Effects of Three Triazine Herbicides on Expression of Critical Peroxide Metabolism Genes in MCF-7 and MCF-10A Cells

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Introduction

Background

- Herbicides are used worldwide in agricultural processes, yet are found in surface runoff and groundwater at alarming rates [1,2].
- Chloro-s-triazine herbicides are among the most widely used throughout the United States [3]. Of these, atrazine, cyanazine, and simazine have been shown to impact specific gene expression in MCF-7 breast cancer epithelium [4].
- Previous, unpublished data from our lab indicates that these herbicides have also demonstrated significant impacts on hydrogen peroxide (H_2O_2) production [5].
- The hydrogen peroxide metabolism pathway is divided into two pathways, either protein or enzymatic metabolism of H_2O_2, following activation of the transcription factor Nrf2. Atrazine has been shown to increase the expression of one of these enzymes, Peroxiredoxin 1 (PRX1) in MCF-7 cells [6].
- We have focused our search on the enzymatic portion, concerning four interconnected enzymes: PRX1 , Thioredoxin 1 (TRX1), Sulfiredoxin (SRX), and Thioredoxin Reductase (TRXR) (Figure 1).

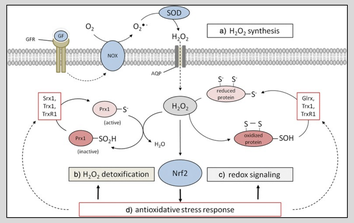

Figure 1. Hydrogen peroxide metabolism pathway [7].

Hypotheses

- We hypothesized that of our three herbicides, atrazine will have the greatest impact on PRX1 expression.
- We hypothesized that since PRX1 should increase, the levels of SRX will also increase while expression of TRX1 and TRXR will decrease.
- We hypothesized that the expression change of these enzymes will be greater in MCF-10A (non-cancerous) cells than in MCF-7 (cancerous) cells.

Methods and Materials

- MCF-7 human breast cancer cells and MCF-10A normal human breast epithelial cells were utilized as models for gene expression analyses.
- Cells were split into 6-well plates and allowed to adhere overnight. Cells were then treated for 24 hours with atrazine, cyanazine, or simazine at three different concentrations: 0.1x, 1.0x, and 10x the EPA maximum exposure limits. DMSO was utilized as a vehicle control.
- RNA was isolated, and samples were treated with DNase (Promega, Madison, WI) to remove contaminating nucleotides. RNA purity and quantity were determined using a spectrophotometer at 260 and 280 nm.
- cDNA was synthesized from RNA using random primers and M-MLV reverse transcriptase (Promega, Madison, WI).
- Real-Time PCR was conducted using SYBR Green master mix, synthesized cDNA, and specific DNA primers for PRX, SRX, TRX, TRXR, and a control gene, 36B4, using the iCycler (BioRad, Hercules, CA).
- Melt curves from PCR reactions were analyzed for primer integrity (Figure 2A). Gene-specific nucleotide amounts were indicated by the cycle number when the reaction reached a threshold absorbance (Figure 2B), as determined through iCycler data analysis software (CFX Manager, BioRad, Hercules, CA).
- 285 individual data points were collected. Using an ANOVA and Tukey's Post Hoc tests, data analysis was conducted using cell type, compound used, and concentration as independent variables, and expression fold as the dependent variable. Untreated cell expression was used as the control.

Results

Real-time PCR analysis relies upon identifying the PCR cycle in which enough product is produced to generate a fluorescent signal above a threshold. A two-fold change in expression can be assumed for every full cycle difference. Cycle values are subjected to numerical conversions and compared to gene controls to ascertain relative expression levels (Figure 2C).

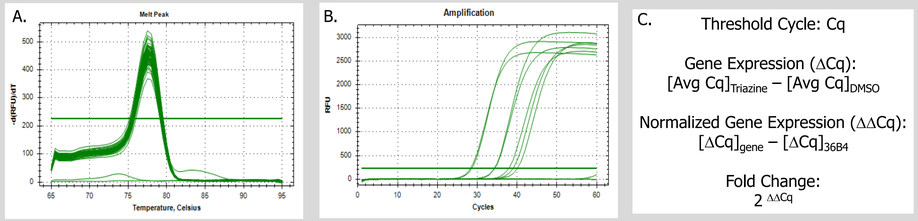

Figure 2. iCycler graph examples from real time PCR reactions. (A) Example melt curve of PCR product demonstrating effective annealing of specific gene. (B) Standard curve of increasing cDNA concentrations. (C) Equations utilized to calculate gene expression.

PRX1:

- Between the two cell lines there was a significant difference in overall expression (p=0.000, a versus b).
- There were three trends, between compound, cell type and compound, and compound and concentration (p=0.091, 0.072, and 0.099 respectively).
- There were no significant differences in expression for the MCF-7 cell line alone.
- There was a significant difference in PRX1 expression in MCF-10A cells based on compound used (p=0.029), specifically between cyanazine and simazine (p=0.025, # versus *).
- There were trends toward higher expression comparing 1.0x concentrations and the control (p=0.062), as well as concentration in general (p=0.093).

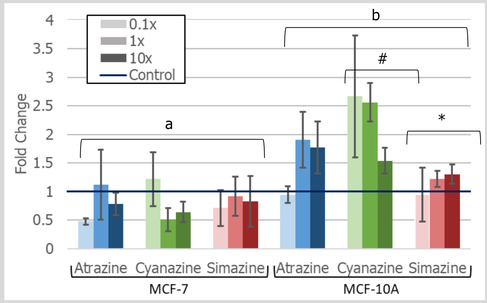

Figure 3. Expression fold change of PRX1 in MCF-7 and MCF-10A cells exposed to three triazine herbicides at 0.1x, 1.0x, and 10x concentrations of EPA maximums. Results represent 3 independent experiments performed in duplicate, ± SEM.

TRX1

- There was a significant difference in expression between cell types (p=0.005, a versus b).
- There were no further significant differences or trends in expression between the cell lines, or within cell lines in regards to either compound or concentration.

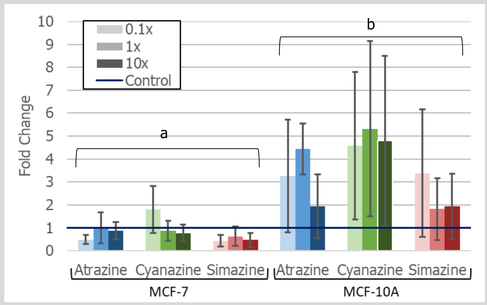

Figure 5. Expression fold change of TRX1 in MCF-7 and MCF-10A cells exposed to three triazine herbicides at 0.1x, 1.0x, and 10x concentrations of EPA maximums. Results represent 3 independent experiments performed in duplicate, ± SEM.

SRX:

- There was a significant difference in expression between the two cell lines (p=0.001, a versus b).
- There was a significant difference between the 1.0x concentration and the control when all data was collectively analyzed (p=0.028, *).
- There were no significant differences in expression within the MCF-7 cell line alone.
- Within the MCF-10A cells, there were trends indicating potential impacts due to concentration (p=0.059), and significant differences between the compiled 1.0x treatments and the control (p=0.033, #).
- Cyanazine concentrations in general demonstrated trends towards significance (p=0.091) and at 1.0x concentrations specifically (p=0.077).

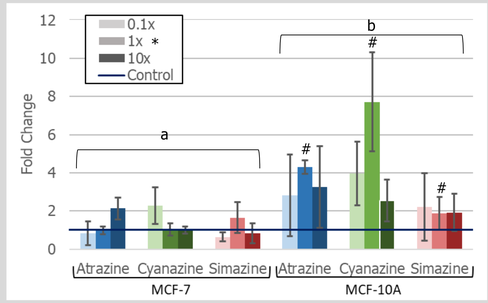

Figure 4. Expression fold change of SRX in MCF-7 and MCF-10A cells exposed to three triazine herbicides at 0.1x, 1.0x, and 10x concentrations of EPA maximums. Results represent 3 independent experiments performed in duplicate, ± SEM.

TRXR:

- There was no significant difference in expression between cell lines (p=0.134), or within the MCF-7 cell line.
- In the MCF-10A cell line, concentration or the ticide used demonstrated trends near significance (p=0.053 and p=0.070 respectively). These occurred between cyanazine and both other herbicides (atrazine p=0.109 and simazine p=0.107), and the 10x vs control concentration (p=0.033, *) which was significant.

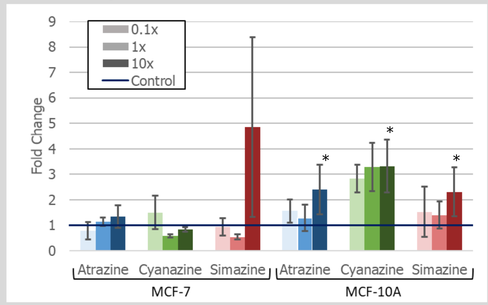

Figure 6. Expression fold change of TRXR in MCF-7 and MCF-10A cells exposed to three triazine herbicides at 0.1x, 1.0x, and 10x concentrations of EPA maximums. Results represent 3 independent experiments performed in duplicate, ± SEM.

Discussion

- Our first hypothesis was not supported. Atrazine did not alter the expression of PRX1, SRX, TRX1, or TRXR. In contrast, cyanazine repeatedly increased expression more than atrazine or simazine.
  - The lack of previous research of cyanazine on H_2O_2 metabolic enzyme expression may have contributed to biased hypotheses. The minute differences in chemical structure between the triazines may contribute to novel chemical interactions resulting in the increased fold change.
- Our second hypothesis was not fully supported. Although PRX and SRX expression did increase in concert with one another, TRX and TRXR expression also increased following treatment with the herbicides.
  - The expression increase in all enzymes may suggest that the herbicides are affecting the pathway earlier in the process, resulting in ubiquitous expression changes.
- Our third hypothesis was supported. MCF-10A cells did have the largest increase in expression. All statistically significant results were found within this cell line.
- Varying increases in expression of all four enzymes in the MCF-10A cells may indicate that the effects of these herbicides only affect healthy metabolic machinery, resulting in an imbalance of antioxidants and free H_2O_2.
- The lack of significant differences or expression trends in the MCF-7 cell line may indicate that these genes are not affected [8], or alternatively, expression levels may already be so high that these genes cannot be induced further. By viewing both cancerous and non-cancerous cell lines, we have avoided missing these effects.
- It may also indicate that we have not pinpointed the optimal exposure time. Previous work indicated H_2O_2 level changes after 48 hours of triazine exposure, while our study was performed after 24 hours [5].

Future research:

- Altered gene expression by these triazine herbicides indicates that the primary pathway activator (Nrf2) may be compromised or the repressor, Keap1, may be inhibited [9].
- We will focus on the expression of Nrf2, the transcription marker responsible for activating the antioxidant expression pathway.
- We will also be focusing on a similar class of enzyme also involved in H_2O_2 metabolism, glutathione peroxidases (GPX) [10].

Literature Cited

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