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Evaluation of transporter expression in HK-2 cells after exposure to free and ester-bound 3-MCPD

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ABSTRACT

3-Monochloropropane-1,2-diol (3-MCPD) is a food processing contaminant in some infant formula products and other foods in the United States. Although rodent studies have demonstrated that 3-MCPD and its palmitic esters have the potential to induce nephrotoxicity, our recent human cell culture studies using the human renal proximal tubule cell line HK-2 have not strongly supported this finding. Considering this disparity, we sought to examine whether changes in transporter gene expression on proximal tubule cells could be modulated by these compounds and allow us to glean mechanistic information on a possible indirect path to proximal tubule injury *in vivo*. If fundamental processes like water and solute transport could be disrupted by 3-MCPD compounds, then a new avenue of toxicity could be further explored in both infant and adult models. In our current study, we used HK-2 cells as an *in vitro* cellular model of human proximal tubule cells to investigate the effects of low (10 μM) and high (100 μM) 3-MCPD compound exposures to these cells for 24 hours (h) on the expression of 20 transporter genes that are known to be relevant to proximal tubules. Although we detected consistent upregulation of AQP1 expression at the RNA transcript level following HK-2 treatment with both low and high doses of several ester-bound 3-MCPD compounds, these increases were not associated with statistically significant elevations in their protein expression levels. Moreover, we observed a lack of modulation of other members of the AQP protein family that are known to be expressed by human proximal tubule cells. Overall, our study suggests the possibility that 3-MCPD-related nephrotoxicity could be associated with indirect modes of action relating to aquaporin homeostasis, but additional studies with other human-derived models would be pertinent to further explore these findings and to better understand transporter expression differences under different stages of proximal tubule development.

1. Introduction

Industrial processing of vegetable oils can lead to the production of unwanted chemical byproducts including 3-monochloropropane-1,2-diol (3-MCPD) and its esterified derivatives. Comprehensive survey data collected since 2013 revealed that in the United States, infant formula and other foods made with refined vegetable oil ingredients contained detectable levels of such food processing contaminants [1–7]. Other reports also revealed detectable levels of free and ester-bound 3-MCPD in baked goods and other commonly consumed food products [8,9]. Questions about the safety of 3-MCPD compounds have been raised by concerned groups based on data from several *in vivo* animal safety studies investigating the potential health effects of consuming free and ester-bound 3-MCPD [10,11]. Although research using non-human primates uniquely showed evidence of hematological problems following the ingestion of free 3-MCPD [12], many studies using rodents that were fed either free 3-MCPD or select ester-bound derivatives revealed a wide variety of negative health outcomes [13–21]. These effects predominantly included elevated kidney weights, kidney tubular necrosis, tubular hyperplasia, renal carcinoma, and chronic progressive

Abbreviations: HK-2, Human Kidney-2; 3-MCPD, 3-Monochloropropane-1,2-diol; 1-Pa, 1-Palmitoyl-3-chloropropanediol; 1-Li, 1-Linoleoyl-3-chloropropanediol; 1-Ol, 1-Oleoyl-3-chloropropanediol; Pa-Li, 1-Palmitoyl-2-linoleoyl-3-chloropropanediol; Pa-Ol, 1-Palmitoyl-2-oleoyl-3-chloropropanediol; Ol-Li, 1-Oleoyl-2-linoleoyl-3-chloropropanediol; Pa-Pa, 1,2-Di-palmitoyl-3-chloropropanediol; Ol-Ol, 1,2-Di-oleoyl-3-chloropropanediol; Li-Li, 1,2-Di-linoleoyl-3-chloropropanediol; Pa, Palmitic Acid; Ol, Oleic Acid; Li, Linoleic Acid; PMA, Phenylmercuric Acetate; VAL, Valproic Acid.

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nephropathy [17,22–25].

Given the presence of free and ester-bound 3-MCPD in the food supply, we recently published research aimed at investigating the potential vulnerability of human proximal tubule cells to these food contaminants. We performed a series of *in vitro* cellular studies to investigate the direct effects free 3-MCPD and nine of its commonly detected esters derived from palmitic, oleic, and linoleic fatty acids on the model human proximal tubule cell line, HK-2 [26]. We evaluated cell viability, reactive oxygen species production, mitochondrial dysfunction, kidney-specific biomarker expression, and even metabolic changes, but we generally found a lack of overt toxicity. Even long-term exposure of HK-2 cells to free 3-MCPD necessitated relatively high treatment concentrations or long durations to induce significant cellular toxicity [27]. Although our findings were consistent with the lack of nephrotoxicity reported from non-human primate research, we set out to investigate the possibility that the mode of action carried out by these compounds *in vivo* were simply not captured in the collection of *in vitro* assays we performed.

Like in most situations, there are multiple mechanisms by which proximal tubules can be injured. Harmful compounds can damage cells of the proximal tubules through obstructive (or other physical) means, such as through the formation of crystals [28–30] or biochemical (ischemic, hypoxic, oxidative, or metabolic) mechanisms, such as through drugs, uremic toxins, or heavy metal-containing chemicals that can inhibit fundamental cellular and mitochondrial processes that are critical to sustaining tubular viability and hence function [31–33]. In the case of 3-MCPD and its ester derivatives, their mechanisms of renal injury are not completely understood and we asked whether directly exposing HK-2 cells to 3-MCPD and several of its esters *in vitro* had other more subtle consequences that would not necessarily damage the cells themselves in the short term, but that would instead have far-reaching implications that could affect proximal tubular function *in vivo*. Given that proximal tubule cells play a major role in reabsorption and transport of solutes and water [32,34], we hypothesized that a preliminary evaluation of transporter gene expression following direct exposure to these compounds could reveal whether treating this model of human proximal tubule cells could induce a different form of disturbance that had not been previously investigated in this context. For this current study, our objective was to perform a preliminary investigation into the short-term (24 -h) effects of free 3-MCPD, nine of its esters derived from palmitate, oleate, and linoleate, their three corresponding free fatty acids, and two control compounds on the expression of 20 genes that are relevant to renal transporter function. We describe the outcomes on their transcribed and protein levels.

2. Methods

2.1. Cell culture and chemical treatment exposure

Human proximal tubule cell line HK-2 was purchased from ATCC (Manassas, VA) and maintained in cell culture medium composed of Keratinocyte-SFM base medium supplemented with 10 % Fetal Bovine Serum, 50 mg/L Bovine Pituitary Extract and 5 μg/L human recombinant Epidermal Growth Factor (all from Gibco, Waltham, MA) as described previously [26,27]. HK-2 cells were plated in T150 Corning flasks (Corning, NY) to reach ~80 % confluence after 24 h of culture. Cells were treated with low (10 μM) or high (100 μM) concentrations of the following chemicals purchased from Toronto Research Chemicals (TRC; Toronto, ON, Canada) unless otherwise indicated: 3-Monochloropropane-1,2-diol (3-MCPD), 1-Palmitoyl-3-chloropropanediol (1-Pa), 1-Linoleoyl-3-chloropropanediol (1-Li), 1-Oleoyl-3-chloropropanediol (1-Ol), 1-Palmitoyl-2-linoleoyl-3-chloropropanediol (Pa-Li), 1-Palmitoyl-2-oleoyl-3-chloropropanediol (Pa-Ol), 1-Oleoyl-2-linoleoyl-3-chloropropanediol (Ol-Li), 1,2-Di-palmitoyl-3-chloropropanediol (Pa-Pa), 1,2-Di-oleoyl-3-chloropropanediol (Ol-Ol), 1,2-Di-linoleoyl-3-chloropropanediol (Li-Li), Palmitic Acid (Pa), Oleic Acid (Ol), Linoleic Acid (Li), Phenylmercuric Acetate (PMA; Sigma, St. Louis, MO), and Valproic Acid (VAL; Sigma). Compounds were dissolved in HK-2 media using the protocol recently described by *Mapa et al. 2019* [52]. Briefly, 10 mM stock solutions were prepared in warm DMSO before diluting them 10-fold in warm FBS and then again in HK-2 media to achieve a final test compound concentration of 10 or 100 μM.

2.2. Transcript screening by Luminex

HK-2 cells that were subjected to high- or low-dose treatments or left untreated were harvested as previously described [35]. Briefly, cell lysates were prepared using protease-containing lysis buffer (Invitrogen; Carlsbad, CA) and kept frozen until further use. Lysed cell homogenate samples were thawed and diluted 50× using Homogenization Solution and added in a multi-well Hybridization Plate and further processed using the RNA probe-conjugated and Capture beads following the manufacturer's recommendations (Invitrogen). After the plate was sealed, incubated overnight 54.4 °C with 600 rpm shaking, and centrifuged at 240×g at room temperature for 1 min, its contents were transferred to a magnetic separation plate and washed by plate-washer (Bio-Rad, Hercules, CA). After the pre-amplifier solution was pipetted into each well and incubated with shaking for 1 h at 50.4 °C, the plate was then washed, treated with Amplifier solution, SAPE solution, SAPE working reagent, with wash steps in between each addition, following the manufacturer's recommendations. At the final step, the plate was washed and read on a Luminex 200 Bio-plex instrument (Bio-Rad) to determine the RNA levels of the following select renal transporter genes: SLC22A1 (OCT1), SLC22A2 (OCT2), SLC22A4 (OCTN1), SLC22A5 (OCTN2), SLC22A6 (OAT1), SLC22A8 (OAT3), SLC22A11 (OAT4), SLC22A12 (URAT1), SLC5A1 (SGLT1), SLC5A2 (SGLT2), SLC9A3 (NHE3), SLC15A1 (PEPT1), SLC15A2 (PEPT2), SLC21A20 (OATP4C1), SLC47A1 (MATE1), SLC47A2 (MATE2K), AQP1, AQP2 and select control genes: PPIA, CDH16, GAPDH, GJB1, SQSTM1, ACTB, RPL19.

2.3. Protein expression detection by Simple Western analysis

Protein lysates were prepared by harvesting treated HK-2 cells in Roche (Basel, Switzerland) complete protease inhibitor lysis buffer and then freezing multiple aliquots of lysates at −80 °C until further use. Protein contents were determined by Sensolyte OPA Protein Quantitation Kit (AnaSpec; Fremont, CA) following the manufacturer's instructions. Briefly, 100 μL of serially diluted stock BSA solution and each lysate were plated in 96-well plates (Greiner Bio-One GmbH; Frickenhausen, Germany) and incubated with shaking at 800 rpm at RT with OPA assay solution, prepared by mixing OPA with reducing solution and assay buffer. After a 2 -h incubation at RT, the final mixtures were read on a FLUOstar plate reader (BMG Labtech; Cary, NC) to quantify protein levels and adjust each lysate to a final stock concentration of 400 μg/mL. Simple Western analyses were then performed on a WES system (Protein Simple; Santa Clara, CA) according to the manufacturer's instructions using a 12–230 kDa Separation Module (Protein Simple SM-W004) and either the Anti-Mouse Detection Module (Protein Simple DM-002) or the Anti-Rabbit Detection Module (Protein Simple DM-001), depending on the primary antibody used: AQP1 (B-11; Santa Cruz Biotechnology; Dallas, TX), AQP7 (D-12; Santa Cruz Biotechnology) or AQP11 (CUSABIO Technology; Houston, TX). In brief, HK-2 cell lysates from different treatments were diluted to 200 μg/mL in 0.1X sample buffer from the separation module. Samples were then mixed with fluorescent master mix and heated at 95 °C for 5 min in a thermocycler (Bio-Rad). The samples, blocking reagent (antibody diluent 2), primary antibodies diluted in antibody diluent 2, HRP-conjugated secondary antibodies, Luminol/peroxide chemiluminescent substrate, and separation and stacking matrices were pipetted into their designated wells in a provided 25-well plate. Using the WES instrument, lysate proteins were separated based on the molecular weight through the stacking matrix at 375 V for 35 min and incubated with blocking reagent for 15 min before the

targeted proteins were immunoprobed with primary antibody for 60 min followed by HRP-conjugated secondary antibody for 30 min. Finally, Luminol/peroxide chemiluminescence was detected and the resulting digital chromatographs were analyzed by Compass software (Protein Simple).

2.4. Statistics

Data were collected and analyzed using Excel (Microsoft, Redmond, WA) as well as GraphPad Prism (GraphPad Software, La Jolla, CA). Using a P-value of less than 0.05 or 0.01 (as indicated), statistical analyses were carried out using student 2-way ANOVA tests to establish the significance of treatment *vs*. no treatment differences.

3. Results

3.1. Effect of free and ester-bound 3-MCPD on transporter gene expression

To gain an understanding of whether free 3-MCPD and its palmitic, oleic, and linoleic-ester derivatives were associated with any changes in transporter expression in HK-2 cells, several genes that encode human renal proximal tubule transporters were evaluated for transcript level modulation. RNA transcripts from a broad selection of transporter genes were quantified following HK-2 cell exposure to low (10 μM) or high (100 μM) treatment doses of free 3-MCPD, three mono-esters (1-Ol, 1-Pa, and 1-Li), three homo-di-esters (Ol-Ol, Pa-Pa, and Li-Li), three hetero-esters (Ol-Li, Pa-Ol, and Pa-Li), their corresponding free fatty acids (Ol, Pa, Li), and two additional compounds, valproic acid (VAL) and phenylmercuric acetate (PMA) that serve as cell viability controls for HK-2 cells. As shown in Fig. 1, when eight prominent members of the SLC22A family of cation and anion transporters were evaluated, none of the low-dose exposures resulted in any appreciable changes in RNA expression levels. Even when the high dose was applied, only mild up-regulation of SLC22A1 (OCT1) expression in the range of about 2- to 3-fold was measured following HK-2 exposure to the mono- and di-esters. As expected, changes in SLC22A members were not significantly different following treatments with control compounds VAL or PMA, relative to no treatment.

Similar to the results obtained from evaluating members of the SLC22A family, when select renal transporter genes from a variety of other families (SLC5A, SLC9A, SLC15A, SLC21A, SLC47A, and AQP) responsible for the transport of many types of ions and molecules (glucose, sodium and hydrogen ions, peptides, and cations, anions, and water) were evaluated, we found that no significant changes were measured, with one exception, as shown in Fig. 2. AQP1 RNA transcript levels were exclusively and highly up-regulated to levels ranging from about 4- to 8-fold ($P < 0.01$) following treatment with two of the three tested mono-esters (1-Li and 1-Ol), all six di-esters, and even one free fatty acid (Ol). Additionally, weaker (about 2-fold) up-regulation in AQP1 was measured in the homogenates of cells treated with free 3-MCPD and Ol free fatty acid. In contrast to this major shift in AQP1 transcript levels, no significant changes in AQP2 expression were detected, as expected, since proximal tubule cells do not express this transporter gene. Although control compound PMA did not induce any substantial changes to AQP1 expression at the RNA level, high-dose VAL induced a 3.1-fold upregulation relative to no treatment ($P < 0.01$). Additionally, no significant down-regulation trends were measured in any of these selected transporter genes.

3.2. Aquaporin protein expression is not significantly up-regulated in free or ester-bound 3-MCPD

Given the very high levels of AQP1 transcript up-regulation following many of the 3-MCPD compound treatments at both low and high treatment doses, we next evaluated (a) whether these changes in

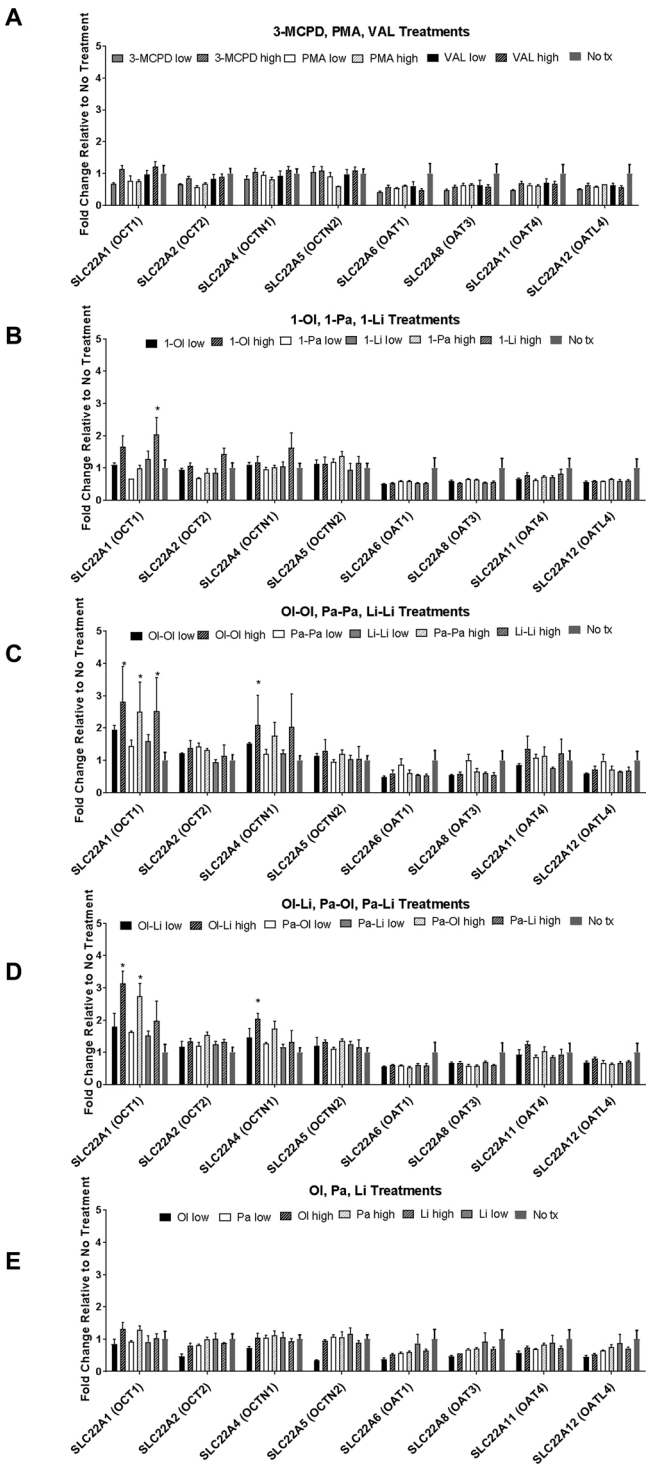

Fig. 1. HK-2 cells were treated with either low (10 μM) or high (100 μM) concentrations of free 3-MCPD, PMA, VAL (A), mono-ester-bound 3-MCPD (B), homo-di-ester-bound 3-MCPD (C), hetero-di-ester-bound 3-MCPD (D) or corresponding free fatty acids (E). Transcript expression levels of SLC22A transporter genes were measured by Luminex in replicates of three. Statistical significance of $P < 0.01$ for treated *vs*. non-treated cell populations is indicated by * symbols.

AQP1 RNA transcript expression were effectively translated to similar changes at protein level and (b) whether other members of the AQP family known to be expressed on proximal tubule cells were also associated with similar changes in their protein expression levels. As such, we treated HK-2 cells with 100 μM of each of the 15 test compounds and

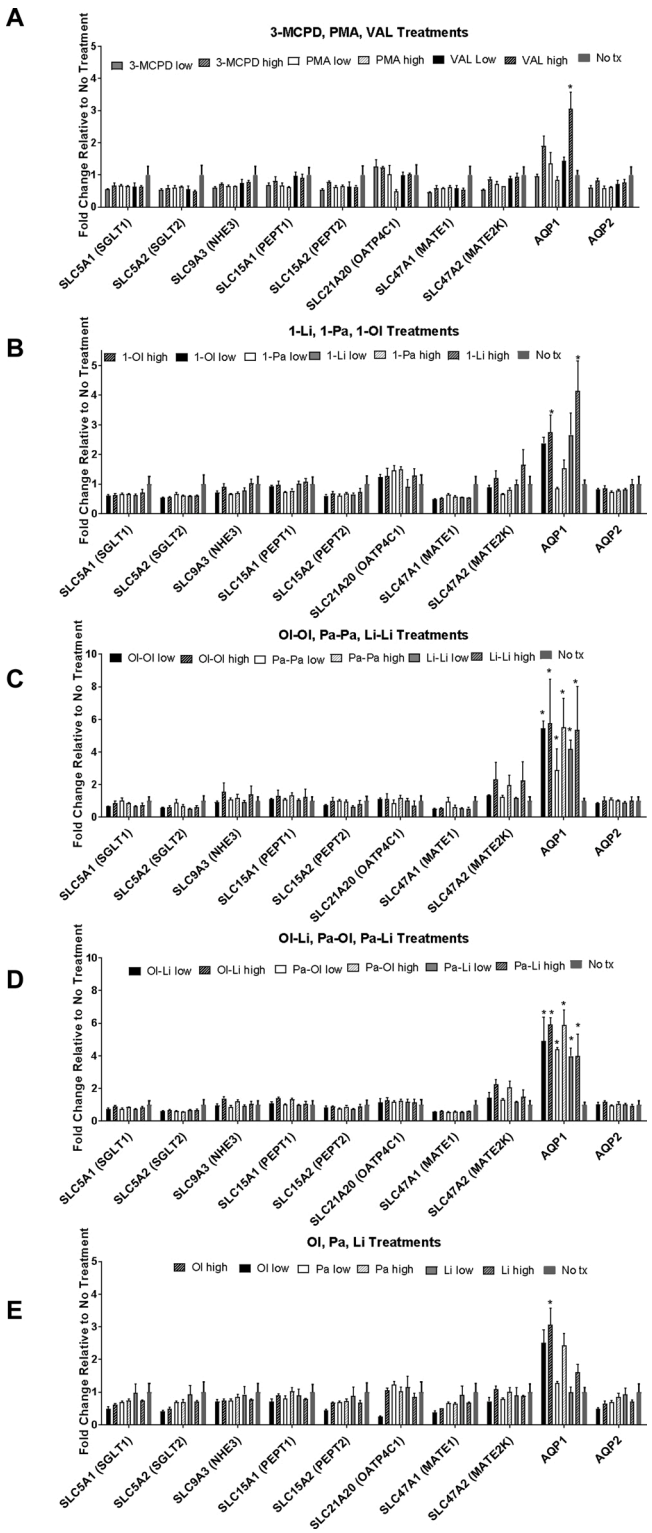

Fig. 2. HK-2 cells were treated with either low (10 μM) or high (100 μM) concentrations of free 3-MCPD, PMA, VAL (A), mono-ester-bound 3-MCPD (B), homo-di-ester-bound 3-MCPD (C), hetero-di-ester-bound 3-MCPD (D) or corresponding free fatty acids (E). Transcript expression levels of SLC5A, SLC15A, SLC21A, SLC47A, and AQP transporter genes were measured by Luminex in replicates of three. Statistical significance of $P < 0.01$ for treated *vs*. non-treated cell populations is indicated by * symbols.

measured AQP1 protein levels by Simple western, along with protein levels of the only other two members of the aquaporin family that are reported to be expressed in proximal tubule cells, AQP7 and AQP11. As shown in Fig. 3, relative to AQP1 protein levels assayed in non-treated cells, only treatment with Ol-Li di-ester yielded a 2.6-fold elevation, but this change in AQP1 was not statistically significant. Furthermore, none of the other treatment conditions tested using free or ester-bound 3-MCPD compounds, free fatty acids, or control compounds yielded significant changes (up or down) in translated AQP1 expression.

We next evaluated AQP7 and AQP11 expression following HK-2 treatment with 100 μM of each test compound. Similar to the pattern we observed with AQP1, AQP7 levels were not strongly affected by any of the treatments, with the exception of Ol-Li exposure (Fig. 3). Levels of AQP7 in HK-2 cells were about 3.6-fold higher when Ol-Li was applied, however, like in the case of AQP1, statistical significance was not reached. By contrast, HK-2 cell exposure to Ol-Li did yield statistically significant up-regulation of AQP11 by a striking 13.6-fold relative to no treatment ($P < 0.01$). Additionally, HK-2 treatment with Li-Li di-ester was associated with a 5.6-fold increase in AQP11, and about 3-fold increases in its levels were measured following HK-2 exposure to 1-Li mono-ester as well as Li and Pa free fatty acids, but without reaching statistical significance. No other exposure condition that we tested resulted in appreciable changes (up or down) in either AQP7 or AQP11 protein expression.

4. Discussion

The presence of free and ester-bound 3-MCPD contaminants in many commonly consumed foods including infant formula in the U.S. has reportedly raised questions about their possible health effects on the human body [10,11]. Since infants are commonly fed formula, the exposure of their developing organs to these types of contaminants underscores the need for safety studies. Several animal studies [13–23] have demonstrated the potential for free 3-MCPD and palmitic-derived 3-MCPD esters to cause some harm, especially to the renal proximal tubules, and therefore greater scrutiny towards the safety of these unwanted byproducts for this specialized renal structure is warranted. In the absence of comprehensive human safety testing *in vitro* or *in vivo* covering a wide breadth of 3-MCPD ester derivatives found in food, we recently published an extensive *in vitro* cellular evaluation of the effects of several 3-MCPD compounds on the human proximal tubule cell line HK-2 [26,27]. To our surprise, exposing HK-2 cells to free 3-MCPD and several esters derived from palmitic, oleic, and linoleic acid did not yield the extensive toxic effects that had been reported in rodent *in vitro* and *in vivo* models, despite testing multiple facets of cellular and organellar toxicity. Given the many important roles of the proximal tubules in reabsorbing about two-thirds of the glomerular filtrate load, which includes bicarbonate, glucose, amino acids, and other solutes that are critical to maintaining solute-water balance homeostasis [36–38], we have taken a first step to investigate whether 3-MCPD compounds had any effect on proximal tubule-associated solute and water transporters. In this work, we predicted that if changes in transporter expression were effectively modulated, then such a result would uncover a novel mechanistic aspect of *in vivo* nephrotoxicity induced by 3-MCPD compounds and support the possibility that human proximal tubules are indeed vulnerable to similar negative effects observed in rodent models. However, the actual findings of our preliminary study do not strongly support these ideas, as solute transporters were not consistently modulated, if at all, and the majority of measured changes in aquaporin family transporters AQP1, AQP7, and AQP11 lacked statistically significant upregulation at the protein level, despite (a) the strikingly high elevation in AQP1 transcript levels up to 8-fold and (b) the consistent pattern of AQP11 protein over-expression ranging from 3- to 13-fold.

The transportation role of aquaporins in facilitating proximal tubule function helps account for the critical role that these specialized renal structures play in the reabsorption of water and some solutes across the

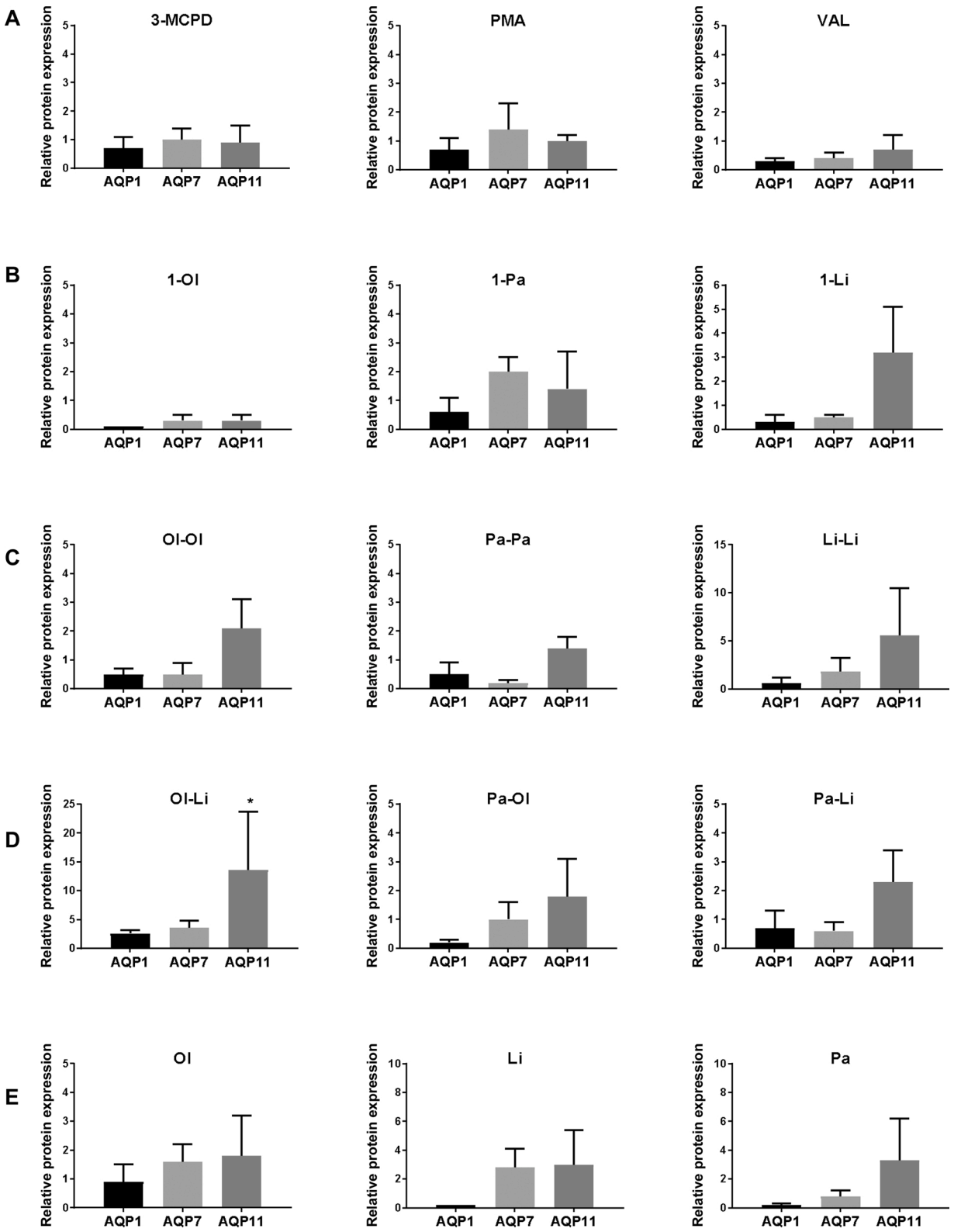

Fig. 3. Protein levels of AQP1, AQP7, and AQP 11 were measured in non-treated HK-2 cells and cells treated with 100 μM of free 3-MCPD, PMA, VAL (A), mono-ester-bound 3-MCPD (B), homo-di-ester-bound 3-MCPD (C), hetero-di-ester-bound 3-MCPD (D) or corresponding free fatty acids (E). The graphs shown are representative of three experiments. Statistical significance of $P < 0.05$ for treated *vs*. non-treated cell populations is indicated by * symbols.

plasma membrane [39]. Most aquaporins, including AQP1 and 7, are expressed in the proximal tubule cell membrane, but unlike AQP7, which is only localized on the proximal tubule apical cell membrane, AQP1 is associated with both apical and basolateral cell membranes. Disturbances in AQP1 expression can affect its role in ensuring that water reabsorption follows Na^+ and other solutes co-transported with this electrolyte and is associated with several types of cancer, including renal cell carcinoma [40,41]. AQP7 appears to be more involved in the

reabsorption of glycerol [42,43]. Interestingly, AQP11 is apparently localized to the cytoplasm, specifically in the region of the endoplasmic reticulum [44]. Although its functions remain unclear, when AQP11 expression is manipulated in mice, one major outcome is the formation of proximal tubule cysts [45].

Since there are no studies on the effects of free and ester-bound 3-MCPD on infant models of proximal tubules, it is important to understand the limitations of extrapolating our findings to the context of 3-MCPD compound exposure to proximal tubules of infants relying on formula as a primary nutrition source. Postnatal renal tubular development is a highly complex process and studies have found that renal transporters are expressed spatially and temporally in a specific age-dependent manner [46]. For human proximal tubules, direct infant to adult comparative expression studies are lacking, but at least one study has clearly demonstrated that AQP1 levels are prominent at the neonatal stage and that levels increase quickly during infancy to levels that are close to those of an adult [47]. Other transporters like NHE3 follow this trend as well [48], but until more comprehensive studies are performed, it will remain unclear whether these trends are truly prevalent in large sample populations of males and females. Interestingly, the expression trends of human aquaporins are mirrored in rat models [49]. Similarities between rat and human aquaporin expression patterns would have implications for the potential for 3-MCPD compound toxicity to be relevant to both species.

Many studies have suggested that the observed *in vivo* toxicity of 3-MCPD compounds is a result of ester-bound 3-MCPD undergoing hydrolysis *in vivo* during digestion and liberating free 3-MCPD, which in turn can be metabolized into β-chlorolactic acid, a purported toxicant [50,51]. We had previously tested the cytotoxicity of β-chlorolactic acid, but we found that it followed the same underwhelming effect as free 3-MCPD in a direct exposure model with HK-2 cells (unpublished data). It would be interesting to test whether the *in vitro* experimental condition of directly exposing HK-2 cells to β-chlorolactic acid would result in a more clear-cut effect on transporter expression than unmetabolized 3-MCPD seemed to have in this study. Future studies would also benefit from screening a wider scope of transporter families, as this study was designed to be preliminary in that respect. Although the number of test compounds we employed in our research is comprehensive relative to other studies, our recent approach to overcoming their insolubility in aqueous cell culture media [52] provides the opportunity to examine an even greater range of ester derivatives beyond those made with Pa, Ol, and Li in the future. Indeed, we expect that similar investigations into detailed aspects of the mode of entry and molecular effects can more easily proceed. Furthermore, although the HK-2 cell line has reliably represented human proximal tubule cells in many *in vitro* toxicity studies [53–58], it would be interesting to use this model under more advanced cultured conditions [59,60] that would achieve cell polarity, since transporter proteins do have their specific localizations relative to apical and basal cell polarity [39]. Such changes could allow a more detailed exploration of where the aquaporin expression changes occurred within the HK-2 cells and could be extended to other possible toxicological investigations [61–64].

Overall, our study points to a possible association between 3-MCPD compound exposure to HK-2 cells and aquaporin expression modulation. Such a mechanistic aspect of toxicity could partly account for why 3-MCPD compounds appear to have dual effect of not strongly inducing cytotoxicity to the individual proximal tubule cells yet still having the ability to induce tubular injury *in vivo* through manipulation of transporter expression. Clearly, extrapolating toxicity data from adult to infant models cannot be done easily, and comparative studies using infant and adult models would be highly valuable to better understand this aspect of 3-MCPD compound exposure. Thus, future studies are needed to study the effects of 3-MCPD compounds and their metabolites on aquaporin and other transporter expression and function using developmentally-appropriate proximal tubule cells models that are cultured to achieve cell polarity.

CRediT authorship contribution statement

Miriam E. Mossoba: Conceptualization, Methodology, Data curation, Writing - original draft. **Mapa S.T. Mapa:** Conceptualization, Methodology, Data curation. **Jessica Sprando:** Methodology, Data curation. **Magali Araujo:** Conceptualization, Writing - review & editing. **Robert L. Sprando:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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