based *in situ* terminal transferase reaction as we described [10].

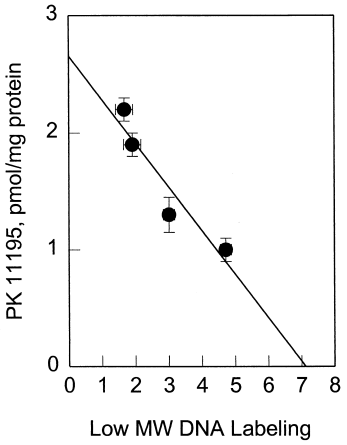

FIG. 1. Correlation of PBR ligand binding (B_{max} using the ligand PK 11195; [8]) and low MW DNA fragment labeling [7] after 4-, 8-, and 24-hr GnRH-Ag treatment. Values are means ± SD (N = 3). The correlation coefficient for DNA fragmentation versus PBR was 0.89.

Ligand Binding Studies

Radioligand binding assays for CL PBR were performed using PK 11195 as the radiolabeled ligand as described [8, 11]. Protein was quantitated using the dye-binding assay of Bradford [12].

RESULTS AND DISCUSSION

GnRH-Ag treatment suppresses serum progesterone levels as early as 8 hr following initiation of the treatment via a miniosmotic pump; maximal reduction in serum progesterone levels occurs at 24 hr [7, 8]. In our previous study, we demonstrated that GnRH-Ag treatment also suppresses the luteal mRNA expression and ligand binding of PBR at all time periods compared with corresponding controls. This effect is time-dependent, with the maximum effect seen at 24 hr after the initiation of the treatment [8]. At the same time we observed that the GnRH-Ag treatment induced an increase in the total CL DNA fragmentation. This effect was also time-dependent, with the maximum effect seen at 24 hr after the initiation of the treatment [7]. Comparing the amount of CL PBR and the low MW DNA fragment labeling in CL, quantitated by liquid scintillation spectrometry counting of low MW radiolabeled DNA fractions (<15 kb) following electrophoresis, at the various times (4, 8, and 24 hr) upon initiation of the treatment indicated that there was an excellent correlation between the two events (coefficient of correlation, $r = 0.89$; Fig. 1). These data also showed that only a certain amount of PBR may be regulated by GnRH because there appears to be a tendency for saturation of the response to GnRH-Ag. The GnRH-Ag-induced CL DNA fragmentation was confirmed using a method of *in situ* detection of DNA breakdown. A 3′-end labeling signal was detected in CL of the GnRH-Ag-treated rats (Fig. 2B), whereas only a small population of luteal

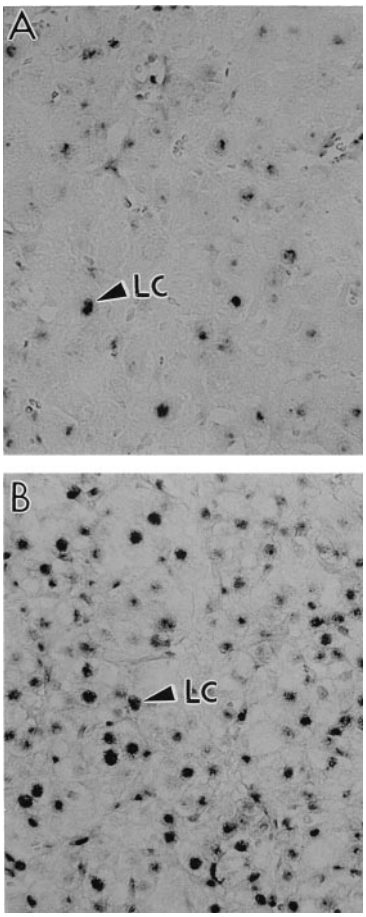

FIG. 2. *In situ* DNA labeling analysis of control (A) and GnRH-Ag-treated (B) CL collected after 24 hr of GnRH-Ag treatment. Darkly stained luteal cells indicate extensive DNA labeling. Magnification: 400x. As controls, the colorimetric reaction did not occur in cells of sections processed without either the terminal transferase enzyme or the labeling nucleotide (data not shown). The data shown are representative of three independent experiments.

cells from control rats showed evidence of 3′-end labeling (Fig. 2A).

PBR is an 18-kDa outer mitochondrial membrane protein that is present in all steroidogenic tissues [13] including the rat ovary [8, 14, 15]. Data generated by us and other laboratories (for review, see Ref. 13) strongly suggest that PBR is rate-limiting for cholesterol transport into the mitochondria of adrenal cortical, Leydig, ovarian, glial, and placental cells (all known steroid-synthesizing cell types). This function of PBR is not limited only to vertebrates but extends to arthropods [16]. Inhibition of PBR expression both *in vivo* [8, 9] and *in vitro* [17] results in decreased steroid production, further supporting the determining role of PBR in steroid biosynthesis.

Considering the above correlation presented between PBR ligand binding expression and DNA fragmentation, the outer mitochondrial membrane localization of PBR [13] and of Bcl-2, Bcl-X_L, and Bax gene products, shown to be involved in the apoptotic process [18], we examined whether GnRH-Ag induced any changes at the mitochondrial protein level 8 hr after the initiation of the treatment, when PBR ligand binding levels were decreased by approximately 50% and DNA fragmentation was increased by approximately 50%. The levels of mitochondrial PBR protein, which functions as a channel for cholesterol [19], were decreased by 50% (Fig. 3). The levels of Bcl-X_L protein, the cell survival gene product that forms cation-selective ion channels in the outer mitochondrial membrane [18], were decreased by 90% (Fig. 3). At the same time, the levels of Bcl-2, which also forms cation-selective ion channels in the outer mitochondrial membrane [18], were not affected by the treatment (Fig. 3). However, the levels of Bax, the cell death gene product that forms anion-selective ion channels in the outer mitochondrial membrane [18], were increased by 50%, although it was found in relatively low basal amounts in CL mitochondria (Fig. 3). These data on the mitochondrial protein levels of PBR, Bcl-2, Bcl-X_L, and Bax gene products closely resembled the changes that occur at the levels of their corresponding mRNAs [7, 8]. In addition, translocation of Bax from the cytosol to mitochondria may also account for the increased levels of Bax found in the CL mitochondria [20].

In support of our findings, a paper recently appeared showing that treatment of various cells of the immune system with the PBR drug ligand PK 11195 facilitated the induction of apoptosis [21]. Considering that PK 11195 in some systems works as a receptor antagonist, these data indicate that PBR may be involved in apoptosis. Moreover, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay for cell proliferation, which measures mitochondrial integrity, we observed that PBR-mutant Leydig cells grow at one-tenth of the rate of the wild-type cells [16]. These data suggest that the absence of PBR results in the disruption of the mitochondrial integrity

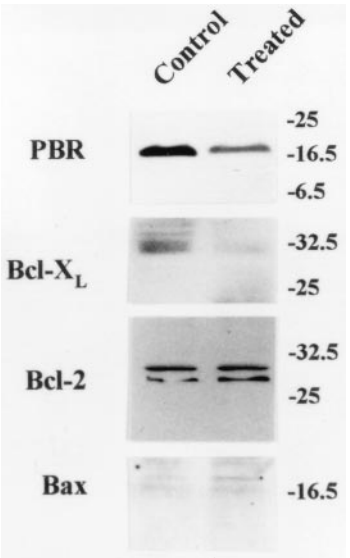

FIG. 3. Immunoblot analyses of the luteal mitochondrial PBR, Bcl-2, Bcl-X_L, and Bax protein content at 8 hr after the commencement of GnRH-Ag treatment. The data shown are representative of two independent experiments.

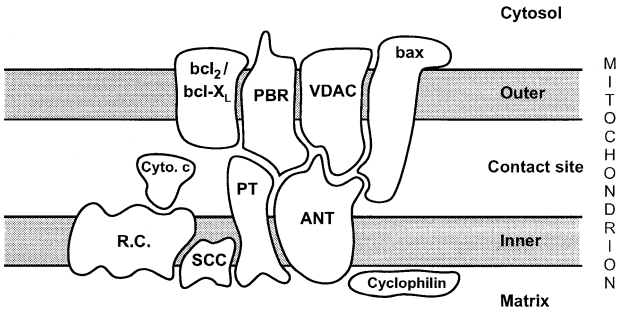

FIG. 4. Model of the mitochondrial protein complex involved in GnRH-Ag-induced apoptosis in the CL. Abbreviations: R.C., respiratory chain; Cyto. C, cytochrome *c*; and SCC, cytochrome P450 side chain cleavage. For other abbreviations, see the text.

associated with either reduced cell proliferation or increased cell death. The results presented herein provide further evidence that the 18-kDa PBR protein is a component of the mitochondrial apoptosis cascade. Whether the loss of PBR is the first, intermediate, or last step in the apoptosis pathway remains to be determined. Moreover, it is not yet clear whether PBR plays any causal role or if it is just one of the gene products affected by apoptosis. It also should be noted that the correlation of PBR decrease and induction of apoptosis observed in the present model might apply only to some tissues and in response to certain stimuli.

Mitochondrial cytochrome *c* release into the cytosol has been established as one of the triggers of stimulus-induced apoptosis [1]. Cytochrome *c* resides in the space between the outer and the inner mitochondrial membrane, and it has been suggested that it is released into the cytosol upon the rupture of the outer mitochondrial membrane, occurring as a consequence of the mitochondrial PT pore (also called inner mitochondrial megachannel) opening [22]. Stimulus-induced mitochondrial swelling and rupture may be another way to explain the cytochrome *c* release [23]. In this model, the mitochondrial contact site protein VDAC may be involved. Interestingly, VDAC has been proposed to be part of the PT pore [24, 25], and PBR has been shown to interact in a structural and functional manner with VDAC [26, 27]. Alternatively, the formation of a large enough channel by the pro-apoptotic Bax in the outer mitochondrial membrane may allow the release of cytochrome *c* [28]. Recently, Marzo *et al.* [29] reported that Bax cooperates with ANT and the PT pore complex to increase mitochondrial membrane permeability and trigger cell death.

It has been suggested that PT is composed of VDAC, ANT, and the 18-kDa PBR protein [24, 25]. Moreover, PBR has been shown to interact with both VDAC and ANT [26, 27]. Furthermore, PT has been shown to be inhibited by PBR ligands [30] and to be sensitive to the immunosuppressant cyclosporin A [31], a noncompetitive inhibitor of PBR (unpublished data), suggesting that PT may regulate PBR in an "allosteric" manner. Considering these observations, we propose a model of the contact sites of mitochondrial membranes, in which the outer membrane cholesterol channel 18-kDa PBR and the ion channel proteins Bcl-2, Bcl-X_L, and Bax, the contact site ion channel protein VDAC, and the two inner membrane proteins ANT and PT may interact in a coordinated stimulus-induced manner (Fig. 4). Changes in the levels and/or ratios of these proteins may lead to breakage of the outer membrane, release of cytochrome *c*, and apoptosis. The PT structure has not been identified yet and may comprise some of the above-mentioned proteins. The exact roles of each of these components in the preapoptotic cascade of events remain to be determined.

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