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Comprehensive Analysis of BIRC Family Gene Expression Changes by Mercury Compounds and Arsenic Exposure in Neuroblastoma, Kidney, and Hepatic Cells

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Several metal(loid) toxicants can trigger cytotoxicity by causing apoptosis. Our previous study demonstrated that cadmium induces apoptosis by suppressing expression of the apoptosis inhibitor, *BIRC3* [Baculoviral inhibition of apoptosis (IAP) protein repeat containing 3], in human proximal tubular cells (HK-2 cells). BIRC3 is a member of the BIRC family, which consists of eight family members in human, and seven in mouse. The observed suppression of *BIRC3* gene expression was mostly specific to cadmium and to HK-2 cells. In this study, we examined whether methylmercury, inorganic mercury, or arsenic may affect the gene expression of other BIRC family members in several different cultured cells. Methylmercury decreased the level of *BIRC2* mRNA and increased the level of *BIRC5* mRNA in human IMR-32 neuroblastoma cells. Methylmercury increased the mRNA levels of *BIRC2*, *BIRC5*, and *BIRC8* and decreased the mRNA levels of *BIRC6* in HK-2 cells. Inorganic mercury increased the mRNA levels of *BIRC2* and *BIRC5* in HK-2 cells. Finally, arsenic increased the levels of *Birc1* and *Birc7* and decreased the levels of *Birc2*, *Birc4*, *Birc5*, and *Birc6* mRNA in mouse normal hepatic AML-12 cells. Taken together, these results indicate that each metal(loid) toxicant may regulate the gene expression of BIRC family members in different manners. Therefore, each BIRC family member may play distinct roles when various tissues are exposed to toxic heavy metals or metalloid toxicants.

Key words BIRC family, gene expression, methylmercury, inorganic mercury, arsenic

Toxic heavy metals and metalloids such as cadmium, mercury, and arsenic can induce apoptotic cell death in their target tissues.[1-4] Our previous study demonstrated that cadmium, which causes chronic renal toxicity, induced apoptosis in human proximal tubular cells (HK-2 cells) and in the mouse kidney.[5,6] Strikingly, cadmium-induced apoptosis involves *BIRC3* gene suppression.[5] BIRC3 is a member of the BIRC family of apoptosis inhibitors, which consists of eight family members in human and seven family members in mouse.[7-9] In HK-2 cells, cadmium induces apoptosis by reducing *BIRC3* expression through transcription suppression.[5] The BIRC family includes BIRC1/NAIP, BIRC2/cIAP1, BIRC3/cIAP2, BIRC4/XIAP, BIRC5/Survivin, BIRC6/Apollon, BIRC7/ML-IAP, and BIRC8/ILP.[7-9] Interestingly, of the eight BIRC family members, cadmium only reduced *BIRC3* mRNA levels in HK-2 cells.[5] BIRC family members possess one or more baculovirus IAP repeat (BIR) domain that selectively inhibits the activity of caspase-9, -3, or -7.[7-9] The activation of caspase-3 is involved in cadmium-induced BIRC3-mediated apoptosis.[5] We examined the effect of mercury compounds and arsenic, which are known to induce apoptosis, on the gene expression of BIRC3 (Birc3) and caspase-3 activity in various cultured cells.[5] Those results showed that suppression of *BIRC3* gene expression was mostly specific to cadmium and to proximal tubular cells. In addition to BIRC3, other BIRC family members are involved in apoptosis inhibition. This suggests that although *BIRC3* may not be involved in apoptosis induced by mercury compounds and arsenic, different BIRC family members may be involved in regulating the apoptosis induced by these toxins. In this study, we examined weather methylmercury, inorganic mercury, and arsenic affect the gene expression levels of BIRC family members using HK-2, IMR-32 (human neuroblastoma), and AML-12 (mouse hepatic) cells.

MATERIALS AND METHODS

Cell Culture and Treatment HK-2 cells were purchased from ATCC (Manassas, MA, USA). HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 25 U/mL penicillin (DS Pharm, Osaka, Japan), 25 μg/mL streptomycin (DS Pharm), 1% Insulin-Transferrin-Selenium-X (Gibco), 10 ng/mL EGF (epidermal growth factor; Sigma-Aldrich), and 5 ng/mL hydrocortisone at 37°C in a humidified incubator containing 5% CO_2.

IMR-32 cells were kindly provided by Dr. Katsuhiko Muraki (Laboratory of Cellular Pharmacology, School of Pharmacy, Aichi Gakuin University, Japan). IMR-32 cells were cultured in DMEM supplemented with 10% FBS, 25 U/mL penicillin, 25 μg/mL streptomycin, and 1% MEM non-essential amino acid solution (Sigma-Aldrich) at 37°C in a humidified incuba-

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tor containing 5% CO_2.

AML-12 cells were purchased from the ATCC, and cultured in DMEM/F-12, supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco), 25 U/mL penicillin, 25 μg/mL streptomycin, 1% Insulin-Transferrin-Selenium-X, and 10 ng/mL EGF at 37°C in a humidified incubator containing 5% CO_2.

HK-2 and AML-12 cells were grown in plates at a density of 250 cells/mm^2 and cultured for 48 h. IMR-32 cells were grown in plates at a density of 500 cells/mm^2 and cultured for 48 h. The culture medium was discarded, and the cells were treated with methylmercury (CH_3HgCl; GL Sciences Inc., Tokyo, Japan), inorganic mercury ($HgCl_2$; Wako Pure Chemical Industries, Osaka, Japan), or arsenic ($NaAsO_2$; Wako Pure Chemical Industries) in serum-free culture medium.

RNA Extraction Cells were washed twice with ice-cold PBS (-) and total RNA was extracted with the PureLink™ RNA Mini Kit (Ambion, Grand Island, NY, USA) or QuickGene-810 (Fujifilm; Wako Pure Chemical Industries) following the manufacturer's instructions. RNA was quantified and purity was determined using BioSpec-nano (Shimadzu, Kyoto, Japan).

Real Time RT-PCR Total RNA was incubated with the PrimeScript reverse transcription (RT) Reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan) to generate cDNA. Real-time PCR was performed with SYBR Premix Ex TaqII (Perfect Real Time) (Takara Bio) and the Thermal Cycler Dice Real Time System (Takara Bio). PCR conditions were as follows: 10 s hot-start at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Gene expression was normalized to *GAPDH* or *β-actin* mRNA levels. The oligonucleotide sequences of the primers were as follows: sense, 5′-CATGTGTGTGGAGGGTGAAG-3′, and antisense, 5′-TTTAACAGGGGACAGCATCC-3′, for the human *BIRC1* gene; sense, 5′-GCATTTTCCCAACTGTCCAT-3′, and antisense, 5′-ATTCGAGCTGCATGTGTCTG-3′, for the human *BIRC2* gene; sense, 5′-TGGGGTTCAGTTTCAAGGAC-3′, and antisense, 5′-TGCAACCAGAACCTCAAGTG-3′, for the human *BIRC4* gene; sense, 5′-GTTGCGCTTTCCTTTCTGTC-3′, and antisense, 5′-TCTCCGCAGTTTCCTCAAAT-3′, for the human *BIRC5* gene; sense, 5′-TGACGCTTTCAACCTCACTG-3′, and antisense, 5′-GTGTCCGCTGGACCAGTTAT-3′, for the human *BIRC6* gene; sense, 5′-TGGCCTCCTTCTATGACTGG-3′, and antisense, 5′-ACCTCACCTTGTCCTGATGG-3′, for the human *BIRC7* gene; sense, 5′-AAGCCCGGCTCATTACTTTT-3′, and antisense, 5′-ATCTTCCTTGGGCTTCCAGT-3′, for the human *BIRC8* gene; sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for the human *GAPDH* gene; sense, 5′-CATGTGTGTGGAGGGTGAAG-3′ and antisense, 5′-TTTAACAGGGGACAGCATCC-3′ for the mouse *Birc1* gene; sense, 5′-GCATTTTCCCAACTGTCCAT-3′ and antisense, 5′-ATTCGAGCTGCATGTGTCTG-3′ for the mouse *Birc2* gene; sense, 5′-TGGGGTTCAGTTTCAAGGAC-3′ and antisense, 5′-TGCAACCAGAACCTCAAGTG-3′ for the mouse *Birc4* gene; sense, 5′-GTTGCGCTTTCCTTTCTGTC-3′ and antisense, 5′-TCTCCGCAGTTTCCTCAAAT-3′ for the mouse *Birc5* gene; sense, 5′-TGACGCTTTCAACCTCACTG-3′ and antisense, 5′-GTGTCCGCTGGACCAGTTAT-3′ for the mouse *Birc6* gene; sense, 5′-TGGCCTCCTTCTATGACTGG-3′ and antisense, 5′-ACCTCACCTTGTCCTGATGG-3′ for the mouse *Birc7* gene; sense, 5′-CCTAAGGCCAACCGTGAAAA-3′, and antisense, 5′-AGCCATACAGGGACAGCACA-3′, for the mouse *β-actin* gene.

Statistical Analysis Statistical analyses were performed using one-way ANOVA. When the F value was significant ($P < 0.05$), Bonferroni's multiple *t*-test was performed for post-hoc comparison ($P < 0.05$).

RESULTS

Effect of Methylmercury on the Expression of BIRC Family Genes in IMR-32 Cells Our previous report demonstrated that treatment with 6 μM methylmercury for 3 h and treatment with 4 μM methylmercury for 6 h slightly, but significantly, decreased the viability of IMR-32 cells.[5] Moreover, 6 h methylmercury treatment increased activated caspase-3 levels. However, we also showed that *BIRC3* mRNA levels were not altered following methylmercury treatment.[5] In this study, we examined the mRNA levels of seven BIRC family genes in IMR-32 cells treated with various concentrations of methylmercury for 6 h. The mRNA levels of *BIRC1*, *BIRC4*, *BIRC6*, *BIRC7*, and *BIRC8* did not change following methylmercury treatment (Figs. 1A, C, E, F, G). However, the mRNA levels of *BIRC5* and *BIRC2* were significantly increased and decreased, respectively, by methylmercury (Figs. 1D, B).

Effect of Methylmercury on the Expression of BIRC Family Genes in HK-2 Cells Methylmercury treatment for 3 h significantly decreased the viability of HK-2 cells, increased activated caspase-3, but had no effect on *BIRC3* mRNA levels.[5] Here, we examined the effect of methylmercury on the mRNA levels of seven BIRC family genes in HK-2 cells. In HK-2 cells, the mRNA levels of *BIRC1*, *BIRC4*, and *BIRC7* showed no change following methylmercury treatment (Figs. 2A, C, F). However, methylmercury significantly increased the mRNA levels of *BIRC2*, *BIRC5*, and *BIRC8* (Figs. 2B, D, G) and significantly decreased the mRNA levels of *BIRC6* mRNA (Fig. 2E) in HK-2 cells.

Effect of Inorganic Mercury on the Expression of BIRC Family Genes in HK-2 Cells We previously demonstrated that although the viability was almost the same as in the control group, inorganic mercury decreased *BIRC3* mRNA level in HK-2 cells. When inorganic mercury treatment reduced the viability of HK-2 cells, cleaved caspase-3 level was unchanged.[5] In this study, the effects of inorganic mercury on the expression levels of the remaining seven BIRC family members were examined in HK-2 cells. Inorganic mercury did not affect the mRNA levels of *BIRC1*, *BIRC4*, *BIRC6*, *BIRC7*, and *BIRC8* in HK-2 cells (Figs. 3A, C, E, F, G). However, inorganic mercury treatment resulted in a significant increase in *BIRC2* and *BIRC5* mRNA levels HK-2 cells (Figs. 3B, D).

Effect of Arsenic on the Expression of Birc Family Genes in AML-12 Cells In AML-12 cells, arsenic decreased cell viability, and significantly increased the mRNA level of *Birc3*.[5] Both caspase-3 and activated caspase-3 levels were slightly increased by arsenic treatment.[5] Here, we examined the effect of arsenic treatment on the mRNA levels of six Birc family genes in AML-12 cells. The mRNA levels of *Birc1* and *Birc7* were significantly elevated by arsenic treatment (Figs. 4A, F). However, the mRNA levels of *Birc2*, *Birc4*, *Birc5*, and *Birc6* were significantly reduced by arsenic (Figs. 4B, C, D, E).

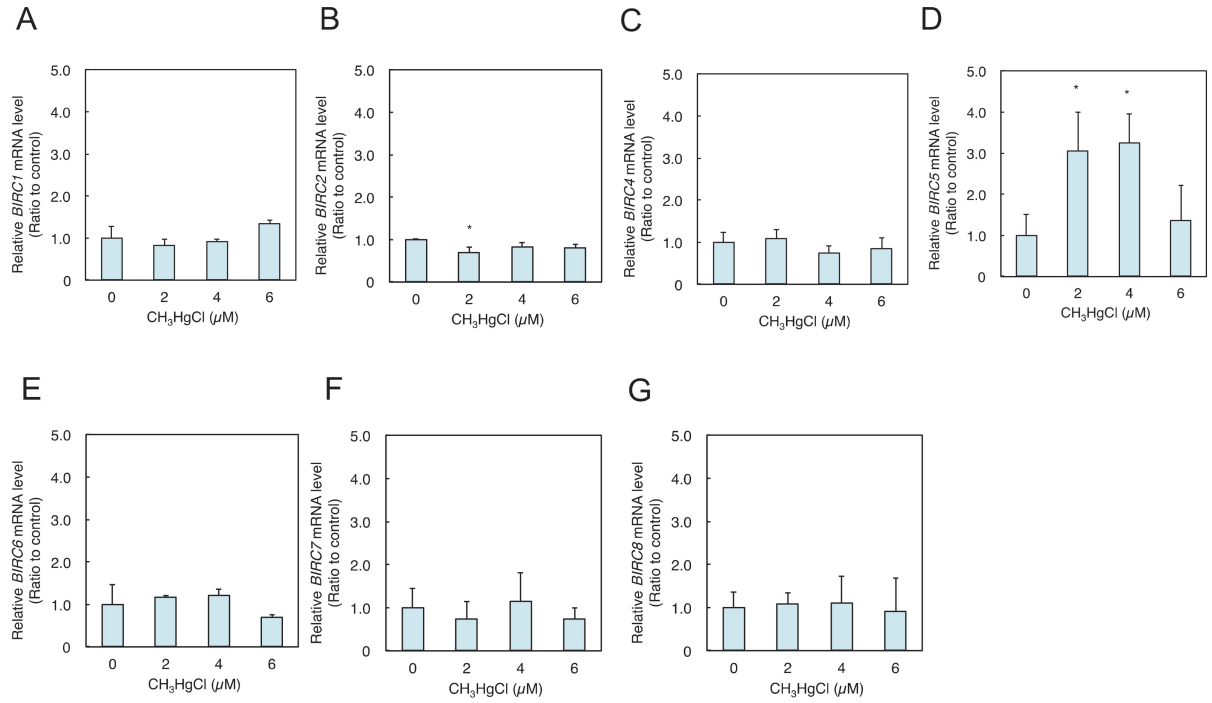

Fig. 1. mRNA levels of BIRC family genes in IMR-32 cells treated with methylmercury for 6 h

IMR-32 cells were seeded onto 6-well plates at a density of 500 cells/mm^2 and cultured for 48 h. The culture medium was discarded, and the cells were treated with methylmercury (CH_3HgCl) in serum-free medium for 6 h. BIRC family gene mRNA levels were examined using real-time RT-PCR. (A) *BIRC1*. (B) *BIRC2*. (C) *BIRC4*. (D) *BIRC5*. (E) *BIRC6*. (F) *BIRC7*. (G) *BIRC8*. Expression levels were normalized to that of *GAPDH*. Values are means ± S.D. (n = 3). *Significantly different from the control group, $P < 0.05$.

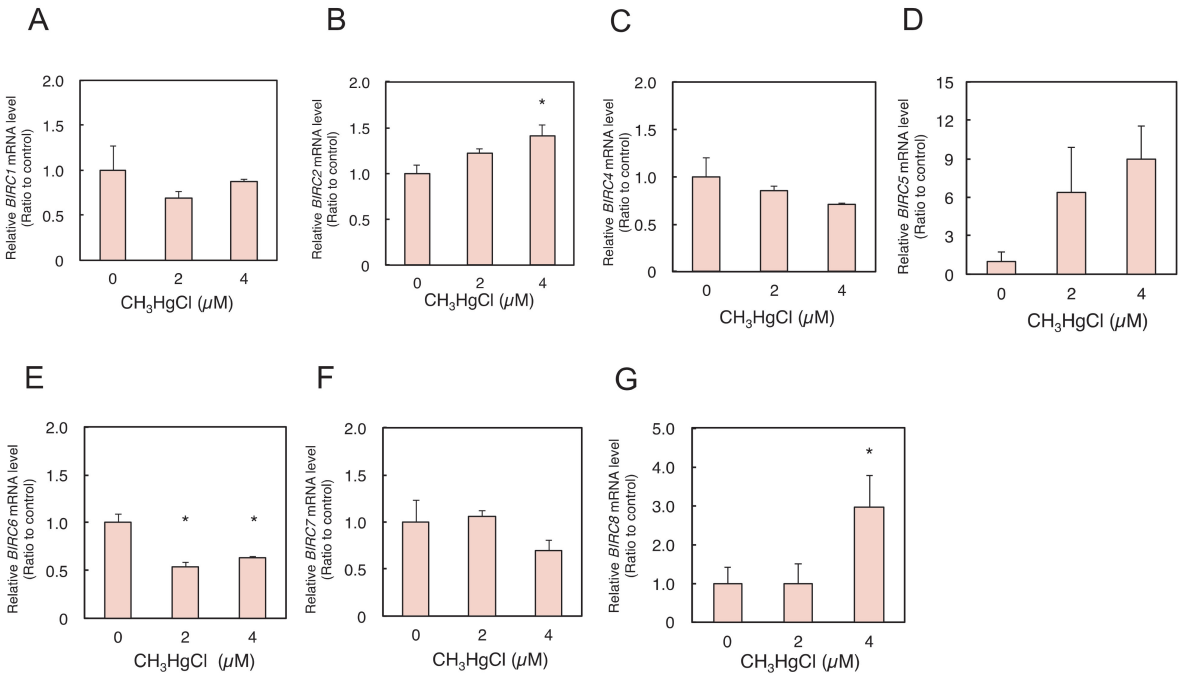

Fig. 2. mRNA levels of BIRC family genes in HK-2 cells treated with methylmercury for 3 h

HK-2 cells were seeded onto 6-well plates at a density of 250 cells/mm^2 and cultured for 48 h. The culture medium was discarded, and the cells were treated with methylmercury (CH_3HgCl) in serum-free medium for 3 h. BIRC family gene mRNA levels were examined using real-time RT-PCR. (A) *BIRC1*. (B) *BIRC2*. (C) *BIRC4*. (D) *BIRC5*. (E) *BIRC6*. (F) *BIRC7*. (G) *BIRC8*. Expression levels were normalized to that of *GAPDH*. Values are means ± S.D. (n = 3). *Significantly different from the control group, $P < 0.05$.

DISCUSSION

Our results indicate that methylmercury, inorganic mercury, and arsenic change the expression of BIRC family genes in various patterns in cultured cells. In HK-2 cells, cadmium markedly decreased *BIRC3* gene expression levels and significantly increased the levels of activated caspase-3.[5] However, cadmium did not induce significant changes in the expression of other BIRC family members in HK-2 cells.[5] Although methylmercury activated caspase-3 in IMR-32 and HK-2 cells,[5] methylmercury increased and decreased mRNA levels of BIRC family member genes in IMR-32 and HK-2 cells

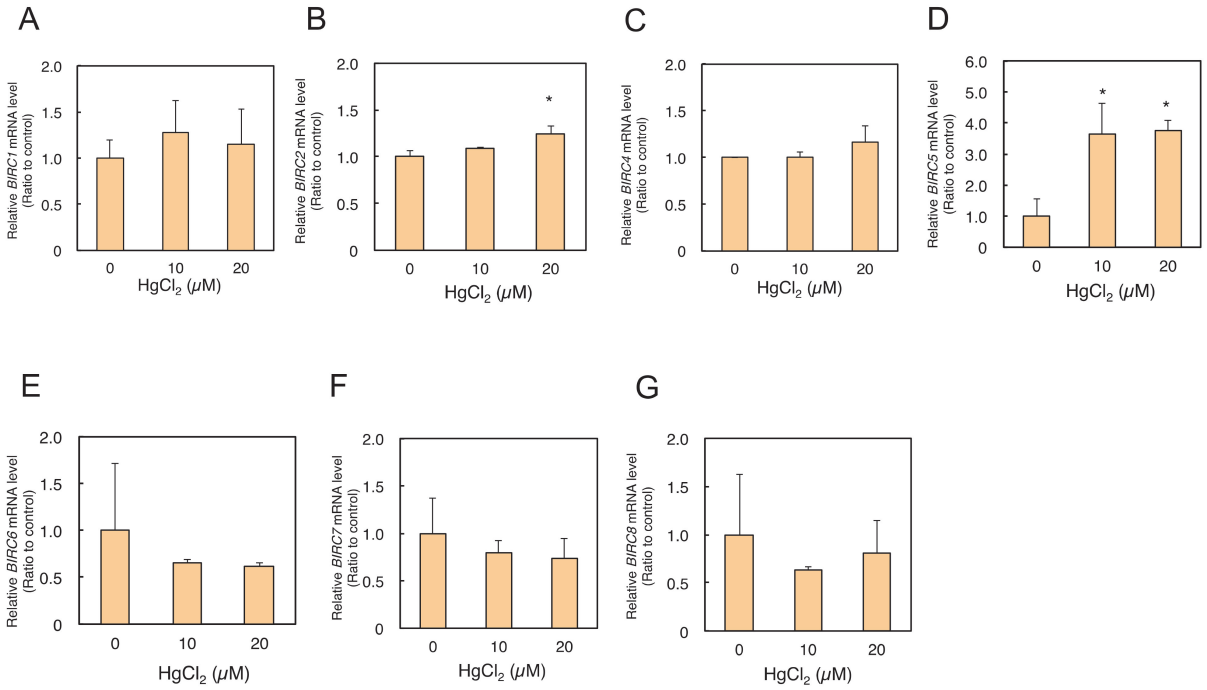

Fig. 3. mRNA levels of BIRC family genes in HK-2 cells treated with inorganic mercury for 3 h

HK-2 cells were seeded onto 6-well plates at a density of 250 cells/mm² and cultured for 48 h. The culture medium was discarded, and the cells were treated with inorganic mercury ($HgCl_2$) in serum-free medium for 3 h. BIRC family gene mRNA levels were examined using real-time RT-PCR. (A) *BIRC1*. (B) *BIRC2*. (C) *BIRC4*. (D) *BIRC5*. (E) *BIRC6*. (F) *BIRC7*. (G) *BIRC8*. Expression levels were normalized to that of *GAPDH*. Values are means ± S.D. (n = 3). *Significantly different from the control group, $P < 0.05$.

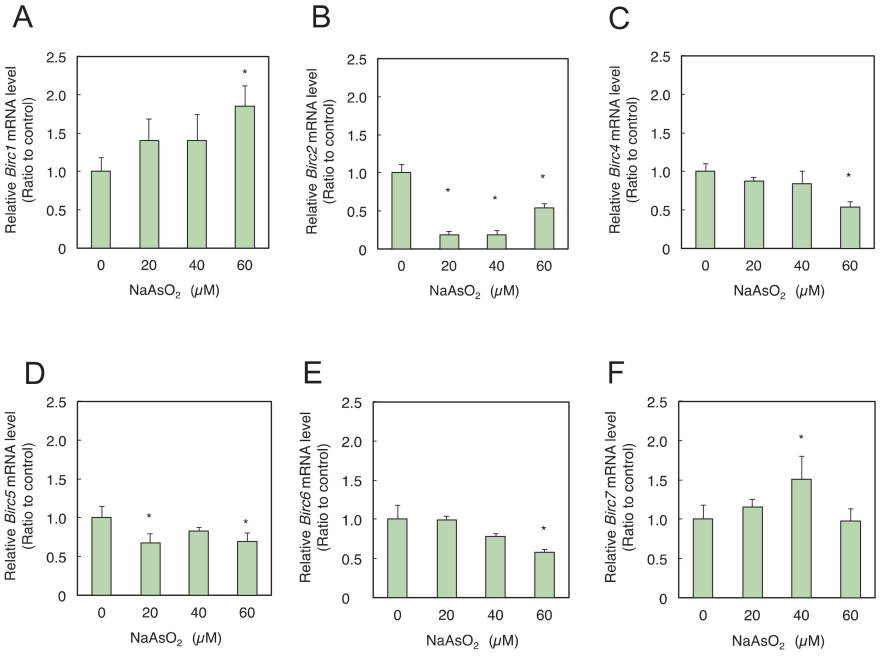

Fig. 4. mRNA levels of Birc family genes in AML-12 cells treated with arsenic for 6 h

AML-12 cells were seeded onto 6-well plates at a density of 250 cells/mm² and cultured for 48 h. The culture medium was discarded, and the cells were treated with arsenic ($NaAsO_2$) in serum-free medium for 6 h. Birc family gene mRNA levels were examined using real-time RT-PCR. (A) *Birc1*. (B) *Birc2*. (C) *Birc4*. (D) *Birc5*. (E) *Birc6*. (F) *Birc7*. Expression levels were normalized to that of *ß-actin*. Values are means ± S.D. (n = 3). *Significantly different from the control group, $P < 0.05$.

(Figs. 1, 2). Moreover, although arsenic treatment resulted in slightly increased activated caspase-3 levels,[5] it altered the expression of all Birc family member genes in AML-12 cells (Fig. 4). Taken together, each metal(loid) toxicant may regulate the expression of BIRC family member genes in different manners. Therefore, each BIRC family member gene may play a distinct role following the exposure of various tissues to toxic heavy metal or metalloid toxicants.

Methylmercury causes central nervous system disorders *via* apoptosis.[2] In this study, methylmercury markedly increased *BIRC5* mRNA levels in IMR-32 cells, even though caspase-3 was profoundly activated by methylmercury. Inorganic mer-

cury, in addition to cadmium, is also known to cause kidney cell damage.[3] Our previous study demonstrated that inorganic mercury induced apoptosis signals, such as DNA fragmentation, in rat proximal tubular NRK-52E cells.[10] In HK-2 cells, inorganic mercury did not activate caspase-3,[5] but significantly increased *BIRC5* mRNA levels (Fig. 3). BIRC5 is an anti-apoptotic factor also known as Survivin, and has been reported to suppress apoptosis in nerve cells.[11] Therefore, BIRC5 may act as a protective factor against inorganic mercury-induced human kidney damage and may perform a different role in the case of methylmercury toxicity. Arsenic exposure can cause liver and skin disorders, Bowen's disease, skin cancer, and apoptosis.[9,12,13] Previously, arsenic markedly increased *Birc3* expression in AML-12 cells.[5] However, we showed here that *Birc2* expression was significantly suppressed by arsenic in AML-12. Furthermore, slight activation of caspase-3 was observed in AML-12 cells treated with arsenic.[5] Therefore, changes in Birc family gene expression following arsenic treatment may be associated with apoptosis in mouse hepatic cells.

BIRC4, known as XIAP (X-linked IAP), is the most studied member of the BIRC family.[14] The BIR2 and BIR3 regions of BIRC4 interact with the active-site pocket of caspases to suppress apoptosis.[15] Therefore, it was proposed that most BIRC family members neutralize caspase activities in the same manner. However, recent reports indicate that BIRC family members have non-equivalent functions and regulate the activities of caspases through distinct mechanisms.[16-18] BIRC2, BIRC3, and BIRC4 contain a C-terminal RING zinc finger domain with E3 ubiquitin ligase activity, which mediates proteasomal degradation of target proteins as well as themselves.[19] BIRC2 independently interacts with caspase-7 at the active-site pocket.[16] Moreover, BIRC2 suppresses apoptosis by ubiquitinating activated caspase-3 and -7 to mediate their proteasomal degradation.[17]

Our previous study demonstrated that cadmium suppresses *BIRC3* gene expression by inhibiting the activity of the ARNT (aryl hydrocarbon receptor nuclear translocator) transcription factor.[5] We used DNA microarrays to screen ARNT knockdown cells for the down-stream targets of ARNT, and identified *BIRC3* among these targets. It has been suggested that the PI3K/Akt signaling pathway regulates BIRC2 expression.[20] Therefore, methylmercury, inorganic mercury, or arsenic may affect other transcription factors in addition to ARNT.

Future detailed studies investigating the involvement of the BIRC family in the toxicity of various harmful heavy metals will clarify the mechanisms of toxicity and/or defense against toxicity.

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Conflict of Interest The authors declare no conflict of interest.

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