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Short Communication

Influence of Deltamethrin and Cypermethrin on the Expression of SUMO Isoforms and UBC9

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Abstract

Deltamethrin and cypermethrin are extensively used insecticides to which humans are widely exposed. The present study is designed to analyze the effects of these two pesticides on HePG2 cell lines and on differentiated 3T3-L1 and C2C12 cells with respect to the expression of small ubiquitin-like modifiers (SUMO) and SUMO-conjugating enzyme UBC9. Treatment of HePG2 cells with either deltamethrin or cypermethrin caused elevated mRNA levels of SUMO 2 and 3 as well as UBC9. This was associated with elevated nitric oxide and nitric oxide synthase mRNA levels and decreased NADPH levels. Differentiated 3T3-L1 cells treated with either deltamethrin or cypermethrin led to increased mRNA levels of inflammatory cytokines IL-Iβ and IL-6, indicating that these pesticides promote the inflammatory response. These observations are significant since previous studies reported that UBC9 and SUMO isoforms are upregulated in many types of cancers and hence the extensive use of these pesticides is a concern.

Keywords: SUMO isoforms, UBC9, deltamethrin, cypermethrin

Introduction

SUMOs are a family of proteins that are similar in structure to ubiquitin [1-2]. In humans, five isoforms (SUMO1, SUMO2/3, SUMO4, and SUMO5) have been identified so far [3-4]. SUMOs covalently conjugate to the target proteins (SUMOylation), a process that requires several enzymes, including the ubiquitin-conjugating enzyme UBC9, which is known to be conserved throughout the eukaryotes. SUMO attachment is transient as specific proteases cleave the target from SUMO [3, 5]. It is well established that SUMO modification occurs for a large number of proteins [6] and the SUMO isoforms exhibit target preferences [7]. Oxidative, osmotic, and heat shock stresses modulate overall cellular SUMOylation pattern [8-10]. SUMOylation imbalance is reported in cancer and neurodegenerative disease [11-12]. Altered SUMO2/3 modification of 15 different targets is reported in breast cancer [13].

High expression of UBC9 is associated with lung, breast, ovarian, head, and neck cancers [14-18]. Guo et al. reported that overexpression of SUMO1 occurred in

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hepatocellular carcinoma clinical samples and that the SUMO1-SiRNA inhibited cancer cell proliferation [19].

Around 520 tons of pyrethroids are used as insecticides worldwide [20]. Deltamethrin and cypermethrin are Type II synthetic pyrethroids [21], to which humans are widely exposed through dietary and nondietary modes [22]. Deltamethrin causes high expression of metallothioneins [23], inhibition of osteoclast differentiation [24], and endocrine disruption [25-26]. Cypermethrin is known to cause impairments in seminiferous tubule structure and spermatogenesis in rats associated with reduced androgen receptor expression (27) and neurodegeneration [28]. Cypermethrin is also reported to cause developmental delays and chromosomal aberrations [29].

It is of interest to see if an extensive use of pesticides has any consequence in enhanced expression of SUMO proteins. In this study, we demonstrate that deltamethrin and cypermethrin affect the expression of SUMO isoforms and UBC9 in hepatic and adipocyte cells (HePG2, 3T3-L1 cell lines) in addition to triggering oxidative stress.

Materials and Methods

Cell Lines and Reagents

Human hepatocarcinoma cells (HePG2), mouse embryonic fibroblast pre-adipocytes (3T3-L1), and myoblasts (C2C12) were obtained from ATCC. Deltamethrin (N-11579) and cypermethrin (N-11061) were purchased from Chem Service Inc., USA. MEM (cat No. M0643), DMSO (D2438), Trypsin, DPBS (Dulbecco's PBS), MTT, Trizol reagent, IBMX (Isobutyl-1-methylxanthine), Hoechst B33258, Griess reagent, and DCF (dichlorofluorescein diacetate) were purchased from Sigma. FBS (Foetal bovine serum), and Penicillin- streptomycin were purchased from Gibco. Dexamethasone (RM4185) and DMEM (AT183) were obtained from Himedia. I script cDNA synthesis kit (1708891) and Bradford reagent were purchased from Bio-Rad. Primary antibodies purchased were: anti-SUMO1 antibody from 'Sigma' (cat No. S8070-200UL), anti-UBC9 antibody from 'Cell Signaling Technology' (cat No. 4918S), and anti-β-actin antibody from 'Santa Cruz Biotechnology' (cat No. sc-47778). PCR- kit was purchased from Kappa labs (SYBR Fast 2X qPCR Master Mix) and ECL- Kit (cat No. 32209) was purchased from Thermo scientific.

Cell Culture

HePG2 cells were cultured in MEM supplemented with 3.5 g/l glucose and 10% FBS. 3T3-L1 cells were cultured in DMEM supplemented with 10% FBS and 3.5 g/l glucose. Post-confluent monolayers were subjected to differentiation into adipocytes in the presence of differentiation medium for the first three days (DMEM supplemented with 500µM IBMX, 1 µM Dexamethasone, and 100 nM Insulin), followed by maintenance medium (DMEM with 100 nM insulin) for the next four days and in culture medium until day 15. C2C12 cells were cultured in DMEM supplemented with 3.5 g/l glucose and 10% FBS. They were differentiated into myotubes by reducing FBS to 2% for seven days. All media contained 100 mg/ml streptomycin and 100 U/ml penicillin. Treatment with pesticides was carried out for 24 hrs. 3T3-L1 and C2C12 were differentiated before treatment.

MTT Assay

For the cytotoxicity assay, cells were seeded into 96 well plates (3T3-L1 and C2C12 were on the 15th and 7th days of differentiation, respectively) and were grown to 70-80% confluence. After 24-hour treatment with either deltamethrin or cypermethrin, 10µl of MTT solution was dispensed into each well (5mg/ml PBS) and incubated at 37°C. The purple formazan crystals formed were dissolved by adding DMSO. OD 560/OD 670 nm [30] was calculated. Six replicas were maintained for each concentration. IC_{50} values for each chemical were calculated and concentrations below the IC_{50} were used for further experiments

Estimating ROS and NADPH

Reactive oxygen species (ROS) was estimated by the Dichlorofluorescein diacetate (DCF) method [31]. After treating cells for 24 hours with deltamethrin or cypermethrin, DCF dye was added (20µM) in phenol-red free medium, incubated for one hour at 37ºC, and OD was read at 485/528 nm. The readings were normalized with that of DNA (read after one-hour incubation with 10 µM Hoechst). In the case of 3T3-L1 and C2C12, cells were differentiated prior to treatment with the pesticides. For NADPH estimation, autofluorescence of the cell lysates was read at 340/420 nm [32].

Nitric Oxide Estimation

Spent media from cell cultures treated with deltamethrin or cypermethrin were mixed with equal volumes of Greiss reagent and incubated for 10 min at RT. Absorbance was measured at 540 nm [33]. Sodium nitrate was used as standard, and Bradford assay was performed to estimate protein for normalization.

Quantitative PCR

For real-time qPCR, RNA was extracted from cells using Trizol reagent, followed by chloroform-isopropanol precipitation and washing with 75% ethanol. cDNA synthesis was done by using i Script cDNA synthesis kit as per manufacturer's protocol. Gene-specific amplification was done using SYBR Fast 2X qPCR Master Mix as per the protocol. The primers used were as follows (5-3'): human GAPDH-FP (ggctgcttttaactctggcaaa) and RP(gtgggtagaatcatactggaacatgt); human NOS2A-FP

(acaagcctacccctccagat) and RP(tcccgtcagttggtaggttc); mouse 18S RNA-FP (ggacacggacaggattgaca) and RP (cgctcca ccaactaagaacg); mouse IL1β-FP (acatcagca cctcacaagca) and RP (gcattagaaacagtccagccca); and mouse IL6-FP (gccttcttgggactgatgct) and RP (agacaggtctg ttgggagtgg). Human SUMO1-FP (tgtggggaagggagaaggat) and RP (aaggttttgcctcctggtca), human SUMO2-FP (atgaaagcctatt gtgaacg) and RP (cttcatcctccatttccaac); human SUMO3-FP (gagaggcagggcttgtcaat) and RP (gaacacgtcgatggtgtcct); human UBC9-FP (cgaaccaccattatttcacc) and RP (ggatctgtttgattgtga tgg); and mouse SUMO1-FP (ctccgaaagaactgggaatgga) and RP (ctaaaccgtcgagtgacccc). Mouse SUMO2-FP (gggacaggatggttctgtgg) and RP (ttccaactgtgcaggtgtgt); mouse SUMO3- FP (cccaaggagggtgtgaag ac) and RP (ttgaactgtaccaccgagcc); and mouse UBC9-FP (ctgtctctgcc actggaaact) and RP (cactacggtggcttggata).

Statistical Analysis

A minimum four replicas were maintained for each concentration and the experiment was repeated twice. Statistical analysis was performed by column statistics in graph pad prism.

Results and Discussion

To determine the IC_{50} value of deltamethrin and cypermethrin to the cell lines under testing (HepG2, 3T3-L1, and C2C12), MTT assay was performed after being treated with either of the pesticides for 24 hours. As shown in Figs 1(a-f), cell viability is reduced in a dose-dependent manner. HePG2 and 3T3-L1 cell lines show IC_{50} between 200-250 μM concentrations for both deltamethrin and cypermethrin. These chemicals were less toxic to C2C12 cells compared to HePG2/ 3T3-L1. Concentrations much below IC_{50} were used for further experiments.

Increased reactive oxygen and nitrogen intermediates can lead to many pathological conditions. In order to study the extent of oxidative stress caused by these pesticides, we measured the ROS/nitric oxide/mRNA levels of nitric oxide synthase2A (NOS2A)/NADPH in (HePG2), differentiated muscle cells (C2C12) and mature adipocytes (3T3-L1). Deltamethrin and cypermethrin caused an increase in ROS and NO accompanied by elevated NOS2A mRNA as well as reduced NADPH levels in HePG2 cells (Figs 2a-f). In differentiated 3T3-L1, concentration-dependent increases in NO levels accompanied by a drastic decrease in NADPH levels (Figs. 3a-b) were observed. A similar pattern was observed in C2C12 (Figs 3c-d). Proinflammatory cytokines such as interleukin-1 β (IL-1β) and interleukin-6 are reported to be elevated in a number of metabolic disorders [34-35]. Our study shows that both deltamethrin and cypermethrin cause a significant increase in mRNA levels of IL-6 and IL-1β in 3T3-L1 cells (Figs 3e-f).

SUMO isoforms and UBC9 are known to be elevated in certain cancers [13, 16, 19]. Hence, we studied the influence of these pesticides on the expression of SUMO

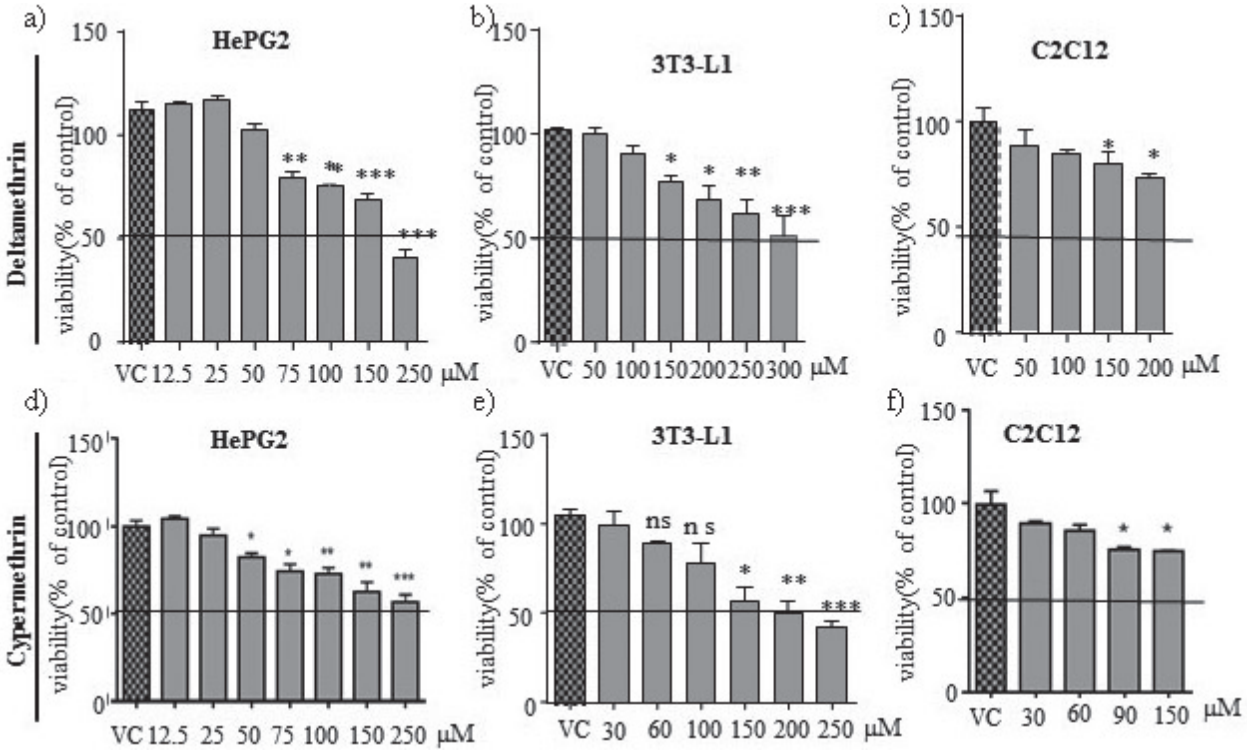

Fig. 1. Cell viability assay by MTT: a) HepG2 cell lines, b) differentiated 3T3-L1, c) differentiated C2C12 induced with deltamethrin for 24 hrs., and d-f) HePG2, differentiated 3T3L1, and differentiated C2C12, respectively, exposed to cypermethrin. Data are expressed as mean ±SEM, and statistical analysis was performed using the unpaired Student's t-test (*P<0.05, **P<0.01, ***P<0.001 and n = 4).

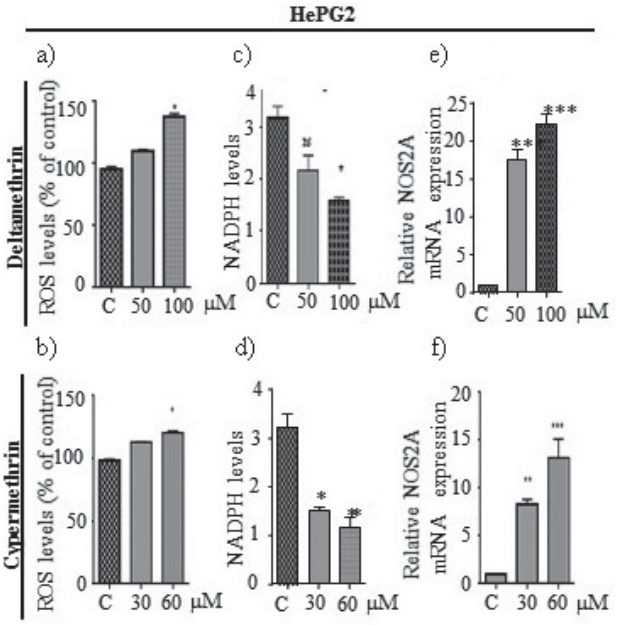

Fig. 2. Deltamethrin and cypermethrin cause oxidative stress in HePG2 cell lines: a) and b) refer to ROS levels on exposure to deltamethrin and cypermethrin, respectively. c) and d) refer to NADPH levels on exposure to deltamethrin and cypermethrin, respectively. e) and f) refer to the expression of NOS2A gene relative to GAPDH mRNA. Gene expression was quantified by RT-PCR and the data expressed as mean ±SEM. Statistical analysis was performed using the unpaired t-test. (n = 4, *P<0.05, **P<0.01, ***P<0.001).

isoforms (1-3) and UBC9. In HePG2 cells, SUMO2 mRNA levels increased upon treatment with deltamethrin, whereas both SUMO2 and 3 mRNA levels were increased with cypermethrin. There was no significant change in mRNA expression of SUMO1 in these cell lines. UBC9 levels also were elevated in HePG2 cells upon treatment with deltamethrin and cypermethrin (Figs 4a-b).

In 3T3-L1 cells, treatment with cypermethrin led to elevated mRNA levels of SUMO1 and SUMO2 (Fig. 4d), with no significant changes in UBC9 mRNA levels. In differentiated C2C12 cells, no significant increase or decrease was observed in mRNA expression levels of SUMOs except in SUMO3 (cypermethrin) (Figs. 4e-f).

The effects of deltamethrin and cypermethrin on immune, reproductive, and nervous systems have been documented [23, 36-37]. Deltamethrin is sprayed for control of malaria in endemic areas. Its concentration in some soils was found to be as high as 8.9 mg/kg. In children living in sprayed houses, the urinary concentration of 3-PBA and Br_2CA (metabolites of deltamethrin) were found to be 27.3 µg and 60 µg per gram of creatinine, respectively [38]. So far, there are no reports available on how these pesticides influence the expression of proteins involved in SUMOylation. Such studies are required in view of the fact that SUMO modification occurs to a large number of cellular proteins and SUMOylation plays an important role in many cellular processes [39-40]. It was previously demonstrated that oxidative stress induced by H_2O_2 led to high levels of free SUMO1 accumulation in HeLa cells [41]. Likewise, in mammalian cells, nitric oxide accumulation caused global hypo-SUMOylation of SUMO1 and SUMO2/3 [42]. In our study we observed that oxidative stress being developed upon treatment with these two pesticides was accompanied by higher

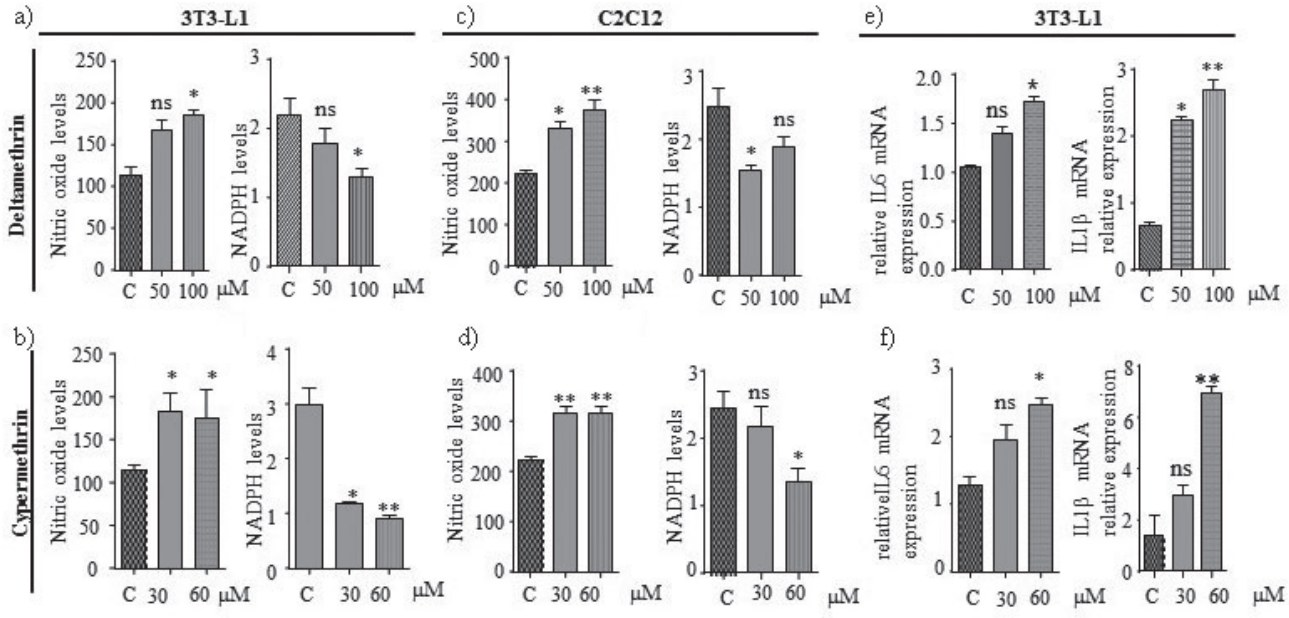

Fig. 3. (a-d) Deltamethrin and cypermethrin caused an increase in nitric oxide levels and a decrease in NADPH in differentiated 3T3L1 and C2C12 cells: NO was estimated in spent medium and NADPH levels were estimated in cell lysates (mean ±SD; * = p<0.05, ** = p<0.01 and n = 4). (e and f) Effect of deltamethrin and cypermethrin on mRNA levels of IL1β and IL6 in differentiated 3T3L1cells as quantified by real time q RT-PCR. (n = 4, * = P<0.05, ** = P<0.01).

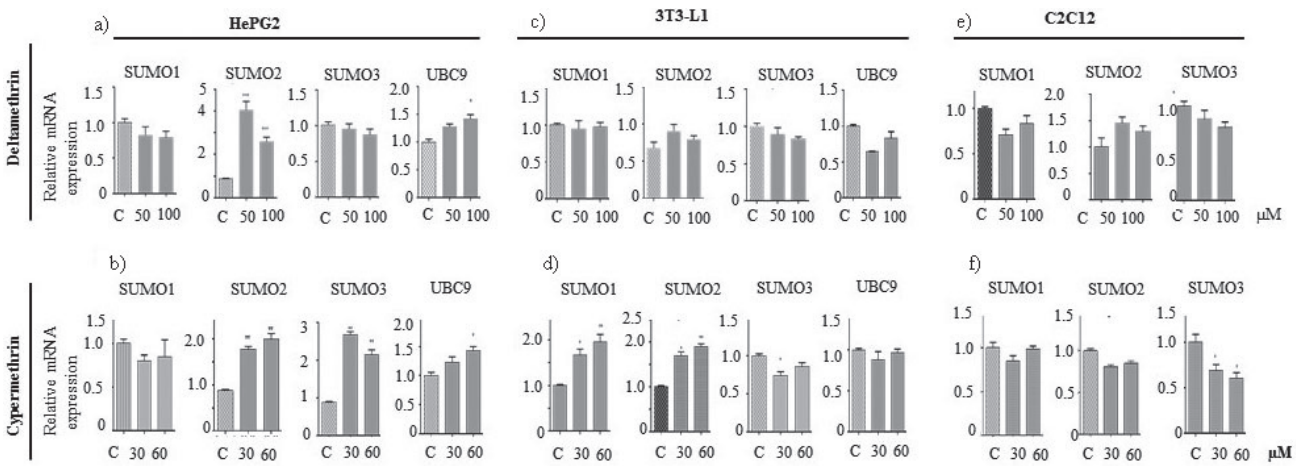

Fig. 4. (a-f) represents SUMO1, SUMO2/3 expression in various cell lines treated with deltamethrin or cypermethrin. (a and b) mRNA expression levels of SUMO1, SUMO2, SUMO3, and UBC9 in HePG2. Fig. (c and d) represents mRNA expression levels of SUMO1, SUMO2, SUMO3, and UBC9 in differentiated 3T3-L1 cell lines. Fig. (e and f) represents mRNA expression levels of SUMO1, SUMO2, SUMO3 in differentiated C2C12 cells, treated with either deltamethrin or cypermethrin as quantified by real time qRT-PCR (n = 4, * = P<0.05, ** = P<0.01).

expression of selective SUMO isoforms in the cell lines tested. Elevated expression of SUMO isoforms indicates the extent of stress created within the cell.

A number of studies have shown that UBC9 is up-regulated in a variety of cancers and can serve as a biomarker [15-16, 36]. Our study demonstrates that UBC9 levels significantly increase in HePG2 on treatment with deltamethrin and cypermethrin. This observation is of significance in view of the fact that higher UBC9 levels were observed in certain cancers.

Elevated mRNA levels of IL-6 and IL-1β in differentiated adipocytes (3T3-L1) on treatment with deltamethrin and cypermethrin demonstrate the ability of these pesticides to trigger the inflammatory response.

In summary, elevated SUMOs and UBC9 and stress induction are indicators of conditions that are favorable for disease development, indicating that the extensive use of these pesticides is of concern.

Conclusions

SUMOs and UBC9 have an important role in cellular homeostasis as most of the cellular processes involve SUMO-modified target proteins. Deltamethrin and cypermethrin build up oxidative stress and influence SUMO and UBC9 expression.

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