Chapter - III
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Effect of Ethanol on Protein Tyrosine Kinases

Protein tyrosine phosphorylation plays an important role in the transduction of extracellular signals. A protein tyrosine kinase (PTK) localized in plasma membrane and coupled to receptor mediates the binding of extracellular factors. Besides, several cytoplasmic kinases enter nucleus and play a major role in the transcriptional regulation and cell cycle control (Hunter and Cooper, 1985; Hunter, 19%). Several PTKs also have been implicated in diseases such as cancer (Boutin, 1994), Alzheimer's disease (Shapiro et al., 1991) and oncogenic activation of transforming retroviruses (Hunter and Cooper, 1985). The presence of high levels of PTK activity in adult brain (Cotton and Brugge, 1983), suggest that PTKs might play specific role in the regulation of neuronal functions. The neuronal and synaptic PTK activity has been further shown to be involved in the modification of synaptic activity particularly depolarization, induction of long term potentiation (LTP) and long term depression (LTD) and ischemia (Gurd, 1997). The high levels of PTK activity in postmitotic neurons resulted in an altered tyrosine phosphorylation in the synaptic proteins (Dasgupta et al., 1994; Cotton and Brugge, 1983). The tyrosine phosphorylation of the synaptic proteins lead to a short term effect due to phosphorylation of acetylcholine, NMDA and GABA_A receptors besides K^+ and Ca^{2+} channels (Gurd, 1997). The long term effects of tyrosine phosphorylation have been shown to be particularly essential for the induction of LTP, LTD and ischemia (Gurd, 1997).

The presence of high PTK activity in adult brain and due to the role of tyrosine phosphorylation in neuronal functions such as LTP and LTD which form the crucial events for the memory in neuronal circuitry, studies were carried out on tyrosine specific phosphorylation in rat CNS using a tyrosine containing synthetic peptide as exogenous substrate. The synthetic polymer poly(Glu_4 Tyr_1) used in this study has also been shown as an effective substrate for various tyrosine specific protein kinases (Braun et al., 1984). Using this substrate the PTK activity was determined for its subcellular distribution as well as for its role in ethanol treated animals as a function of development.
MATERIALS AND METHODS

Materials
Sodium orthovanaodate, poly (Glu₄ Tyr). Phenylmethylsulfonyl fluoride (PMSF), Dithiothreitol (DTT), reagents for sodium dodecyl sulfate (SOS) - polyacrylamide gel electrophoresis (PAGE), 5-bromo-4-chloro-3-indoylphosphate / nitroblue tetrazolium chloride (BCIP/NBT) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ³²P] ATP (3000 Ci/mmol) was from Board of Radiation and Isotope Technology (Mumbai, India). Phosphocellulose filter (P81) was obtained from Whatman (Canlab Corp., Mississauga, ON, Canada). Nitrocellulose sheets were from Schleicher and Schuell (Keene, NH, U.S.A.). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Monoclonal antibody (PY20) against phosphotyrosine was a generous gift from Dr. Nagaraju, USA.

Assay of Protein tyrosine Kinases (PTKs) Activity

The endogenous protein tyrosine kinases activity was determined in the membrane and cytosolic fractions from control and alcohol induced cerebral cortex using synthetic peptide poly (Glu₄Tyr₁). Phosphorylation of the synthetic substrate was carried out by the procedure described (Trembley et al., 1994). The reaction mixture consisted of 10mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, 50 μg poly (Glu₄Tyr₁), 10 μM ATP, [γ³²P] ATP (1 μCi per sample) (3000Ci/mmol), 200μM sodium orthovanadate and 10μg protein from membrane and cytosolic fraction. Phosphorylation was performed for 10 min at 30°C and stopped by spotting the sample on Whatman No 1 (2 x2 cms) filter papers and precipitating the protein with 10%TCA/1% Na₂P₂O₇ . The filter papers were washed twice with the same solution for 10 min. each, rinsed with ethanol and dried. The radioactivity associated with the filters was measured in a liquid scintillation spectrometer. Specific tyrosine kinase activity was calculated by subtracting the incorporation of labeled phosphate into the endogenous protein from the total activity measured in the presence of endogenous protein and Poly(Glu₄Tyr₁) and expressed in pmol/min/mg protein.

Detection of Tyrosine Phosphorylated Endogenous Proteins

Endogenous phosphorylation of membrane and cytosolic proteins was carried out as described above except that the poly(Glu₄Tyr₁) was omitted and unlabelled ATP (100μM) was used instead of labeled ATP. The reaction was terminated by
adding sample buffer (2X) (Laemmli, 1970) and western blot was performed using anti-phosphotyrosine monoclonal antibodies (PY20) to detect the phosphorylated tyrosine residues in proteins.

**Western analysis for the detection of phosphotyrosine proteins**

Western blot was performed by the method of Towbin et. al,(1979). The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose sheet (0.45µm) using TGSM buffer (25mM Tris, 192mM Glycine, 0.1% SDS and 20% Methanol) as electrode buffer at 0.8mA/cm² transunit (total gel length x breadth) in a LKB-Multiphor II Nova blot for 4 hrs. Blots were stained with ponceau S solution to make molecular weights markings, washed with TBS (Tris-buffered-saline) and then were blocked with 5% milk powder made in TBS for 2 hrs. After blocking, primary antibody was diluted (1:100) in TBS and blots were incubated over night at 4°C on a shaker. The antibody solution was removed and the blots were washed thrice with TBS, followed by incubation with alkaline phosphatase coupled secondary antibodies for 2 hrs. Finally, the nitrocellulose sheet was washed thrice with TBS and visualized using 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride (BCIP/NBT) chromogen system.

**RESULTS**

**Effect of ethanol treatment on protein tyrosine kinase (PTK) enzyme**

Phosphorylation of the synthetic peptide substrate poly (Glu₁ Tyr₁) was used to measure the endogenous protein tyrosine kinase activity in the membrane and cytosolic fractions of control and alcohol fed rat brain cerebral cortex (Fig. 29, 30). The activity was measured in the presence and absence of sodium orthovanadate, a specific protein tyrosine phosphatase inhibitor. During the course of development PTK activity decreased both in the membrane and cytosolic fractions from 8 to 90 days of age. Maximum activity was shown to be associated at the age of 8 days and was gradually declined in the later ages (30 and 90 days) of postnatal development and this data agrees with the previously reported literature (Cudmore and Gurd, 1991). The PTK activity in the alcohol exposed rat cerebral cortex was decreased when compared to control in all the ages of postnatal development (8, 30 and 90 days) in membrane as well as in cytosolic fractions. In the presence of sodium orthovanadate the PTK activity was increased in the membrane and cytosolic fractions of control and alcohol samples. However, the enzyme showed a decreased
activity over development only in the absence of orthovanadate. This trend is abolished once orthovanadate is included in the assay even though it contributes a overall significant increase in the PTK activity. Since orthovanadate is a potent inhibitor of tyrosine phosphatase, it is likely that the balance between tyrosine phosphorylation and dephosphorylation is involved at this cellular context.

**Effect of ethanol treatment on phosphorylation of endogenous substrate proteins of protein tyrosine kinases**

To examine the endogenous substrate proteins of tyrosine specific protein kinases, the membrane and cytosolic fractions of control and ethanol fed rat cerebral cortex were incubated under phosphorylating conditions with unlabelled ATP and analyzed by immunoblotting technique using monoclonal antibody specific for phosphotyrosine residues (Fig. 31, 32). The phosphotyrosine proteins with molecular weight 114, 70, 36, 34, 32, 20 and 14 kDa in the rat brain cerebral cortex membrane fraction were detected both in the control and alcohol fed rat brain during the course of postnatal development from 8 to 90 days of age. However, a higher level of immunoreactivity of these proteins was found in the alcoholic fractions when compared to control fractions particularly at the age of 30 and 90 days of age. Two phosphotyrosine proteins with molecular weight 40 and 38 kDa showed decreased immunoreactivity at the age of 90 days in the cytosolic fraction of alcohol fed rat brain. Several other phosphotyrosine proteins were also detected in the membrane and cytosolic fractions of both control and alcohol fed rat cerebral cortex but the comparison was restricted only to the phosphotyrosine proteins showing a clear change during the course of development and prenatal alcohol exposure.
FIG. 29. Membrane associated PTK activity in control and ethanol treated rat
cerebral cortex at 8, 30 and 90 days of development. Membrane was prepared
and assayed for PTK activity in absence and presence of vanadate as described
in Materials and Methods. Activity is expressed as picomoles of PO₄³⁻
incorporated into the exogenous histone minute milligram of protein. The
data points are mean±SEM (bars) values of five separate experiments. \( P<0.05 \)
as compared with the control.

CM = Control membrane fraction
EM = Ethanol treated cytosolic fraction
Fig. 29. Protein Tyrosine Kinase activity in Membrane
FIG. 30. Cytosol associated PTK activity in control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Cytosol was prepared and assayed for PTK activity in absence and presence of vanadate as described in Materials and Methods. Activity is expressed as picomoles of PO₄⁻³ incorporated into the exogenous histone¹minute⁻¹milligram of protein. The data points are mean±SEM (bars) values of five separate experiments. *P*<0.05 as compared with the control.

CM = Control membrane fraction
EM = FAhanol treated cytosolic fraction
Fig. 30. Protein Tyrosine Kinase activity in cytosol
FIG. 31. Immunoreactivity of phospho tyrosine containing proteins in the absence of vanadate from membrane and cytosolic fractions of control and ethanol treated rat cerebral cortex. The blot was probed with phosphotyrosine antibodies (PY20) and visualized by alkaline phosphatase-BCIP/NBT detection system.

FIG. 32. Immunoreactivity of phospho tyrosine containing proteins in the presence of vanadate from membrane and cytosolic fractions of control and ethanol treated rat cerebral cortex. The blot was probed with phosphotyrosine antibodies (PY20) and visualized by alkaline phosphatase-BCIP/NBT detection system.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.
DISCUSSION

In the present study, the subcellular fractions of membrane and cytosol showed considerable levels of protein tyrosine phosphorylation based on the PTK assay. The cytosolic fraction relatively had higher activity compare to membrane. In most tissues including brain, the increase in the cytosolic tyrosine kinase activity coincides with the decrease in membrane associated tyrosine kinase activity which is in agreement with the previous data (Pamela, 1991). An overall gradual decrease in the tyrosine phosphorylation was noticed during postnatal development of cerebral cortex. The low levels of tyrosine kinase activity during development could be due to a decrease in the enzyme synthesis or due to a posttranslational modification which causes a decrease in enzyme specific activity. If these two possibilities are excluded, the development of cerebral cortex might involve the synthesis of a specific PTK inhibitor (Swarup et al., 1983; Okada and Nakagawa, 1988). Inspite of decreased tyrosine phosphorylation in adult cerebral cortex (90 days), it showed considerable activity that could still account for the endogenous phosphorylation of tyrosine residues.

The inclusion of vanadate in both control and ethanol treated samples of membrane and cytosol not only relieved the inhibition of PTK activity but shooted it up almost two fold. The outburst in the PTK activity in the presence of vanadate could be attributed partially to the potent inhibition of tyrosine phosphatase activity by vanadate. This would explain the inhibition of tyrosine phosphatase activity, which might promote PTK activity so that protein substrates would remain phosphorylated on tyrosine residues for relatively longer duration i.e. as long as the vanadate concentration prevails. The data from this study suggest that the PTK activity is modulated during development and is amenable for the action of agents like tyrosine phosphatase inhibitors. The addition of vanadate showed an increase in tyrosine kinase activity both in the control and ethanol treated group irrespective of different developmental stages studied, indicating the modulation of protein tyrosine kinase activity, directly or indirectly by the protein tyrosine phosphatases. However, tyrosine kinase activity in ethanol treated group remained always lesser than the control group. One plausible explanation seems apparent in this case. Earlier studies have shown that the brain may contain endogenous inhibitors of tyrosine kinases (Pamela, 1991) and further in a recent study it was demonstrated that the induction
and higher levels of **pag** protein, a known physiological inhibitor of nuclear 
**c-abl** tyrosine kinase using compounds inducing oxidative stress (Prosperi et al., 1998). Therefore, it is quite tempting to speculate that certain endogenous inhibitors of tyrosine kinase in the cytosol too might be induced that could serve to **down** regulate the tyrosine kinase activity. Further, ethanol has been shown to induce oxidative stress reactions in brain through its lipophilic and free radical generating properties (Renis et al., 1996). The consequence of protein phosphorylation both in control and ethanol treated samples are noticed in such a way that tyrosine phosphorylation events are tightly controlled in development as they show gradual decrease.

To examine the major substrates of PTKs, the membrane and cytosolic fractions from control and ethanol treated samples from various developmental stages were incubated under phosphorylation conditions using **unlabeled** ATP and analyzed by immunoblotting techniques by monoclonal antibody specific to phosphotyrosine. The tyrosine phosphorylation of endogenous membrane substrate proteins showed selective proteins of less than 29 kDa in ethanol treated samples from 8 and 30 days. However, inclusion of vanadate along with ATP lighted up considerably significant number of tyrosine phosphorylated proteins in the membrane fraction. The cytosolic fractions did not show significant changes.

The presence of high levels of protein tyrosine kinase and their endogenous substrates in membrane and cytosolic fractions is consistent with tyrosine phosphorylation being involved in the neuronal activities like neurotransmitter release, modulation of neurotransmitter receptors and ion channels (Ilirano et al., 1988). Further, the vanadate-induced increase in the PTK activity could possibly underlie potent protein tyrosine phosphatase (PTPs) inhibition. These data could also be interpreted in such a way that the endogenous protein tyrosine phosphatase levels in ethanolic samples must have been reasonably high enough so that in the absence of vanadate tyrosine phosphorylation is **masked** by the high activities of tyrosine phosphatase. In turn all these data points to a possibility of an altered **homeostatic** balance between the activities of PTKs and PTPs that requires further detailed analysis.