MELAMINE: AN EMERGING NEUROTOXICANT?

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ABSTRACT
Melamine is an organic compound used in combination with formaldehyde in the manufacture of plastics and also used as a flame retardant especially in buses and aircrafts. In the summer of 2008, multiple illness and deaths of children in China was associated with melamine-tainted powdered infant milk. Ever since, there has been serious public health concern over the toxic potentials of melamine especially amongst growing infants. We therefore set to investigate the neurotoxicity of melamine using transient transfection-based reporter gene assay and found that low dose melamine suppressed thyroid hormone receptor (TR)-mediated gene expression at 1μM. We further examined the effect of melamine on TR-thyroid hormone response (TRE) binding and found no dissociation of TR from TRE. Melamine did not also suppress co-activator (Steroid receptor co-activator 1; SRC-1) binding with TR, neither did it recruit co-repressors (nuclear co-repressor; N-CoR) to TR in the presence of thyroid hormone. Taken together, our data shows that melamine can interfere with TR-mediated gene expression and consequently may be inimical to the developing brain.

INTRODUCTION
Melamine is an organic compound commonly used in combination with formaldehyde in the manufacture of plastics including kitchen wares, whiteboards and commercial filters which can withstand temperature up to 120 °C. Melamine plastics are thermostatic and as such, are very difficult to recycle. Melamine is also used in a wide variety of flame retardant to reduce the damaging effect of fire outbreak especially in buses and aircrafts.

Melamine's high nitrogen levels (approximately 70% by mass) confers on it the analytical characteristics of protein molecules and as such can be used to adulterate commercial products to give spurious high readings for protein as was the case of the tainted milk scandal in the summer of 2008 in China, and also the incidence of thousands of pet deaths in the United States after consumption of imported pet food. Symptoms associated with melamine consumption included anorexia, vomiting, lethargy, polyuria and
polydypsia, clinical chemistry revealed high blood urea nitrogen and creatinine levels which is suggestive of renal failure. Studies have revealed that chronic exposure to melamine may cause reproductive damage. Oral administration of melamine and cyanuric acid resulted in loss of body weight, bladder and kidney stones, crystalluria, and epithelial hyperplasia of urinary bladder, induce extensive gold brown renal stones in fish, pigs and cats and caused diuresis and bladder calculi in rats and mice following acute, sub-chronic and chronic exposure. However, there are no literatures to our knowledge detailing the neuro-toxic effect of melamine especially on TR-mediated gene expression and possible mechanism.

Thyroid hormones (TH) are important for normal neuronal development and function in animals and humans during the fetal and neonatal periods. They control neuronal and glial proliferation in definitive brain regions, and mediate neuronal migration and differentiation, cause outgrowth of neuronal processes, and regulate synaptogenesis. Hypothyroidism especially during the period of brain growth spurt is associated with decreased intelligence quotient.

The action of TH are primarily mediated by TRs which belong to the family of nuclear hormone receptors containing a DNA-binding and a ligand-binding and they are widely expressed in different brain regions. By interacting with TRE upstream of the DNA, and recruiting coactivator or corepressor proteins, TR can mediate transcriptional regulation of target genes. Disruption of TR-TRE binding or recruitment of corepressors to TR will lead to inhibition of TR-mediated transcription. This mimick conditions of hypothyroidism, and could impair normal brain development and function.

We therefore aimed to investigate the effect of melamine on TR-mediated gene expression and possible mechanisms involved.

MATERIALS AND METHODS

**Chemicals:** Tri-iodothyronine (T3) was purchased from Sigma Chemical Co. (St. Louis, USA). Melamine sulfate was purchased from WAKO Chemicals (Tokyo, Japan).

**Plasmids:** Expression vectors of TRα1 and glucocorticoid receptor (GR) are described elsewhere. The luciferase (LUC) reporter constructs, the chick lysozyme (F2)-thymidine kinase (TK)-LUC, was previously described elsewhere. 5x upstream activating sequence (UAS)-TK-LUC in the PT109 vector and 2x glucocorticoid response element-LUC reporter in pTAL-LUC (BD Biosciences Clontech, Palo Alto, CA) was described previously. Expression vector of Human SRC-1 have been described previously. Expression vector of GAL4-DNA-binding domain (DBD)-fused SRC-1-nuclear receptor binding domain (NBD)-1 (aa 595-780) (otherwise described as nuclear receptor-interacting domain) was previously reported. VP16-TRB1-ligand binding domain (LBD) was constructed by inserting PCR-generated fragments inflame downstream of the VP16 activation domain in AASV-VP16. The Gal4-blank and GAL4-N-CoR (aa 1552-2453) was previously described.

**Cell culture:** CV-1 cells were incubated in Dulbecco's modified eagle's medium supplemented with 5 µg/mL penicillin/streptomycin and 10% fetal bovine serum at 37°C, 5% Co2 atmosphere as previously reported.

**Transient transfection-based reporter gene assays:** Cells were incubated in 24-
well plates 2 days before transfection by calcium-phosphate precipitation method. Cytomegalovirus-β-galactosidase plasmid was used as internal control. Sixteen to twenty-four hours after, wells were re-filled with fresh medium containing the indicated concentration of ligand and/or melamine for 24 hours. Cells were then harvested to measure the luciferase activities as described previously. Total amounts of DNA per well was balanced by adding pcDNA3 plasmids (Invitrogen, San Diego, CA, USA). The LUC activities were normalized to β-galactosidase activity and then calculated as relative LUC activities. All transfection studies were repeated at least twice in triplicate. Data shown represent mean ± S.E.M. of triplicate experiment. The data were analyzed using ANOVA.

Trypan blue exclusion: Trypan blue exclusion was previously described.

Liquid Chemiluminescent DNA pull down Assay (LCDPA): Liquid chemiluminescent DNA pull down assay to examine nuclear receptor-DNA binding in solution was previously described. Briefly, a glutathione S-transferase (GST)-fused TH receptor (GST-TR) bound to glutathione-Sepharose beads was incubated with a digoxigenin (DIG)-labeled double-stranded DNA fragment containing a TH response element (TRE) in protein-DNA binding buffer and 10⁻⁶ M Melamine. After extensive washing, protein-DNA binding on beads was detected using anti-DIG antibody conjugated to alkaline phosphatase. This is then measured by a chemiluminescent reaction using a luminometer. We perform LCDPA at least three times and data shown represent means ± S.E.M. The data were analyzed by ANOVA.

RESULTS

Melamine suppressed TR-mediated transcription in the presence of TH
We examined the effect of melamine on TR-mediated transcription using the transient transfection-based studies in CV-1 cell (Figure 2). Suppression of TR-mediated expression was seen on the F2-TRE-LUC at 10⁻⁶ M. The effect of melamine suppression on TR-mediated transcription was not as a result of cell death as confirmed by trypan blue exclusion (data not shown). Also, melamine did not suppress GR-mediated transcription (Figure 3) indicating that the suppression was TR-specific.

Melamine did not prevent SRC-1 binding to TRβ1 in the presence of TH
We examined the effect of melamine on binding between TRβ1 and SRC-1 in CV-1 cells using mammalian two-hybrid assay. In this assay, the interaction between SRC-1-NBD-1 and TRβ1-LBD with or without T and or melamine was examined. The NBD-1 of SRC-1 was fused to the Gal-4-DNA binding domain, and the LBD of TRβ1 was fused to VP16 transactivation domain. Transactivation mediated by Gal4- SRC-1-NBD-1 and VP16-TRβ1-LBD proceeded in the presence of T₃ (Figure 4; column 4).

Transcriptional activation caused by SRC-1-NBD-1 and VP16-TRβ1-LBD interaction with T₃ was not affected by melamine at concentrations of 10⁻⁵ M and 10⁻⁶ M (Figure 4; columns 5-8). These results indicate that melamine did not inhibit the binding between SRC-1 and TRβ1-LBD.

Melamine did not recruit N-CoR to TRβ1 in the presence of TH
We examined the effect of melamine on binding between N-CoR and TRβ1 in CV-1 cells using mammalian two hybrid assay.
Fig. 1  Structure of Melamine and T3

Melamine

T3

Fig. 2

<table>
<thead>
<tr>
<th>TR 1</th>
<th>Melamine (M)</th>
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<td></td>
<td>-</td>
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Relative Luciferase Activity

- T3
+ T3
10^{-7} M
F2 - TRE-LUC
Fig. 5

![Graph showing relative luciferase activity with multiple bars for different conditions.]

<table>
<thead>
<tr>
<th>Condition</th>
<th>T3 Control</th>
<th>T3 + 10^-7M</th>
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<tr>
<td>VP-16TR 1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gal4-NCoR</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Melamine (M)</td>
<td>-</td>
<td>10^-9 10^-8 10^-7 10^-6</td>
</tr>
</tbody>
</table>

5x UAS-LUC CV-1

Fig. 6

![Graph showing relative light activity with bars for different conditions.]

<table>
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<tr>
<th>Condition</th>
<th>GST alone</th>
<th>TR 1</th>
</tr>
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<tbody>
<tr>
<td>T3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Melamine (M)</td>
<td>-</td>
<td>10^-6</td>
</tr>
</tbody>
</table>

- T3
+ T3
10^-7M
Gal4-N-CoR, VP16-TRβ1 and 5x UAS-TK-LUC were transfected together into CV-1 cells. Gal4-N-CoR and VP16-TRβ1 precedes the transcriptional activity (Figure 5, column 4) in the absence of T₃, while no activation was observed with T₃. Transcriptional activities were not markedly altered in the presence of 10⁻⁶ M and 10⁻⁷ M melamine regardless of T₃ (Figure 5, column 5-8), neither was there any significant dissociation of N-CoR from TR in the absence of T₃ (Figure 5) suggesting that melamine may not recruit N-CoR to TR in the presence of T₃.

**Melamine has no effect on TR-TRE binding in the presence of TH**

Finally, we performed liquid chemiluminescent DNA pull down assay to examine the effect of melamine on TR binding to TRE. We have confirmed previously that the results of this assay were compatible to those of electrophoretic mobility gel shift assay. 10⁻⁶ M melamine did not dissociate TR from TRE in the presence of T₃, 10⁻⁷ M (Figure 6, column 6), indicating that the suppression of TR-mediated transcription by melamine was not due to partial TR-TRE dissociation.

**DISCUSSION**

In the present study, we show that melamine induced suppression of TR-mediated gene expression in the presence of T₃, and may consequently disrupt normal brain development and function especially during the critical period of brain development in children fed with melamine tainted-milk formulas.

Suppression of TR-mediated gene expression by melamine was not as a result of cell death because trypan blue exclusion showed that melamine did not affect cell viability under our experimental conditions (data not shown). Also, the suppression seen with melamine was TR-specific because melamine did not suppress GR-mediated gene expression in the presence of TH.

We initially hypothesized that melamine effect on TR-mediated gene expression could be by dissociation of the coactivator complex from TR or through the recruitment of a corepressor complex to TR, because TR function was regulated in a ligand-dependent manner involving these nuclear cofactors. However, melamine did not recruit complexes containing N-CoR to TR, neither did it dissociate SRC-1 from TR indicating mechanisms other than interaction with nuclear cofactors located upstream of the target gene could be involved.

Liquid chemiluminescent DNA pull down assay also did not show any dissociation of TR from TRE in the presence of melamine and T₃ indicating that DNA-protein interaction involving response elements may not be principally involved in melamine effect`s on TR-mediated transactivation. This suggests that melamine may act via other mechanisms to suppress TR-mediated gene expression.

TH is essential for normal brain development, growth and function and any condition that induces hypothyroidism especially during the perinatal period have been known to induce cretinism with severe cognitive and/or mental disorders in the off springs. Since TH also tightly regulate fundamental gene expression both directly and indirectly in vast neuronal regions, the disruptive effects of melamine on TH homeostasis may disrupt normal brain development via TH-dependent gene regulations. More studies are however required to further elucidate in details the mechanism by which
Melamine inhibits TR-mediated gene expression.

In conclusion, our study shows that melamine suppressed TR-mediated gene expression thereby disrupting TH homeostasis and could consequently interfere with normal brain development and function. Given the widespread contamination of consumer products by melamine especially infant milk formula and the specter of abnormalities that could arise from disruption of TH homeostasis especially on the developing brain, there is urgent need to carefully monitor consumer products to make sure they are melamine-free.

REFERENCES


