Research Article

Effects of Ethylene Glycol Monomethyl Ether and Its Metabolite, 2-Methoxyacetic Acid, on Organogenesis Stage Mouse Limbs In Vitro

Caroline Dayan and Barbara F. Hales*

Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada

Exposure to ethylene glycol monomethyl ether (EGME), a glycol ether compound found in numerous industrial products, or to its active metabolite, 2-methoxyacetic acid (2-MAA), increases the incidence of developmental defects. Using an in vitro limb bud culture system, we tested the hypothesis that the effects of EGME on limb development are mediated by 2-MAA-induced alterations in acetylation programming. Murine gestation day 12 embryonic forelimbs were exposed to 3, 10, or 30 mM EGME or 2-MAA in culture for 6 days to examine effects on limb morphology; limbs were cultured for 1 to 24 hr to monitor effects on the acetylation of histones (H3K9 and H4K12), a nonhistone protein, p53 (p53K379), and markers for cell cycle arrest (*p21*) and apoptosis (cleaved caspase-3). EGME had little effect on limb morphology and no significant effects on the acetylation of histones or p53 or on biomarkers for cell cycle arrest or apoptosis. In contrast, 2-MAA exposure resulted in a significant concentration-dependent increase in limb abnormalities. 2-MAA induced the hyperacetylation of histones H3K9Ac and H4K12Ac at all concentrations tested (3, 10, and 30 mM). Exposure to 10 or 30 mM 2-MAA significantly increased acetylation of p53 at K379, *p21* expression, and caspase-3 cleavage. Thus, 2-MAA, the proximate metabolite of EGME, disrupts limb development in vitro, modifies acetylation programming, and induces biomarkers of cell cycle arrest and apoptosis. *Birth Defects Res (Part B)* 101:254–261, 2014. © 2014 Wiley Periodicals, Inc.

Key words: ***teratogen; histone deacetylase inhibition; lysine hyperacetylation; p53***

INTRODUCTION

Ethylene glycol monomethyl ether (EGME) is a glycol ether compound found in a variety of industrial products, including paints, inks, hydraulic fluids, and jet fuels (Johanson, 2000). Exposure to EGME causes various male and female reproductive defects as well as developmental abnormalities (Miller et al., 1983; Foster et al., 1984; Terry et al., 1996; Weng et al., 2010). Gestation day 8 mice treated with EGME exhibited neural tube malformations, including exencephaly and spina bifida occulta (Terry et al., 1996). EGME is oxidized by alcohol dehydrogenase to form methoxyaldehyde (MALD); MALD is rapidly oxidized by aldehyde dehydrogenase to form the stable metabolite 2-methoxyacetic acid (2-MAA; Welsch, 1995). The oxidative metabolism of EGME to 2-MAA is a prerequisite for embryotoxicity (Sleet et al., 1988). In utero 2-MAA exposure increases the incidence of hydrocephalus, hydronephrosis, heart, tail and limb malformations in species ranging from rodents to nonhuman primates (Brown et al., 1984; Scott et al., 1989). 2-MAA is also a developmental toxicant in vitro (Foster et al., 1987). Indeed, 2-MAA was selected as a class II test chemical for the European Centre for the Validation of Alternative Methods (ECVAM) international validation study on in vitro embryotoxicity tests (Brown, 2002).

Although 2-MAA is a well-established teratogen, its molecular mechanism of action is unclear. Disturbances in folate-dependent one-carbon transfer reactions (Welsch et al., 1987), chondrogenesis (Scofield et al., 2006), and apoptosis (Bagchi et al., 2011) have all been implicated in the teratogenicity induced by 2-MAA. An additional possibility is that the teratogenicity of 2-MAA is mediated by disturbances in acetylation programming. In vivo and in vitro studies have shown that 2-MAA inhibits histone deacetylase (HDAC) activity (Jansen et al., 2004). The significant hyperacetylation of core histones in testicular

*Correspondence to: Barbara F. Hales, Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, QC H3G 1Y6, Canada. E-mail: Barbara.Hales@mcgill.ca

Grant sponsor: Canadian Institutes of Health Research; Grant number: MOP-86511.

Received 28 January 2014; Accepted 12 March 2014

Published online in Wiley Online Library (wileyonlinelibrary.com/journal/bdrb) DOI: 10.1002/bdrb.21108

germ cells after 2-MAA exposure was attributed to both an increase in histone acetyltransferase (HAT) activity and a decrease in HDAC activity (Wade et al., 2008); furthermore, it was suggested that the 2-MAA-induced increase in histone acetylation played a role in spermatocyte death.

The acetylation of core histones by HATs and their deacetylation by HDACs are necessary to balance the opening and closing of chromatin structure that is responsible for the expression of various genes, many of which are important in embryonic development (Lehrmann et al., 2002). Both HATs and HDACs play an important role during development, as observed from different gene knockout studies. For example, mice lacking both p300 alleles are embryonic lethal at midgestation (Yao et al., 1998). The deletion of class 1 HDACs in transgenic mice is embryolethal at early stages of development (Lagger et al., 2002). Deletion of HDAC class 2 produced mice with phenotypic changes in cartilage development (Zhang et al., 2002). There is also growing evidence that HDAC inhibition is an important mechanism for teratogenesis. A study by Menegola et al. (2005) demonstrated that trichostatin A and valproic acid, known HDAC inhibitors, caused axial malformations in CD-1 mice after treatment on gestation day 8. Furthermore, Paradis and Hales (2012) showed that exposure to valproic acid caused significant limb abnormalities in vitro.

HAT and HDAC enzymes have both histone and nonhistone protein substrates (Gregoretti et al., 2004). The tumor suppressor transcription factor p53 is an example of a nonhistone protein target (Jin et al., 2013). Although p53 knockout mice are viable, they exhibit a gender-dependent increase in the incidence of developmental abnormalities such as neural tube defects (Sah et al., 1995). Either the overexpression or underexpression of wild-type p53 may have adverse effects on development (Choi and Donehower, 1999). Furthermore, p53 may have a role in protecting the embryo against teratogens. Moallem and Hales (1998) demonstrated that limb buds from homozygous *p53*-knockout mice were more affected by exposure to a preactivated form of the anticancer drug cyclophosphamide (4-hydroperoxycyclophosphamide), then were limbs from p53 wild-type mice.

The stability and function of p53 are regulated in part by acetylation (Luo et al., 2000). Acetylated p53 not only enables the recruitment of important transcriptional complexes to the promoter region, but also allows for the transcription of p53 target genes (Singh et al., 2010). Such p53 target genes include cyclin-dependent kinase inhibitor 1A (*Cdnk1a* or *p21*) and BLC2-associated X protein (*Bax*), genes that are important for the regulation of cell cycle arrest and apoptosis, respectively (Roy et al., 2005). However, the cellular response to undergo cell cycle arrest or apoptosis depends on the "acetylation signature" of p53 (Sykes et al., 2006; Tang et al., 2008). For example, acetylation of p53 at K320 leads to apoptosis, while ubiquitylation at this same site leads to cell cycle arrest (Le Cam et al., 2006).

We hypothesize that the adverse effects of EGME on limb development are mediated by 2-MAA-induced dysregulation of the acetylation of histone and nonhistone proteins, resulting in changes in cell cycle arrest and programmed cell death. To test this hypothesis, we determined the effects of in vitro exposure to increasing concentrations of EGME and 2-MAA on developing mouse limb buds and on the acetylation of histones H3K9 and H4K12 and of a nonhistone protein, p53K379. Furthermore, we elucidated the effects of EGME and 2-MAA on downstream markers of cell cycle arrest (*p21*) and apoptosis (cleaved caspase-3).

MATERIALS AND METHODS

Limb Bud Cultures and Drug Treatments

All animal studies complied with the guidelines established by the Canadian Council on Animal Care under protocol 1825. Timed-pregnant CD1 mice (20–25 g; Charles River Canada, St-Constant, QC, Canada) were mated between 0900 and 1100 h. The mating day was considered to be gestation day 0. On gestation day 12, between 0900 and 1100 h, females were euthanized by CO_2 inhalation and cervical dislocation and their embryos were explanted in sterile Hank's balanced salt solution (Sigma Aldrich Canada, Oakville, ON). The embryonic forelimbs were cultured, as previously described (Huang and Hales, 2002). Briefly, embryonic forelimbs were excised immediately lateral to the somites, pooled and cultured in roller bottles containing 6 ml culture medium, consisting of 75% BGJb Medium (GIBCO BRL Products, Burlington, ON, Canada), 25% salt solution, ascorbic acid (160 μg/ml), and gentamycin (1 μl/ml, GIBCO BRL Products). Each culture was gassed with a mixture of 50% O_2, 5% CO_2, and 45% N_2 for 2 min. Different concentrations (3, 10, or 30 mM) of EGME (99% pure, Sigma Aldrich) or 2-MAA (98% pure, Aldrich Chemical, Milwaukee, WI lot S45804–438, pH adjusted to 6.2 with 10 M NaOH) were added to designated cultures.

Limb Morphology

Forelimbs were cultured for 6 days at 37°C, with a one-time change of medium and oxygenation on day 3 to replenish the nutrient supply, without further addition of either EGME or 2-MAA. Forelimbs were then fixed overnight in Bouin's fixative (Harleco, Gibbstown, NJ), stained overnight with 0.1% toluidine blue (Fisher Scientific, Montreal, QC, Canada) in 70% ethanol, dehydrated using an ethanol gradient, cleared, and stored in cedarwood oil (Fisher Scientific). Limbs were observed using a dissecting microscope and the morphology and differentiation of each limb were assessed using the limb morphogenetic differentiation scoring system (Neubert and Barrach, 1977). Briefly, this system attributes a score to the radius, ulna, carpalia, and each one of the five digits according to the differentiation status of the limb. Six to eight independent replicates (n = 6–8 bottles/group; 7–10 limbs per bottle) were completed.

Western Blotting

Forelimbs were cultured at 37°C for 1, 3, 6, or 24 hr. Forelimbs were then placed in liquid N_2 and stored at −80°C. Limbs were later homogenized by sonication in lysis buffer containing protease inhibitors (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5, 40 μg/ml bestatin, 0.2M phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 6 μg/ml aprotinin). Total protein was extracted

and content quantified using spectrophotometric Bio-Rad Protein Assays (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins (15–30 μg per sample) were separated by SDS-PAGE acrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Sciences, Baie d'Urfé, QC, Canada). Precision standards (Bio-Rad Laboratories) were used as molecular weight markers. Membranes were blocked in 5% nonfat dried milk in TBS-T (137 mM NaCl, 20 mM Tris [pH 7.4], 0.05% Tween 20) at room temperature for 1 hr, probed overnight at 4°C with primary antibodies, washed in TBS-T, and incubated with the secondary antibody for 1 to 2 hr at room temperature. Immunoblotting was done using a polyclonal antibody against acetyl-histone H4 (Lys 12; H4K12ac, EMD Millipore, Billerica, MA, 04–119, 1:5000), acetyl-histone H3 (Lys 9; H3K9ac, EMD Millipore, 9649, 1:5000), acetyl-p53 (Lys 379; P53K379ac, Cell Signaling, Pickering, ON, 2570, 1:250), p53 (p53 [FL-393]; Santa Cruz Biotechnology, Santa Cruz, CA, SC-6243, 1:150), cleaved caspase-3 (Asp 175; Cell Signaling, 9661, 1:500), or beta-actin (Santa Cruz Biotechnology, SC-1616, 1:10,000). The secondary antibodies, donkey anti-rabbit antibody (GE Healthcare Limited, Baie d'Urfé, QC, Canada, NA-934, 1:5000) and anti-goat antibody (Santa Cruz Biotechnology, SC-2056, 1:10,000) conjugated to horseradish peroxidase (HRP), were used to detect specific antibody interactions. Western blots were visualized with the Enhanced Chemiluminescence Plus Kit (GE Healthcare Limited) and quantified by densitometry with the Chemi-Imager 4000 imaging system (Alpha Innotech, San Leandro, CA) and AlphaEase 3.3b software. Each sample consisted of an average of six to eight forelimbs; three to six separate Western blotting experiments were done for each antibody.

Real-Time qRT-PCR

Total RNA from homogenized limbs (four to five limbs per group, $n = 6$) was extracted using an RNeasy Microkit or RNeasy Minikit (Qiagen, Mississauga, ON, Canada). The RNA concentration and purity of each sample were assessed by spectrophotometry using a NanoDrop1000 spectrophotometer (Fisher Scientific). The samples were diluted to a working concentration of 10 ng/μl and transcripts were quantified using Quantitect One-Step SYBR Green RT-PCR (Qiagen). Primers for *p21* (QT00137053) and *18S* rRNA (QT01036875) were purchased from Qiagen. The reactions were done in a final volume of 20 μl that was composed of 10 μl SYBR Green Master Mix, 2 μl of the primer, 0.2 μl Quantitect Reverse Transcriptase mix, 6.8 μl RNase-DNase-free water, and 1 μl sample. RT-PCR was done under the following conditions: 30 min at 50°C followed by 40 cycles of 95°C for 10 min, 95°C for 15 sec, 50°C for 1 min, and 60°C for 1 min. Serial dilutions of nontreated forelimb RNA samples were used to create a standard curve. Each reaction was done in triplicate, averaged, and normalized to the amount of 18S rRNA transcripts.

Statistical Analyses

All morphology and protein quantification datasets were analyzed statistically using the Mann–Whitney U test and Bonferroni multiple-comparison correction with Systat 10.2 (Systat Software, Point Richmond, CA). The minimum level of significance was $p < 0.05$. Data are expressed as means ± SEM.

RESULTS

The Effects of EGME and 2-MAA on Limb Morphology

The effects of EGME and 2-MAA on the morphology of embryonic forelimbs cultured in vitro are shown in Figure 1. In both groups, the control limbs appeared normal, with properly developed radius, ulna, carpals, metacarpals, and phalanges. Treatment of limbs with increasing concentrations of EGME resulted in limb reduction at the highest concentration (30 mM) after 6 days in culture (Fig. 1A). In comparison, the limbs treated with increasing concentrations of 2-MAA showed a concentration-dependent response starting at the lowest concentration (3 mM), with the most dramatic effects observed at the highest concentration (30 mM, Fig. 1B). The defects observed in the limbs included a size reduction of the ulna and radius, poorly differentiated carpals, shortened metacarpals, fused digits, and missing digits. In EGME-treated limbs, the limb score was only significantly affected at the highest concentration (14% at 30 mM, Fig. 1C), whereas in 2-MAA treated limbs there was a significant concentration-dependent decrease in limb score (12% at 3 mM; 40% at 10 mM; 64% at 30 mM; Fig. 1D). These results reveal that in vitro exposure to 2-MAA, the proximate metabolite of EGME, had a dramatic impact on limb development.

Effects of EGME and 2-MAA on H3K9Ac and H4K12Ac

The effects of EGME and 2-MAA exposure on two specific histone acetylation sites, histone 3 lysine 9 (H3K9Ac) and histone 4 lysine 12 (H4K12Ac), were assessed as marks for effects on global HDAC activity and acetylation programming. The exposure of limbs to EGME did not alter the expression of either H3K9Ac or H4K12Ac at any concentration or time point (Fig. 2A, C). However, exposure of limbs to 2-MAA resulted in a significant increase in H3K9Ac at 1 hr (3, 10, and 30 mM), 3 hr (3, 10, and 30 mM), 6 hr (30 mM), and 24 hr (3, 10, and 30 mM; Fig. 2B). 2-MAA-exposed limbs also demonstrated a significant increase in H4K12Ac at 1 hr (3, 10, and 30 mM), 3 hr (3, 10, and 30 mM), 6 hr (10 and 30 mM), and 24 hr (3, 10, and 30 mM; Fig. 2D).

Effects of EGME and 2-MAA on the Acetylation of p53K379Ac

Neither EGME nor 2-MAA altered p53 protein levels compared to controls at any concentration or time point (Fig. 3A, B). To determine the effects of EGME and 2-MAA on p53 acetylation, we used the commercially available antibody that detects murine p53 acetylated at lysine 379 (p53K379, equivalent to lysine 382 in human p53; Feng et al., 2005). EGME exposure did not affect the expression of p53K379Ac in limbs at any concentration or time point examined (Fig. 3C). However, limbs exposed to 2-MAA showed a significant increase in the expression level of p53K379Ac at 3, 6, and 24 hr (10 and 30 mM, Fig. 3D, E). Thus, 2-MAA exposure induced a significant increase in

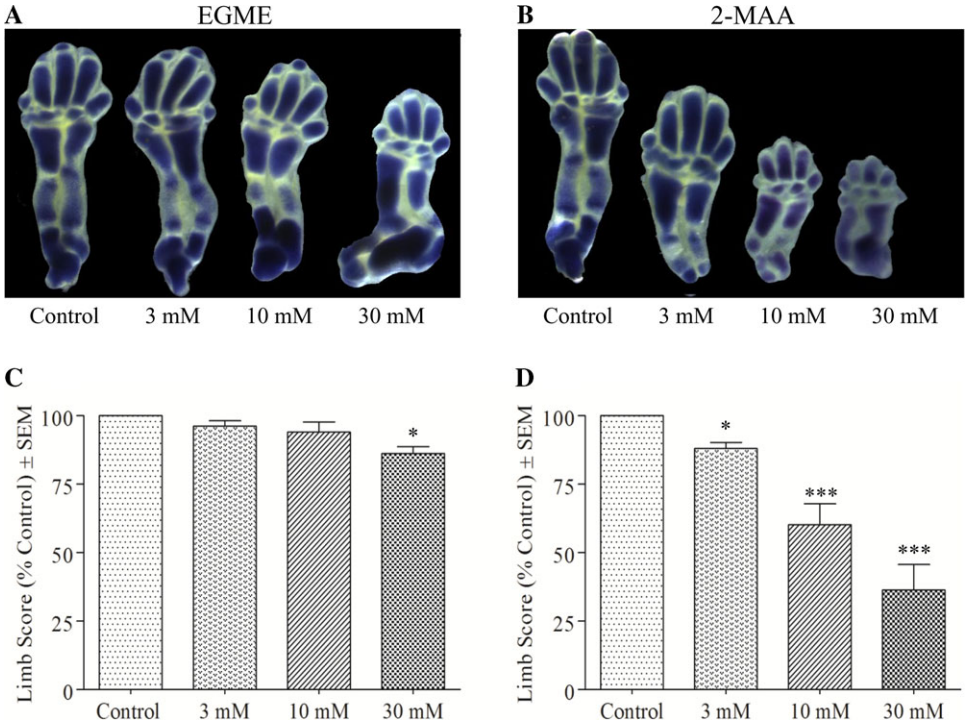

Fig. 1. Embryonic day 12 forelimbs were cultured for 3 days in the presence of increasing concentrations of either EGME or 2-MAA (0, 3, 10, or 30 mM); on day 3 the culture medium was changed without further addition of EGME or 2-MAA and the bottles were oxygenated. Limbs remained in culture for three more days. They were stained with 0.1% toluidine blue (A and B) to visualize cartilage formation and scored according to their morphology (C and D). $n = 6$–8. $^{*}p < 0.05$, $^{***}p < 0.005$.

p53 acetylation in the absence of an overall effect on p53 protein concentrations.

Effects of EGME and 2-MAA on p21 Steady-State Transcript Concentrations

Since acetylation of p53 at K379 has been demonstrated to be required for p53 binding at the *p21* promoter region (Zhao et al., 2006), we determined if *p21* expression levels were correlated with acetylated p53K379Ac. No changes in *p21* expression were observed at 3 hr in EGME treated samples (Fig. 4A). In contrast, 2-MAA exposure resulted in the upregulation of *p21* mRNA expression at 3 hr (10 and 30 mM, Fig. 4B). Therefore, there was a direct correlation between p53K379 acetylation and induction of *p21* expression.

Effects of EGME and 2-MAA on Caspase-3 Cleavage

Since p53 acetylation at K379 has been linked to the regulation of apoptosis (Zhao et al., 2006), we examined the effects of EGME and 2-MAA on protein levels of cleaved caspase-3, a marker of apoptosis. EGME exposure did not alter the amount of cleaved caspase-3 protein detected at any time point (Fig. 5A). However, limbs exposed to 2-MAA showed a significant increase in the amounts of cleaved caspase-3 at 3, 6, and 24 hr (10 and 30 mM, Fig. 5B). Thus, there was a direct correlation between p53K379Ac and cleaved caspase-3.

DISCUSSION

Although EGME and its primary metabolite, 2-MAA, are established teratogens, their mechanisms of action are still poorly understood. In this study, we demonstrated that exposure to 2-MAA caused a concentration-dependent increase in limb malformations in an in vitro limb bud culture system, whereas EGME exposure had only minimal effects at the highest concentration examined. Furthermore, this study provides the first evidence that the 2-MAA-induced limb malformations are strongly correlated with a deregulation of acetylation programming, suggesting that HDAC inhibition is the predominant mechanism of action.

A strong correlation between acetylated p53 K379 (equivalent to lysine 382 in human p53; Feng et al., 2005) and an increase in *p21* expression and in cleaved caspase-3 levels was observed after exposure to 2-MAA. Zhao et al. (2006) demonstrated in A549 cells that HDACi depsipeptide induced acetylation of p53 at K373/382 sites but not at K320. Acetylation at K373/K382 was necessary for p53 binding at the *p21* promoter, thus promoting the induction of *p21* expression. Furthermore, Oh et al. (2012) demonstrated that HDACi CG200745-induced cell death was resistant in a cell line in which p53 was mutated at K382. Therefore, the acetylation at p53 K379 plays an integral role in both cell cycle arrest and apoptosis.

EGME testicular toxicity in vivo was observed after dosing rats with 500 mg/kg for 2 days (Foster et al., 1984). These authors reported that up to 50 mM EGME had no effect on aggregates of Sertoli and germ cells in culture, whereas in vitro exposure to 5 mM 2-MAA

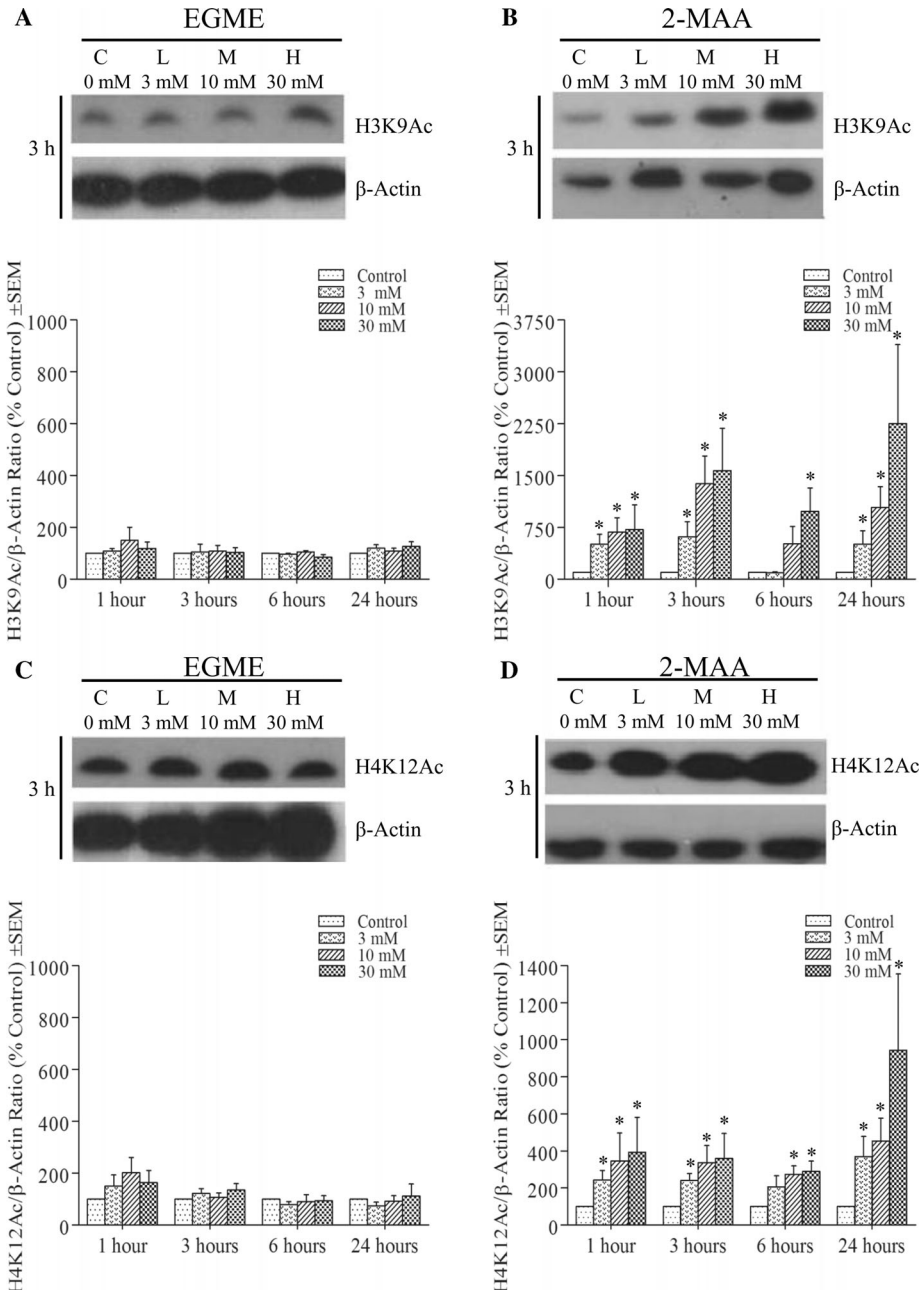

Fig. 2. Western blot analysis of the effects on histone 3 lysine 9 acetylation (H3K9Ac, A and B) and on histone 4 lysine 12 acetylation (H4K12Ac, C and D) following a 3-hr exposure to EGME or 2-MAA. H3K9Ac and H4K12Ac were normalized to β-actin and quantified by densitometry in protein extracts from limbs cultured for 1, 3, 6, or 24 hr in the absence or presence of EGME or 2-MAA. Differences were analyzed by Mann–Whitney U test, followed by Bonferroni correction. $N = 5$; $^*p < 0.05$.

affected pachytene spermatocytes (Foster et al., 1984). Yan et al. (2000) demonstrated that 2-MAA-induced apoptosis in spermatocytes was correlated with the increased expression of the proapoptotic factors BAX and BAK. In another study, 2-MAA exposure resulted in an increase in the death of rat germ cells that was correlated with cytochrome C release and the activation of caspases 9 and 3 (Rao and Shaha, 2002). Here, we report that 2-MAA exposure increased cleaved-caspase-3 in developing limbs. In addition, 2-MAA exposure increased *p21* expression, an important marker for cell cycle arrest. Therefore, the combined effects of cell cycle arrest and increased apoptosis in the developing limb may lead to the limb malformations observed.

Exposure to 3-mM 2-MAA did not affect the acetylation of p53 at K379. At this concentration 2-MAA may not inhibit the HDACs that deacetylate p53, HDAC1, and SIRT1 (Brook and Gu, 2011). There was a strong correlation between the effects of 2-MAA on p53 (K379) acetylation and downstream activation of markers of cell cycle arrest and apoptosis. Overall, this study demonstrates that 2-MAA-induced disruption of limb development in vitro

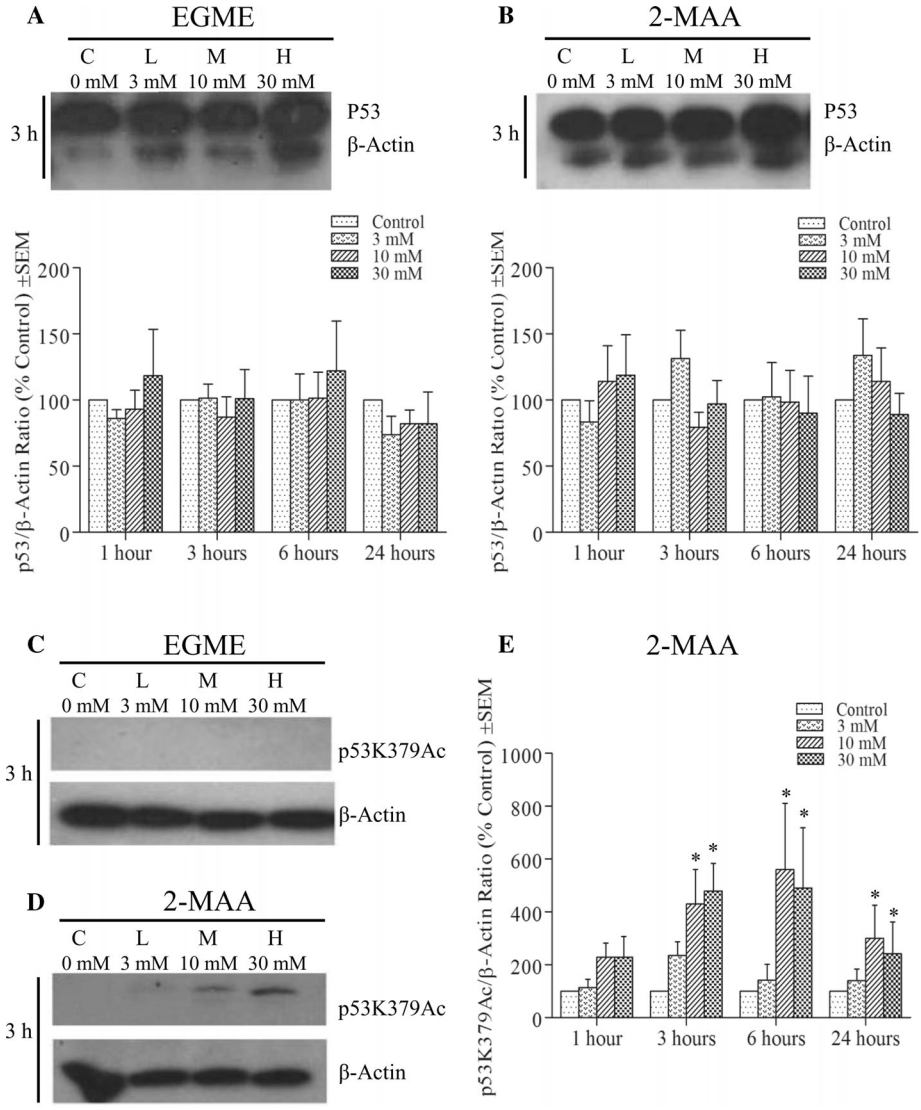

Fig. 3. Western blot analysis of the effects of a 3-hr exposure to EGME or 2-MAA on total p53 (A and B) and on acetylated p53 at lysine 379 (p53K379Ac; C, D, and E). No p53K379Ac was detected in control or EGME-exposed limbs. p53K379Ac in limbs cultured for 1, 3, 6, or 24 hr in the presence of 2-MAA was normalized to β-actin and quantified by densitometry in protein extracts from (E). Differences were analyzed by Mann–Whitney U test, followed by Bonferroni correction, $N = 5$; $^*p < 0.05$.

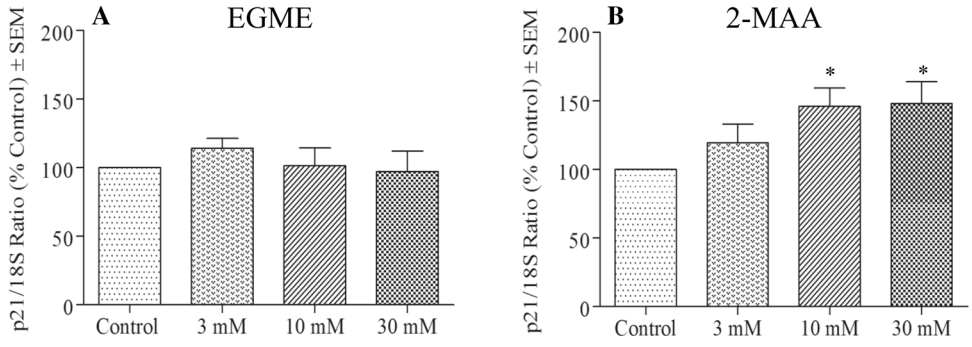

Fig. 4. *P21* gene expression following EGME (A) or 2-MAA (B) exposure. Limbs were cultured with either chemical for 3 hr. *p21* mRNA was quantified by qRT-PCR, normalized to 18S rRNA, and expressed as a percentage of control. Differences were analyzed by Mann–Whitney U test, followed by Bonferroni correction, with $N = 6$; $^*p < 0.05$.

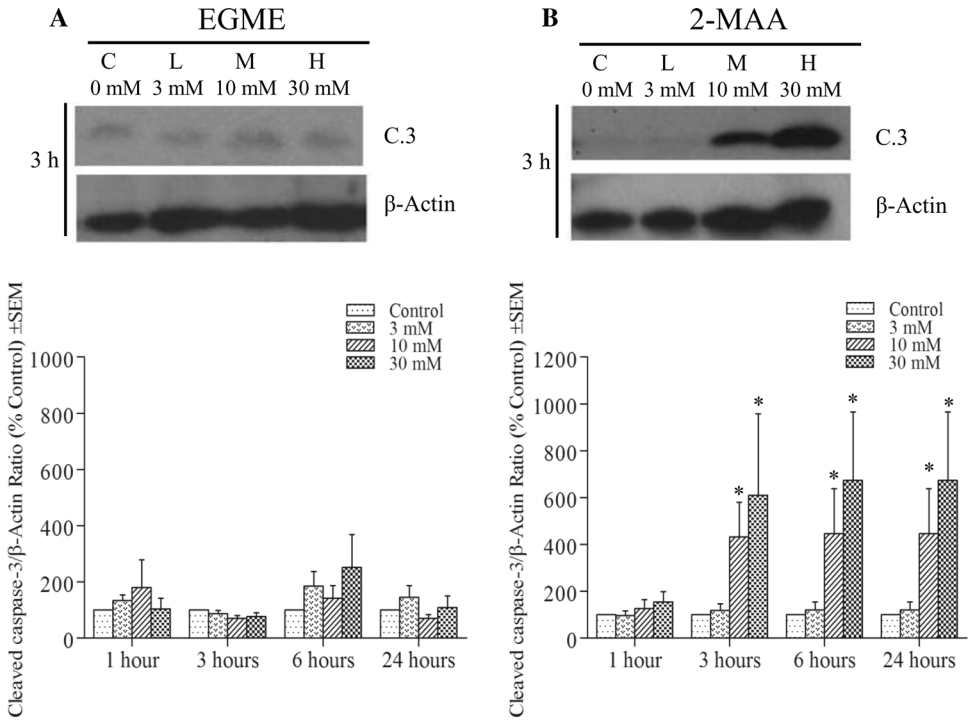

Fig. 5. Western blot analysis of the effects of exposure to EGME (A) and 2-MAA (B) on cleaved caspase-3 (C.3), a marker of apoptosis. C.3 was normalized to β-actin and quantified by densitometry in protein extracts from limbs cultured for 1, 3, 6, or 24 hr in the absence of presence of EGME or 2-MAA. Differences were analyzed by Mann–Whitney U test, followed by Bonferroni correction. $N = 5$; $^*p < 0.05$.

is associated with a dysregulation of acetylation as early as 3 hr after exposure. Therefore, we propose that modifications in acetylation programming underlie the teratogenicity of 2-MAA.

ACKNOWLEDGMENTS

The authors have no conflict of interest to declare. This work was supported by Canadian Institutes of Health Research (CIHR) operating grant MOP-86511. C. Dayan received partial support from the CIHR Drug Development Training Program. B. F. Hales is the recipient of a James McGill Professorship.

REFERENCES

Bagchi G, Zhang Y, Stanley KA, Waxman DJ. 2011. Complex modulation of androgen responsive gene expression by methoxyacetic acid. Reprod Biol Endocrinol 9:42.

Brooks CL, Gu W. 2011. The impact of acetylation and deacetylation on the p53 pathways. Protein Cell 2:456–462.

Brown, NA. 2002. Selection of test chemicals for the ECVAM international validation study on in vitro embryotoxicity tests. European Centre for the Validation of Alternative Methods. Altern Lab Anim 30:177–198.

Brown NA, Holt D, Webb M. 1984. The teratogenicity of methoxyacetic acid in the rat. Toxicol Lett 22:93–100.

Choi J, Donehower LA. 1999. p53 in embryonic development: maintaining a fine balance. Cell Mol Life Sci 55:38–47.

Feng L, Lin T, Uranishi H, Gu W, Xu Y. 2005. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 25:5389–5395.

Foster PM, Creasy DM, Foster JR, Gray TJ. 1984. Testicular toxicity produced by ethylene glycol monomethyl and monoethyl ethers in the rat. Environ Health Perspect 57:207–217.

Foster PMD, Loyd SC, Blackburn DM. 1987. Comparison of the *in vivo* and *in vitro* testicular effects produced by methoxy-, ethoxy- and n-butoxyacetic acids in the rat. Toxicology 43:17–30.

Gregoretti IV, Lee YM, Goodson HV. 2004. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol 338:17–31.

Huang C, Hales BF. 2002. Role of caspases in murine limb bud cell death induced by 4-hydroperoxycyclophosphamide, an activated analog of cyclophosphamide. Teratology 66:288–299.

Jansen MS, Nagel SC, Miranda PJ, Lobenhofer EK, Afshari CA, McDonnell DP. 2004. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proc Natl Acad Sci USA 101: 7199–7204.

Jin H, Liang L, Liu LF, Deng WP, Liu JW. 2013. HDAC inhibitor DWP0016 activates p53 transcription and acetylation to inhibit cell growth in U251 glioblastoma cells. J Cell Biochem 114:1498–1509.

Johanson G. 2000. Toxicity review of ethylene glycol monomethyl ether and its acetate ester. Crit Rev Toxicol 30:307–345.

Lagger G, O'Carrol D, Rembold M, Khier H, Tischler J, Weitzer G, Schuettengruber B, Hauser C, Brunmeir R, Jenuwein T, Seiser C. 2002. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 21:2672–2681.

Le Cam L, Linares LK, Paul C, Julien E, Lacroix M, Hatchi E, Sardet C. 2006. E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. Cell 127:775–788.

Lehrmann H, Pritchard LL, Harel-Bellan A. 2002. Histone acetylatransferase and deacetylases in control of cell proliferation and differentiation. Adv Cancer Res 86:41–65.

Luo J, Su F, Chen D, Shiloh A, Gu W. 2000. Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature 408:377–381.

Menegola E, Di Renzo F, Broccia ML, Prudenziati M, Minucci S, Massa V, Giavini E. 2005. Inhibition of histone deacetylase activity on specific embryonic tissues as a new mechanism for teratogenicity. Birth Defects Res B Dev Reprod Toxicol 74:392–398.

Miller RR, Hermann EA, Langvardt PW, McKenna MJ, Schwetz BA. 1983. Comparative metabolism and disposition of ethylene glycol monomethyl ether and propylene glycol monomethyl ether in male rats. Toxicol Appl Pharmacol 67:229–237.

Moallem SA, Hales BF. 1998. The role of p53 and cell death by apoptosis and necrosis in 4-hydroperoxycyclophosphamide-induced limb malformations. Development 125:3225–3234.

Neubert D, Barrach HJ. 1977. Techniques applicable to study morphogenetic differentiation of limb buds in organ culture. In: Neubert D, Merker H-J, Kwasigroch T, editors. Methods in pre-natal toxicology. Stuttgart, Germany: Georg Thieme Publishers. p. 241–251.

Oh ET, Park MT, Choi BH, Ro S, Choi EK, Jeong SY, Park HJ. 2012. Novel histone deacetylase inhibitor CG200745 induces clonogenic cell death by modulating acetylation of p53 in cancer cells. Invest New Drugs 30:435–442.

Paradis FH, Hales BF. 2012. Exposure to valproic acid inhibits chondrogenesis and osteogenesis in mid-organogenesis mouse limbs. Toxicol Sci 131:234–241.

Rao AV, Shaha C. 2002. N-acetylcysteine prevents MAA induced male germ cell apoptosis: role of glutathione and cytochrome C. FEBS Lett 527:133–137.

Roy S, Packman K, Jeffrey R, Tenniswood M. 2005. Histone deacetylase inhibitors differentially stabilize acetylated p53 and induce cell cycle arrest or apoptosis in prostate cancer cells. Cell Death Differ 12:482–491.

Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T. 1995. A subset of p53-deficient embryos exhibit exencephaly. Nat Genet 10:175–180.

Scofield EH, Henderson WM, Funk AB, Anderson GL, Smith MA. 2006. Diethylene glycol monomethyl ether, ethylene glycol monomethyl ether and the metabolite, 2-methoxyacetic acid affect in vitro chondrogenesis. Reprod Toxicol 22:718–724.

Scott WJ, Fradkin R, Wittfoht W, Nau H. 1989. Teratologic potential of 2-methoxyethanol and transplacental distribution of its metabolite, 2-methoxyacetic acid, in non-human primates. Teratology 39:363–373.

Singh BN, Zhang G, Hwa YL, Li J, Dowdy SC, Jiang S. 2010. Nonhistone protein acetylation as cancer therapy targets. Expert Rev Anticancer Ther 10:935–954.

Sleet RB, Greene JA, Welsch F. 1988. The relationship of embryotoxicity to disposition of 2-methoxyethanol in mice. Toxicol Appl Pharmacol 93:195–207.

Sykes SM, Mellert HS, Holbert MA, Li K, Marmorstein R, Lane WS, McMahon SB. 2006. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. Mol Cell 24:841–851.

Tang Y, Zhao W, Chen Y, Zhao Y, Gu W. 2008. Acetylation is indispensable for p53 activation. Cell 133:612–626.

Terry KK, Stedman DB, Bolon B, Welsch F. 1996. Effects of 2-methoxyethanol on mouse neurulation. Teratology 54:219–229.

Wade MG, Kawata A, Williams A, Yauk C. 2008. Methoxyacetic acid-induced spermatocyte death is associated with histone hyperacetylation in rats. Biol Reprod 78:822–831.

Welsch F. 1995. The mechanism of ethylene glycol ether reproductive and developmental toxicity and evidence for adverse effects in humans. Toxicol Lett 156:13–28.

Welsch F, Sleet RB, Greene JA. 1987. Attenuation of 2-methoxyethanol and methoxyacetic acid-induced digit malformations in mice by simple physiological compounds: implications for the role of further metabolism of methoxyacetic acid in developmental toxicity. J Biochem Toxicol 2:225–240.

Weng SP, Wu TC, Chen SU, Wu J, Lin CC, Yang YC, Chen PC. 2010. The impact of ethylene glycol monomethyl ether on ovarian function may extend to the next generation in female mice: a preliminary study. Reprod Toxicol 29:452–457.

Yan W, Samson M, Jégou B, Toppari J. 2000. Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Mol Endocrinol 14:682–699.

Yao T-P, Oh SP, Fuchs M, Zhou N-D, Ch'ng L-E, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R. 1998. Gene dosage–dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell 93:361–372.

Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 110:479–488.

Zhao Y, Lu S, Wu L, Chai G, Wang H, Chen Y, Sun J, Yu Y, Zhou W, Zheng Q, Wu M, Otterson GA, Zhu WG. 2006. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21 (Waf1/Cip1). Mol Cell Biol 26:2782–2790.