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Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate

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Gene expression is altered in mammalian cells (MCF-7 cells), by exposure to a variety of chemicals that mimic steroid hormones or interact with endocrine receptors or their co-factors. Among those populations chronically exposed to these endocrine disruptive chemicals are persons, and their families, who are employed in agriculture or horticulture, or who use agricultural/horticultural chemicals. Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized *in vitro* DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes, determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed *in utero*. *Human & Experimental Toxicology* (2007) **26,** *747–752*

Key words: agricultural chemicals; DNA microarray analysis; gene expression; glyphosate herbicide; real-time quantitative PCR

Introduction

Glyphosate [*N*-(phosphonomethyl) glycine], an organic compound containing an ionizable phosphate moiety, is usually found as a water soluble formulation of the isopropylamine salt of glyphosate.[1] Typically, glyphosate is applied as either 1–2% for home use, or 41–46% for commercial use, and the vast majority has been sold as Roundup™. In plants, glyphosate is a competitive inhibitor of 5-enolpyruvylshimikate-3-phosphate synthesis,[2] without which the plant dies. Gyphosate has not been shown to be a specific endocrine disruptive chemical (EDC), but has been extensively applied to crops and lawns, and as such may have extensive human exposure as a contact or inhaled toxicant.[3] Minimal acute toxicity has been determined for glyphosate, with a reported oral LD_{50} of >5.0 g/kg body weight.[4,5] The chronic, low-level, toxicity of glyphosate in animals has not been definitively determined, but it is generally considered to be potentially toxic and a probable endocrine disrupter.[6] Studies of chronic toxicity completed on glyphosate have been confusing and inconclusive.[1,6]

Although glyphosate has not been reported to bind endocrine receptors, it is obviously capable of interfering with endocrine-regulated gene expression, potentially by interacting with one or more of the steroid hormones or their receptors or co-factors. Since the expression of an enormous number of genes is regulated by 17β-estradiol (E2), we considered the possibility of glyphosate interfering with estrogen-regulated gene expression.[6]

Steroid hormone receptors are members of the family of cellular receptors that bind with high specificity to both steroid hormones, as natural ligands, and to

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specific DNA elements upstream from regulated genes. Steroid receptor function is essential for maintaining endocrine-interactive regulation of physiology in eukaryotic organisms,[7–10] and extensive studies have led to a proposed cause and effect relationship between exposure to chemicals that have endocrine disrupting activities and altered endocrine-regulated physiological function in animals. Chemically initiated changes in gene expression lead to changes in protein synthesis, which may result in altered metabolism, altered function of physiological systems, morphological, neurological and neurodevelopmental changes, decreased immune surveillance-associated protection against transformed cells and/or decreased normal immune system responses to a variety of microorganisms.[11–13] Exposure of pregnant animals to EDCs may cause variable altered developmental changes in the embryo resulting in a variety of birth defects that may occur, even though concentrations of the offending chemicals may be quite low in the environment.[14–21] In this study, the toxicity of glyphosate was examined as a function of its capacity to alter gene expression in the presence or absence of E2. The authors present data resulting from an investigation of the potential endocrine disruptive activities of a commercially available, unregulated, glyphosate herbicide.

Materials and methods

Materials
MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). All media were from Gibco (Gaithersburg, MD, USA). Fetal bovine serum was from Summit Biotechnology (Ft. Collins, CO, USA). E2 was purchased from Sigma (St. Louis, MO). A 15% home use preparation of glyphosate was purchased from a retail supplier and used from a stock solution, $X = 0.15$. RZPD microarray chips were purchased from Deutsches Ressourcentrum fur Genomforshung GmbH (Berlin, Germany).

Cell culture
MCF-7 cells were grown in minimal essential medium (MEM) as given previously.[22]

To expose MCF-7 cells to glyphosate, the cells were grown in T-150 vented culture flasks. Upon reaching 60% confluency, the medium was removed and replaced with phenol red-free MEM containing 10% stripped fetal bovine serum (SFBS), to reduce the E2 available to the cells. Twenty-four hours later, the cells were treated with glyphosate at 0.1, 0.01, 0.001 or 0.0001% dilutions of the stock (e.g., 0.001×0.15) with or without 3×10^{-10} M E2 for 18 h and total RNA was isolated.

Charcoal-dextran stripped FBS
FBS was steroid-stripped as given previously.[22]

RNA isolation
Cells were harvested after 18 h of treatment and total RNA was purified as previously described.[22]

Generating cyanine-3- or cyanine-5-labeled anti-sense RNA for microarray
Closed DNA (cDNA) was generated from MCF-7 RNA from cells treated with glyphosate or from MCF-7 cells grown in SFBS medium (control) using Roche's cDNA synthesis kit. Cyanine-5- and cyanine-3-labeled anti-sense RNA was generated and hybridized using Wellmer's protocol.

Hybridization of labeled RNA to the array slide
After an equal aliquant of labeled test sample was combined with the labeled control sample, the contents were loaded onto a clean 25×50 mm coverslip and evaluated as previously described.[22]

Array scanning
Microarray slides were scanned in an Axon Genepix 4000B as previously described.[22] Statistical analysis utilized one-way ANOVA followed by Dunnett's test to analyse differences between control and chemically treated samples, with $P < 0.05$ considered to be statistically significant.

Quantitative real-time PCR
About 23 μL of a cocktail containing Universal PCR master mix, no-UNG, from ABI (Branchburg, NJ, USA), pre-made ABI expression assays (primers) specific for the genes of interest, hypoxia-inducible factor 1 (HIF1), chemokine ligand 12 (CXCL12) or early growth response 1 (EGR1), and RNAse/DNAse-free water were combined with 2 μL of each reverse transcription reaction, plated in triplicate in a semi-skirted 96 well PCR plate and sealed with optically clear mylar film. qrtPCR employed an Applied Biosystems 7500 Real-Time PCR System thermocycler (Foster City, CA, USA) and used a three-step cycling program recommended by ABI: one cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s, then 60°C for 1 min.

Results

DNA microarray analysis indicated that a large number of genes, 680 out of 1550 on the chip, were

dysregulated by *in vitro* exposure to the commercial glyphosate herbicide. Of the enormous number of dysregulated genes we selected 29 to show both up- and down-regulation (Table 1). Of these 29 genes, seven were chosen for qrtPCR analysis to corroborate the array data. Glyceraldehyde-3-phosphate dehydrogenase (GADPH), von Hippel– Landau (VHL) tumor suppressor gene, TAP1 (transporter, ATP-binding cassette 1), and inositol polyphosphate-1-phosphatase (INPP1) were not corroborated as being significantly dysregulated by treatment with glyphosate. That is, they were not down-regulated by at least 50% (a haploid level) or up-regulated by a minimum of 100% (a tetraploid level). Neither VHL nor TAP1 were dysregulated significantly at any glyphosate concentration examined by qrtPCR. INPP1 was up-regulated 2.664-fold by glyphosate at 0.023%, a concentration that is likely not reasonable for cellular exposure.

HIF1 (Figure 1), CXCL12 (Figure 2) and EGR1 (Figure 3) were significantly dysregulated by 0.00023% glyphosate. HIF1 was up-regulated to 2.176 and CXCL12 and EGR1 were down-regulated to 0.462 and 0.490, respectively. qrtPCR expression analysis of these three genes corroborated the microarray data, showing up-regulation by more than two-fold for HIF1 and down-regulation by more than one cycle (50%) for both CXCL12 and EGR1.

For each of the genes, treatment with estrogen at 3×10^{-10} M was intermediate between SFBS and

Table 1 Genes found on the RZPD chip that were dysregulated by glyphosate as determined by DNA array analysis

Gene names	*Ratio up*	*Gene names*	*Ratio down*
TAP1	3.478	ACTA1	0.492
INPP1	3.186	EGR1	0.490
VHL	3.002	SLC25A3	0.463
PFN2	2.881	CXCL12	0.462
C2	2.537	GNB2L1	0.458
TRAF4	2.450	CD63	0.448
EST (26)	2.423	DAPK1	0.425
ITGAE	2.397	BDNF	0.288
TIMP3	2.379		
DUSP11	2.29		
ATR	2.238		
ARF4	2.212		
ATP9A	2.195		
PPP3CB	2.184		
GATA4	2.179		
HIF1	2.176		
PIK3R3	2.175		
EPB41L2	2.113		
RAC1	2.103		
BCL2L1	2.057		
MADH4	2.056		
GADPH	2.035		

A ratio above 2 or below 0.5 is considered significant.

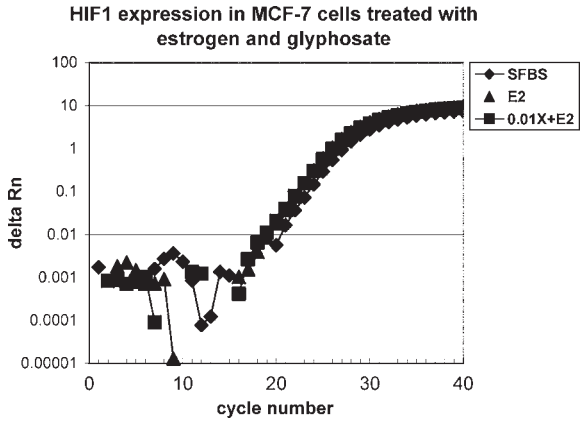

Figure 1 HIF1 expression in MCF7 cells treated with estrogen and glyphosate, as determined by qrtPCR [0.01*X*; + E2 ($X = 0.15$), 0.01×0.15; + E2].

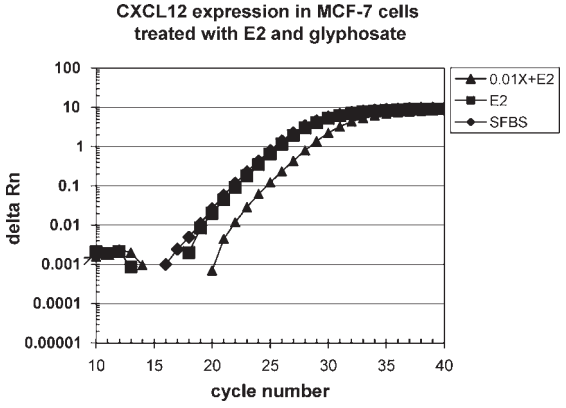

Figure 2 CXCL12 expression in MCF7 cells treated with estrogen and glyphosate, as determined by qrtPCR [0.01*X*; + E2 ($X = 0.15$), 0.01×0.15; + E2].

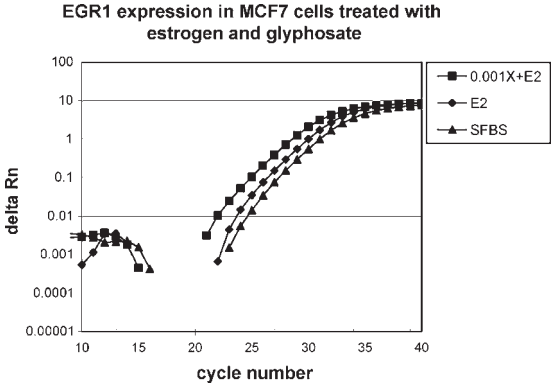

Figure 3 Expression of EGR1 in MCF7 cells treated with glyphosate and estrogen, as determined by qrtPCR [0.01*X*; + E2 ($X = 0.15$), 0.01×0.15; + E2].

treatment with glyphosate plus estrogen. This suggests that estrogen alone had an effect on the expression of these genes that were apparently synergistic with glyphosate.

Discussion

HIF1 was up-regulated to 2.176, slightly more than double the normal homozygous level of gene product, in cells treated *in vitro* with glyphosate at 0.00023%. This is equivalent to a tetraploid (autotetraploid) level of expression. HIF1 plays a key role in intracellular responses to hypoxia.[23] The regulation of HIF1 was reported to be associated in some way with the VHL gene, with HIF alpha subunits being stabilized in VHL-defective cells,[23] where the natural expression level of the HIF1 protein was oxygen-dependent. HIF1 activity is controlled by the oxygen-regulated expression of its 1-alpha subunit,[24] with mutations resulting in its constitutive expression, transcriptional activity being abnormally increased in cells in the absence of hypoxia. In hypoxic conditions, HIF1 primes cells for the initiation of apoptosis,[25] and therefore plays a key role in cell death resulting from cerebral or myocardial ischemia. The question is are elevated levels of HIF1 initiated by exposure to glyphosate sufficient to initiate apoptosis in the absence of hypoxia. Although generalized protein synthesis is inhibited under conditions of hypoxia, HIF1 binds to hypoxia response elements initiating transcription of at least 28 direct HIF1 target genes associated with energy metabolism, iron metabolism, angiogenesis and cell viability.[26] Thus, elevated levels of HIF1 may be associated with a variety of hypoxia-initiated pathophysiological states, including myocardial ischemia, cerebral ischemia, retinal ischemia, pulmonary hypertension, pre-eclampsia and intrauterine growth retardation.

Koshiji reported[27] that HIF1 is responsible for, or an initiator of, genetic instability of cells under hypoxic conditions and that elevated HIF1 levels inhibit the mismatch recognition essential for DNA mismatch repair. The interaction of HIF1 with additional gene expression was further demonstrated by Gustafson,[28] who showed that hypoxia blocked differentiation of mammalian neuronal and myogenic progenitor cells, and was associated with recruitment of HIF1 to promoter sites.

The expression of CXCL12 also called stromal cell-derived factor 1 and pre-beta cell growth-stimulating factor, as measured by qrtPCR, was decreased to a threshold level of 0.462, slightly less than half the normal expression level, by exposure to glyphosate. This is the CXCL12 expression level anticipated for cells heterozygous for a CXCL12 mutation. CXCL12 has been reported to be a very efficient lymphocyte chemoattractant[29] and speculated to play a role in lymphocyte activation. The implantation of a mutant form of CXCL12 into basal ganglia of mammals initiated neuronal cell death and hyperinflammation resulting in neurobehavioral deficit[30] and dementia, difficult to distinguish from that detected for virally infected neuronal cells. Pablos reported[31] that there was sufficient evidence to conclude that CXCL12 was associated with cellular hyperplasia in the perivascular lining and blood vessel endothelium in both rheumatoid arthritis and osteoarthritis, that CXCL12 was a participant in the angiogenesis associated with chronic inflammation and that it was a critical protein for mobilization of cells of the hematopoietic tissues into peripheral blood.[32] Further, Butler reported[33] that CXCL12 levels were elevated in diabetic patients with proliferative retinopathy and that blocking CXCL12 function with specific antibodies prevented neuronal damage associated with diabetic retinopathy.

EGR1, which was down-regulated to 0.490 in cells treated with 0.00023% glyphosate, directly regulates transforming growth factor beta-1 (TGFB1) gene expression, and decreased levels of EGR1 have been reported to decrease levels of TGFB1, altering growth in a human cell line.[34] EGR1 was reported[35] to not be expressed in a variety of human transformed cell lines, to be involved in suppression of growth and transformation and to be pivotal in the initiation of apoptosis.[36] The growth control activity of EGR1 was reported by Liu to be a function of binding to TGFB1 and fibronectin promoters that result in normal cell growth. Elevated levels of EGR1 were reported to be associated with microvascular endothelial cell replication and migration and with microtubular network formation on basement cell matrices.[37] Fahmy also reported that appropriate (diploid) EGR1 levels are essential for regulating endothelial cell growth, neovascularization, tumor initiated angiogenesis and tumor growth. Lastly, Virolle[38] showed that EGR1 was able to confer resistance to apoptosis by inhibiting FAS expression leading to insensitivity to FAS, implying that decreased levels of EGR1 could result in increased initiation of apoptosis. The question is whether or not EGR1 is a haploinsuffiency gene that would be expected to have an effect in heterozygous or down-regulated persons.

The three genes – HIF1, CXCL12 and EGR1 – determined by DNA microarray analysis and quantitative real-time PCR to be dysregulated by exposure to glyphosate, combine to give a bewildering array

of potential altered gene regulation effects. These include initiation of apoptosis in cells of cerebral and myocardial tissues, increased angiogenesis in tumors, retinal ischemia, hypertension, pre-eclampsia, fetal growth retardation and inactivation of tumor repressor genes. In addition, altered levels of CXCL12 may contribute to disruption of immune surveillance and basal extravasation of both monocytes and lymphocytes, and may be associated with neuronal death, perhaps by promotion of apoptosis.

Estrogen-regulated gene expression has been proposed to be a major factor, if not the major factor, in regulating levels of an enormous number of gene products essential for appropriate physiological function in human cells. An investigation of the effects of glyphosate on estrogen regulation of gene expression was, therefore, of interest. Data presented here suggest that physiological levels of estrogen slightly dysregulated each of the three genes in question and that glyphosate plus estrogen increased that dysregulation. A interest in chronic, low-level, exposure to a potential toxicant, and its possible interaction with estrogen, was of greater interest than in acute toxicity. Altered EGR1 levels in response to glyphosate salts are less clear than for HIF1 and CXCL12, but appear to potentially impact rates of apoptosis initiation and alter the levels of vascularization associated with tumor formation. There remains an unclear pattern of very complex events following exposure of human cells to low levels of glyphosate, but events surrounding the altered levels of expression of only three genes – EGR1, CXCL12 and HIF1 – out of the entire battery tested, are both complicated and potentially damaging to adult and fetal cells.

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