Regulation of cytochrome P4501A by protein kinase C: the role of heat shock protein70

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Abstract Carbofuran is a pesticide, which is used throughout the world as a nematicide and an acaricide. This pesticide integrates into living organisms through aquatic ecosystem. In earlier report, we had demonstrated that cytochrome P4501A was induced in cultured catfish hepatocytes in response to carbofuran, which might be responsible for the detoxification of this pesticide. As the underlying signaling mechanism associated with induction and regulation of cytochrome P4501A has not yet been well defined, we therefore in the present study have investigated to identify the regulatory network of cytochrome P4501A in catfish liver or cultured hepatocytes by targeting several key signaling molecules such as phosphatidyl inositol (PI) or protein kinase C (PKC), which are critical molecules for many important pathways. PKC and heat shock protein70 (HSP70) have been shown to be induced in response to carbofuran in catfish hepatocytes. Results also indicate that induction of CYP1A is modulated by HSP70 and PKC in fish hepatocytes. Thus our data shed light on the regulation of EROD activity, which has been used as a bio-monitoring tool for measuring aquatic pollution.

Keywords Carbofuran · Fish · Cytochrome P450 · Liver · HSP70 · PKC

Abbreviations

Cyp1a	Cytochrome P4501A
PKC	Protein kinase C
HSP70	Heat shock protein70
PIP	Phosphatidylinositol phosphate
EROD	Ethoxyresorufin-O-deethylase
PIP2	Phosphatidyl inositol-bisphosphate
CF	Carbofuran
BNF	Beta naphthoflavone
HSF1a	Heat shock factor 1- α

Introduction

Cytochrome P450 (CYP450) is a group of enzymatic system that catalyzes the oxidation reactions of more than 200,000 compounds including drugs, xenobiotics and eubiotics such as steroids, fatty acids, eiconosanoids, retinoids, prostaglandins in the body (Lewis and Pratt 1998). CYP1A is mostly expressed in the liver, however, its localization in other tissues have also been reported. Furthermore although the induction of cytochrome P450 is dependent on receptor-ligand interactions but the regulation of this enzyme system is still incompletely understood. Accumulated evidences indicate that many receptors including aromatic hydrocarbon receptor (Ahr), vitamin D receptor (VDR), retinoic acid receptor (RAR) and glucocorticoid receptor (GR) are involved in its regulation. The Ahr is a class of transcription factor belonging to the PAS (Per-ARNT-Sim) family that controls the expression of CYP1A1, CYP1A2 and CYP1B1, the three important

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groups of cytochrome P450 responsible for detoxification of numerous compounds in our body. It is reported that after binding of Ahr with the ligand, its downstream signaling depends on its phosphorylation status, which is mediated by protein kinase C (PKC) (Denison and Nagy 2003; Denison et al. 2002).

Carbofuran (CF) is a neurotoxic carbamate pesticide that kills the pests by irreversible blocking of acetylcholinesterase, an essential enzyme of the neuromuscular system. The pesticide has high adverse effects on the ecological fauna and flora and induces lethal effects on the biodiversity after coming in contact through surface run off or by other means (Adhikari et al. 2004; Anton et al. 1993; Begum 2004; Begum 2008; Gupta 1994; Hohreiter et al. 1991; Ram et al. 2001; Senger et al. 2005). The inducibility of this pesticide on CYP1A1 enzyme through binding of Ahr in the primary culture of hepatocytes has been studied and reported earlier from our laboratory and by others (Ghosh et al. 2000; Guruge et al. 2009). Involvement of cytochrome P4503A4 in detoxification of carbosulfan has been reported (Abass et al.). Recent report also implicated that induction of cytochrome P450 could be used as a biomarker for carbofuran pollution (Hernandez-Moreno et al. 2008).

In the present study, we investigated to test whether there is any relationship between the expression of heat shock protein70 (HSP70) and carbofuran- induced CYP1A1 because only few reports have indicated that HSP70 could be involved in the regulation of EROD (Ferraris et al. 2002; Rajagopalan et al. 2005; Rios-Sicairos et al. 2010). In our study, it has been observed that induction of CYP1A1 is associated with the increase of PKC and HSP70 and both have parallel dose dependent function with CF. It is evident from our data that PKC can be an important factor, which modulates the expression of HSP70 and thus influencing the CYP1A1 metabolizing enzyme during detoxification. For the first time, our study has provided answers to the long standing question on the impact of HSP70 that can regulate the CYP1A1 in fish hepatocytes.

Materials and methods

Carbofuran (98.5% pure) was obtained as a generous gift from Rallis India Inc. (Karnataka, India). Diacyl glycerol (DAG); ethoxyresorufin, a substrate for estimating the ethoxyresorufin-O-deethylase (EROD); beta-naphthoflavone (BNF), a known inducer of cytochrome P4501A and standard for phosphatidyl inositol phosphate (PIP), phosphatidyl inositol bis-phosphate (PIP₂) used in thin layer chromatography (TLC) were purchased from Sigma chemical (St. Louis, MO). Staurosporine, a blocker of PKC, was purchased from Calbiochem (Darmstadt, Germany). Mouse

monoclonal antibody for HSP70 was purchased from BD Biosciences Pharmingen (San Diego, CA). Biotin conjugated secondary antibody specific for the primary antibody, the fluorochrome fluorescein-isothiocyanate (FITC) conjugated avidin to detect the biotinylated secondary antibody, were purchased from different company with highest purity.

Culture of catfish hepatocytes

Maintenance of catfish in institute aquarium, treatment, sacrifice and collection of liver were undertaken in compliance with the regulatory rules set by Bose Institute Animal Ethics Committee (*No#95/99/CPCSEA*). Isolation of hepatocytes from catfish liver and their culture were done following the methods routinely used in our laboratory (Ghosh et al. 2000).

Treatment of the hepatocytes with carbofuran (CF), β -naphthoflavone (BNF) or staurosporine (ST) and extraction of total lipid from cultured catfish hepatocytes membrane

CF, BNF and ST were dissolved in minimum amount of DMSO. Carbofuran and β -naphthoflavone were added at a concentration of 1 μ M to the culture media and incubated for 24 h. Hepatocytes were pretreated with staurosporine at a concentration of 200 nM for 1 h followed by change and addition of fresh media and subsequently incubated for 24 h. Thereafter, pretreated hepatocytes with staurosporine were exposed to CF or BNF at a concentration of 1 μ M for 24 h. Treated hepatocytes were harvested and pooled for further experiments. Microsome was prepared from the sonicated hepatocytes by ultracentrifugation at 100000 $\times$ g for 1 h. The total lipid was extracted from cell membranes according to the method of Bligh and Dyer (Bligh and Dyer 1959). The amount of total phospholipid was estimated from the total lipid according to the method of Rouser et al. (Rouser et al. 1970).

Sample preparation from the cultured hepatocytes membrane and estimation of phosphatidyl inositol phosphate (PIP) and phosphatidyl inositol bisphosphate (PIP₂) by TLC

Extraction of phosphoinositides from the total lipid was done according to the method of Augert et al. (1989) using neomycin column. The collected phosphoinositides were run in thin layer chromatography (TLC) of silica gel (60 plates, impregnated with 1% potassium oxalate) using a developing solution of CHCl₃/acetone/MeOH/acetic acid/H₂O (12: 45: 25: 30: 20). About 100 μ g of phospholipid (here mixture of phosphoinositides) in terms of inorganic phosphate content was added on the TLC plate from treated

as well as control samples. After development, the plates were dried and stained. Phosphoinositide species were localized by their comigration against the standard. One plate was stained by iodine vapor and the other plate was stained with a solution of 10% CuSO₄ and 8% H₃PO₄ followed by charring at 180°C for 20 min. Iodine stained plates was used for quantitative estimation by scraping the band from the plate.

Iodine staining of the TLC plates

Four or five crystals of iodine were taken into the air tight chamber and the TLC plate was placed into it. After 30 min, yellow spots appeared on the plates. The spots were scrapped from the plate based upon the comigration against standard and taken into separate glass tube for further analysis.

Quantification of phosphatidyl inositol phosphate (PIP) and phosphatidyl inositol bisphosphate (PIP₂) by inorganic phosphate estimation

An improved method was developed for the determination of lipid inorganic phosphorous in the sub-microgram range by Zhou et al. (Zhou and Arthur 1992). This method is based on the formation of a complex between phosphomolybdenum and malachite green and its resultant shift is absorption maximum at 620 nm.

Assay of protein kinase C (PKC)

PKC was assayed according to the method of Kajikawa et al. (Kajikawa et al. 1983), with minor modifications using a PKC measuring kit obtained from GIBCO, BRL (San Francisco, CA). The kit contained various chemicals as mentioned in the assay protocol. γ -³²P ATP was purchased

from BITS (Hyderabad, India). The assay protocol including the components of reaction mixtures was presented in the Table 1.

Estimation of heat shock protein70 (HSP70) from cultured catfish hepatocytes by FACS

Expression of HSP70 from fish hepatocytes was performed according to the method described in earlier work with minor changes (Bachelet et al. 1998). One million cells were taken into 1 ml of PBS. The cells were fixed in 3% paraformaldehyde overnight at 4°C. After incubation in permeabilization buffer, cells were washed three times and pellet was collected into 100 μ l of staining buffer. Then primary antibody (1:100) for HSP70 was added and incubated in room temperature. The cells were washed for 3 times followed by addition of biotinylated secondary antibody (1:1000) and finally FITC labeled avidin (1:1000). Cells were washed and reading was taken in fluorescence activated cell sorter (FACS) using FL1 detector (FACS Calibur, Becton-Dickinson) within 2 h of avidin addition.

Assay of ethoxyresorufin-O-deethylase activity (EROD) activity

The procedure of EROD activity assay was followed as described before (Ghosh et al. 2000).

Statistical analysis of the data

Each experiment was repeated at least three times. Statistical analysis of the data was done by Student's *t*-test between two groups and Duncan's Multiple Range Test among various groups and significance level was considered at $p < 0.05$.

Table 1 Composition and amount of reagents used to assay the PKC. The total volume of reaction mixture was 50 μ l and several controls were run with or without the critical components. Finally, the reaction mixture was adsorbed to the phosphocellulose paper. Rest of the procedure was followed according to the description in "Materials and methods"

Constituents	Blank (μ l)	Membrane	
		Without Ca/PS/DAG(μ l)	With Ca/PS/DAG(μ l)
Tris-HCl (20 mM)	—	10	10
MgCl ₂ (10 mM)	—	5	5
CaCl ₂ (0.1 mM)	—	—	5
PS 0.2 mg/ml	—	—	5
DAG 0.1 mg/ml	—	—	5
Histone 2 mg/ml	5	5	5
ATP (0.05 μ Ci/ μ l)	10	10	10
Membrane suspension (5 μ g)	—	5	5
H ₂ O	35	15	—
Total	50	50	50

Results

Carbofuran induces membrane phospholipids, PIP and PIP₂ and activity of PKC in cultured catfish hepatocytes

In thin layer chromatography plate (TLC) plate, the amount of two precursors of PI3-kinase signaling pathway, phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂) increased significantly in CF (1 μ M) treated hepatocytes membrane in comparison to the control (DMSO-treated) (Fig. 1a). These results were consistent when spots were scraped from the plate and the amount was estimated analytically. The amount of PIP and PIP₂ were calculated as 9.61 and 7.38 microgram per 100 microgram of total membrane phospholipid respectively which was almost 1.5 and 2 times higher ($p < 0.05$) in comparison to vehicle treated control (Fig. 1b). Protein kinase C (PKC)

activity was measured in hepatocytes membrane treated with different doses (0.01–100 μ M) of CF. Maximum activity of PKC was found at 1.0 μ M dose of CF, thereafter higher doses (10 and 100 μ M) showed attenuation of the PKC activity (Fig. 1c). β -naphthoflavone (BNF), a known inducer of cytochrome P450 (Stegeman et al. 1989), also induced PKC activity, which was blocked by staurosporine (ST), an inhibitor of PKC. Staurosporine alone showed the PKC activity very similar to control level (Fig. 1d).

Induction of cytochrome P4501A by CF is PKC dependent event in cultured catfish hepatocytes

Similar to PKC, EROD activity demonstrated a dose dependent increase with the amount of CF and BNF that were added to the media of cultured hepatocytes. For both,

Fig. 1 **A** Estimation of phosphatidyl inositol phosphate (PIP) and phosphatidyl inositol bisphosphate (PIP₂) from cultured hepatocytes. Hepatocytes were cultured and treated with CF (1 μ M). Total lipid extraction, isolation of phosphoinositides (PIP and PIP₂) and analytical estimation were done. S indicated the Standard, C and T indicated the phosphoinositides from control and treated respectively. **B** The spots were scrapped from the TLC plate based upon the migration of standard on the plate and the amount was quantified. The amount was expressed as μ g of PIP or PIP₂/100 μ g of total phospholipid. The data represent the average of three observations. * indicated the value was statistically significant in comparison to control value. **C** The dose dependent induction of PKC by CF in primary culture of catfish hepatocytes. Hepatocytes were cultured and acclimatized. Then hepatocytes were exposed to different concentration of CF for 24 h. The cells were harvested and total PKC was.

Each value indicated the mean $\pm$ SE of six culture wells from three independent experiments. * indicated the values were statistically significant in comparison to control value. **D** Effect of staurosporine on the CF and BNF induced PKC in cultured catfish hepatocytes. Hepatocytes were cultured and acclimatized. Cells were exposed to CF and BNF with dose of 1 μ M each. Cells were pretreated with ST (200 nM) for 1 h followed by washing and then left alone in fresh media. In parallel, hepatocytes pretreated with ST with 1 h were exposed to CF or BNF for 24 h. Cells were harvested and PKC was assayed. Each value indicated the mean $\pm$ SE of six culture wells from three independent experiments. * indicated the values were statistically significant in comparison to control value. # indicated the values were not significant in comparison to control value. + indicated the values were statistically significant in comparison to CF or BNF respectively

1 μM dose stimulated the maximal activity of EROD, which was about 3 fold higher than the vehicle treated control ($p < 0.01$) (Fig. 2a). Addition of staurosporine, a PKC inhibitor, demonstrated a significant attenuation of EROD activity ($p < 0.001$). However, treatment with only staurosporine to the hepatocytes did not show any significant change in EROD activity in comparison to the control (Fig. 2b).

Fig. 2 **a** The dose dependent induction of EROD activity by CF and BNF in primary culture of catfish hepatocytes. Hepatocytes were cultured and acclimatized. Then hepatocytes were exposed to different concentration of CF and BNF for 24 h. The cells were harvested and EROD activity was measured. The correlation coefficient (r) of the two curves were calculated which was statistically significant ($p < 0.05$). Each value indicated the mean $\pm$ SE of six culture wells from three independent experiments. * indicated the values were statistically significant in comparison to control value. **b** Effect of staurosporine on the CF and BNF induced EROD activity in cultured catfish hepatocytes. Hepatocytes were cultured and acclimatized. Then they were exposed to CF, BNF with dose of 1 μM each. Cells were pretreated with ST (200 nM) for 1 h followed by washing and then left alone in fresh media. In parallel, hepatocytes pretreated with ST with 1 h were exposed to CF or BNF for 24 h. Hepatocytes were harvested and EROD activity was assayed. Each value indicated the mean $\pm$ SE of six culture wells from three independent experiments. * indicated the values were statistically significant in comparison to control value. # indicated the values were not significant in comparison to control value. + indicated the values were statistically significant in comparison to CF or BNF respectively

Carbofuran induces the heat shock protein-70 (HSP70) and the induction is mediated by PKC

At 1 μM dose of CF or BNF, the level of HSP70 was elevated 8–11 folds ($p < 0.001$) in comparison to the vehicle treated control (Fig. 3a). Treatment with different doses of CF (0.01, 0.1, 1.0 and 10 μM) demonstrated a clear dose response relationship in expression of HSP70 in culture catfish hepatocytes (Fig. 3b). Staurosporine, a PKC blocker, was able to block the expression of HSP70 in comparison to CF-induced expression at 1 μM dose (Fig. 3c). The final results from several experiments demonstrated a consistent higher expression of HSP70 in hepatocytes treated with CF and BNF. The statistical analysis of the results also confirmed it. The maximum amount was expressed at 10 μM of CF ($p < 0.001$), which gradually diminished at lower dose such as 1.0 ($p < 0.01$), 0.1 and 0.01 μM ($p < 0.05$). In addition, the increase of HSP70 by BNF ($p < 0.01$) was significantly higher in comparison to vehicle treated control. There was no significant difference in expressions of HSP70 between staurosporine alone and pre-treated staurosporine followed by CF treatment (Fig. 3d).

Discussion

Several reports indicated that phosphorylation of Ah receptor or nuclear translocator protein (Arnt) influenced the heterodimerization and binding of the heterodimer to DNA for initiation of transcription (Okino et al. 1992; Pollenz et al. 2002) (Schafer et al. 1993). Chen and Tukey had also shown that inhibition of PKC activity blocks the transcriptional activation of CYP1A1 gene directly, indicating a role of PKC in Ahr-mediated transcriptional activation process (Chen and Tukey 1996; Machemer and Tukey 2005). Using human Hepatoma cells HepG2, they had shown that transcription of CYP1A1 was potentiated by phorbol ester indicating cross talk between PKC and other regulatory pathways that depend on Ahr signaling. In our present study, it was revealed that PKC maintained a dose dependent relationship with the CF where the log phase of the curve goes to peak at 1 μM of CF followed by a sharp decline. The curve of EROD with same condition showed similar results. Furthermore the dose where the curve declines was not found to be toxic for the hepatocytes as shown by trypan blue exclusion test. The EROD activity in the hepatocytes treated with the same dose of CF was in parallel with the PKC activity. Data in both the cases revealed peak activities at 1 μM dose of CF followed by a steady fall, however, no detectable mortality of the hepatocytes was demonstrated at these doses. Responsiveness of PKC to the CF treatment was further supported by determining phosphatidyl inositol phosphate (PIP) and

Fig. 3 **a, b** and **c** FACS of HSP70 from CF treated cultured hepatocytes. Hepatocytes were cultured and acclimatized. Then hepatocytes were exposed to different concentration of CF, BNF (1 μ M). Cells were pretreated with ST (200 nM) for 1 h followed by washing and then left alone in fresh media. In parallel, hepatocytes pretreated with ST with 1 h were exposed to CF or BNF for 24 h. In other condition, hepatocytes were exposed to a serial dose of CF (0.01–10 μ M) to test the dose responsiveness on the HSP70. Hepatocytes were harvested and FACS was done for HSP70. Different conditions were described in each inset of histogram. NC, indicated the Negative control. **d** Average values of three independent experiments were presented with standard error. * indicated the values were statistically significant in comparison to control value. # indicated the values were not significant in comparison to only staurosporine (ST)

phosphatidyl inositol biphosphate (PIP₂), the two precursor molecules essential for the activation of PKC. In our present investigation, PIP and PIP₂ were significantly higher in the CF treated hepatocytes membrane than the control, which was indicative of stronger responsiveness of CF via activation of PKC through classical membrane lipid signaling. Our observation was also strongly supported by the reports of Reiners et al. who had demonstrated that the induction of EROD could be inhibited in murine hepatoma cell line (1C1C7) when these cells were treated with PKC blocker, staurosporine. In addition, PKC activator tetradecanoylphorbol-13-acetate (TPA) also inhibited the induction of EROD. TPA induced down regulation of PKC and subsequent inhibition CYP1a gene in rainbow trout hepatocytes was also reported by Lee and Dasmahapatra (Lee and Dasmahapatra 1993). However, the cause of attenuation of CYP1a by both activator and inhibitor of PKC was not evident from those studies (Reiners et al. 1993). Based on different reports, it might be conceivable that activator of PKC such as PMA might induce hyperphosphorylation of PKC, which in turn negatively regulates the heat shock factor-1 (HSF-1), a transcription factor known to be involved in regulation of heat shock protein70 (HSP70) (Ding et al. 1998). HSP70 is a molecular chaperon that safeguards conformation of protein in cellular milieu during toxic stress (Cheng et al. 2007; Jacquier-Sarlin et al. 1995). Less amount of

HSP70 in the cytosol indicated less chaperon activity of HSP70, which in turn affects the stability of EROD during toxic stress.

Heat shock protein is a family of broad spectrum protein expressed in cells during stress and acts as molecular chaperon, which maintains normal conformity of other protein. Jacquier- Sarlin et al. (Jacquier-Sarlin et al. 1995) reported that HSP70, as well as HSP90 expression might be

Fig. 4 Schematic presentation of hypothetical function of induced heat shock protein70 (HSP70) through activation of phosphatidylinositol (PI) in our work. Treatment of carbofuran to the cultured hepatocytes triggered the membrane signal through activation of PI that ultimately activated the PKC. That activated PKC was thought to be involved in induction of heat shock protein70 which was shown to control the activity of EROD

associated with the status of cellular PKC. They showed that mRNA of both proteins were elevated compared to the controls in human peripheral blood monocytes exposed to 12-phorbol 13-myristate acetate (PMA), a PKC activator. Later, studies had explored the regulatory role of PKC on the expression of HSP. It was gradually understood that induction of heat shock gene was mediated by specific heat shock transcription factors (HSFs), but the signaling pathways leading to the activation of HSF are still obscure (Ding et al. 1998). Upon blocking of PKC with staurosporine, we had shown that the expression of heat shock protein70 (HSP70) was inhibited (Fig. 3c). As mentioned before, HSP70 is a molecular chaperon that helps in maintaining conformity of many cellular proteins (Hartl and Martin 1995; Martin and Hartl 1997). In fact the expression of HSP70 remained high throughout the year in livers of fishes that live in water polluted with hydrocarbon (Rios-Sicairos et al.). This observation indicated that HSP70 was required for maintaining conformity of cytochrome P450 protein including EROD that might be required for detoxification of pollutants. Our observations were in strong agreement with these findings that blocking of PKC with staurosporine led to less expression of HSP70 and that might result the less activity of EROD.

Taken together, these investigations indicated that HSP70 might be responsible for the regulation of EROD activity through PKC. Based on our data, it was clear that CF induced the PKC through activations of PIP and PIP2 and also had the ability to directly regulate EROD activity. Furthermore, blocking of HSP70 induction by staurosporine indicated that PKC was involved in the regulation of HSP70. The association of heat shock protein in modulation of cytochrome P450 had also been previously shown by others (Rajagopalan et al. 2005). Our investigation thus represented a functional role of PKC, which was the missing link between the modulation of HSP70 and EROD during toxic stress in liver (Fig. 4).

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Conflict of Interest Authors declared that there was no 'conflict of interest' in this work

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