Glycoconj J (2006) 23: 199–207
DOI 10.1007/s10719-006-7925-3

Increased expression of peripheral benzodiazepine receptor (PBR) in dimethylbenz[a]anthracene-induced mammary tumors in rats

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Abstract Expression of peripheral benzodiazepine receptors (PBR) has been found in every tissue examined; however, it is most abundant in steroid-producing tissues. Although the primary function of PBR is the regulation of steroidogenesis, its existence in nonsteroidogenic tissues as well as in other cellular compartments including the nucleus suggests that there may be other roles for PBR. Our laboratory reported earlier a significant increase of PBR density in the nucleus of DMBA-induced malignant submandibular glands of rats, suggesting a role of PBR in nuclear events of peripheral tissues. Since then numerous studies have demonstrated the abundance of PBR in tumors.

Numerous studies implicate a role for cholesterol in the mechanisms underlying cell proliferation and cancer progression. Based on studies with a battery of human breast cancer cell lines and several human tissue biopsies, Hardwick *et al*. suggested that PBR expression, nuclear localization, and PBR-mediated cholesterol transport into the nucleus are involved in human breast cancer cell proliferation and aggressive phenotype expression. The purpose of the present study is to confirm this hypothesis by developing an animal breast cancer model and correlating the above events with the breast cancer.

Weanling rats were maintained on a diet containing animal protein (casein) for 30 days and then a single dose of DMBA in sesame oil (80 mg/kg) was administered by gavage to the animals. Control animals received the vehicle only. After 122 days of DMBA administration, the animals were sacrificed. All tumors were detected by palpation. B_{max} of PBRs was 52.6% and 128.4% higher in the non-aggressive and aggressive cancer tissues, respectively, than that in normal tissues. Cholesterol uptake into isolated nuclei was found to be higher in both non-aggressive and aggressive tumor breast tissue than that in control tissue. There was also corresponding increase in B_{max} of PBRs in the nucleus of cancer tissues. Furthermore, the nuclear nucleoside triphosphatase (NTPase) activity was found to be higher in aggressive tumor tissues than that in non-aggressive tumor tissues. In conclusion, these data suggest that PBR ligand binding, and PBR-mediated cholesterol transport into the nucleus may be involved in the development of mammary gland adenocarcinoma, thus participating in the advancement of the disease.

Keywords Peripheral benzodiazepine receptor · Benzodiazepine · Cholesterol · Mammary gland

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Introduction

Benzodiazepine (BZ) exerts its anxiolytic effects via specific binding sites coupled to neuronal $GABA_A$ receptors [1]. In addition to these GABA receptor-linked central types of BZ receptors, a second type [peripheral benzodiazepine receptor (PBR)] has been identified. This PBR has been found throughout the body including the nervous tissue [2–5]. The PBR is a heteromeric complex of three different subunits including a 18 kDa isoquinoline-binding subunit and is predominantly located in the outer mitochondrial membrane associated with voltage-dependent anion channels [6]. Further studies demonstrated that pharmacologically induced reduction of adrenal PBR levels *in vivo* resulted in decreased circulating glucocorticoid levels [7]. Targeted disruption of the

PBR gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation. In addition, transfection of the mutant cells with a PBR cDNA rescued steroidogenesis [8].

Although the exact function of PBRs is still not clear, attention has been focused on the involvement of PBR in cell proliferation and differentiation. Several neoplastic tissues possess altered binding characteristics of PBR [9,10]. PBR ligands have been shown to have an effect on differentiation and proliferation of normal and malignant cells *in vitro* [11–14]. Initial studies using high concentrations of drug ligands indicated their binding to PBR could inhibit DNA synthesis in different kinds of mammalian cell lines [15,16]. Now it has been shown that low concentrations of diazepam, PK 11195, and Ro5-4864 may induce cell proliferation [17,18]. A biphasic effect on cell proliferation depending on the ligand concentration shows that concentrations in the receptor binding range (nanomolar range) stimulate DNA synthesis, whereas PBR ligands are effective as antiproliferative agents in experiments with approximately 1000-fold higher concentrations [15,18,19]. In human astrocytomas, PBR expression has been correlated with tumor malignancy grade, proliferation index, and patient survival [20]. In brain tumors, increased PBR concentration has been used for diagnostic imaging and as a target for antineoplastic agents [21]. In comparison to normal tissues and to benign tumors, elevated PBR density has been demonstrated in ovarian carcinomas [9]. In highly aggressive cell lines relative to non-aggressive breast cancer cell lines, ligand binding and PBR-mRNA was dramatically increased [22]. The supposed endogenous ligand of PBR, a polypeptide named DBI, is capable of binding acyl-CoA and may, therefore, play an important role in the regulation of mitosis. In cells with a fast turnover that are able to metabolize fatty acids, acyl-CoA may be the primary energy supplier [23].

Nuclear localization of PBR was first reported by studies in our laboratory [24,25]. It was found that malignancy induced by Dimethyl-benz(a) anthracene (DMBA) implantation into a submandibular gland showed an increase in the density of PBR in the nucleus [24]. Later studies in our laboratory revealed that in addition to the predominant existence of PBR in the mitochondrial membrane, substantial density of PBR existed in the nucleus of guinea pig lungs [25]. Recently [22], subcellular localization studies of PBR revealed that the highly aggressive MDA-231 human breast cancer cell line had PBR localized in the nucleus and the perinuclear region. This nuclear localization was in contradiction to the highly studied mitochondrial localization of tumor leydig cells and the largely cytoplasmic localization in the normal breast tissue and non-aggressive MCF-7 cell line. In steroid synthesizing cells, PBR is primarily located in the outer mitochondrial membrane, where it is involved in the uptake of cholesterol. Cholesterol is a precursor of steroids formed in the mitochondria [8,26]. Mitochondrial PBR has also been involved in other functions such as respiration [27,28] and apoptosis [29,30]. In proliferating cells however, PBR was found in the nucleus, where it is involved in the uptake of cholesterol into the nucleus and the regulation of cell proliferation [22,31,32]. These studies suggest that PBR may serve a more general function in cholesterol compartmentalization [33]. Li *et al*. [34] demonstrated that deletions in the cytoplasmic carboxyl terminus of PBR dramatically reduced the cholesterol uptake function of PBR, even though the capacity to bind drug ligands such as PK11195 was retained. In the same study [34], it was also proposed that there exists a cholesterol recognition and interaction amino acid consensus (CRAC) site at the carboxyl end of the PBR protein. Tyr 153 and Arg 156 were found to be critical for the interaction of the receptor with cholesterol.

In this study we tested the hypothesis that PBR expression in breast cancer can be correlated to its function in nuclear cholesterol transport. Our studies have focused on the notion that PBR could play a role in breast cancer with many reports implicating its over-expression in many cancer states. The objective of this study was to verify whether this high expression could be a result of PBR function in other biological membranes such as the nuclear membrane.

Materials and methods

Development of breast tumor in female rats

Adult female Sprague Dawley rats were purchased at 22 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed individually in polycarbonate cages. Animals were divided into two groups, group 1 and group 2. All animals were maintained on standard AIN-76A diet containing 20% casein (Harlan Teklad, Madison, WI). The animals were placed on the test diets at 25 days of age and remained on the diet for the rest of the study. Rats were allowed to feed and drink *ad libitum*. Mammary tumors were induced on rats of group 2 at 50 days of age by a single gavage administration of dimethylbenz[a]anthracene (DMBA) in sesame oil (80 mg/kg b.wt). Control animals (group1) received only the vehicle by gavage.

Animals were weighed and also palpated twice weekly to detect tumors beginning four weeks after the administration of carcinogen. At 122 days post-injection, animals were killed by CO_2 asphyxiation. All tumors were weighed and processed for histological grading [35] and biochemical analysis.

Receptor binding assay

The breast tissue was homogenized in 50 mM Tris-HCl using a Brinkman Sonifier at settings 6–7. The resulting

homogenate was centrifuged initially at 600 × g for 30 min, and then at 20,000 × g for 15 min to acquire membrane. The membrane sample was resuspended in assay buffer (50 mM Tris-HCl pH 7.4). Protein was measured following the method of Lowry *et al*. [36]. The binding of the PBR ligand [^{3}H] Ro5-4864 was determined in a volume of 1 ml containing 0.1 ml of total protein (100 μg), 0.1 ml of radioligand [^{3}H] Ro5-4864, 0.1 ml Ro5-4864 (10 μM) or buffer, and 0.7 ml assay buffer (50 mM Tris-HCl pH 7.4) as described earlier [25]. The reaction was initiated by the addition of membrane protein and incubation at 0 to 4°C for 60 min with agitation. The reaction was then terminated by rapid filtration over Whatman GF/B strips using Millipore Brandel M-24R filtering manifold, with two 5 ml washes of ice-cold buffer (50 mM Tris-HCl). The filter strips were used to retain receptors bound to ligand. The specific binding of [^{3}H] Ro5-4864 was defined as the difference in binding obtained in the presence (non-specific binding) and absence (total binding) of excess cold Ro5-4864 (10 μM). The radioactivity retained by the filters was measured in a Beckman LS-355 liquid scintillation counter. Scatchard analysis [37] and determination of binding constants was performed using Graphpad Prism 4®.

Isolation of nucleus

The breast tissue was homogenized in 10 volumes of 0.25 M sucrose, 1 mM EDTA (pH 7.4) in a Potter-Elvehjem homogenizer and filtered through 100-μm nylon mesh. The nuclear fraction was obtained by centrifuging the filtrate at 600 × g for 10 min in a refrigerated Beckman TJ-6 centrifuge. The nuclear pellet was washed once with respective homogenizing buffer. The crude nuclei were purified by layering on top of a 30% (w/v) sucrose solution in 0.1 M Tris-HCl, 1 mM EDTA (pH 7.4) and centrifuging in a Beckman L8 M ultracentrifuge at 500 × g for 30 min in an SW 40 rotor. The pellet (pure nuclei) was washed with Tris-HCl/EDTA buffer and resuspended in a known volume of ice-cold 1 × PBS buffer.

Nuclear receptor binding assay

The method was the same as described in the previous "Receptor binding assay". Here nuclear pellet was used for this experiment.

Nuclear cholesterol transport

A nuclear pellet was used to determine cholesterol transport. [^{3}H] Cholesterol uptake in breast cancer and normal tissue was examined in the presence of 10 nM [^{3}H] cholesterol. Nuclei were incubated in 1 × PBS containing [^{3}H] cholesterol for 60 min at 37°C [22]. Samples were then centrifuged, pelleted, and washed 3 times with 1 × PBS and resuspended in a final volume of 1 ml. This suspension was mixed with 5 ml scintillation cocktail. Radioactivity was measured in liquid scintillation fluid using Beckman LS-355 liquid scintillation counter.

Assay of nucleoside triphosphatase (NTP) enzyme activity

A nuclear pellet was used to determine the NTPase activity. NTPase was measured according to the method of Kim *et al*. [38]. Nuclear protein was incubated with buffer containing 20 mM Tris-acetate, pH 8.5, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 100 mM NaCl and 2 mM ATP in a total reaction volume of 60 μl for 45 min at room temperature. The reaction was stopped with 60 μl of 10% TCA. 100 μl of the stopped reaction was then mixed with 100 μl of a colorimetric reagent (6N H_2SO_4, 0.8% ammonium molybdate, 10% ascorbic acid in 1:3:1 ratio). This mixture was incubated for 30 min at 37°C and absorbance at 655 nm was measured with a microplate colorimeter. Standard curves generated with a dilution series of KH_2PO_4 were used to convert the O.D. values to amounts of inorganic phosphate (P_i) released [39].

Measurement of endogeneous cholesterol

Lipids were extracted from nuclear pellet with chloroform:methanol (2:1 v/v) by the method of Folch *et al*. [40]. The quantity of total cholesterol was determined in the total chloroform elute by adopting the procedure of Courchaine *et al*. [41].

Western blot analysis

Breast tissue was homogenized in a 5 volumes of ice cold 210 mM Manitol, 70 mM Sucrose, 10 mM Tris-HCl, 1 mM EGTA, 2 mM $CaCl_2$ Buffer, pH 7.2 containing protease inhibitor cocktail (0.01%) by using a Brinkman Polytron (setting 6–7, 30 sec only). Proteins were measured by Lowry method [36] and 50 μg protein was separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred electrophoretically onto PVDF membranes Immobilon-P (Millipore, Bedford, MA). The membrane was immunoblotted by using the polyclonal PBR-specific antibody raised in rabbit (SantaCruz Biotechnology Inc., CA, and Trevigen Inc., MD) as first antibody, and Horseradish Peroxidase (HRP)-conjugated anti rabbit antibody as secondary antibody. Binding of antibodies to the blots was detected with an ECL-detection system (Perkin Elmer, Boston, MA) following manufacturers instructions. Stripped blots were re-probed with β-actin specific polyclonal antibodies (SantaCruz Biotechnology Inc., CA) to enable normalization of signals between samples. Band intensities were analyzed

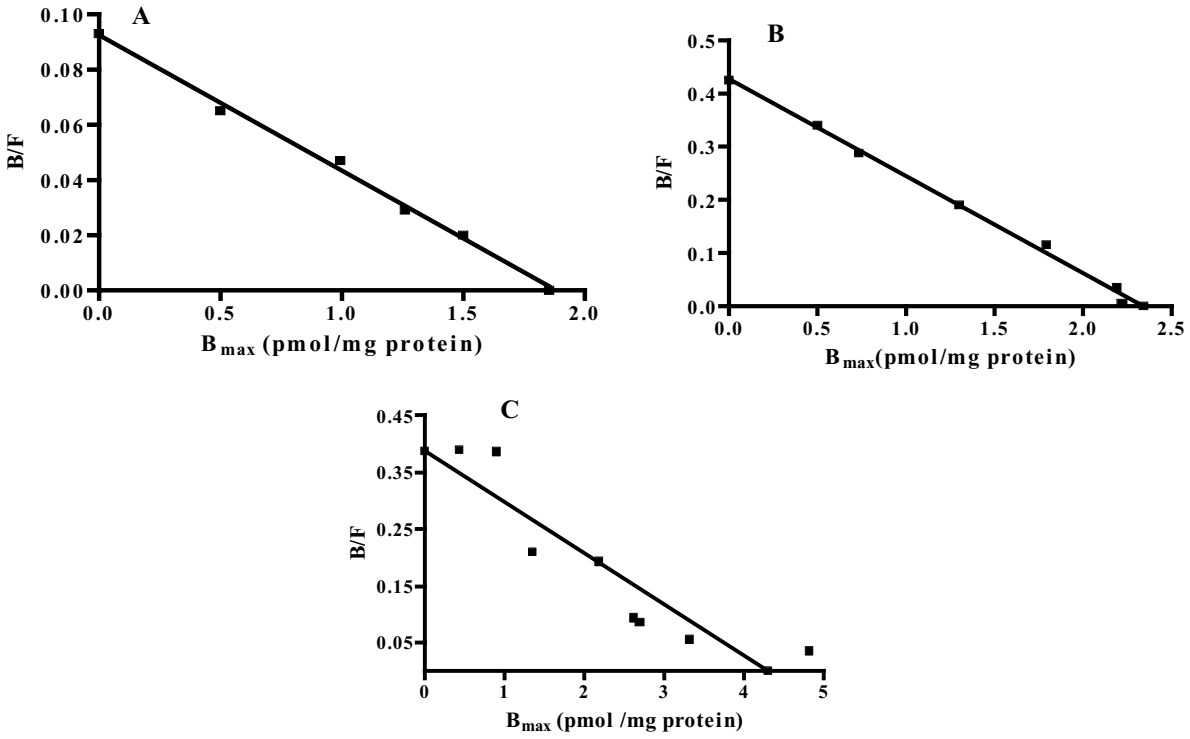

Fig. 1 Representative Scatchard Analysis of [^{3}H] Ro5-4864 Binding to PBR in Rat Mammary Gland Membranes: A – Normal, B – Non-aggressive tumor, C – Aggressive tumor

using Kodak image analyzer (Kodak, Perkins Elmer, Boston, MA).

Statistical analysis

Differences between control and breast cancer samples were assessed by using student *t*-test, and the significance level was set for $p \leq 0.05$.

Results

According to histological grading all the tumors were divided into two groups. One group had grade I (non-aggressive) mammary gland adenocarcinoma, and the second group had highly aggressive (grade II and III) mammary gland adenocarcinoma (data not shown).

Binding of ^{3}[H] Ro5-4864 to PBR in mammary gland

Differential ligand binding was observed in mammary glands. [^{3}H] Ro5-4864 bound specifically to PBR. PBR binding was characterized by Scatchard analysis described under "materials and methods". The observed k_d values of breast tissue suggest an increase of affinity for the receptor in cancer tissues (Fig. 1 and Table 1). B_{max} was significantly higher in breast tumor than that in normal breast. However, in comparison to normal tissues, the PBR density was significantly higher in aggressive tumor tissues (2.8 fold) than non-aggressive tumor tissues (1.4-fold).

Cholesterol transport

Transport of cholesterol was determined as the incorporation of [^{3}H] cholesterol in intact nucleus acquired from normal and cancer tissue. Experiments were carried out using 10 μM [^{3}H] cholesterol. Significant increase of cholesterol transport was observed in both non-aggressive and aggressive breast cancer tissue compared to normal (Fig. 2).

Table 1 Binding Characteristics of PBRs in Normal and DMBA-induced Rat Mammary Gland

		Cancer	
Parameters	Normal	Non-aggressive	Aggressive
B_{max} (pmol/mg protein)	1.94 ± 0.24	2.96 ± 0.44*	4.43 ± 0.33*
K_d (nM)	42.90 ± 7.63	2.29 ± 0.25*	5.93 ± 0.98*

Results are expressed as mean ± SEM of 5 different observations.
*Significantly different from Normal ($p < 0.05$).

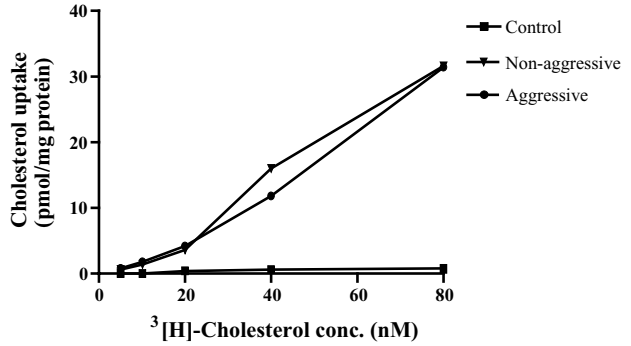

Fig. 2 Nuclear Cholesterol Transport in Normal and DMBA-induced Rat Mammary Glands. Data is representative of 5 independent experiments reported as means ± SEM. $^{*}P < 0.05$

Binding of ^{3}[H] Ro5-4864 to nucleus PBR in mammary gland

The observed k_d values suggest an increase of affinity for the nuclear receptor in cancer tissues (Fig. 3 and Table 2). B_{max} was remarkably higher in both aggressive and non-aggressive tumor tissue than in normal tissue.

NTPase activity

According to Fig. 4, NTPase activity was higher (130%) in aggressive cancer tissue in comparison to normal mammary gland. No significant change was observed in a non-aggressive tumor compared to normal.

Table 2 Binding Characteristics of PBRs in Nucleus of Normal and DMBA-induced Rat Mammary Gland

		Cancer	
Parameters	Normal	Non-aggressive	Aggressive
B_{max} (pmol/mg protein)	0.65 ± 0.05	21.70 ± 3.406*	44.87 ± 6.74*
K_d (nM)	8.91 ± 7.81	6.828 ± 1.887	5.56 ± 1.31

Results are expressed as mean ± SEM of 5 different observations.
* Significantly different from Normal ($p < 0.05$).

Endogenous nuclear cholesterol level

26% increase in cholesterol level was observed in aggressive cancer tissues compared to a normal gland (Fig. 5). There was no significant change in cholesterol level in non-aggressive mammary gland compared to normal mammary gland.

Western blot analysis

At the level of protein regulation, we measured changes of the 18-kDa and 32-kDa subunits of PBR separately (Fig. 6). We found significant changes in the levels of both subunits when

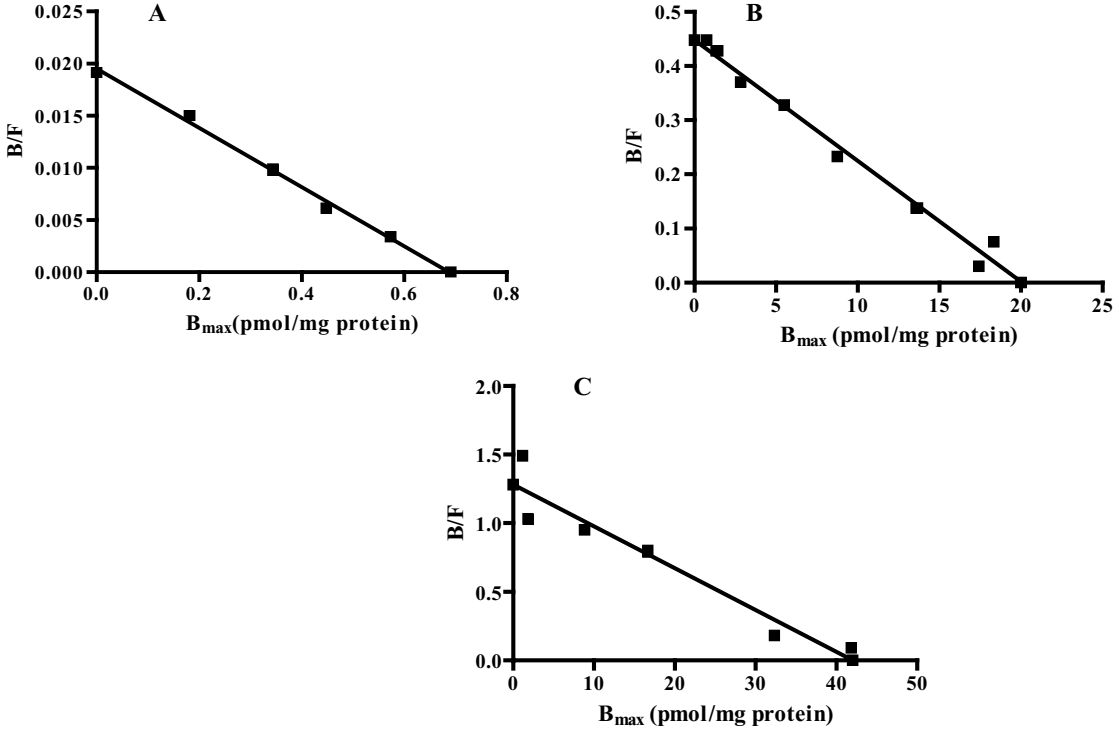

Fig. 3 Representative Scatchard Analysis of [^{3}H] Ro5-4864 Binding to PBR in Nucleus of Rat Mammary Gland: A–Normal, B–Non-aggressive tumor, C–Aggressive tumor

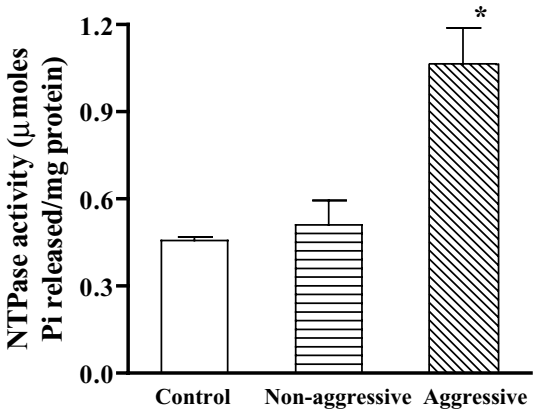

Fig. 4 Nuclear NTPase Activity in Normal and DMBA-induced Rat Mammary Glands. Results are expressed as mean ± SEM of 5 different observations. $^{*}P < 0.05$

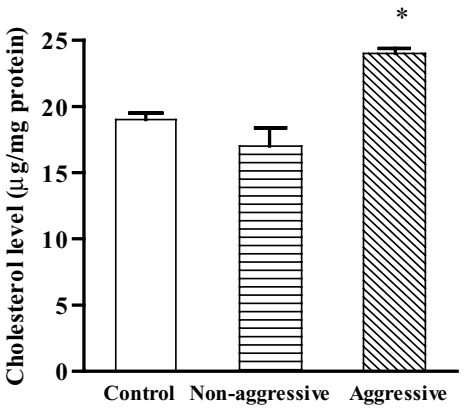

Fig. 5 Endogenous Nuclear Cholesterol Levels in Normal and DMBA-induced Rat Mammary Glands. Results are expressed as mean ± SEM of 5 different observations. $^{*}P < 0.05$

comparing the normal vs tumor tissues. In case of 32-kDa subunits, PBR expression was increased by 42.9% in aggressive tumor than normal mammary gland (Fig. 6), whereas between control and non-aggressive tumor tissue no significant difference was observed. The expression of 18-kDa subunit of PBR was increased (45.5%) in aggressive mammary tissue in comparison to the normal. Contrary to the 32-kDa subunit, a significant decrease (31.82%) of the expression of 18-kDa subunit was observed in non-aggressive mammary gland in comparison to the control animal.

Discussion

PBR and its endogenous ligand DBI have been detected in many benign and malignant tissues of various species. PBR

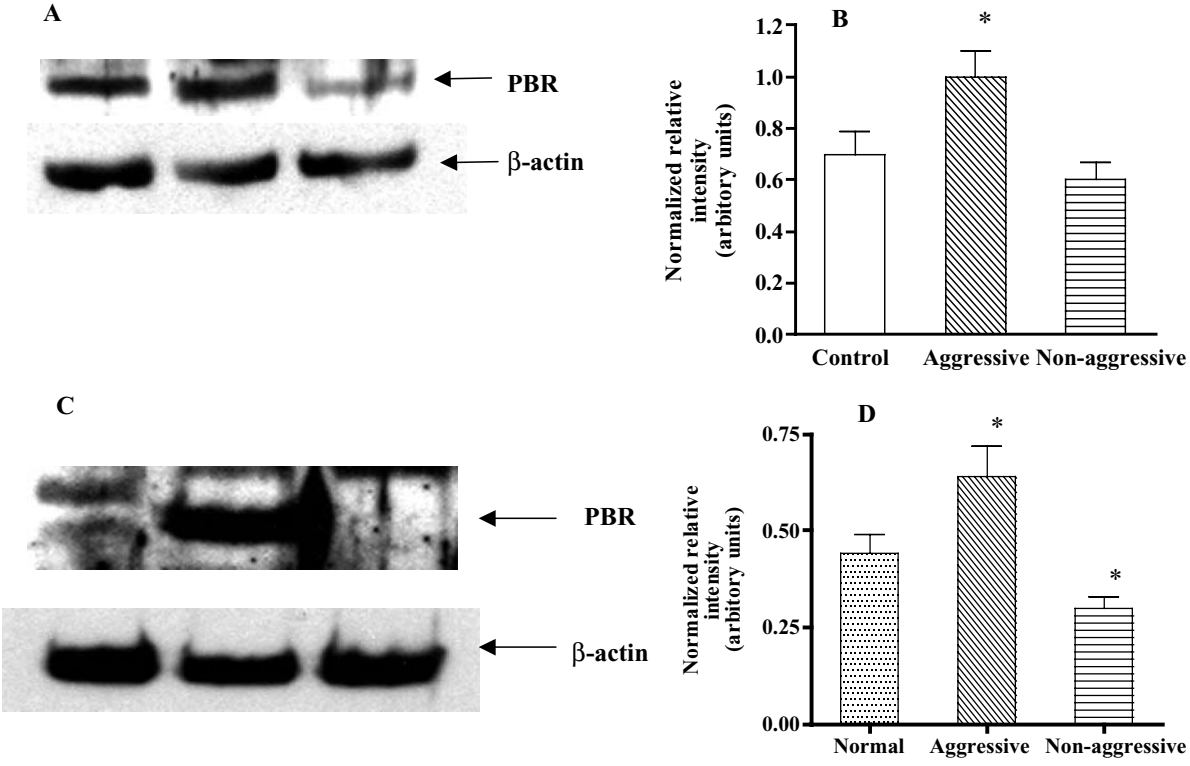

Fig. 6 **(A).** Western blot analysis of the 32-kDa PBR subunit ($n = 5$ in each group) of control and DMBA-induced rat mammary gland. Lane 1 – Normal; lane 2 – Aggressive; lane 3 – Non-aggressive. **(B).** Histograms summarizing western blot analysis data for the 32-kDa PBR subunit, $^{*}P < 0.05$. **(C).** Western blot analysis of the 18-kDa PBR subunit ($n = 5$ in each group) of control and DMBA-induced rat mammary gland. Lane 1 – Normal; lane 2 – Aggressive; lane 3 – Non-aggressive. **(D).** Histograms summarizing western blot analysis data for the 18-kDa PBR subunit, $^{*}P < 0.05$

and DBI have previously been detected in acinar cells of rat breast tissue and at a higher density in DMBA-induced breast tumors [42]. In our experiments, we also find an increase in the number of receptors available for binding (B_{max}) (Fig. 1, Table 1). In fact, tumor tissue shows lower k_d indicating higher affinity of ligand to these tissue. Based on these data the involvement of PBR and DBI in the regulation of function and growth of rat mammary cells may be suggested.

The physiological role of PBR is still debated. Its major function in endocrine tissues and some cell lines seems to be associated with cholesterol transport and steroidogenesis [26]. We have found an increase in the density of PBR in nucleus of breast tumors (Fig. 3 and Table 2), which may be responsible for increased transport of cholesterol into the nuclei of breast cancer tissue (both aggressive and non-aggressive) in comparison to normal tissue (Fig. 2). Even though endogenous nuclear cholesterol level was not different between normal and non-aggressive breast tumor, there was a significantly higher level of cholesterol in nucleus of aggressive breast tumors. We do not have any explanation at this time why basal cholesterol level was also high in normal tissue. Cholesterol is a lipid found in many biological membranes. Studies have also implicated a role of nuclear cholesterol in mechanisms underlying cell proliferation and cancer progression [42,43]. We suggest that endogenous PBR ligands bind to PBR found on the nuclear membrane and facilitate cholesterol transport into the nucleus (Fig. 2, Table 2). Cholesterol is then mobilized into the nucleus. Cholesterol's presence in the nucleus may change the dynamics of the nuclear membrane, such as fluidity, or associate itself as part of the nuclear membrane. When membrane fluidity is altered, signals that direct cell proliferation pathways indicate numerous signaling cascades in the cell [43].

Our results clearly demonstrate that the nuclear NTPase is sensitive to the cholesterol content of the nuclear membrane in aggressive breast tumors (Figs. 4 & 5). Czubryt *et al*. [44] showed that the nuclear membrane cholesterol increased *in vivo* and the NTPase activity increased with it. The incorporation of cholesterol into the nuclear membrane in the present study may alter NTPase activity via a change in membrane rigidity. The response of cholesterol-enriched nuclei suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli like cancer.

The expression of 32-kDa subunit of PBR protein increases only in aggressive tumors and not in non-aggressive tumors (Fig. 6). Furthermore, 18-kDa subunit of PBR also increases in aggressive mammary tissues whereas it is decreased in non-aggressive mammary tissues (Fig. 6). However, it should be noted that the commercially available 18 kDa PBR antibody did not give a clean band in comparison to 32 kDa PBR antibody. Even though the exact mechanism is not known at this time, it may be suggested that 32-kDa proteins may be a polymorphic form of 18-kDa PBR proteins. It is known that aggressive human breast cancer cells contain mainly a PBR dimer, which increases cholesterol transport into the nucleus and cell proliferation [22]. Delavoie *et al*. [45] also proposed that PBR polymer might be the functional unit responsible for ligand-activated cholesterol binding, and that PBR polymerization is a dynamic process modulating the function of this receptor in cholesterol transport and other cell-specific PBR-mediated functions.

PBR nuclear localization and increase in cholesterol transport in breast cancer implicates that PBR has a role in nuclear functions. Many molecular and cellular changes are currently used as a factor in diagnosing breast cancers as prognostic indicators. Effective anticancer therapies are key in treating breast cancer. This study clearly indicates that PBR is an important molecule in cancer diagnosis and progression. Data on this study will provide a better understanding of the interplay involving PBR and other molecules, especially cholesterol, in the breast cancer signaling cascade.

Acknowledgments Supported by grants from the Department of Army (DAMD 17-03-1-0352 and DAMD 17-03-2-0054)

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