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Peripheral benzodiazepine receptor protein expression in cells treated with alcohol and cytokines; a study on alcoholism

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Keywords: Alcohilism, Neurogegeneration.

ABSTRACT

Alcoholism is a complex disease that impacts the CNS through tolerance, dependence, brain damage and neurological and cognitive deficits. Chronic exposure to ethanol brings neurodegeneration and an increase in the level of an outer mitochondrial membrane protein, called the peripheral benzodiazepine receptor, PBR for short. In this study, western blotting and immunocytochemical methods were used to detect the effects of ethanol and cytokine treatments on astrocytes, astrocytomas, and microglia. Although ethanol seemed to have led to a decrease in PBR in the astrocytes and microglia, it did produce an increase in astrocytomas. Additionally, LPS and cytokine mix seemed to have at least physically altered the appearance of these ethanol-exposed cells.

INTRODUCTION

Chronic ethanol exposure leads to impairments of learning and memory. In fact, rats exposed to a 4-day ethanol binge showed neurogengeration in the olfactory bulb, piriform cortex, perirhinal cortex, entorhinal cortex, and the dentate gyrus using silver staining. Since binge ethanol exposure resulted in neurodegeneration of a corticolimbic circuit and was associated with perserverative responding on a spatial learning task, a single binge may very well cause neurodegeneartion and cognitive dysfunction in humans (Obernier et al., 2002).
The PBR, peripheral benzodiazepine receptor, is an outer mitochondrial membrane protein found in many places including the heart, liver, adrenal and testis, as well as hemopoietic and lymphatic cells. Originally discovered as an additional binding site for diazepam, which usually binds to GABAA receptors.

PBR is a critical component of the mitochondrial permeability transition pore, MPTP, a multiprotein complex located at the contact site between inner and outer mitochondrial membranes, which is involved in apoptosis. PBR presents many future implications as it is widely distributed throughout the body and has been associated with numerous biological functions including apoptosis, regulation of cell proliferation, stimulation of steroidogenesis, immunomodulation, porphyrin transport, anion transport and regulation of mitochondrial functions. PBR activity may have potential therapeutic applications and might be of significant clinical benefit in the management of a large spectrum of different indications including cancers and neurodegenerative diseases.

The binding of specific ligands to PBR has been implicated in several physiological functions, including steroidogenesis (Papadopoulos et al., 1990), cell growth and differentiation (Wang et al., 1984), chemotaxis (Ruff et al., 1985), and mitochondrial respiratory control (Hirsch et al., 1989). More importantly, benzodiazepines are often used for alcohol withdrawal treatment, as they can improve most conditions of physically dependent animals (Goldstein, 1972; Okamoto et al., 1983).

Chronic exposure to ethanol increases PBR levels throughout different regions in the brain (Obernier et al., 2002). While the brain PBR of alcohol-dependent mice increased after 7 days of liquid diet treatment, no change was found in central type benzodiazepine receptors (Schoemaker et al., 1983). Another study showed that

animals made physically dependent on ethanol showed only an increase in PBR in the brain, but not in the kidney, and that a single ethanol injection did not affect brain central or peripheral type benzodiazepine receptors (Tamborska and Marangos, 1986). Multiple studies have suggested that ethanol exposure causes time-dependent changes in brain PBR that may be linked to the development of physical dependence (Syapin and Alkana, 1988).

This study attempted to measure the amount of PBR in human astrocytes and microglial cells in culture. Treating the cells with various cytokines causes the astrocytes and mitochondria to change shape, affecting the level of PBR. Although PBR is proposed to be used to measure neurodamage in humans, the nature of its role in alcoholism and withdrawal remains unknown. By attempting to the measure the level of PBR in cells treated with ethanol and various cytokines, this study traces the activity of the protein, especially in observance of PBR's function as a marker of activated cells during brain damage.

More specifically, this study uses cell culture replications and western blots to detect PBR levels and observe its activity under alcohol and cytokines.

MATERIALS AND METHODS

Several methods were used in this study to detect the levels of PBR.

Western Blotting Procedure

Preparation of Samples: Whole cell extracts made using triple-detergent buffer were prepared as described by Sambrook et al., 1889. Mitochondrial pellets were prepared as described previously (Karri, et al, 2007).

Gel Electrophoresis: The Biorad system was used. For PBR, both the 12% and 15% Lower Gels were used. For the 12% Lower Gel, 4.66 mL of 30% Acrylamide/Bis Solution, 3.0 mL of Lower Tris Solution, 3.95 of mL DI Water, 50 µL of Ammonium Persulfate solution, and 10 µL of TEMED were used. For the 15% Lower Gel, 5.83 mL of 30% Acrylamide/Bis Solution, 3.0 of mL Lower Tris Solution, 2.78 of mL DI Water, 50 µL of Ammonium Persulfate solution, and 10 µL of TEMED were used.

Using a Pasteur pipet and bulb, the space between the two glass plates up to about ½ cm below the bottom of the wells was filled. The same was repeated for the second gel and the leftover lower gel solution was left in the tube to tell when the gel was fully polymerized by checking the leftover in the test tube.

In another 16x100 mm glass test tube, the stacking gel was mixed and vortexed. The stacking gel was made after the lower gel polymerized, with 0.8 mL of 30% Acrylamide, 1.5 mL of Upper Tris Buffer, 3.64 of mL water, 50 µL of Ammonium Persulfate solution, and 10 µL of TEMED.

The Pasteur pipet was left with the leftover solution to tell when the gel was fully polymerized.

Once the gel was fully polymerized, the comb was carefully removed by pulling it straight up out of the gel. The chambers between the gel sandwich and the cooling tower were then filled with running buffer until it fills the sample wells. Each well was then gently flushed with the running buffer to remove unpolymerized acrylamide. The buffer reservoir tub was filled with enough running buffer to cover about the bottom 1.5 cm of the gel sandwiches. The samples, diluted in loading buffer, were then placed in a boiling water bath for five minutes. The samples were then removed from the water bath and centrifuged for a few seconds to collect all parts of the samples at the bottoms of the tubes.

First, some of the running buffer was pulled up into the pipet to lubricate the samples. Then the samples were loaded by carefully injecting the 10 µL of each sample into the appropriate wells. Once all the samples were loaded, the electrophoresis hood was placed over the cooling tower and reservoir, the wires were hooked up red to red, and black to black. The ends of the wires were then plugged into the power source and turned on 125V, for about 30-60 minutes, until the electrophoretic separation by molecular weight takes place. The power supply was shut off once the dye front reached about 1 cm from the bottom of the gel.

Transfer: Three larger tubs were filled with about 1 cm deep of methanol, deionized water, and cold transfer buffer. Two PVDF membranes were removed from their bags and the plastic separator sheets were then thrown away. One membrane was soaked in the methanol for about 10 seconds and then removed and placed in the water tub and soaked on top of the water for 5 minutes.

After removing the membrane from the water tub, it was completely submerged in the cold transfer buffer tub for at least 5 minutes. The washing was then repeated for the second membrane in the same tubs. The gel sandwiches were then removed from the cooling tower and T-spacers from the sides of the sandwich were pulled out.

After pulling the glass plate off the top of the sandwich, the glass plate was used to cut through the stacking gel (the wells) to discard it. The plate and the gel were then soaked in a tub of cold transfer buffer with the gel facing up. With a cut weighing boat spatula, the gel was carefully separated from the glass plate. Then the glass plate was removed from the tub without allowing the gel to flip upside down.

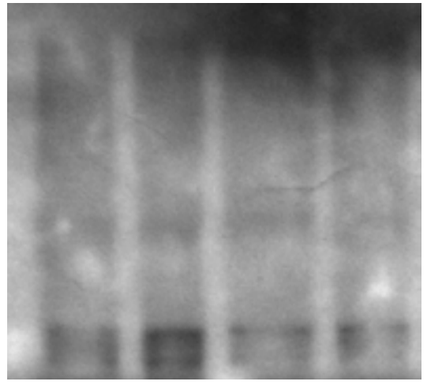
A piece of 3 MM paper was then floated on top of the gel. The paper was then gently pressed upon and then peeled off of the bottom of the tub. The gel was then stuck to the 3 MM paper.

The washed PVDF membrane was placed on top of the gel and the 3 MM paper and was centered. A second piece of 3 MM paper was wetted and centered on top of the PVDF membrane as well. A glass test tube was used to gently smooth out all the air bubbles. Two of the transfer sponges in the cold transfer buffer tub was wetted. A wet transfer sponge was place on the black side of the cassette.

The PVDF:3MM:gel sandwich on this sponge had the gel facing the black side of the cassette and the PVDF membrane facing the clear side of the cassette. The second wet transfer sponge was placed on top of the sandwich and the cassette was then closed.
The cassette was then placed in the cassette holder with the correct colors facing the sides of the holder (red to clear, black to black).

The final configuration of the sandwich:

□□□□□□□□: clear side of the cassette
xxxxxxxxxx: wet transfer sponge
==========: wet 3 MM paper
----------: PVDF membrane
––––––––––: PAGE gel
==========: wet 3 MM paper
xxxxxxxxxx: wet transfer sponge
□□□□□□□□: black side of the cassette



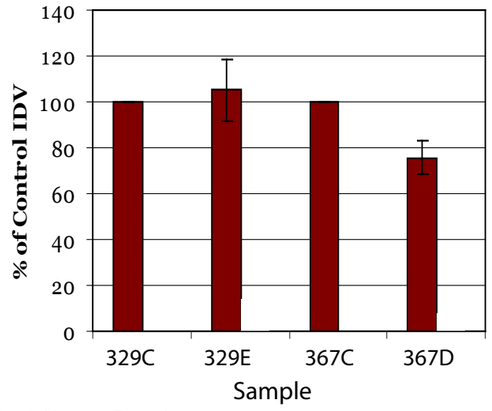
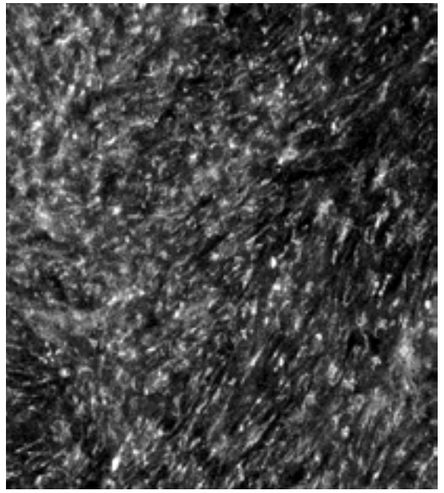


Fig. 1: Human Fetal Astrocytes
The effect of ethanol on PBR in human astrocytes. Top: autoradiogram of a western blot for PBR. The arrow indicates the bands quantified. Bottom: graph of IDV from western blots for PBR (mean plus SEM, N=4). In the 329 sample, the ethanol seemed to have increased the amount of PBR. In the 367 sample however, the ethanol seemed to have decreased the amount of PBR.



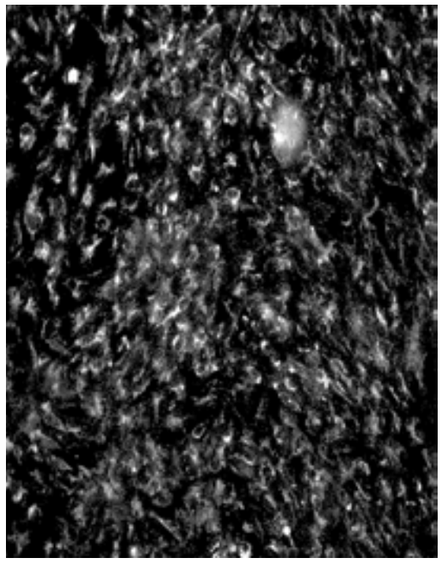


Fig. 2: Immunocytochemistry of 367 cells
Top: the 367 is ethanol treated. Bottom: is 367 control. These are greyscale conversions of MitoTracker fluorscence. The Cells with ethanol were more condensed.

These steps were repeated for the second gel. The cassette holder in the transfer tub was then placed so that the current plugs are in the center of the tub. A magnetic stir bar was dropped in the bottom of the tub and an ice block was placed next to the sandwiches. The tub was filled with 4°C transfer buffer up to the last row of holes on the cassettes. The cover was placed over the tub so that the leads match, red to red, black to black. The whole tub on a stir plate was put inside the cold room (4°C) and turned on a spin setting of 3. The lid wires were then connected to the power source and turned on. Transfer took place for 2 hours at 87V and the ice pack was changed in the middle of the 2 hours to ensure that the tub remains as cold as possible.

Blocking: Once the transfer took place, the cassettes were opened and the PVDF membranes were removed and placed in separate tubs containing 25 mL of the TST buffer (4.844g of Tris base, 23.376g of NaCl, 4mL of Tween 20 brought to 4.0L with good water and to a pH of 7.5) with 1% bovine serum albumin. The blocking took place for 1 hour at room temperature with rocking at 25 revolutions per minute. The tubs were covered to prevent evaporation.

After that, the blocking buffer was poured off and then the membranes were placed in tubs holding 25 mL of the primary antibody (1:250 or 1:500 dilution of goat anti-serum against human PBR (Santa Cruz Biotechnology Inc.) dissolved in blocking buffer solution), and were rocked in the cold room overnight.

The next day, the primary antibody solution was poured off and the membranes were washed in 25 mL of TST and allowed the tubs to rock for 5 minutes. The washing with TST was then repeated for 4-6 times, with 5 minutes each time.

Then, 25 mL of the secondary antibody (1:5,000 dilution of Alexa-488 conjugated donkey anti-goat IGg dissolved in the blocking buffer solution) was used for each membrane. The tubs were then rocked for at least 3 hours before the secondary antibody solution was poured off. The same 4-6 TST washings then took place.

After the final TST wash, the membranes were put in separate tubs containing 10 mL of the chemiluminescent substrate (5 mL of each bottle). The membranes were then incubated in this substrate, without rocking, for five minutes.

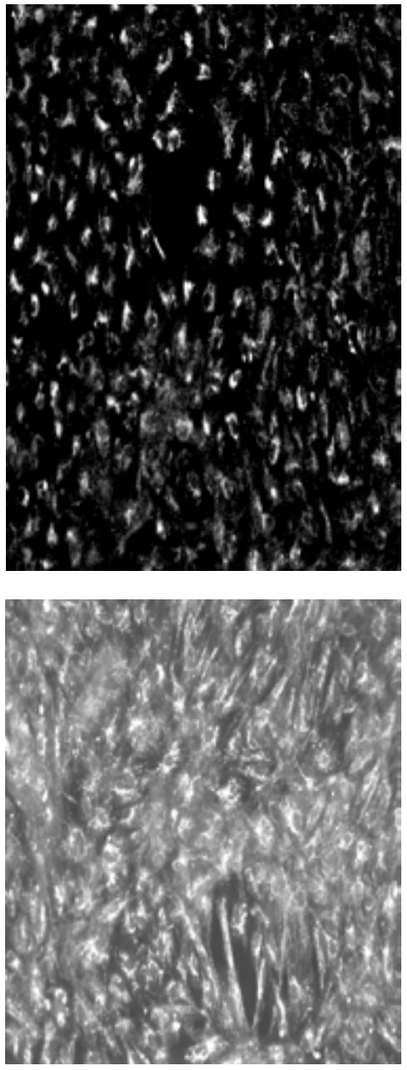


Fig. 3: Immunochemistry of 329 Cells
Top: the 329 is ethanol treated. Bottom: is 329 control. These are grey scale conversions of MitoTracker fluorescence. The cells treated with ethanol seem less condensed.

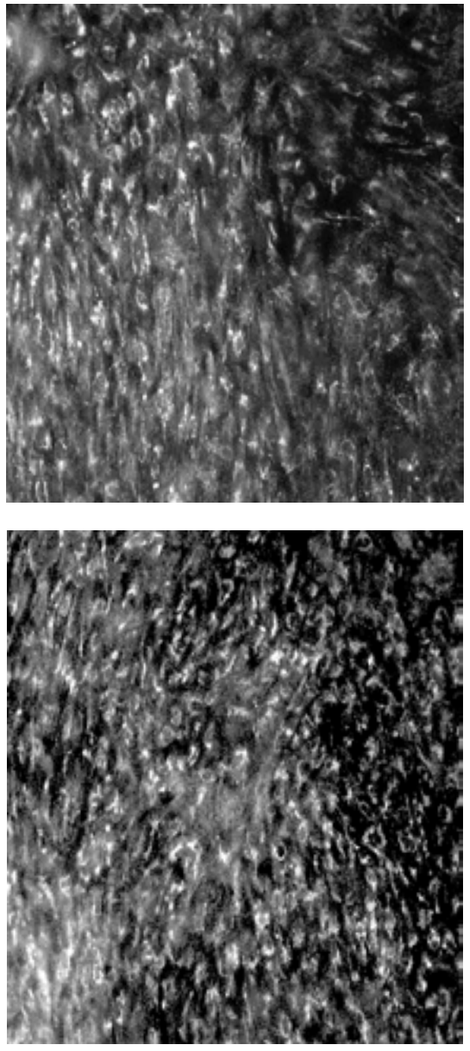


Fig. 4: Immunochemistry of 367 Cells
Top: the 367 is ethanol treated, along with lipopolysaccharides. Bottom: is the 367 control with lipopolysaccharides. These are grey scale conversions of MitoTracker fluorescence. These two pictures do not show that much difference in appearance.

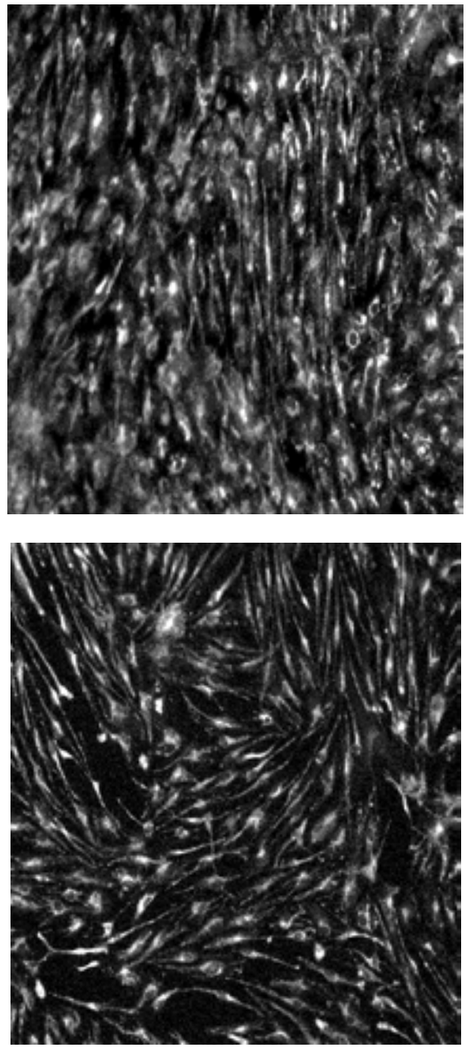


Fig. 5: Immunochemistry of 367 cells
Top: the 367 is ethanol treated along with CM (cytokine mix). Bottom: it the 367 controll with CM. These are grey scale conversions of MitoTracker fluorescence. The cytokine mix seemed to have changed the cells drastically by elongating them.

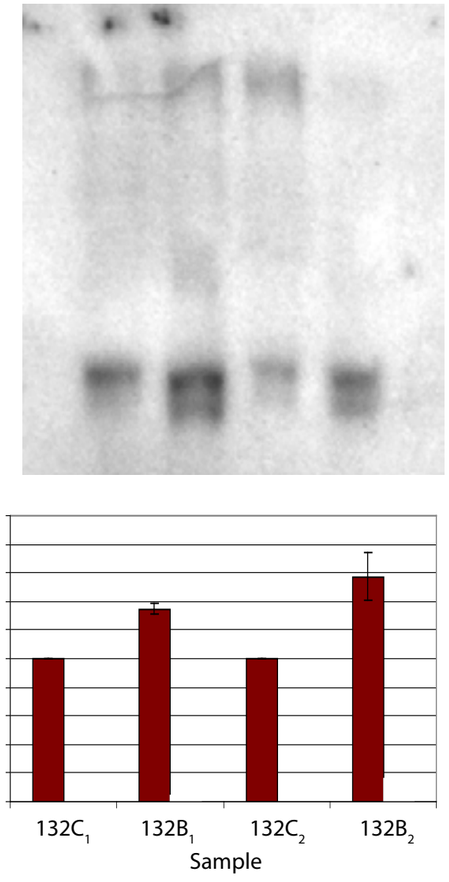
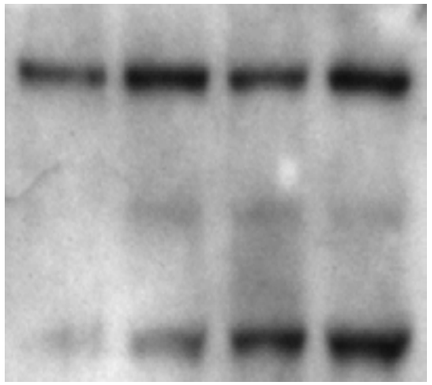


Fig. 6: The effect of ethanol on PBR in astrocytoma
Top: autoradiogram of a western blot for PBR. The arrow indicates the bands quantified. Bottom: graph of IDV from western blots for PBR (mean plus SEM, N = 2). In both samples of 132, the ethanol seemed to have increased the amount of PBR.

The membranes were then removed and drained by being held over a paper towel with the forceps and touching just one corner of the membrane to the towel.

Detection: Then the membranes were placed on a piece of transparency and aligned in the developing cassette, with their protein side facing upwards. Then the membranes were exposed to a piece of X-ray film in the dark room for 15, 30, 45, 60, 90 seconds, depending on the quality of the signal. After developing the films and marking the molecular weight bands, the membranes were submereged in a tub filled with 25 mL of TST and 30 µL of Black ink. The membranes were then left to rock at room temperature overnight.



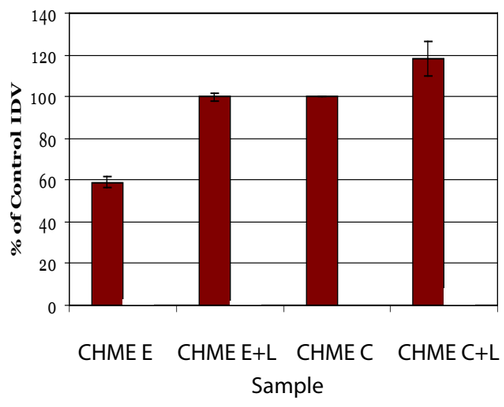


Figure 7: The effect of ethanol on PBR inCHME Microglia
Top: autoradiogram of a western blot for PBR. The arrow indicates the bonds quantified. Bottom: graph of IDV from western blots for PBR (means plus SEM, N = 2). In the CHME samples (CHME E and CHME E+L), the ethanol seemed to have decreased the amount of PBR. The lipopolysaccaride (L) in both cases has increaseed the level of PBR.

Analysis: The AlphaEase detection system was used to capture an image of the autoradiogram and to quantify the intensity (in IDV, which is Integrated Density Value) of the bands of interest. Then the IDV's were used to construct a graph to visually display the differences between the control and the treatment.

Immunocytochemistry/Cell Culture

Growth of Human Astrocytes: Human fetal astrocytes, lot numbers 329 and 367, were from two different donors and were grown according to the directions provided by Sciencellonline.com. Poly-L-lysine coated flasks (2 µg/cm2, T-75) were prepared and left in the 37°C incubator overnight.

Complete medium was prepared to contain 2% fetal bovine serum, astrocyte growth factors, and antibiotics. Cells were seeded at the recommended density of 5,000 cells/cm2 and grown at 37°C in 5% CO2. The medium was changed every other day.

The cells were subcultured when they were over 90% confluent using trypsin/EDTA solution, trypsin neutralization solution, and DPBS to room temperature. The harvested cell suspension was centrifuged at 860 rpm for 5 min and resuspended in growth medium.

Cells were counted with a hemocytometer and plated in new, poly-L-lysine coated flasks or glass coverslips (for immunocytochemistry) at the recommended cell density.

Cell Culture of CHME Microglia: Human transformed microglia, CHME-5 cells, SV40-immortalized microglial cell lines (Janabi et al., 1995) were seeded at 0.25 x 106 cells in a 75 cm2 polystyrene culture flask and incubated at 37oC with 5% CO2. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics/antimycotic was used as growth media to maintain the cells in culture. Media was changed on the fifth day of incubation. After a week period, stocks were harvested using 10x EDTA and reseeded. Microglia used in experiments were grown in separate 10 cm dishes seeded at 1x106 cells/dish. These microglia were maintained as previously described until use.

Cell Culture of 132-1N1 astrocytoma cells: The human astrocytoma cell line 132-1N1 was grown in 75 cm2 polystyrene culture flasks and incubated at 37oC with 5% CO2. Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum and antibiotics was used as the growth medium.

Treatment with ethanol and cytokines: Cells were exposed to 44 mM ethanol in the presence or absence of various cytokines and lipopolysaccharide (500 ng/mL) in serum-free culture medium (SFM) for 1-5 days (see Results for details). For the Tnfα, the concentration used was 6 ng/mL, with a dilution of 1:1000. For the lipopolysaccharide (LPS), the concentration was 500 ng/mL with a dilution of 1:2000. For the interleukin and interfeuron group (I + I), the concentrations were 2 ng/mL and 100 ng/mL, respectively. For the cytokine mix (CM), which consisted of I + I + Tnfα, the concentrations used were the same as mentioned above.

Immunocytochemistry: The solutions prepared include Triton (0.1 – 0.3%) and 0.05% Triton in PBS, 3% BSA (for diluting primary and secondary antibodies), and 0.1% glycine in PBS (to quench fluorescence background due to aldehyde fixation). The primary antibody is prepared by diluting it first in 3% BSA. Antibody concentration was determined by published protocols, vendor's recommendation, or empirically by testing a range of concentrations (dilution curve). The secondary antibody was Alexa Fluor diluted 1:500 in 3% BSA in PBS. Generally, a sufficient volume is needed to evenly coat a coverslip surface (500 µL per coverslip if staining inside a 24-well plate, less if inverting coverslips on parafilm).

Cell for immunocytochemical analyses were first incubated with 50 nM MitoTracker CMXRos to label mitochondria. Following MitoTracker labeling they were rinsed in warm SFM followed by warm PBS containing Mg+2 and Ca+2. Cells were than fixed in 4% Formalin for 15 minutes at 37 °C. Following fixation they were rinsed four times in PBS + Ca/Mg.

The cells were then incubated in 0.1% glycine for 5 minutes at room temperature. Then the cells were washed with 0.1 M PBS. After that, all cell membranes in the cells were made permeable with cold 0.1 – 0.3% Triton for 5 – 15 minutes. The cells were then washed twice for one minute each with PBS.

Blocking buffer is subsequently used to block nonspecific binding sites for 15 – 30 minutes. Then the cells were incubated with diluted primary antibody for 1 – 2 hours at room temperature. The cells were then washed thrice for five minutes each with PBS. The cells were then incubated with the secondary antibody for 45-90 minutes at room temperature.

After cells were once again washed thrice for five minutes each with PBS, they were incubated with DAPI for five minutes at room temperature. Finally, the cells were rinsed with pure water and mounted (inverted, with cells down) on glass slides with Mowiol/DABCO.

BCA (bicinchoninic acid) Protein Assay in 96 Well Plates: This procedure was used to determine the concentration of protein in samples of cells or tissues prepared in 0.5 M NaOH (final concentration). The common solutions used include the Bovine serum albumin standard (Sigma A-7638: a 2 mg/mL stock solution, deionized water, Bicinchoninic acid (BSA protein assay reagent A, Pierce No. 23223), and 4% cupric sulfate pentahydrate solution (Sigma C-2284) and 2.5 M NaOH diluted from 10 M stock, which is kept at room temperature.

The BSA concentrations used include 0, 2, 4, 6, 8, 12 and 16 µg.

RESULTS

Human Astrocytes: Two samples of human astrocytes were studied, the 367 and 329 cells. For western blots and immunocytochemistry, the cells were treated for five days with 44 mM ethanol. The whole cell extracts of human astrocytes were studied.

In the 329 samples, there was 5.2% PBR increase in the presence of ethanol, with a standard error margin of 13.5%. In the 367 samples, there was a 24.4% PBR decrease in the presence of ethanol, with a standard error margin of 7.5.

The immunocytochemical methods used were unable to detect PBR, but the cytokine treatments and possibly the ethanol appeared to have affected the cells. The CM seemed to have affected the cells the most, changing the shapes and sizes of the cells (see Figure 5). The cells looked more different in the 329 sample under ethanol than the cells in the 367 sample (compare Figures 2 and 3). Adding lipopolysaccharide to the cells did not seem to produce any significant physical change to the cells (see Figure 4).

For the 367 cells treated with LPS, there does not seem to be any noticeable difference in appearance between the control and ethanol treated cells. The same is true for the 367 cells treated with the interleukin and interferon (results not shown).

For the 329 cells treated with Tnfα, there does not seem to be any noticeable difference in appearance between the control and ethanol treated cells (results not shown).

132 Astrocytoma: Samples of human astrocytoma were studied, with C1 and C2 grown and prepared separately. For western blots, the cells were treated for seven days with 44 mM ethanol. Whole cell extracts were used.

In both samples of the 132 cells, ethanol seemed to have increased the concentration of PBR. In C1, the ethanol increased the PBR concentration by 34.9%. In C2, the ethanol increased the PBR concentration by 57.4%.

CHME Microglia: Samples of immortalized microglia were studied. For western blots, the cells were treated for four days with 44 mM ethanol. Mitochondria prep was also used.

Ethanol alone decreased the concentration of PBR in microglia by 41.0%. The addition of lipopolysaccharide alone increased the concentration of PBR in microglia by 18.3%, while the addition of both ethanol and lipopolysaccharide decreased PBR by 0.2%. The interaction of lipopolysaccharide with ethanol seemed to have weakened, almost cancelled out, the effects of ethanol on PBR, since ethanol alone would have decreased the level of PBR by almost half, even though adding lipopolysaccharide alone only increases PBR levels by less than 20%. The cause and significance of this phenomenon remain unclear.

DISCUSSION

Human Astrocytes: The results produced by the human astrocytes were inconsistent––the ethanol seemed to have increased the level of PBR in 367 and decreased the level of PBR in 329. The differences may be attributed to the natural variations in fetuses between the two samples, the 367 and 329 lots. The 329 and 367 cells were harvested during separate time periods from two different fetuses, which may be differently gendered. More trials would have to be conducted to confirm the effects of ethanol on human astrocytes in the future.
Although the immunocytochemistry did not successfully detect PBR, even with staining methods, the images did show that the cytokine treatments did alter the physical appearance of the cells. The addition of ethanol to the 367 cells did not seem to have changed the cells much, whereas the changes to the 329 cells seem to be much more significant. While the effects of LPS, Interleukin and interferon, and Tnfα on the appearance of the astrocytes seemed to have been negligible in the immunocytochemistry, LPS did increase the concentration of PBR detected in the cells in the western blots. What these physical changes in shapes and sizes and what this inconsistency may mean remain unclear.

132 Astrocytoma: In the 132 cells, the addition of ethanol increased the concentration of the PBR.

CHME Microglia: In the immortalized microglial cells, ethanol decreased the level of PBR.

CONCLUSIONS

Ethanol only seemed to have consistently increased the level of PBR in astrocytoma cells. In the human astrocytes harvested from two fetuses, ethanol produced contradicting results in the level of PBR. In the CHME microglia, the ethanol decreased the level of PBR consistently.

More trials and other methods (immunohistochemistry, mRNA, etc) should be considered for future studies.

Although many gaps still remain in the knowledge of the relationship between PBR and ethanol, this study does show that ethanol increases the concentration of PBR in certain cases as much as it increases them. Also, the addition of cytokines also seems to have some observable effects on the appearance of the cells, though those effects remain unknown

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