Effect of sub-toxic chlorpyrifos on redox sensitive kinases and insulin signaling in rat L6 myotubes

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Abstract

Objectives Sub-chronic exposures to chlorpyrifos, an organophosphorus pesticide is associated with incidence of diabetes mellitus. Biochemical basis of chlorpyrifos-induced diabetes mellitus is not known. Hence, effect of its sub-toxic exposure on redox sensitive kinases, insulin signaling and insulin-induced glucose uptake were assessed in rat muscle cell line.

Methods In an in vitro study, rat myoblasts (L6) cell line were differentiated to myotubes and then were exposed to sub-toxic concentrations (6 mg/L and 12 mg/L) of chlorpyrifos for 18 h. Then total anti-oxidant level in myotubes was measured and insulin-stimulated glucose uptake was assayed. Assessment of activation of NFκB & p38MAPK and insulin signaling following insulin stimulation from tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and serine phosphorylation of Akt were done in myotubes after chlorpyrifos exposure by western blot (WB) and compared with those in vehicle-treated controls.

Results The glucose uptake and total antioxidant level in L6-derived myotubes after sub-toxic exposure to chlorpyrifos were decreased in a dose-dependent manner. As measured from band density of WB, phosphorylation levels increased for redox-sensitive kinases (p38MAPK and IκBα component of NFκB) and decreased for IRS-1 (at tyrosine 1222) and Akt (at serine 473) on insulin stimulation following chlorpyrifos exposure as compared to those in controls.

Conclusion We conclude that sub-toxic chlorpyrifos exposure induces oxidative stress in muscle cells activating redox sensitive kinases that impairs insulin signaling and thereby insulin-stimulated glucose uptake in muscle cells. This probably explains the biochemical basis of chlorpyrifos-induced insulin resistance state and diabetes mellitus.

Keywords Chlorpyrifos · Type 2 diabetes mellitus · Insulin signaling · Insulin resistance · NFκB · p38MAPK

Key message

1. Sub-toxic chlorpyrifos exposure imparts oxidative stress and thus activates redox sensitive kinases.
2. The activated redox sensitive kinases impair insulin signaling, that in turn induce insulin resistance in muscle cells leading to decreased glucose uptake.

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Abbreviations

T2DM	Type 2 diabetes mellitus
OP	Organophosphate
IRS-1	Insulin Receptor Substrate-1
RSKs	Redox sensitive kinases
NFκB	Nuclear factor kappa B
p38 MAPK	p38 Mitogen-Activated Protein Kinase
MTT	3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum

Introduction

Epidemiological reports have incriminated pesticides exposures as risk factors for insulin resistance and development of type 2 diabetes mellitus (T2DM) [1, 2]. Chlorpyrifos (0, 0-diethyl 0-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate) is a broad spectrum chlorinated organophosphate (OP). This synthetic pesticide is widely used in agriculture and domestic purposes [3]. Besides the effects that are caused due to inhibition of acetylcholinesterase, chlorpyrifos exposure was found to induce hyperlipidemia in experimental animals and to modulate apoptosis pathway [4–6]. Chlorpyrifos is reported to influence expression of various genes in different tissues [7]. The epigenetic mechanism through differential methylation of genes has been proposed for regulations of some genes [8, 9]. Agricultural Health Study (AHS), a large prospective cohort study with male farmers reported a positive association between chlorpyrifos exposures and incidence of diabetes mellitus [10]. Few experimental studies have reported disturbances in glucose metabolism following sub-chronic chlorpyrifos exposure to rodents [11–13].

Evidences indicate that insulin resistance in skeletal muscle leads to hyperglycemia and T2DM [14, 15]. Skeletal muscle maintains glucose homeostasis by insulin-mediated glucose uptake and disposal. Under normal physiological condition, glucose uptake by skeletal muscle is the rate limiting step in glucose metabolism [16, 17]. Any reduction in the rate of its uptake gives rise to a proportional decline in muscle glucose disposal. Glucose uptake in skeletal muscle requires phosphorylation at tyrosine moieties of insulin receptor (IR) following insulin binding and Insulin Receptor Substrate-1 (IRS-1) for downstream signal propagation to Akt that is phosphorylated at its serine moieties [18]. Impairment of phosphorylation at IRS-1 and thereby of Akt by various mechanism lead to the state of insulin resistance [19, 20].

Several in vitro [21, 22] and in vivo [23, 24] studies have demonstrated that chlorpyrifos exposure induces oxidative stress. Oxidative stress is known to activate redox sensitive kinases (RSKs) like NFκB (Nuclear Factor kappa B) and p38 MAPK (p38 Mitogen-Activated Protein Kinase) [25–27].

NFκB has three sub-units: Rel A (p65), p50 and inhibitory Kappa Bα (IκBα). During activation, IκBα gets phosphorylated by various stimuli and separated out to release p65/p50 complex to perform its functions [28]. p38MAPK is also activated by phosphorylation in oxidative stress condition [29]. These activated redox sensitive kinases affects insulin signaling by impairing tyrosine phosphorylation and causing serine phosphorylation of IRS-1 resulting in insulin resistance [30, 31]. Increased levels of the cytokine TNF-alpha (TNFα) is also seen to cause insulin resistance in skeletal muscle by activating NFκB [32]. The effects of chlorpyrifos exposure on insulin signaling and redox sensitive kinases in skeletal muscle are not clearly known to the best of our knowledge. Hence the present study is designed to explore the effect of sub-toxic chlorpyrifos exposure on insulin stimulated glucose uptake, total anti-oxidant level, insulin signaling, redox sensitive kinases (e.g. NFκB and p38 MAPK) and TNFα in myotube derived from rat L6 cell-line.

Materials and methods

Reagents and antibodies

Chlorpyrifos (99% pure) was obtained as a free sample from Coromandel International Limited. (Gujarat, India). 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), Trypsin EDTA solution were purchased from Hi-media (Mumbai, India). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin were purchased from Gibco Invitrogen (Barcelona, Spain). Dimethyl sulfoxide (DMSO), insulin, protease-phosphatase inhibitor cocktail (PPI), radioimmunoprecipitation assay (RIPA) buffer with ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid were purchased from Sigma Aldrich (Missouri, USA). 3,3',5,5'-tetramethylbenzidine (TMB) reagent was purchased from GeNei (Bangalore, India). Polyvinylidene difluoride (PVDF) membrane was purchased from Merck Millipore (Darmstadt, Germany). Bicinchoninic Acid (BCA) based protein assay kit was purchased from Thermo Scientific Pierce (Massachusetts, USA). Total antioxidant kit was purchased from Randox (Crumlin, UK). Rat TNF alpha PicoKine™ ELISA Kit was purchased from Boster Biological Technology (Pleasanton, CA, USA). Glucose uptake colorimetric assay kit was purchased from BioVision (Milpitas, CA, USA).

A primary antibody from rabbit against phospho-Akt (S473) was purchased from Abcam (Cambridge, MA). Anti-phospho-IRS-1(Y1222) and secondary anti-rabbit antibodies tagged with horse raddish peroxidase (HRP) were purchased from Cell Signaling Technologies (Berberly, MA). Primary mouse antibodies for β-actin, phospho-p38MAPK (T180/Y182), phospho-IKBα (S32/S36) and secondary anti-

mouse antibody tagged with HRP were purchased from Sigma Aldrich (Massachusetts, USA).

Culture of L6 cell line and differentiation

Rat skeletal myoblast (L6) cell line was obtained from the National Centre for Cell Sciences (Pune, India) and propagated at 37 °C in 5% CO₂ incubator in DMEM supplemented with 100 units/ml of penicillin G, 100 µg/ml of streptomycin sulfate, 0.25 µg/ml amphotericin B and 10% FBS. Once the myoblasts reached 70–80% confluence, the L6 myoblasts were allowed to differentiate into myotubes for 5–7 days in differentiation medium (DMEM supplemented with 100 units/ml penicillin, 100 µg/ml of streptomycin sulfate, 0.25 µg/ml amphotericin B and 2% FBS) spontaneously. The differentiation of myoblasts into myotubes was checked by the morphology and fusion of myotubes under the microscope. All the following experiments were carried out in the differentiated myotubes. For assay of insulin signaling, redox sensitive kinases, antioxidant assay and TNF α , cells were grown and differentiated in 75 cm² culture flasks and for glucose uptake and MTT assay, 96 well culture plates were used (Tarsons India Pvt. Ltd., Kolkata, India).

Determination of sub-toxic dose

A stock solution of chlorpyrifos (1000 mg/mL) was prepared in DMSO and diluted in DMEM media with 2% FBS. Myotubes differentiated in 96 well plates were treated for 18 h with various concentrations of chlorpyrifos (1, 3, 6, 10, 30, 60, 100, 300, 600, 1000 mg/L) for cytotoxicity assay. The cells from the control group received vehicle (DMSO). MTT (stock 1 mg/mL) was prepared in phosphate buffered saline. At the end of the treatment, 60 µL treated media were discarded from all wells except blank, then 60 µL of MTT solution from stock solution was added (final concentration 0.3 mg/mL) and cells were incubated further for 3 h. For the analysis, the medium was removed and formazan crystals were dissolved in DMSO (100 µL). The absorbance was measured at 570 nm using a microplate reader (Biorad 680 XR, CA, USA).

Glucose uptake assay

The stock solution of the chlorpyrifos was prepared in such a way that the final concentration of DMSO in incubation media was less than or equal to 0.1%. For glucose uptake assays, the cells were grown in 96-well plates and treated with 6 or 12 mg/L chlorpyrifos for 18 h, followed by serum starvation for 40 min and 1 µM insulin stimulation for 20 min. Two concurrent controls (vehicle treated) were treated with insulin and two wells of controls received no insulin stimulation. Glucose uptake colorimetric assay kit was used for the glucose

uptake determination and done as per the instruction provided with the kit. Absorbance was measured at 412 nm by a microplate reader (Biorad 680 XR, CA, USA). Glucose uptake was expressed in pmol/well.

Treatment of myotubes with chlorpyrifos and preparation of cell lysate

L6 myotubes grown and differentiated in 75 cm² culture flasks were treated with chlorpyrifos at concentration of 6 mg/L and 12 mg/L (18 h) followed by serum starvation and stimulation by 1 µM insulin for 15 min. Concurrent vehicle-treated controls were also treated with insulin in a similar way. Adherent cells were then harvested using cell scraper and lysed in RIPA lysis buffer containing 1 mM PPI cocktail on ice. Cells were further lysed using hand held homogeniser and centrifuged at 10,000 g for 20 min at 4 °C. Protein concentrations were determined by bicinchoninic acid (BCA) based protein assay kit.

Total antioxidant status and TNF α determination

Total antioxidant of cell lysates were quantified by using kit in a random clinical chemistry analyzer (Randox RX Imola, Bangalore, India) and was expressed in mmol/g of proteins. The differentiation media (i.e., DMEM with 2% FBS in which pesticide was dissolved and treatment to cells was administered for 18 h) was collected for the assay of TNF α . TNF α in collected DMEM media and FBS as mentioned above was measured by using ELISA kit and was expressed in pg/ml.

Assay of phosphorylation of IRS-1, Akt and redox sensitive kinases

Equal amount of proteins (20 µg/well) from above-mentioned cell lysates was separated using 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes using semi-dry blotter (Merck, New Jersey, USA). Antibodies were diluted as recommended by the producers. The membranes were incubated with different primary antibodies at 4 °C overnight, washed thrice in PBS and then dipped in a secondary antibody coupled to horseradish peroxidase for 1 h. Immunoreactivity was visualized using TMB with ChemiDoc XRS⁺ (Bio-Rad) and band density measured using Image lab software (Bio-Rad). For β -actin, PVDF membrane was stripped of in stripping buffer (10% SDS, 0.5 M Tris-buffer pH 6.8 and β mercaptoethanol) at 50 °C, hybridized similarly with primary & secondary antibodies and visualized as mentioned above. Band density of phosphorylated (IRS-1, Akt, p38MAPK, and I κ B α) was normalized with band density of β -actin.

Statistical analyses

The data are presented as means $\pm$ SE. All data shown are the representative results of three independent experiments. Data were analyzed by one-way ANOVA followed by post-hoc (Tukey HSD) test using the IBM SPSS version 17 software. Significance of differences was defined at the $p < 0.05$ level.

Results

Effects of chlorpyrifos on viability of L6 derived myotubes

Chlorpyrifos-induced cytotoxicity was measured by MTT assay. NOEL (dose, no observable effect level) was 60 mg/L. One fifth (i.e. 12 mg/L) and 1/10th (i.e. 6 mg/L) of NOEL was considered as sub-toxic dose of chlorpyrifos for myotube and used for experiment in the present study.

Effects of chlorpyrifos on insulin-stimulated glucose uptake in rat L6 derived myotubes

Glucose uptake with insulin stimulation was significantly decreased in chlorpyrifos-exposed myotubes in comparison to those in control myotubes ($p < 0.05$) and chlorpyrifos exposure dose-dependently suppressed insulin-stimulated glucose uptake in L6 derived myotubes ($p < 0.05$) (Table 1).

Effects of chlorpyrifos on total antioxidant level

The level of total antioxidant status in chlorpyrifos exposed myotubes was significantly decreased (1.93 ± 0.03 mmol/g and 1.75 ± 0.03 mmol/g for 6 mg/L and 12 mg/L chlorpyrifos exposed myotubes respectively) than that of controls (2.52 ± 0.06 mmol/g) ($p < 0.05$). The effect was dose-dependent ($p < 0.05$).

Table 1 Non-specific and insulin-stimulated glucose uptake in controls and sub-toxic chlorpyrifos treated myotubes derived from rat L6 cell line

	Non-specific (without insulin stimulation) Controls	Insulin stimulated glucose uptake after subtracting non-specific glucose uptake		
		Controls	Chlorpyrifos treated myotubes (6 mg/L)	Chlorpyrifos treated myotubes (12 mg/L)
Glucose uptake (pmol/well) (n=3 wells in each group) Mean $\pm$ SE	2.23 ± 0.11	21.62 ± 0.26	$17.7 \pm 0.11^*$	$12.66 \pm 0.27^{*\dagger}$

* $p < 0.05$ in comparison to insulin stimulated glucose uptake in control

$^\dagger p < 0.05$ in comparison to insulin stimulated glucose uptake by chlorpyrifos (6 mg/L) treated myotubes by one way ANOVA followed by Tukey HSD post hoc test

Effects of Chlorpyrifos on TNF α level

TNF α in FBS was 4 ng/mL. In post treated 2% DMEM with 2% FBS, it was 0.08 ng/ml without significant effect of sub-toxic pesticide treatment. So, TNF α measured in post-treated media was contributed only by FBS added to it and not by the myotubes.

Effects of chlorpyrifos on insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt

Exposure of L6 myotubes to sub-toxic doses of chlorpyrifos significantly reduced insulin stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt following insulin stimulation ($p < 0.05$) (Figs. 1 and 2).

Effects of chlorpyrifos on phosphorylation of I κ B α and p38MAPK

Sub-toxic chlorpyrifos exposure to L6 derived myotubes significantly increased phosphorylation of p38MAPK and I κ B α ($p < 0.001$) (Figs. 3 and 4). Increased phosphorylation of p38MAPK was dose-dependent ($p < 0.01$) (Fig. 3).

Discussion

Treatment of L6 cell line derived myotube with sub-toxic dose of chlorpyrifos was found to decrease the glucose uptake in a dose-dependent manner (Table 1). This indicates sub-toxic exposure to chlorpyrifos induces insulin resistance in muscle cells. Previous studies have also shown that sub-toxic chlorpyrifos exposures perturb the glucose homeostasis [13, 33–35]. Our results re-affirm the above contention. In an in vitro experiment on whole blood, chlorpyrifos was found to decrease the glucose uptake by blood cells only at toxic doses [36]. Sub-toxic dose didn't have any effect on glucose

Fig. 1 Bar diagram showing the effect of Chlorpyrifos exposure for 18hrs on the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) in L6 myoblast derived myotubes after normalized with beta actin by western blot hybridization. Data shown are means with standard errors of three independent experiments. C+i = Control with insulin, CPF6+i = Chlorpyrifos (6mg/L) exposure for 18 hrs followed by treatment with insulin (1uM for 20min), CPF12+i = Chlorpyrifos 12mg/L exposure for 18hrs followed by treatment with insulin (1uM for 20min). † $p < 0.05$ in comparison to control, * $p < 0.05$ in comparison to Chlorpyrifos 6mg/L exposure groups by one way ANOVA followed by Tukey HSD post-hoc test

Fig. 2 Bar diagram showing the effect of Chlorpyrifos exposure for 18hrs on the serine phosphorylation of Akt in L6 myoblast derived myotubes after normalized with beta actin by western blot hybridization. Data shown are means with standard errors of three independent experiments. C+i = Control with insulin, CPF6+i = Chlorpyrifos (6mg/L) exposure for 18 hrs followed by treatment with insulin (1uM for 20min), CPF12+i = Chlorpyrifos 12mg/L exposure for 18hrs followed by treatment with insulin (1uM for 20min). † $p < 0.05$ in comparison to control, * $p < 0.05$ in comparison to Chlorpyrifos 6mg/L exposure groups by one way ANOVA followed by Tukey HSD post-hoc test

Fig. 3 Bar diagram showing the effect of Chlorpyrifos exposure for 18hrs on the phosphorylation of p38 MAPK in L6 myoblast derived myotubes after normalized with beta actin by western blot hybridization. Data shown are means with standard errors of three independent experiments. C+i = Control with insulin, CPF6+i = Chlorpyrifos (6mg/L) exposure for 18 hrs followed by treatment with insulin (1uM for 20min), CPF12+i = Chlorpyrifos 12mg/L exposure for 18hrs followed by treatment with insulin (1uM for 20min). †p<0.05 in comparison to control, *p<0.05 in comparison to Chlorpyrifos 6mg/L exposure groups by one way ANOVA followed by Tukey HSD post-hoc test

uptake in whole blood. Impairment of glucose uptake by whole blood was reported to be attributed to hemolysis and metabolic impairment induced by chlorpyrifos. As glucose

uptake in whole blood is mostly insulin-independent, low dose of chlorpyrifos didn't alter its glucose uptake. Hence, whole blood experiment was not suitable for screening insulin

Fig. 4 Bar diagram showing the effect of Chlorpyrifos exposure for 18hrs on the phosphorylation of IκBα in L6 myoblast derived myotubes after normalized with beta actin by western blot hybridization. Data shown are means with standard errors of three independent experiments. C+i = Control with insulin, CPF6+i = Chlorpyrifos (6mg/L) exposure for 18 hrs followed by treatment with insulin (1uM for 20min), CPF12+i = Chlorpyrifos 12mg/L exposure for 18hrs followed by treatment with insulin (1uM for 20min). †p<0.05 in comparison to control, *p<0.05 in comparison to Chlorpyrifos 6mg/L exposure groups by one way ANOVA followed by Tukey HSD post-hoc test

resistance inducing property of pesticides at sub-toxic concentrations. However, the present data indicate that L6 derived myotubes can be used for evaluation of insulin resistance inducing property of pesticides as insulin receptor and insulin signaling pathways are well expressed in these myotubes. Hence, they behave like muscle cells and the observed effect can be very well extrapolated for muscles.

The present study showed that there was no increase in TNF α in the supernatant of L6 myotubes following pesticide exposure. Pesticides are claimed to induce insulin resistance by increasing TNF α in blood in human beings [37]. The present data indicate that TNF α is not probably involved in inducing insulin resistance at sub-toxic dose of chlorpyrifos in in vitro experiment on this cell-line. It is known that the major source of TNF α is adipocytes and immunocytes. In muscle cells, TNF α gene is not probably expressed. Hence, chlorpyrifos treatment didn't alter TNF α level in decanted DMEM in which chlorpyrifos treatment was administered. This experiment should ideally be carried out in adipocyte cell line to know if chlorpyrifos exposure increases the expression of TNF α gene and thereby contribute to its insulin resistance-inducing property.

Antioxidant level from cell lysates of L6 myotube was decreased following sub-toxic chlorpyrifos exposure in a dose-dependent manner. This indicates that sub-toxic exposure to chlorpyrifos induces oxidative stress in L6 myotubes. Oxidative stress is known to contribute to the pathogenesis of insulin resistance by activation of redox sensitive kinases [38]. Hence, insulin resistance induced by sub-toxic chlorpyrifos might be attributed to the oxidative stress.

The present study showed the impairment of insulin signaling as revealed by decrease phosphorylation of IRS-1 and Akt, following insulin stimulation of L6 derived myotube after sub-toxic exposure to chlorpyrifos (Figs. 1 and 2). These data clearly demonstrate that chlorpyrifos-induced insulin resistance as revealed by decreased glucose uptake following insulin stimulation is associated with reduced IRS-1 and Akt phosphorylation. The redox-sensitive kinases (p38MAPK and I κ B α) were phosphorylated at higher levels after sub-toxic exposure to chlorpyrifos (Figs. 3 and 4). These results suggest the activation of redox-sensitive NF- κ B and p38MAPK pathways by chlorpyrifos. Others also reported activation of redox sensitive kinases on chlorpyrifos exposure [39, 40]. Upon phosphorylation of I κ B α , p50-p65 complex gets separated from whole NF- κ B and migrates to nucleus to activate various genes to carry out its functions. These activated redox-sensitive kinases are known to interfere with the insulin signaling [41–43]. Oxidative stress is known to phosphorylate (activate) these kinases [27, 44–46]. Hence, we conclude that sub-toxic chlorpyrifos exposure induces insulin resistance in muscle cells by impairment of insulin signaling at the level of IRS-1 that occurs due to activation of RSKs by oxidative stress imparted by chlorpyrifos. This might be the probable

explanation for chlorpyrifos being a risk factor for the development of insulin resistance state and diabetes mellitus.

In real life situation, exposure duration of chlorpyrifos is very long but the tissue concentration is very low. Hence, one of the limitations of the study is that the concentrations of chlorpyrifos used in this experiment were sub-toxic but supra-physiological and the exposures given to myotubes is for only 18 h. It cannot be prolonged (as in real life situations) beyond a limit because of technical limitation of such in vitro experiments.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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