Purified plasmid DNA concentrations were assessed by spectrophotometry at 260 nm and then serially diluted.

2.4.1. Analysis with cultured VSMCs

2.4.1.1. Cell culture. Two types of human vascular smooth muscle cells (CRL-2481 derived from human umbilical vein, and CRL-1999 derived from human aorta) were commercially obtained from American Type Culture Collection (ATCC) (Manassas, VA). They were cultured to sub-confluence (24,000 cells/cm^2) in 75 cm^2 flask in ATCC 30-2004 medium, respectively, at 37 °C in a 5% CO_2 atmosphere. We examined whether these cell lines expressed both subtypes of ERs using RT-PCR analysis. In addition, we further examined other characterization of these cell lines utilizing other methods, such as morphology and immunostaining.

2.4.1.2. Cell proliferation assay. Cultured cells were seeded in 25 cm^2 flask at an initial concentration of 100,000 cells/flask containing 5% FBS per well and cultured until sub-confluency. The medium was then replaced with phenol red-free medium (Modified Eagle's Medium, Sigma, St. Louis, USA) to slow the growth of the cells. After 24 h, the medium was replaced again with phenol red-free medium containing 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) with E_2 (100 pM, 10 nM), E_2 (100 pM, 10 nM) with ICI 182780 (Wako, Tokyo, Japan) (1 μM), or vehicle (0.1% ethanol). After incubation for 48 h, the cells were trypsinized and suspended. We then employed a Cell Counting Kit-8 system (Wako) to measure the number of cells in each sample (Ishiyama et al., 1997). A 100 μl aliquot of each sample was incubated onto a 96-well microtiter plate, and the plate pre-incubated for 24 h in a CO_2 incubator at 37 °C. Ten microliter of a working solution containing WST-8 was added to each well and incubated for an additional 2 h. The absorbance of each well was measured at 450 nm with a reference wavelength of 650 nm with an M-Uvmax microscope reader (Molecular Devices Corp., Menlo Park, USA). An aliquot was taken from the medium to count the number of the cells with a Burker-Turk counter (Nitirin, Tokyo, Japan).

2.5. Real-time PCR for proliferating cell nuclear antigen (PCNA) mRNA

In order to further objectively examine the effects of estrogens on cell proliferation of VSMCs, we examined the levels of proliferating cell nuclear antigen (PCNA) mRNA (Dzau et al., 2002). PCNA primers employed in this study are summarized in Table 2. These cells were seeded in a 25 cm^2 flask at an initial concentration of 100,000 cells/flask containing 5% FBS per well and cultured until sub-confluency. The medium was then replaced with phenol red-free medium to arrest the growth of the cells. After 24 h, the medium was replaced again with phenol red-free medium containing 5% DCC–FBS with E_2 (100 pM, 10 nM), E_2 (100 pM, 10 nM) with ICI 182780 (1 μM), or vehicle (0.1% ethanol). Following incubation for 48 h, the cells were subsequently subjected to total RNA extraction for RT/real-time PCR analysis for PCNA mRNA expression.

2.6. Statistical analysis

Values for the results are presented as mean ± standard error of means (S.E.M.). For comparisons between the two groups, we used a one-way analysis of variance followed by Tukey–Kramer multiple comparisons post-test for immunohistochemistry and quantitative RT-PCR data, or Dunnett multiple comparisons post-test for cell study data. *P*-values <0.05 were considered significant in this study.

3. Results

3.1. Immunohistochemistry

Results of immunohistochemistry are summarized in Figs. 1 and 2.

Both ERα and ERβ immunoreactivity were detected in the nuclei of VSMCs in both the neointima and media in all specimens examined. In addition, a low level of expression of both ERs was detected sporadically in endothelial cells, but cells positive for both ERs were not detected in macrophages (data not shown). Fig. 1 shows representative illustrations of an abdominal aorta specimen obtained from a 45-year-old woman with a mild degree of atherosclerosis (Group A_3), and that of a 76-year-old woman with a severe degree of atherosclerosis (Group A_4). Double immunostaining for ERs and α-SMA demonstrated the presence of immunopositive ERs in VSMCs of both the neointima and media. In addition, the relative abundance of ERα and β immunoreactivity was more marked in VSMCs in the neointima of Group A_3 than that in Group A_4. However, the status of ERβ immunoreactivity in the media of VSMCs in Group A_4 was not different from that of Group A_3.

Fig. 2 shows the relative immunoreactivity of ERs in VSMCs among the five groups. The number of ERα positive cells in the neointima was significantly smaller in the post-menopausal female aorta with a severe degree of atherosclerotic change (Group A_4) (24.3 ± 4.1 H-score) than in the pre-menopausal aorta with a mild degree of atherosclerosis (Group A_3) (89.1 ± 19.1 H-score) and the post-menopausal aorta with a mild degree of atherosclerosis (Group A_5) (79.7 ± 6.8 H-score) ($P < 0.05$). The abundance of ERα positive cells in the neointima was significantly higher in the pre-menopausal female aorta with a mild degree of atherosclerotic change (Group A_3) than in the male aorta with a mild degree of atherosclerosis (Group A_1) (42.7 ± 12.3 H-score) and the male aorta with a severe degree of atherosclerosis (Group A_2) (43.4 ± 8.7

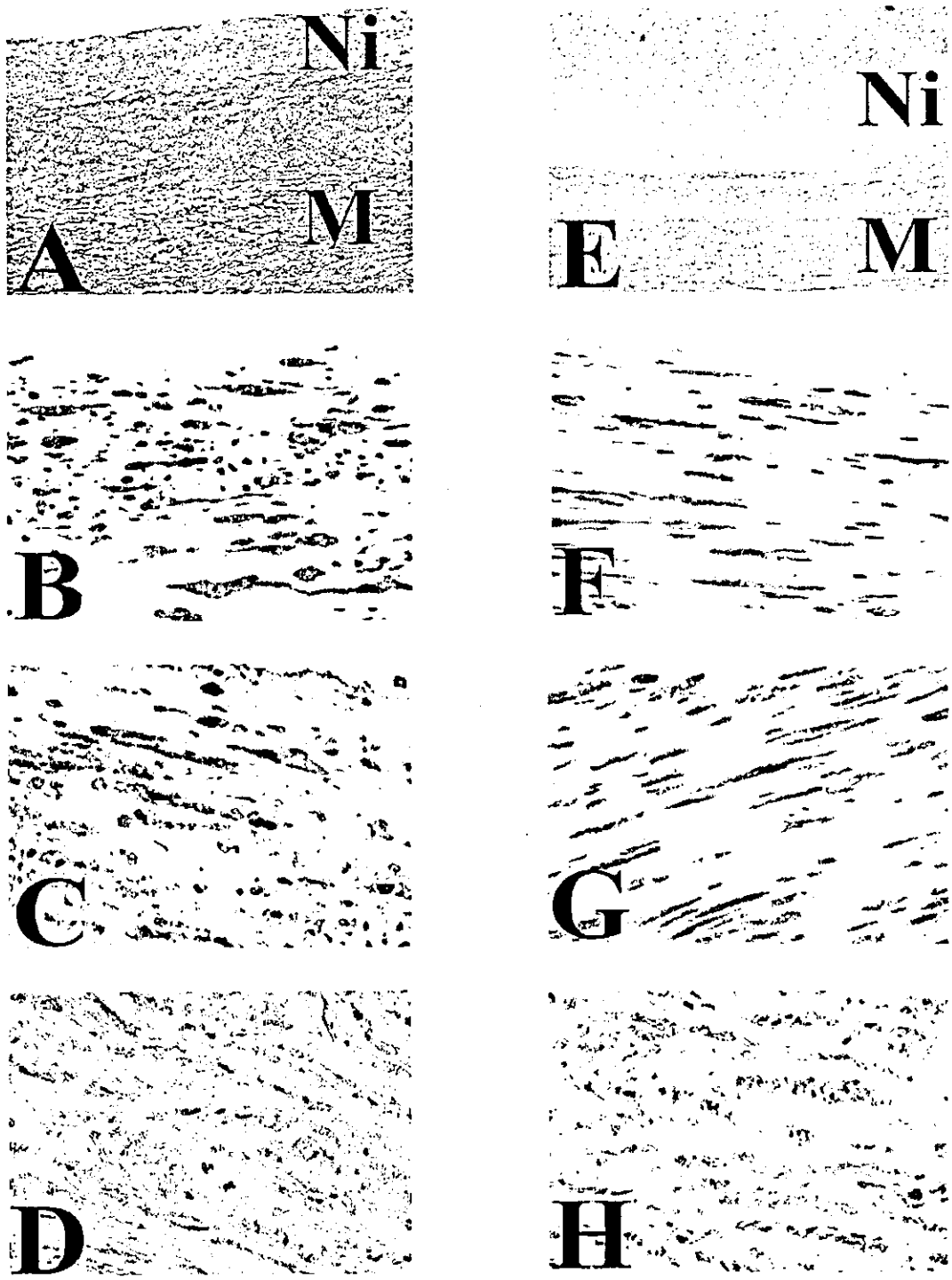

Fig. 1. Modified Masson Goldner's stains (A) and double immunohistochemical staining illustrations for α-SMA and ERα in the neointima (B), ERβ in the neointima (C), and in the media (D), of an abdominal aorta specimen obtained from a 45-year-old pre-menopausal woman with a mild degree of atherosclerosis (Group A_3). In addition, Modified Masson Goldner's stains (E), and double immunohistochemical staining illustrations for α-SMA and ERα in the neointima (F), ERβ in the neointima (G), and in the media (H) of an abdominal aorta specimen obtained from a 76-year-old woman with a severe degree of atherosclerosis (Group A_4). Immunopositive cells for ERs appear brown as a result of DAB colorimetric reaction. Immuno-positive cells for α-SMA appear blue as a result of Vector-blue colorimetric reaction. Double immunopositive cells are confirmed, respectively. Original magnification, 100× for modified Masson Goldner's stains, and 400× for double immunohistochemical staining, respectively (Ni: neointima; M: media).

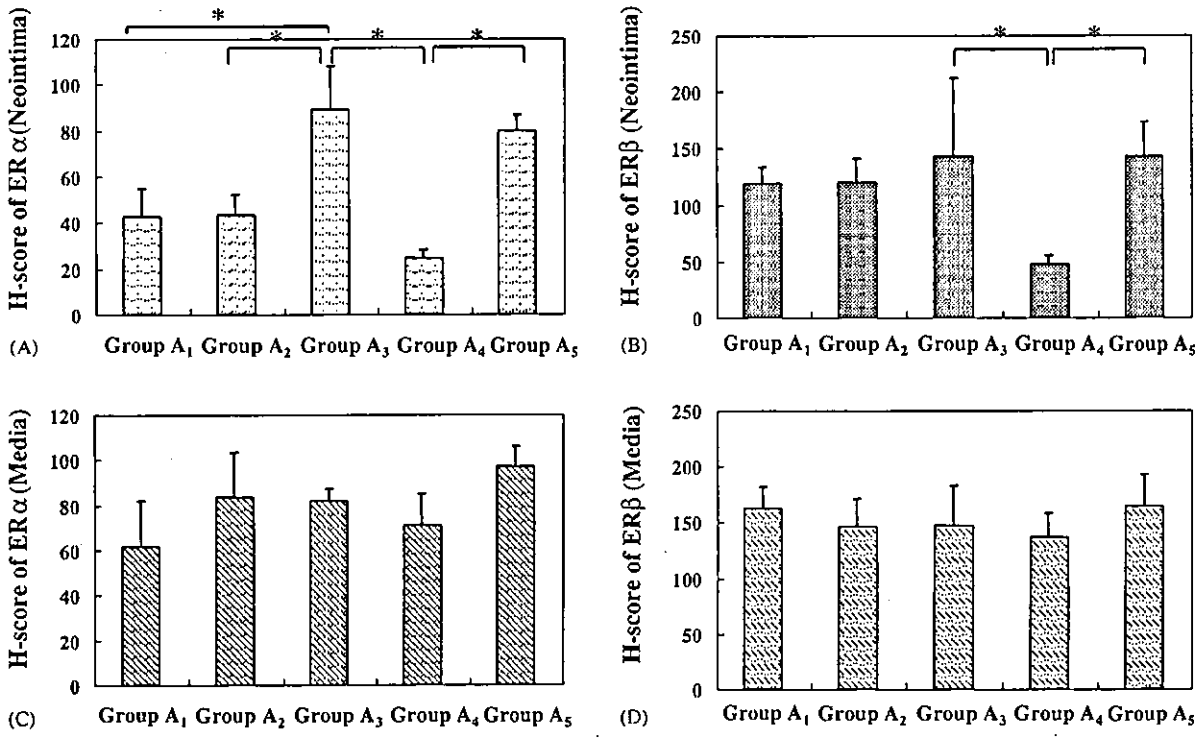

Fig. 2. The results for H-score analysis of ERα (A), ERβ (B) in the neointima, and ERα (C), and ERβ (D) in the media among five groups (Group A_1, A_2, A_3, A_4, and A_5) (*$P < 0.05$).

H-score) ($P < 0.05$). In addition, the number of ERβ positive cells in the neointima was significantly smaller in the post-menopausal female aorta with a severe degree of atherosclerotic change (Group A_4) (47.2 ± 8.2 H-score) than in the pre-menopausal aorta with a mild degree of atherosclerosis (Group A_3) (142.5 ± 39.7 H-score) and the post-menopausal aorta with a mild degree of atherosclerosis (Group A_5) (143.1 ± 29.8 H-score) ($P < 0.05$). In VSMCs of the female neointima with a severe degree of atherosclerosis, the number of ER positive cells was markedly low. However, H-scores for ERs in the male neointima and/or male and female media were not significantly correlated with sex or the degrees of atherosclerotic change. In addition, the relative immunoreactivity of both ER in the neointima and media were not correlated with ages (data not shown).

3.2. Real-time PCR for ER mRNA

Results are summarized in Fig. 3.

Both ERα and ERβ mRNAs were detected in all the specimens examined. The relative abundance of ERα mRNA determined by real-time PCR analysis was significantly lower in the post-menopausal female aorta with a severe degree of atherosclerotic change (Group A_4) (1.3 ± 0.3%) than in the pre-menopausal female aorta with a mild degree of atherosclerosis (Group A_3) (10.4 ± 3.1%) and in the post-menopausal female aorta with a mild degree of atherosclerosis (Group A_5) (8.9 ± 1.4%) ($P < 0.05$). The relative abundance of ERα mRNA was significantly higher in the pre-menopausal female aorta with a mild degree of atherosclerotic change (Group A_3) than in the male aorta with a mild degree of atherosclerosis (Group A_1) (2.6 ± 0.5%) and in the male aorta with a severe degree of atherosclerosis (Group A_2) (3.0 ± 0.7%) ($P < 0.05$). In addition, the level of ERβ mRNA was significantly lower in the post-menopausal female aorta with a severe degree of atherosclerotic change (Group A_4) (1.3 ± 0.2%) than in the pre-menopausal female aorta with a mild degree of atherosclerosis (Group A_3) (4.7 ± 0.2%) and in the post-menopausal aorta with a mild degree of atherosclerosis (Group A_5) (7.2 ± 1.7%) ($P < 0.05$). The relative abundance of both ER mRNAs in the male aorta determined by real-time PCR analysis was not correlated with the degree of atherosclerosis. In addition, the expression levels of both ER mRNAs were not correlated with ages (data not shown).

3.2.1. Cell study

3.2.1.1. Characterization of cell lines. Using RT-PCR analysis, CRL-2481 cells were found to be positive only for ERα, whereas CRL-1999 cells were found to be positive only for ERβ (Fig. 4). Expression level of ERα mRNA in CRL-2481 cells was 0.21 ± 0.03% (adjusted by GAPDH mRNA level) in ERα, and that of ERβ mRNA in CRL-1999 cells was 0.14 ± 0.01% (adjusted by GAPDH mRNA level)

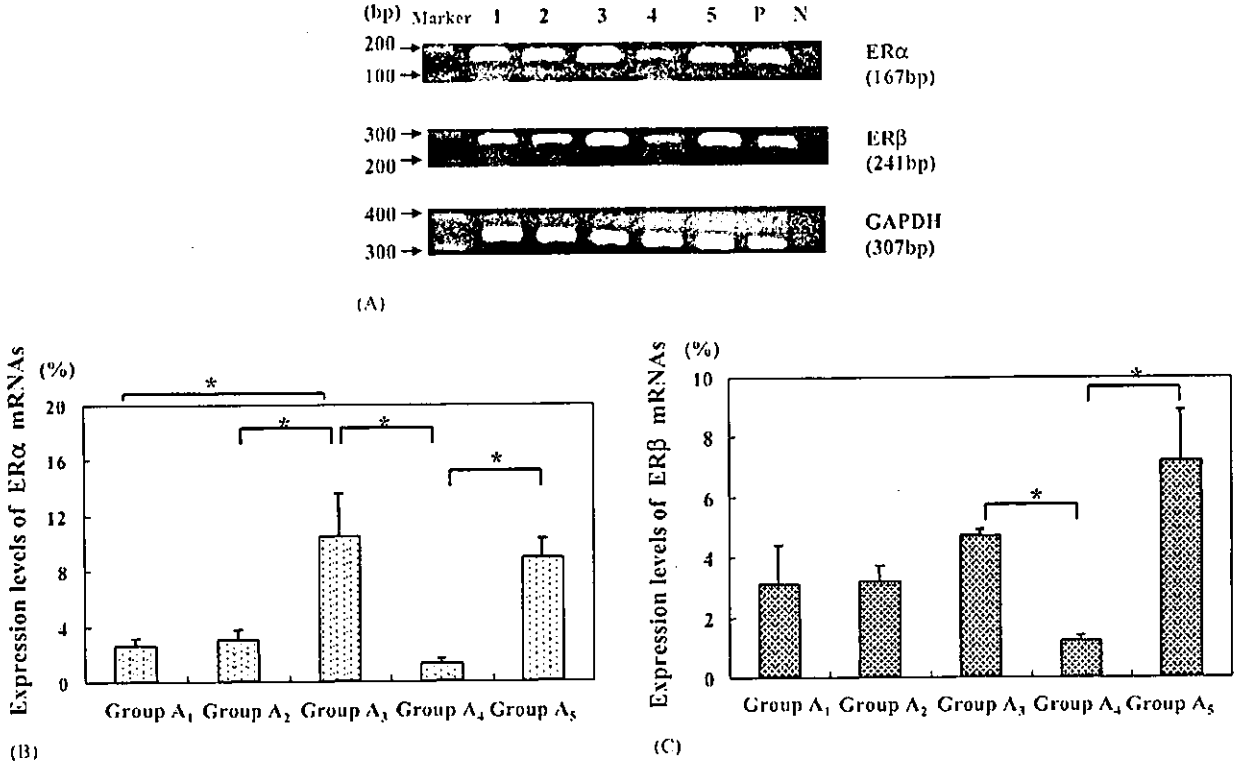

Fig. 3. (A) Results of RT/real-time PCR analysis for both ERs in human aorta. Bands for PCR products were detected as specific single bands (167bp for ERα, 241 bp for ERβ, and 307 bp for GAPDH). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these RT/real-time PCR gene products are shown. 1, an aorta of a 32-year-old man with mild atherosclerotic change (Group A_1); 2, A, an aorta of a 65-year-old man with severe atherosclerotic change (Group A_2); 3, an aorta of a 38-year-old pre-menopausal woman with mild atherosclerotic change (Group A_3); 4, an aorta of a 76-year-old post-menopausal woman with severe atherosclerotic change (Group A_4); 5, an aorta of a 71-year-old post-menopausal woman with mild atherosclerotic change (Group A_5); P, positive controls (T-47D cell lines); N, negative controls (no cDNA substrates). (B-C) The results for mRNA expression levels for ERα (B), and ERβ (C) among five groups (Group A_1, A_2, A_3, A_4, and A_5) ($^*P < 0.05$).

in ERβ, respectively, (data not shown). In addition, we confirmed that although these cells were positive for α-SMA immunoreactivity, both were generally regarded as similar types of undifferentiated VSMCs in view of morphology and specific markers such as caldesmon and α-tropomyosin (Kashiwada et al., 1997). Furthermore, their growth rates were not significantly different when they were exposed by 5% DCC-FBS (data not shown).

3.3. Cell proliferation assay

Results are summarized in Figs. 5 and 6.

E_2 significantly inhibited cell proliferation of ERα positive VSMCs compared to controls (71.2 ± 5.1% by E_2 100 pM, and 66.4 ± 5.9% by E_2 10 nM, respectively) ($P < 0.05$). E_2 together with ICI 182780, a specific ER inhibitor, did not suppress the cell proliferation of VSMCs. On the

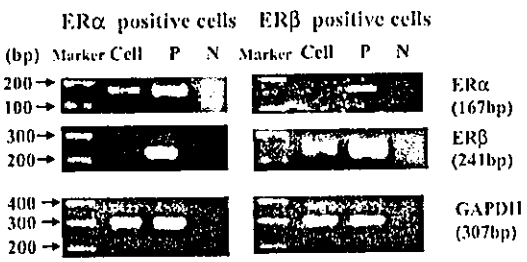

Fig. 4. Results of RT/real-time PCR analysis for ERα and ERβ in human VSMC cell lines. These results demonstrate that CRL-2481 cells are positive only for ERα, whereas CRL-1999 cells are positive only for ERβ (cell, vascular smooth muscle cells; P, positive controls; N, negative controls).

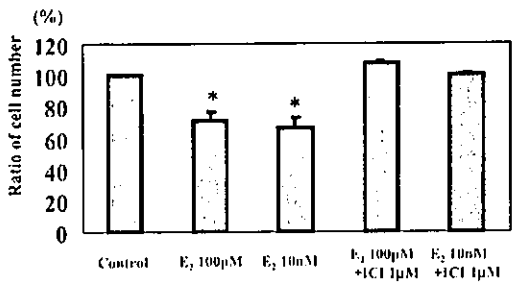

Fig. 5. The relative levels of cell numbers in ERα positive cells among cells treated with vehicle (control), or estrogen (E_2) alone (100 pM, 10 nM), or E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Significantly decreased compared to control ($^*P < 0.05$).

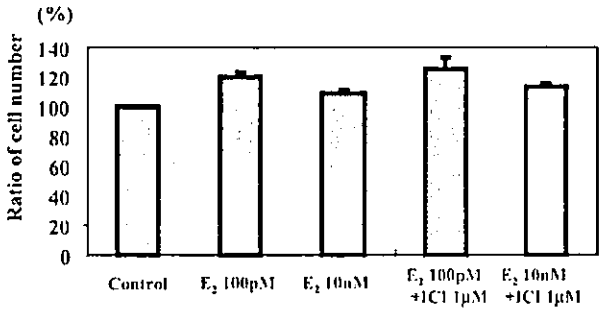

Fig. 6. The relative levels of cell numbers in ERβ positive cells among cells treated with vehicle (control), or estrogen (E_2) alone (100 pM, 10 nM), or E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Significantly decreased compared to control (*$P < 0.05$).

other hand, E_2 did not significantly inhibit the proliferation of ERβ positive VSMCs compared to controls.

3.4. Relative expression levels of PCNA mRNA

Results are summarized in Figs. 7 and 8.

E_2 significantly suppressed PCNA mRNA levels of ERα positive VSMCs compared to controls (41.1 ± 5.3%, by E_2 10 nM) ($P < 0.05$). E_2 together with ICI 182780 did not suppress PCNA mRNAs. On the other hand, E_2 did not significantly inhibit PCNA mRNA levels of ERβ positive VSMCs compared to controls.

4. Discussion

This is the first study to demonstrate the relative abundance of ERα and ERβ expression in VSMCs among different subjects and different degrees of atherosclerosis. The relative abundance of both ER subtypes in the neointima of the female aorta decreased in number and intensity in proportion to the development of atherosclerosis. In addition, based on the results of cell proliferation analyses, including the analyses of PCNA mRNA expression in VSMC cells, estrogen signals via ERα may be considered more important for the inhibition of atherosclerosis pathogenesis in VSMCs of the human CVS than those ligands interacting with ERβ.

Several recent studies have demonstrated that estrogens inhibit the proliferation and migration of transformed VSMCs in vitro (Okubo et al., 2000; Seeger et al., 2001). Results of the present study have also demonstrated that estrogens might play an important role in anti-atherogenic effects possibly by suppressing cell proliferative activity of transformed VSMCs directly through ERs present in these cells. The presence of not only ERα, but also of ERβ mRNA has been reported in VSMCs of the human aorta (Hodges et al., 2000; Register and Adams, 1998). In addition, ERβ was reported to be predominantly expressed in VSMCs (Hodges et al., 2000). On the other hand, Pare et al. demonstrated that ERα mediates inhibition of the vascular injury response by estrogen in a study of ERα-null mice, suggesting that ERα is required for the suppression of atherosclerosis (Pare et al., 2002). Results from the present study are consistent with the results of these aforementioned reports above, such as in the relative importance of signals through ERα compared to ERβ in suppressing the cell proliferation of VSMCs and subsequently in athero-protection. This could be explained by a previously published study which Hodges et al. reported that ERα is a more potent transactivator than ERβ at low receptor concentrations in response to E_2 (Hodges et al., 2000).

In this study, we demonstrated that ER expression levels in VSMCs of the neointima of the female aorta with severe atherosclerotic changes were lower than in those of the female aorta with mild atherosclerotic changes. This may represent the following two possibilities; one is that decreased

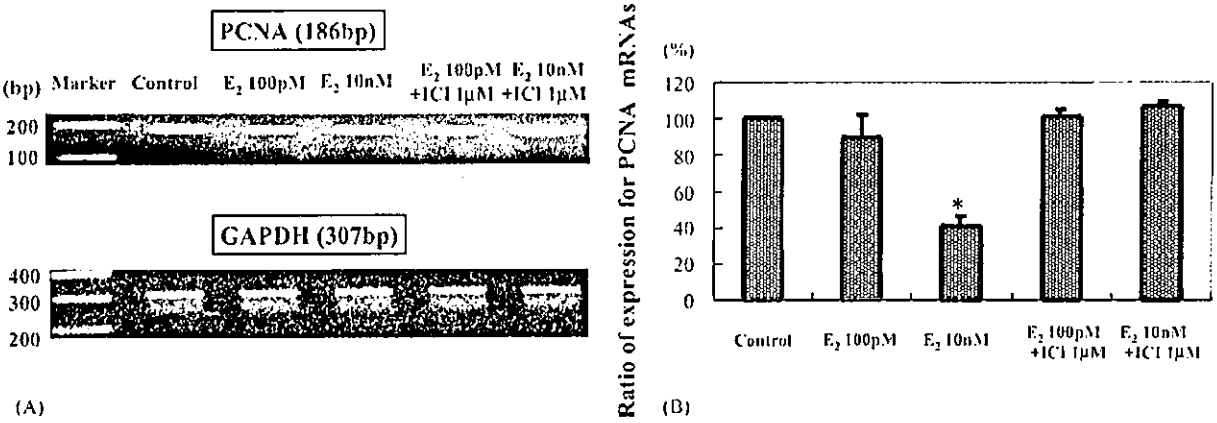

Fig. 7. (A) Results of RT/real-time PCR analysis for proliferating cell nuclear antigen (PCNA) mRNA in ERα positive cells among cells treated with vehicle (control), E_2 alone (100 pM, 10 nM), and E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Bands for PCR products were detected as specific single bands (186 bp for PCNA and 307 bp for GAPDH). The amplified products were run on a 2% agarose gel stained with ethidium bromide. (B) The relative levels of PCNA mRNA expression in ERα positive cells among cells treated with vehicle (control), or E_2 alone (100 pM, 10 nM), or E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Significantly decreased compared to control (*$P < 0.05$).

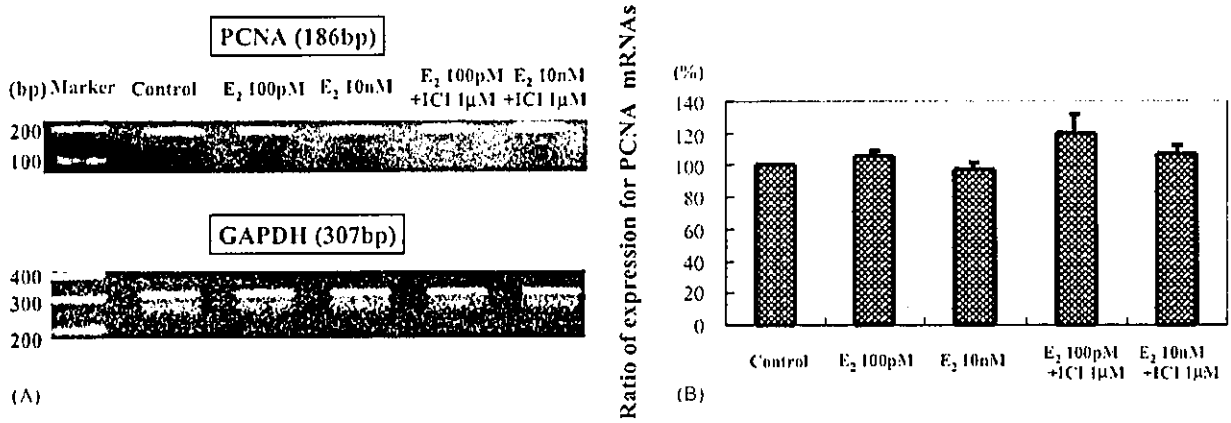

Fig. 8. (A) Results of RT/real-time PCR analysis for PCNA mRNA in ERβ positive cells among cells treated with vehicle (control), E_2 alone (100 pM, 10 nM), and E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Bands for PCR products were detected as specific single bands (186 bp for PCNA and 307 bp for GAPDH). The amplified products were run on a 2% agarose gel stained with ethidium bromide. (B) The relative levels of PCNA mRNA expression in ERβ positive cells among cells treated with vehicle (control), or E_2 alone (100 pM, 10 nM), or E_2 (100 pM, 10 nM) with ICI 182780 (1 μM) after 48 h. Significantly decreased compared to control (*$P < 0.05$).

ERs expression in the neointima of the post-menopausal female aorta, especially ERα, may result in the progression of neointimal formation against estrogenic anti-atherogenic effects and promote atherogenesis; the other is that when neointimal formation progresses, VSMCs without ERs become more abundant than those with ERs and could suppress estrogenic anti-atherogenic effects in the neointima of the post-menopausal aorta. However, further investigations are required for clarification. In addition, in this study, we have demonstrated that ER expression levels in VSMCs of the neointima of the female aorta with mild atherosclerotic changes were not different between pre-menopausal and post-menopausal subjects. This may be due to in situ estrogen biosynthesis in the human aorta, which has been suggested by several previous reports that have demonstrated the presence of aromatase and steroid sulfatase (STS), producing estrogen in situ (Diano et al., 1999; Murakami et al., 2001; Nakamura et al., in press). On the other hand, the expression levels of both ERs did not change in the neointima of the male aorta when atherosclerotic change progressed. These findings appear to suggest that anti-atherogenic effects by estrogens may be weaker in the male aorta than in the female aorta. This observation may be related to the higher risks of progression of atherosclerosis in men. On the other hand, the relative abundance of both ERα and ERβ in the media of the human aorta in this study was not significantly correlated with the sex and/or degree of atherosclerosis. In previously published studies, both ERs have also been shown to regulate the expression of a number of vasodilator and vasoconstrictor proteins, including multiple components of the renin-angiotensin system (RAS) (Mendelsshon and Karas, 1999). Especially, ERβ has recently been demonstrated to play an essential role in the regulation of vascular function and blood pressure in ERβ-deficient mice (Zhu et al., 2002). These results, as well as those of our present study, suggest that estrogenic actions via ERs, especially ERβ, in the tunica media of the human aorta may be required for the maintenance of blood vessel tone, but its relative abundance may not be directly related to the development of atherosclerosis and/or sex. Further investigations are required, however, to clarify the specific roles that ER subtypes play in the pathogenesis of atherosclerosis and related cardiovascular pathologies.

In our study, we could not examine the pre-menopausal subjects with severe atherosclerotic changes because of an unavailability of these specimens in a series of autopsy materials available for examination, resulting in insufficient number of these cases in our present study. In addition, the specimens for immunoblotting studies were not available for examination. Therefore, further investigations are required to clarify the possible association between direct estrogen actions, and the progression of atherosclerosis in the human vascular system in much larger number of cases. In addition, further investigations for cell proliferation experiment using other procedures are required for clarification, such as cell cycle assay.

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Behavioral Alterations in Response to Fear-Provoking Stimuli and Tranylcypromine Induced by Perinatal Exposure to Bisphenol A and Nonylphenol in Male Rats

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The purpose of this study was to examine whether perinatal exposure to two major environmental endocrine-disrupting chemicals, bisphenol A (BPA; 0.1 mg/kg/day orally) and nonylphenol [NP; 0.1 mg/kg/day (low dose) and 10 mg/kg/day (high dose) orally] daily from gestational day 3 to postnatal day 20 (transplacental and lactational exposures) would lead to behavioral alterations in the male offspring of F344 rats. Neither BPA nor NP exposure affected behavioral characteristics in an open-field test (8 weeks of age), in a measurement of spontaneous motor activity (12 weeks of age), or in an elevated plus-maze test (14 weeks of age). A passive avoidance test (13 weeks of age) showed that both BPA- and NP-treated offspring tended to delay entry into a dark compartment. An active avoidance test at 15 weeks of age revealed that BPA-treated offspring showed significantly fewer avoidance responses and low-dose NP-treated offspring exhibited slightly fewer avoidance responses. Furthermore, BPA-treated offspring significantly increased the number of failures to avoid electrical unconditioned stimuli within 5-sec electrical shock presentation compared with the control offspring. In a monoamine-disruption test using 5 mg/kg (intraperitoneal) tranylcypromine (Tcy), a monoamine oxidase inhibitor, both BPA-treated and low-dose NP-treated offspring at 22–24 weeks of age failed to show a significant increment in locomotion in response to Tcy, whereas control and high-dose NP-treated offspring significantly increased locomotion behavior after Tcy injection. In addition, when only saline was injected during a monoamine-disruption test, low-dose NP-treated offspring showed frequent rearing compared with the control offspring. The present results indicate that perinatal low-dose BPA or NP exposure irreversibly influenced the reception of fear-provoking stimuli (e.g., electrical shock), as well as monoaminergic neural pathways. *Key words:* behavior, bisphenol A, fear, learning, monoamine, nonylphenol. *Environ Health Perspect* 112:1159–1164 (2004). doi:10.1289/ehp.6961 available via *http://dx.doi.org/* [Online 26 May 2004]

Recently, there has been increasing concern about the exposure of the developing fetus to environmental endocrine-disrupting chemicals (EDCs). The disruption of cognitive function and various behavioral traits due to EDC exposure has been suspected (Schantz and Widholm 2001) because the development of the central nervous system (CNS) is highly regulated by endogenous hormones directly, including gonadal hormones, and by hormonally regulated events that occur early in development. The main purpose of this study was to examine whether perinatal exposure to two well-known environmental EDCs, bisphenol A (BPA) and nonylphenol (NP), can lead to behavioral alterations in the male offspring of F344 rats.

BPA (4,4′-isopropylidene-2-diphenol) is a high-production-volume chemical used in the manufacture of polycarbonate plastics, epoxy resins, and polyester resins. Worldwide production of BPA has increased and will most likely continue to increase in the future (Staples et al. 1998). Human exposure to BPA can occur via BPA-containing products included in certain baby bottles, food containers, resin-based food can linings, and dental sealants. Previous reports revealed that BPA had estrogenic (Gaido et al. 1997; Laws et al. 2000), and antiandrogenic (Sohoni and Sumpter 1998) activity in *in vitro* and *in vivo* assays. More recent studies have also identified BPA as an antiandrogen by a yeast two-hybrid system (Lee et al. 2003) and as an antagonist to thyroid hormone activity (Moriyama et al. 2002). These various activities of BPA might exert complicated adverse effects on CNS development because endogenous hormones at appropriate levels at certain limited developmental stages are essential for normal CNS development.

NP (4-nonylphenol) is another environmental EDC with week estrogenic activity (White et al. 1994). NP is used as an additive or surfactant in the manufacture of plastics, and it is a degradation product of nonylphenol polyethoxylates, which are widely used. NP has been shown to have equal or even more estrogenic activity than BPA in *in vitro* and *in vivo* assays (Laws et al. 2000). Although weak androgenic NP activity was identified by Sohoni and Sumpter (1998), a more recent study using a yeast two-hybrid system revealed the antiandrogenic effects of NP (Lee et al. 2003). NP may thus have different effects according to the experimental conditions of each assay system. It is therefore also possible that NP exerts a variety of activities under *in vivo* conditions.

There have been a number of reports suggesting the adverse effects of perinatal exposure to BPA on various behavioral traits in laboratory rodents. In mice, exposure to BPA during fetal development was shown to alter maternal behavior (Palanza et al. 2002) and enhance a methamphetamine-induced abuse state (Suzuki et al. 2003). In rats, alteration of sociosexual behavior (Farabollini et al. 2002), play behavior (Dessi-Fulgheri et al. 2002), and impulsive behavior (Adriani et al. 2003); reduced response to amphetamines (Adriani et al. 2003); and reduced behavioral sexual differentiation (Kubo et al. 2001, 2003) have been demonstrated after perinatal BPA exposure. In our previous report using F344 rats (Negishi et al. 2003b), perinatal exposure to 4 mg/kg/day BPA significantly affected the appropriate avoidance responses of offspring at 8 weeks of age in a shuttle-box avoidance test, suggesting that some alteration took place in response to fear-provoking stimuli; these responses are furthermore known to be controlled by the monoaminergic system (Gingrich 2002; Giorgi et al. 2003; Inoue et al. 1994). When these results are taken together, it appears that perinatal exposure to BPA can interfere with the development of monoaminergic systems, which might in turn be responsible for subtle behavioral changes.

In contrast, only a few studies have reported the effects of perinatal NP exposure on the behavioral traits of the offspring of experimental animals. Ferguson et al. (2000) demonstrated toxicity of NP to mothers and offspring, but found no alterations in

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open-field activity and running wheel activity in offspring after perinatal NP exposure. Latendresse et al. (2001) reported that the intake of a sodium solution was increased in offspring perinatally treated with NP (2,000 ppm in diet; > 200 mg/kg/day), but instead of focusing on CNS alterations, the authors' focus was the relationship between this increased intake and the renal toxicity of NP. However, the possibility remains that NP at a much lower (≤ 10 mg/kg/day) dose alters certain cognitive functions and/or fine behavioral characteristics, including the response to fear-provoking stimuli, but without being associated with general motor dysfunction.

In the present study, we examined the adverse effects of low-dose (0.1 mg/kg/day) perinatal BPA or NP exposure on behavioral characteristics. To this end, we performed a series of behavioral tests: an open-field test, a measurement of spontaneous activity during a dark phase, a step-through passive avoidance test, an elevated plus-maze test, and a two-way shuttle-box avoidance test. In addition, in order to evaluate suspected alterations in the monoaminergic system, we investigated behavioral responses to tranylcypromine (Tcy), a monoamine oxidase inhibitor.

Materials and Methods

Animals and treatments. Male and female F344/N rats were purchased from SLC (Sizuoka, Japan). The animals were maintained under controlled temperature (24 ± 1°C) and humidity (55 ± 5%), on a 12-hr light (09:00–21:00 hr):12-hr dark (21:00–09:00 hr) cycle. Food and water were freely available. After acclimatization for 1 week, female rats were placed with males. Vaginal smears were examined daily: a sperm-positive smear determined gestational day (GD)0. After detection, the pregnant dams were housed individually and were randomly assigned to an exposure condition (n = 10–11/condition). The dams were orally exposed to BPA (0.1 mg/kg/day; Tokyo Kasei Kogyo, Tokyo, Japan) or NP (0.1 or 10 mg/kg/day; Tokyo Kasei Kogyo) dissolved in corn oil, or to corn oil alone (vehicle control; 2 mL/kg/day) from GD3 until postnatal day (PND)20. Oral administrations of BPA and NP were performed by gavage. Because animals were trained to receive the feeding needle before mating, this procedure was not stressful. The dams were examined for clinical signs of toxicity and were weighed daily before dosing. After parturition (PND0), the pups were counted, weighed, and assigned to groups of six pups per litter, maintaining equivalent sex distributions when possible. Pups remained with their biological mother. Offspring were weighed and the body weights recorded on PND0, 3, 7, 10, 14, and 21 and again at 8 and 13 weeks of age. We included the mean weight of each littermate in the statistical analysis. Male pups were marked with ink for identification; On PND21, the marked male pups were gathered from different litters and housed together according to treatment group (7–8/cage). The dams were anesthetized with diethyl ether and then sacrificed by exsanguination; the body weights and organ weights (liver, kidney, spleen, and thymus) were then recorded.

We randomly selected one male pup per litter to undergo a series of behavioral tests (n = 9–10/group). The remaining male pups in the litter were subjected to the measurement of organ weights (liver, kidney, spleen, thymus, brain, and testis) at weaning (PND21) or at 8 weeks of age. Although male rats were usually housed in groups according to experimental treatment, rats were housed individually for some behavioral tests. At the end of each behavioral test, rats were again housed in a group according to treatment. In this study, we excluded female pups from behavioral tests because the estrous cycle in mature females affects various behavioral characteristics. When using female animals, it is important to consider the estrous cycle in evaluating the results of behavioral tests that require several consecutive days. This study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, University of Tokyo.

Open-field behavior test. At 8 weeks of age, animals were subjected to an open-field test. Each subject was housed individually for 24 hr before the test. The open-field apparatus was a rectangular field (56 × 39 cm); none of the animals was familiar with this apparatus. Open-field behavior was recorded for 5 min by a video camera positioned above the apparatus during the dark phase (21:30–23:00 hr) under a low white light; responses were automatically analyzed by a computer-assisted system, which classified observed behavior as locomotion, rearing, or "other" behaviors.

Spontaneous motor activity. We measured spontaneous motor activity in 12-week-old male offspring using a Supermex (Muromachi Kikai, Tokyo, Japan) (Masuo et al. 1997). The Supermex consisted of a sensor monitor, which was mounted above the cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses. The body heat radiated by the animal was detected with the sensor head of the monitor, which contained paired infrared pyroelectric detectors. In this manner, the system allowed the monitoring and counting of all spontaneous movements. Each animal was housed individually in the experimental cage—a differently arranged housing cage with food and water freely available—for 24 hr before the measurement to become accustomed to this experimental condition; spontaneous activity was then measured for about 12 hr (starting at 21:00 hr). All counts were automatically totaled and recorded in 2-min intervals. We defined "immobile time" as 2 min with no signal (count = 0).

Passive avoidance test. We conducted the step-through passive avoidance test when the animals were 13 weeks of age. The test was carried out during the light phase (13:00–17:00 hr), and each animal was housed individually during the test. The passive avoidance apparatus consisted of light and dark compartments. The first time each animal was placed in this apparatus, an electric foot shock (0.25 mA, 3 sec) was delivered to the animal through the grid floor just after the animal had completely left the light compartment for the dark compartment. We recorded the latency period required before each animal entered the dark compartment after having been placed in the light compartment. Twenty-four hours later, a retention trial (with no shock) was performed, and the latency period before entering the dark compartment was recorded. In addition to the traditional measure, we recorded the frequency and percentage of duration of poking into the dark compartment until the animal completely entered the dark compartment in the retention trial. If an animal failed to enter the dark compartment within 20 min, the test was terminated.

Elevated plus-maze test. The elevated plus-maze apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm, with 50-cm high walls) extending from a central square platform (10 × 10 cm); arms were arranged so that those of the same type were opposite each other. The apparatus was elevated 60 cm above the floor. At 14 weeks of age, each animal was placed in the central square facing an open arm during the dark phase (21:30–23:00 hr). We then recorded standard spatiotemporal factors for 5 min (i.e., the frequency of entries into the open arms and the closed arms was recorded, whereby "arm entry" was defined as moving the head into an open arm).

Active avoidance test. At 15 weeks of age, animals were subjected to an active avoidance test in a two-way shuttle-box (Muromachi Kikai, Tokyo, Japan) consisting of two compartments connected to each other by a hole in the wall; this test was carried out during the light phase (13:00–17:00 hr). Each animal was housed individually through the active avoidance test. Each animal was allowed to become accustomed to the shuttle-box apparatus for 5 min before every session; the animal was then subjected to 25 daily trials/session of avoidance conditioning in four consecutive sessions (acquisition test). For each trial, a 5-sec conditioned stimulus (CS), consisting of a buzzer and light, was followed by a 5-sec unconditioned stimulus (UCS), which included a scrambled shock of 0.2 mA delivered through the floor grid. In addition, on the day after the

fourth session, each rat performed the extinction test, which is basically the avoidance text without the UCS. Each trial was separated by variable intertrial intervals (10–90 sec between trials; total of 1,250 sec/session). During the acquisition tests in sessions 1–4 and the extinction test, we recorded the percentage of correct avoidance responses, in which the animals moved to the other compartment of the shuttle box within a 5-sec CS in each block of 25 trials. To evaluate further behavioral characteristics in this procedure, we recorded the percentage of failures to avoid the stimulus within 5-sec UCS and the latency periods associated with both the CS and UCS throughout the four acquisition sessions.

Monoamine-disruption test. Disturbances of the monoaminergic system in the CNS were induced by a single intraperitoneal (i.p.) injection of Tcy (*trans*-2-phenylcyclopropyl-amine hydrochloride; Sigma-Aldrich, St. Louis, MO, USA). At 22–24 weeks of age, BPA- or NP-treated male offspring were subjected to the monoamine-disruption test. Before the monoamine-disruption tests, we determined the optimal dose of Tcy for the monoamine-disruption test in a different set of male F344 rats (n = 15) at 9 weeks of age. We injected (i.p.) Tcy solution in 0.9% saline at 0, 2, 5, and 10 mg/mL (1 mL/kg) at 16:00 hr (n = 4, 4, 4, and 3, respectively) and then measured spontaneous motor activity as described above. We confirmed that animals treated with 5 mg/kg Tcy showed a high increment of activity at 21:30 hr, when open-field behavior was recorded.

Saline challenge. On the first day of the monoamine-disruption test, we injected 0.9% saline (1 mL/kg; i.p.) as a vehicle into each rat; 5.5 hr after the injection, we observed and recorded the behavior of the animals in the open-field apparatus for 4 min.

Tcy challenge. On the day after the saline challenge, we injected 5 mg/kg Tcy (i.p.) into the same animal and recorded open-field behavior for 4 min, as described for the saline challenge. Behavioral analyses in the monoamine-disruption test were performed as described for the open-field test.

Statistical analyses. We conducted statistical analyses using StatView, Version 5.0 (SAS Institute, Cary, NC, USA). We analyzed the effects of perinatal BPA or NP exposure on maternal body weight increase and the body weight of male offspring by analysis of variance (ANOVA) with one between-subject factor (treatment) and one repeated-measures factor (days). The number of total, male, and female pups;, the organ weights of dams at weaning; and the organ weights of male offspring at PND21 and at 8 weeks of age were analyzed by one-way ANOVA. Behavioral measurements were analyzed by one-way ANOVA, except for the percentages of correct avoidance in the shuttle-box avoidance test, which were assessed by repeated measures of ANOVA over days (sessions). In the analysis of latency in the passive avoidance test, data processed through logarithmic transformations were used for the ANOVA because of their significantly inappropriate distributions with respect to the normal distributions. In each statistical analysis, the effects of BPA and NP exposure were analyzed with respect to the control in the same ANOVA. When the ANOVA produced significant results, we then performed the post hoc Fisher's protected least-significant difference test for comparisons between groups. The significance level for all tests was set at $p < 0.05$.

Results

Maternal toxicity and reproductive results. Oral exposure to BPA or NP showed no statistically significant effect on maternal body weight increase during pregnancy and lactation or on the number of total, male, and female pups (data not shown). All dams in this study delivered their offspring on GD22. There was no significant effect of 40-day exposure to BPA or NP on either body weight or organ weights (data not shown).

Development of male offspring. Perinatal exposure to BPA or NP had no significant effect on either body weight gain or organ weights of male offspring on PND21 or at 8 weeks of age (data not shown). No male offspring died during the course of the study (> 25 weeks of age).

Open-field test. In the open-field test, neither BPA nor NP exposure significantly affected the percentage of locomotion [$F_{(3,32)}$ = 0.271, $p > 0.5$] or the number of rearings [$F_{(3,32)}$ = 0.189, $p > 0.5$; data not shown].

Spontaneous motor activity. To assess general motor activity under nonstress conditions, we recorded spontaneous motor activity of male offspring. Neither BPA nor NP exposure had any effect on the rhythm of activity, the total counts of activity [$F_{(3,32)}$ = 0.554, $p > 0.5$], or the immobile time [$F_{(3,35)}$ = 0.078, $p > 0.5$] during the 12-hr dark phase (data not shown).

Passive avoidance test. On shock-presenting day of the passive avoidance test, the subjects readily entered the dark compartment (< 30 sec). During the retention trial 24 hr after shock presentation, ANOVA [$F_{(7,63)}$ = 12.174, $p < 0.001$] and multiple comparisons revealed that the subjects showed significant hesitation ($p < 0.01$) to enter the dark compartment compared with the short latency during shock presentation in all of the experimental groups (Figure 1A). Although both BPA- and NP-treated groups tended to remain in the light compartment longer than the control offspring, there was no significant difference in latency periods during the retention trial among the experimental groups. Neither the frequency of poking into the dark [$F_{(3,28)}$ = 1.166, $p > 0.1$; Figure 1B] nor the percentage of duration of poking into the dark [$F_{(3,28)}$ = 1.919, $p > 0.1$; Figure 1C] during the retention trial was affected by chemical exposure.

Elevated plus-maze test. Neither BPA nor NP exposure significantly altered the frequency of entering the open arms [$F_{(3,27)}$ = 0.571, $p > 0.5$] or the closed arms [$F_{(3,27)}$ = 0.139, $p > 0.5$] of the elevated plus-maze test, although the frequency of entering the open arms was slightly higher in the BPA-treated group than in the controls (data not shown).

Active avoidance test. BPA and low-dose NP exposure significantly affected the avoidance responses of the male offspring in the active avoidance test. Repeated-measures one-way ANOVA showed a significant effect of chemical exposure [$F_{(3,35)}$ = 5.724, $p < 0.01$] and number of sessions [$F_{(3,105)}$ = 107.322, $p < 0.0001$], as well as a significant interaction between chemical exposure and the number of sessions [$F_{(9,105)}$ = 3.536, $p < 0.001$]. One-way ANOVAs and post hoc multiple comparisons for each session indicated significantly fewer avoidance responses in BPA-treated offspring at the first, second, and third sessions than in the control offspring (Figure 2A). Low-dose NP-treated offspring showed a lower avoidance

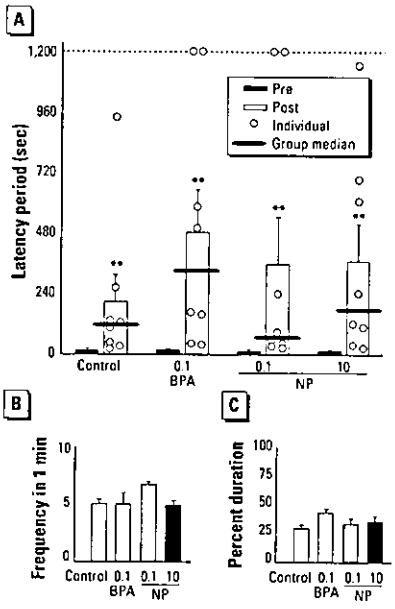

Figure 1. Effect of perinatal exposure (mean ± SE) to BPA or NP (mg/kg/day) on the behavioral characteristics in a passive avoidance test (n = 8/group). Abbreviations: Post, latency during the retention trial; Pre, latency on shock-presenting day. (*A*) The latency period until the animals completely entered the dark compartment. (*B*) The frequency of poking into a dark box until complete entrance. (*C*) The percent duration of poking into a dark box until complete entrance.
**$p < 0.01$ compared with Pre for same treatment.

rate than the control offspring, but only in the first session (Figure 2B). In the fifth extinction session (i.e., without electrical shocks as the UCS), the BPA- and NP-treated offspring showed slightly less correct avoidance behavior than the control offspring, although the effect of chemical exposure, as determined by one-way ANOVA, was not statistically significant [$F_{(3,35)} = 0.571$, $p = 0.104$]. One-way ANOVA indicated a significant effect of chemical exposure and that the frequency of failure of avoidance within 5 sec of shock presentation—in which one-way ANOVA indicated a significant effect of chemical exposure [$F_{(3,35)} = 3.700$, $p < 0.05$]—in BPA-treated offspring was significantly higher ($p < 0.001$) than that in the control offspring; low-dose NP-treated offspring showed a similar tendency (Figure 2C). We found no significant effect of chemical exposure on the mean of the latency periods associated with CS [$F_{(3,35)} = 0.722$, $p > 0.5$; Figure 2D] and UCS [$F_{(3,35)} = 1.186$, $p > 0.1$; Figure 2E] in 100 trials of four sessions.

Monoamine-disruption test. Tcy injection led to a large, slow increase in motor activity at 5 and 10 mg/kg compared with the saline control, although 2 mg/kg Tcy did not induce an increase (Figure 3A). We confirmed that the animals administered 5 mg/kg Tcy showed a high increase of activity 5.5 hr after injection, which corresponded to the schedule for the monoamine-disruption test. One-way ANOVA and post hoc tests about locomotion behavior [$F_{(7,58)} = 2.498$, $p < 0.05$] and the number of rearing behaviors [$F_{(7,58)} = 9.629$, $p < 0.01$] yielded the following results. In the monoamine-disruption test, control and high-dose NP-treated offspring showed a significant increase in locomotion behavior resulting from Tcy injection ($p < 0.01$; Figure 3B). However, BPA-treated or low-dose NP-treated offspring failed to show a clear increase in locomotion ($p > 0.1$). Tcy also caused significant decreases in the number of rearing behaviors in all experimental groups (Figure 3C) in the monoamine-disruption test. When only saline was administered, low-dose NP-treated offspring showed a significantly increased number of rearing behaviors, compared with those of the control offspring in the saline challenge; in addition, the BPA-treated offspring also appeared to have a similar tendency ($p < 0.1$), which was abolished by monoamine disruption by Tcy (Figure 3C).

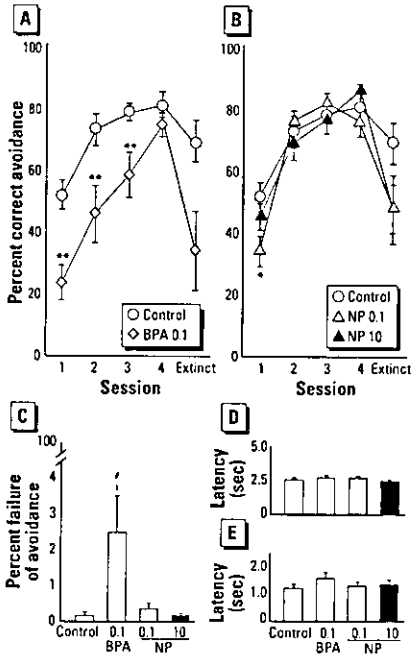

Figure 2. Effects of perinatal exposure to BPA or NP (mg/kg/day) on behavioral characteristics in a shuttle-box avoidance test (mean ± SE; n = 9–10/group). Avoidance learning curves of male offspring perinatally exposed to BPA (*A*) or NP (*B*). (*C*) Percentage of failure of avoidance when an electrical shock was presented for 5 sec among 100 trials of four sessions. Length of latency period associated with a CS (*D*) and a UCS (*E*) in four sessions.
*$p < 0.05$; **$p < 0.01$; and #$p < 0.001$ compared with control.

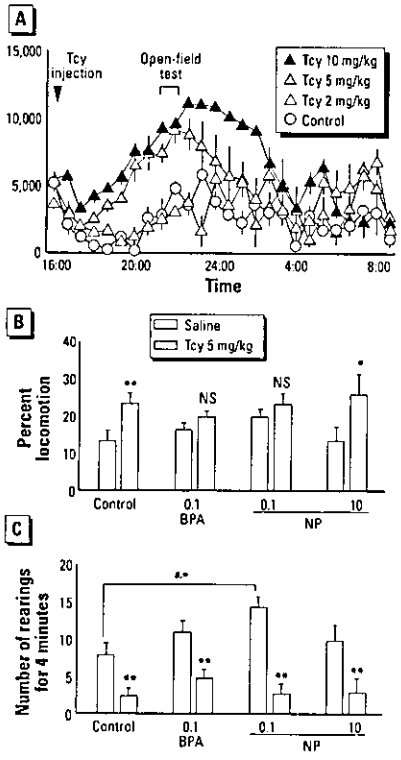

Figure 3. Effects of perinatal exposure to BPA or NP (mg/kg/day) on behavior in the monoamine-disruption test 5.5 hr after Tcy treatment. NS, not significant. (*A*) Locomotor activity (mean ± SE) after a single injection with 2, 5, or 10 mg/kg/day Tcy (n = 3–4/group). (*B*) Effect of perinatal BPA and NP on Tcy (5 mg/kg)-induced increases in locomotion behavior in an open-field apparatus (mean ± SE; n = 7–9/group). (*C*) Effects of perinatal BPA and NP on Tcy (5 mg/kg)-induced suppression of rearing in an open-field apparatus (mean ± SE; n = 7–9/group).
#The bracket indicates the comparison of offspring exposed to BPA and low-dose NP in the saline challenge. *$p < 0.05$. **$p < 0.01$ compared with saline control.

Discussion

In the present study, we carried out a series of behavioral tests and demonstrated the subtle and complex functional effects of perinatal exposure to BPA and NP on the behavior of male rat offspring. Behavioral alterations by perinatal exposure to BPA and NP were detected only in specific challenges involving fear-provoking stimuli and pharmacologic disruption of monoaminergic system, whereas spontaneous explorative behavior and responses to novelty were not affected by the exposure to these chemicals.

Evaluation of the toxicity of BPA as well as that of NP on maternal body weight, parturition, maternal organ weights at weaning (PND21), and general development (body weight and organ weights) of the offspring confirmed that there were no adverse effects of BPA at 0.1 mg/kg/day or of NP at 0.1 and 10 mg/kg/day, which was consistent with the findings of previous reports (Ferguson et al. 2000; Kwon et al. 2000).

In the present study, we detected no statistically significant alterations by BPA or NP in the spontaneous activity and behaviors of rats in the open-field test at 8 weeks of age and in the elevated plus-maze test at 14 weeks of age. This suggests that these chemical exposures induce no severe abnormalities in general behavior.

In the passive avoidance test, offspring perinatally exposed to BPA or NP seemed to be more sensitive to fear-inducing shock than were the control offspring, which might have led to the somewhat stronger retention in the chemical-treated groups; however, such changes were statistically ambiguous because of the large individual differences in the experimental conditions used in this study.

In the active avoidance test, BPA and low-dose NP showed clear or partial adverse effects on behavior, respectively. In particular, BPA-treated offspring may have been less able to learn than the control offspring in terms of causality. Low-dose NP-treated offspring were also affected to some extent. Although the possibility remains that BPA-treated offspring were more insensitive to electrical shock than were the control offspring, the slight elongation of the latency period in the passive avoidance test would have excluded this possibility. If BPA-treated offspring had found the electrical stimuli less fear-provoking and/or painful, they would have entered the dark compartment more quickly than the control offspring in the passive avoidance test. It would also be unlikely that less learning took place as a result of motor dysfunction or sensory abnormality

because there was no alteration in the spontaneous activity during the dark phase and in the duration of locomotion in the open-field test, as well as in the latency periods associated with CS and UCS in the active avoidance test. When electrical shock was presented as a UCS, BPA-treated offspring failed to enter the opposite compartment within 5 sec more frequently than the control offspring, and NP-treated offspring showed a similar tendency. BPA-treated offspring tended to stiffen in the corner of the box during the UCS, and these animals appeared to stop avoiding the UCS more often than did the control offspring, as determined by direct observation (data not shown). It is possible that excessive fear of the UCS would interfere with the smooth progression of avoidance learning. Perinatal BPA exposure may render male offspring exceedingly vulnerable to intolerable levels of fear. Interestingly, this hypothesis may be supported by a previous study (Aloisi et al. 2002), which indicated that perinatal BPA exposure increased the sensitivity of the central neural pathways for nociception in male offspring. Farabollini and colleagues reported the details of various behavioral changes observed due to perinatal exposure to BPA at 0.04 or 0.4 mg/kg/day in rats (Adriani et al. 2003; Aloisi et al. 2002; Dessi-Fulgheri et al. 2002; Farabollini et al. 1999, 2002). In the present study we also provided new evidence of the behavioral adverse effects of perinatal exposure to BPA at a low dose of 0.1 mg/kg/day on electrical UCS-related responses in an active avoidance test.

In the monoamine-disruption test, Tcy-induced increases in locomotion were significantly less marked in BPA-treated and low-dose NP-treated offspring, but not in the high-dose NP-treated offspring, compared with the control offspring. This is the first study reporting behavioral alterations due to perinatal BPA and NP exposure shown by responses to the disruption of monoaminergic systems, with Tcy having clear and straightforward pharmacologic effects as a monoamine oxidase inhibitor. Previous studies have reported changes after BPA exposure in a mouse model of psychostimulant abuse (methamphetamine) (Suzuki et al. 2003) and in a rat model of an increment in activity by amphetamine (Adriani et al. 2003), which have complicated pharmacologic effects on CNS function. It is possible that BPA-treated and low-dose NP-treated offspring might be insensitive to monoamines overflowing in excess into the extrasynaptic space. Such animals might show abnormal expressions of each type of monoamine receptor (dopamine, serotonin, and noradrenaline receptors, including the subtypes of each receptor class) or monoamine oxidase in certain region(s) of the CNS. Further investigations considering each of the monoamergic systems (dopaminergic, serotoninergic, and noradrenalinergic) are likely to produce more insight into the mechanism of traces induced by perinatal BPA and NP exposure in the CNS. Although monoamine disruption by Tcy significantly reduced the number of rearing behaviors, the response to Tcy was not influenced by perinatal chemical exposure. There was a discrepancy between the results of behavior in the open-field apparatus at 8 weeks of age and > 22 weeks of age. When the animals were > 22 weeks of age, the observed significant increase in the number of rearing behaviors suggested that low-dose NP-treated offspring might have experienced less anxiety than the control offspring in the open-field apparatus. The results furthermore suggested that these behavioral alterations caused by perinatal low-dose NP exposure might appear only at a stage of advanced age, that is, at a time when rats are relatively slow in their movements and rarely show rearing compared with juveniles. Further investigations will be required in this regard. In any case, the effective dose of NP from a neurobehavioral standpoint is much lower than the dose associated with general physical toxicity, as observed in the case of BPA in our previous study of that substance (Negishi et al. 2003b).

We cannot address differences in sexes in the present study because we limited this behavioral study to the male offspring; our primary goal was to detect behavioral alteration by perinatal BPA exposure in male offspring. However, studies in rats by Kubo et al. (2001, 2003) have demonstrated that perinatal exposure to BPA removed the differences between the sexes in the volume of locus ceruleus and in behavioral characteristics. In addition, some studies have reported sex differences in the effects of BPA (Dessi-Fulgheri et al. 2002; Farabollini et al. 1999, 2002). Further experiments using the active avoidance test and the monoamine-disruption test on both male and female offspring would be informative.

In the present study, one animal per litter sequentially underwent all of the behavioral tests. It is possible that an experience in an earlier behavioral test influenced the results of the subsequent behavioral tests. For example, a painful experience immediately after exploratory behavior in the passive avoidance test might interfere with the behavioral propensity in the elevated plus-maze test. However, we believed that using sequential behavioral tests in the same animal would not obstruct the evaluation of effects of perinatal exposure to chemicals because all of the animals in the four treatment groups experienced the stimuli.

In summary, perinatal exposure to BPA and NP, both at 0.1 mg/kg/day, affected the extent of shock-related behavior and affected responses to the disruption of the monoaminergic system, although the direct mechanisms of these alterations remain unclear at present. Moreover, the neurobehavioral toxicity of both BPA and NP may be out of proportion with the *in vitro* and *in vivo* estrogenic potency of these compounds determined by certain simple assay systems. It may be useful to consider other potencies and/or metabolites of BPA and NP (Moriyama et al. 2002; Yoshihara et al. 2001) in addition to their weak estrogenic activity. We suggest that there may be a causal relationship between behavioral alterations in response to fear-provoking stimuli and abnormality in the monoaminergic system because both dopamine and serotonin play important roles in the processing of fear-provoking and/or stressful stimuli in the CNS (Gingrich 2002; Giorgi et al. 2003; Inoue et al. 1994). In our recent study using primary cultured neurons (Negishi et al. 2003a), BPA and NP inhibited staurosporine-induced neuronal cell death, interfering with caspase-3 activation. BPA and NP may, in this manner, disrupt programmed neuronal cell death during development, which would irreversibly lead to an abnormal neural network—including the monoaminergic system—and cause behavioral abnormalities in adulthood.

Conclusion

We conclude that perinatal BPA and NP exposure, even at slightly higher doses than those associated with environmental exposure in humans, had adverse behavioral effects on rats, especially when the animals were forced to avoid fear-provoking stimuli such as electrical shocks. Perinatal exposure to BPA and NP disrupted the reception of intolerable stress, which may be due to the alterations in monoaminergic system.

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—Note—

Comparative Study on Toxicokinetics of Bisphenol A in F344 Rats, Monkeys (Macaca fascicularis), and Chimpanzees (Pan troglodytes)

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Abstract: *We compared the toxicokinetics of bisphenol A (BPA) among three animal species: rats, cynomolgus monkeys and chimpanzees. Rats and monkeys were administered BPA orally or subcutaneously at 10 or 100 mg/kg body weight, while chimpanzees were administered only 10 mg/kg of BPA. BPA in serum was measured by ELISA. In oral administration of BPA at 10 mg/kg, both C_{max} and AUC were rats < chimpanzee < monkeys. In oral administration of BPA at 100 mg/kg, both C_{max} and AUC were rats < monkeys. Subcutaneous BPA administrations also revealed similar results, although the values of toxicokinetic parameters in subcutaneous administration were higher than those in oral administration. These results suggest that orally or subcutaneously administered BPA in primates is more easily absorbed than that in rats. We conclude that there are considerable differences in distribution, metabolism, and excretion of BPA between rodents and primates.*

Key words: *bisphenol A, chimpanzee, cynomolgus monkey*

Recently, there is an increasing concern about the risk of human exposure to bisphenol A (4,4'-isopropylidene-2-diphenol, BPA) which is a volume chemical used in the manufacture of polycarbonate plastics. BPA is known to have various hormone disrupting effects, such as estrogenic [4], anti-androgenic [14], and anti-thyroid activities [8]. Studies using rodents suggested that perinatal exposure to BPA results in abnormalities in reproductive function [16], hormonal function [11], central nervous system development [5, 12] and behavior [2, 9].

In toxicological studies, rodents, especially rats, are the most popular experimental animals. Considering human risk, however, primates would be more useful

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because of their similarities to humans in their physiological characteristics. Chimpanzees, our closest relative, are considered to be the best models for humans among the non-human primates. Although there are a number of reports on the bioavailability of BPA in rats [6, 10, 15, 17] and monkeys [7], we tried for the first time to compare the toxicokinetics of BPA among three species, i.e., rats, cynomolgus monkeys and chimpanzees, to consider the human risk of BPA exposure. BPA was administered to animals of three species orally or subcutaneously at low (10 mg/kg) or high (100 mg/kg) doses to evaluate and compare dependency on dose as well as route of BPA administration among the three species.

Seventy-eight female F344/N rats (140–150 g body weight) were purchased from SLC (Shizuoka, Japan) and used for administrations of BPA. Collections of whole blood and serum preparations (see below) in rats were performed at the University of Tokyo (Tokyo, Japan). BPA administrations, collections of whole blood, and serum preparations in six female cynomolgus monkeys (*Macaca fascicularis*) (4.0 to 5.0 kg body weight), and the two female Western chimpanzees (*Pan troglodytes verus*) (40 to 50 kg body weight), were performed at Shin Nippon Biomedical Laboratory (Kagoshima, Japan) and Sanwa Kagaku Kenkyusho (Kumamoto, Japan), respectively. BPA (Tokyo Kasei Kogyo, Tokyo, Japan) was first dissolved in distilled water with 0.5% CM-cellulose (Wako Pure Chemical, Osaka, Japan) for oral administrations, and in a mixture of dimethylacetamide (Wako) and polyethylene glycol (Wako) (1 : 1) for subcutaneous administrations. BPA at 10 mg/kg or 100 mg/kg was administered to rats and monkeys by oral gavage or dorsal subcutaneous injection, while only 10 mg/kg BPA was administered to chimpanzees by the same ways as rats and monkeys for the ethical reason of avoiding any acute adverse effect at the higher dose of BPA (100 mg/kg). This study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. Rats were euthanatized by drawing whole blood under diethylether anesthesia before and 0.5, 1, 2, 4, 6, and 24 h after BPA administration (n=3 at each point). Monkeys (n=3 for 10 mg/kg and n=3 for 100 mg/kg) and chimpanzees (n=2 for 10 mg/kg) were first orally administered BPA at the two dosages and low dose (10 mg/kg), respectively. After one week for entire excretion of BPA [3], the above dosages were repeated subcutaneously. Serial blood samples were taken from each monkey before and 0.5, 1, 2, 4, 6, and 24 h after administration or from each chimpanzee before and 0.25, 0.5, 1, 2, 3, 4, 8, and 24 h after administration. Serum samples were harvested by centrifugation at 3,000 g for 15 min and were kept at –20°C until analysis. BPA in serum was measured by BPA ELISA kit (Japan EnviroChemicals, Japan). Methanol was first added to the serum (1:5, methanol: serum), and then the sample was centrifuged at 10,000 g for 15 min. Supernatant was diluted with the same volume of purified water. Prepared samples were subjected to ELISA according to product manuals. Briefly, the sample and the antigen (BPA)-enzyme complex solution were mixed and added to each microplate well, the inside of which was coated with the BPA-specific antibody. After 60 min competitive assay, unbound or excess reagents were washed out and substrate chromogen was added to each well to develop the color. The optical density at 490 nm was measured to determine the amount of BPA in the sample. In the present method, the detection limit of BPA in serum was 12.5 μg/L. Toxicokinetic parameters were determined from the individual serum BPA concentration-time curves. Peak serum concentrations (C_{max}) and the time to reach C_{max} (T_{max}) were obtained from observed data. The area under the serum BPA concentration-time curves for 4 h ($AUC_{0-4\,h}$) and for 24 h ($AUC_{0-24\,h}$) were calculated by the linear trapezoidal method.

The concentration-time profiles of oral BPA administration at 10 mg/kg in rats, monkeys and chimpanzees, and those at 100 mg/kg in rats and monkeys are shown in Figs. 1A and B, respectively. These profiles indicate lower BPA bioavailability in rats compared to that in both monkeys and chimpanzees. Indeed, no sample of rats contained detectable levels of BPA except for one (33 μg/L) of three samples at 2 h after oral BPA administration at 10 mg/kg, thus C_{max}, $AUC_{0-4\,h}$, and $AUC_{0-24\,h}$ could not be calculated (Table 1), and T_{max} was not defined. In oral administration of BPA at 10 mg/kg, C_{max}, $AUC_{0-4\,h}$, and $AUC_{0-24\,h}$ showed the same tendency, that is, rats < chimpanzees < monkeys (Table 1). In oral administration of BPA at 100 mg/kg, C_{max}, $AUC_{0-4\,h}$, and $AUC_{0-24\,h}$ were all rats < monkeys (Table 1). Subcutaneous BPA administrations at 10 or 100

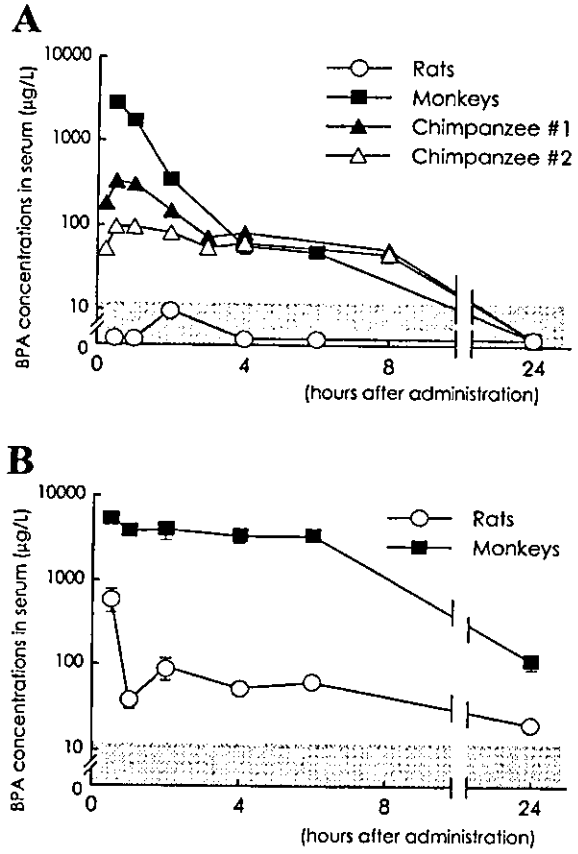

Fig. 1. Serum levels of bisphenol A after oral administration at 10 mg/kg in female rats, monkeys and chimpanzees (A), and at 100 mg/kg in rats and monkeys (B). Data represent the mean ± SEM of three rats and three monkeys. Data of chimpanzees are indicated as plots from each subject (chimpanzee #1 and #2). No error bar in rats or monkeys indicates that the error is included within the symbol. Plots in gray area mean that serum BPA concentration is under the detection limit in this study.

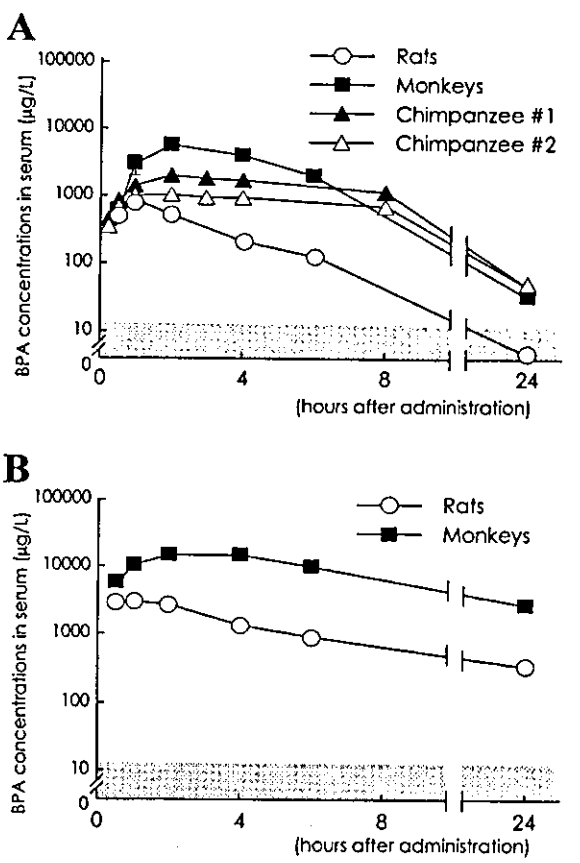

Fig. 2. Serum levels of bisphenol A after subcutaneous administration at 10 mg/kg in female rats, monkeys and chimpanzees (A), and at 100 mg/kg in rats and monkeys (B). Data represent the mean ± SEM of three rats and three monkeys. See also the figure legend of Fig. 1.

mg/kg showed similar results, although the values of toxicokinetic parameters were higher than those in oral administrations at each dose (Fig. 2 and Table 1).

We demonstrated the direct comparison of toxicokinetics of oral or subcutaneous administration of BPA among F344 rats, cynomolgus monkeys, and Western chimpanzees. Oral administration resulted in lower availability of BPA when compared to subcutaneous administration in all species examined in this study, suggesting a route dependency common to mammals, which is consistent with a previous report [10]. Fast-pass metabolism by the intestine and/or liver as well as intestinal secretion probably contributed to the lower bioavailability of orally administered BPA. We also observed clear dose dependency in toxicokinetic parameters between 10 and 100 mg/kg administrations in rats and monkeys as expected. Irrespective of route of administration, bioavailabilities of BPA at 10 or 100 mg/kg were rats < chimpanzees < monkeys, or rats < monkeys, respectively, in this study. These results suggest that either orally or subcutaneously administered BPA in primates is more easily absorbed than in rats. This might be due to the differences in the abilities of metabolism and/or excretion of BPA between rats and monkeys. Further researches about the species differences in alteration of hepatic and intestinal enzyme expression and function accompanying BPA disposi-

Table 1. Toxicokinetic parameters in rats, monkeys, and chimpanzees after oral or subcutaneous injection of BPA at 10 or 100 mg/kg

	10 mg/kg BPA			100 mg/kg BPA	
	Rats (n=3)[a] (mean±SD)	Monkeys (n=3) (mean±SD)	Chimpanzees (#1, #2)	Rats (n=3) (mean±SD)	Monkeys (n=3) (mean±SD)
Oral injection					
C_{max} (μg/L)	N.C.[b]	2,793 ± 920	325, 96	580 ± 398	5,732 ± 525
T_{max} (h)	N.D.[c]	0.7 ± 0.2	0.5, 0.5	0.5	0.7 ± 0.2
$AUC_{0-4\,h}$ (μg·L^{-1}·h)	N.C.	3,209 ± 536	491, 235	506 ± 313	14,747 ± 2,495
$AUC_{0-24\,h}$ (μg·L^{-1}·h)	N.C.	3,247 ± 587	1,167, 813	1,353 ± 462	52,595 ± 8,951
Subcutaneous injection					
C_{max} (μg/L)	872 ± 164	57,934 ± 1,902	2,058, 1,026	3,439 ± 679	10,851 ± 3,915
T_{max} (h)	1.0	2.0 ± 0.0	2.0, 2.0	1.0	2.0 ± 0.0
$AUC_{0-4\,h}$ (μg·L^{-1}·h)	1,912 ± 262	15,316 ± 5,856	5,658, 3,109	9,314 ± 2,634	48,010 ± 11,641
$AUC_{0-24\,h}$ (μg·L^{-1}·h)	3,377 ± 334	39,040 ± 10,738	21,141, 12,492	23,001 ± 6,387	189,627 ± 21,790

[a] Note that T_{max}, in rats was determined by using the mean of each time point. C_{max} in rats represents mean ± SD calculated by the serum BPA concentrations of three animals at T_{max}. [b] N.C.: Not calculated. [c] N.D.: Not defined.

tion, such as CYP450 and UDP-glucuronosyl transferase (UDPGT), among rodents and primates are important, since BPA is metabolized principally to its monoglucuronide conjugate and is excreted via feces or urine [13]. It is possible that humans might also absorb BPA more easily than experimental rats. Both behavioral and neurological alterations in rodents by perinatal low-dose BPA exposure per os even at < 100 μg/kg/day [1] would strongly suggest potential adverse effects of chronic environmental exposure to BPA in humans (< 10 μg/kg/day), if the differences in bioavailability between rodents and primates including humans also applies to much lower dose exposure to BPA. We conclude that there are considerable differences in distribution, metabolism, and excretion of BPA between rodents and primates.

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PROMOTING EFFECTS OF MONOMETHYLARSONIC ACID, DIMETHYLARSINIC ACID AND TRIMETHYLARSINE OXIDE ON INDUCTION OF RAT LIVER PRENEOPLASTIC GLUTATHIONE S-TRANSFERASE PLACENTAL FORM POSITIVE FOCI: A POSSIBLE REACTIVE OXYGEN SPECIES MECHANISM

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Dimethylarsinic acid (DMA) is a major metabolite of inorganic arsenicals, which are epidemiologically significant chemicals in relation to liver cancer in mammals. The present study was conducted to determine the promoting effects of organic arsenicals related to DMA [monomethylarsonic acid (MMA) and trimethylarsine oxide (TMAO)] on rat liver carcinogenesis using a liver medium-term bioassay (the Ito test). Male, 10-week-old, F344 rats were given a single i.p. injection of diethylnitrosamine at a dose of 200 mg/kg b.w. as an initiator. Starting 2 weeks thereafter they received 100 ppm of MMA, DMA or TMAO in their drinking water, or no supplement as a control, for 6 weeks. All animals underwent 2/3 partial hepatectomy in week 3 after initiation. Quantification of glutathione S-transferase placental form (GST-P)-positive foci as preneoplastic lesions in liver sections revealed significantly increased numbers and areas in all 3 treated groups compared with controls. Hepatic microsome cytochrome P-450 content was markedly increased with all 3 arsenic treatments. Markedly elevated CYP 2B1 protein levels and *CYP 2B1/2* mRNA levels were thus observed in all cases. The potency of promotion was similar for MMA, DMA and TMAO. Since hydroxyradicals were found to be generated in the relatively early phase while methylated arsenicals were metabolized in liver, the resultant oxidative stress might have promoted lesion development.

Key words: *arsenic; hepatocarcinogenesis enhancement; oxidative stress; cytochrome P-450*

Drinking water contamination by arsenicals remains a major public health problem in many parts of the world. Epidemiologic research on arsenic has demonstrated significantly higher standardized mortality rate for cancers of the kidney, liver and colon, with especially high rates in the skin, urinary bladder and lung.[1,2] For causal factors in arsenic epidemiology, the focus has been on inorganic arsenite (AsIII) and arsenate (AsV), but experimental evidence was limited until recently.[3] Methylation of inorganic arsenics has been considered to result in detoxication. Thus inorganic arsenicals are methylated into dimethylarsinic acid (DMA), which is then excreted from the human body *via* the urine.[4] However, DMA may induce mitotic arrest in V79 cells, with potent clastogenic effects in human fibroblast cells and DMA has caused aneuploidy in mouse bone marrow cells.[5–7] Recent research demonstrated a promotion potential for DMA in the urinary bladder, kidney, liver and thyroid gland using a multiorgan carcinogenesis bioassay in rats, and also for skin tumorigenesis in mice.[8,9] We have also established that DMA promotes urinary bladder and liver carcinogenesis in a dose-dependent manner.[10,11] Moreover, DMA was found to be carcinogenic for the urinary bladder on long-term exposure to rats.[12] Monomethylarsonic acid (MMA) and trimethylarsine oxide (TMAO) are also related methylated metabolites of inorganic arsenate and arsenite.[13–15]

In the present study the 3 organic arsenics MMA, DMA and TMAO were tested in a rat medium-term cancer bioassay to clarify their carcinogenic effects in the liver.[16] We also analyzed hepatic levels of 8-hydroxydeoxyguanosine (8-OHdG) in DNA and microsomal cytochrome P-450 by Western blotting and RT-PCR. One aim of our work was to evaluate the role of reactive oxygen species in arsenic carcinogenesis.

MATERIAL AND METHODS

Animals

A total of 80 10-week-old, male F344 rats (Charles River Japan, Atsugi, Japan) were used. They were housed (with wood chips for bedding) in an animal room with a 12 hr light/dark cycle at 23 ± 2°C and relative humidity at 55 ± 5%. Their general condition was checked carefully every day, and body weights and drinking water consumption were measured weekly throughout the test compound administration period. Diet and water were available to animals *ad libitum*.

Chemicals

MMA and TMAO (purity ≥99.9 %) were purchased from Tri Chemical Laboratory (Yamanashi, Japan), and DMA (purity ≥98 %) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Diethylnitrosamine (DEN) was from Sakai Research Laboratories (Fukui, Japan).

Treatment

The experimental design is shown in Figure 1.The rats were divided into 4 groups (groups 1–4, 20 rats each) for the liver medium-term bioassay (the Ito test[16]). All animals were given a single i.p. injection of DEN (200 mg/kg b.w.) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks on a basal pellet diet (CE2, Clea Japan, Tokyo) and water, animals in groups 1, 2, 3 and 4 were given either 100 ppm MMA, DMA, TMAO or no arsenic (the control group), respectively, in the drinking water for

Abbreviations: DEN, diethylnitrosoamine; DMA, dimethylarsinic acid; MMA, monomethylarsonic acid; GST-P, glutathione S-transferase placental form; 8-OHdG, 8-hydroxydeoxyguanosine; TMAO, trimethylarsine oxide.

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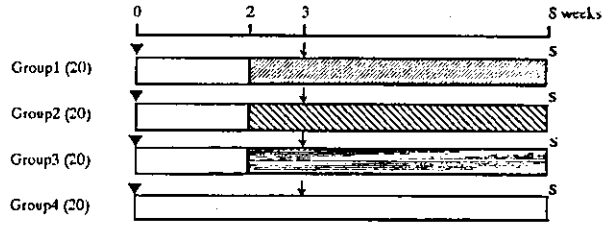

FIGURE 1 – Experimental design of the study. Triangles down, DEN, 200 mg/kg body weight (i.p.); arrows down, 2/3 partial hepatectomy; S, animals that were sacrificed. Three arsenicals of MMA in group 1, DMA in group 2 and TMAO in group 3 were administered, with the same dosage of 100 ppm in drinking water from week 2 to the end point of the experiment. Tap water without supplement was provided for the rats in group 4.

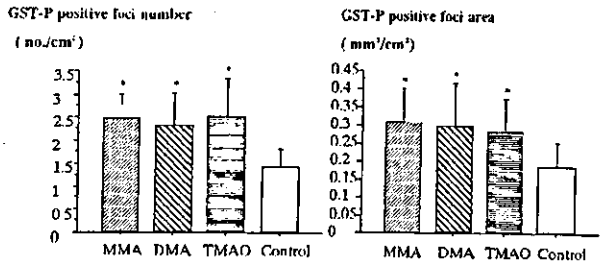

FIGURE 2 – Numbers and areas of GST-P-positive foci (per cm^2 liver tissue) in the liver. *, $p < 0.05$ *vs.* control.

6 weeks *ad libitum*. The dose of 100 ppm was determined from preliminary experiments (data not shown). All animals were subjected to 2/3 partial hepatectomy under ether anesthesia at week 3 and were sacrificed under ether anesthesia for examination of a putative preneoplastic lesion, glutathione S-transferase placental form (GST-P)-positive foci in the liver at week 8. Increases in this type of lesion are known to correlate well with known potency to promote hepatocarcinogenesis.[16]

Tissue processing

At autopsy, livers were quickly dissected out and weighed, and 2–3 mm thick sections from 3 lobes were fixed in 10% buffered formalin and embedded in paraffin wax. Sections cut at 4 μm were used for immunohistochemistry. The remaining liver tissue was preserved in liquid nitrogen and stored at −80°C for DNA assessment and microsome isolation.

Immunohistochemistry for GST-P

The avidin-biotin complex method was used to demonstrate GST-P-positive liver foci. After deparaffinization, liver sections were treated sequentially with 3% H_2O_2, normal goat serum and then rabbit anti-rat GST-P (the primary antibody; 1:2,000, 37°C, 2 hr; Medical and Biological Laboratories, Nagoya, Japan). Reactivity with the primary antibody was demonstrated with biotin-labeled goat anti-rabbit IgG (the second antibody) and finally the avidin-biotin-peroxidase complex (ABC kit; Vector, Burlingame, CA.). The sites of peroxidase binding were visualized with the substrate diaminobenzidine tetrahydrochloride (DAB), and the tissue sections were lightly counterstained with hematoxylin to facilitate orientation under microscopic examination.

Quantitative assessment of GST-P-positive foci

Quantitation of GST-P-positive foci was performed using 2-dimensional evalution only. The numbers and areas of GST-P-positive foci greater than 0.2 mm in diameter and the total areas of the liver sections were measured using a color image processor (IPAP; Sumica Technos, Osaka, Japan) to give values per cm^2 of liver section.

Analysis of hepatic cytochrome p-450, CYP 2B1 and 2E1 proteins

Rat liver microsomes were separately isolated from 5 rats in each group and used for the determination of cytochrome P-450 total content and Western blotting.[17,18] Total protein levels in liver microsomes were analyzed according to Lowry *et al.*[19] Antibodies to CYP 2B1 and 2E1 generated by Funae and Imaoka[20] were used for this purpose. SDS-PAGE was performed by the method of Laemmli.[21]

Analysis of hepatic P-450 mRNA levels

Determination of CYP 2B1-mRNA expression was performed by competitive RT-PCR. Total RNA was isolated from rat liver using ISOGEN (Nippon Gene, Toyama, Japan) and isopropanol precipitation, with diethylpyrocarbonate (DEPC)-treated distilled water and then stored at −80°C until use. Reverse transcription of *CYP 2B1/2* mRNA and then PCR with a rat cytochrome P-450 competitive RT-PCR set (Takara, Osaka, Japan) was performed. PCR products were separated on gel electrophoresis with 3% NuSive GTG Agarose gel (Takara) and analyzed using a FMBIO II Multi-View Image Analyzer Scanning Unit (Hitachi, Yokohama, Japan).

Detection of 8-OHdG formation in DNA

Liver samples of approximately 2 g wet weight were taken from 10 animals at random from each treatment group. The DNA was isolated and digested into deoxynucleosides by combined treatment with nuclease P1 (Yamasa Shoyu, Chiba, Japan) plus alkaline phosphatase (Sigma, St. Louis, MO).[22] The level of 8-OHdG in each resulting preparation was determined by high-performance liquid chromatography using an adaptation of the method of Floyd *et al.*[23] and Kasai *et al.*[24] as described elsewhere in detail.[25] The level of 8-OHdG formation was expressed as the number of 8-OHdG residues/10^6 of total deoxyguanosine.

Statistical evalution

Statistical analysis of our data was conducted with the Stat-View-J 4.02 program using Fisher's PLSD method (Abacus Concepts, Berkeley, CA).

RESULTS

The experimental design contained partial hepatectomy in all 4 groups. Two animals in group 2, 2 in group 3 and 1 in group 4 died in weeks 3–6, mainly because of bleeding from insufficient ligating of the hepatic vein. All other rats survived the experimental period. Final numbers of survivors were 20 in group 1, 18 in group 2, 18 in group 3 and 19 in group 4. Final average body weights were significantly decreased in the MMA (249.6 ± 13.9 g, $p < 0.05$) and DMA (257.1 ± 13.2 g, $p < 0.05$) groups but not in the TMAO group (262.3 ± 18.0 g) compared with the control group (268.6 ± 13.7 g). Water consumption of rats exposed to DMA was greater (20.5 g) than in the control group (18.1 g), but this was not found for MMA (17.9 g) or TMAO (16.5 g) groups. Food intake did not vary significantly among the groups (data not shown).

Data for numbers and areas of GST-P-positive foci are illustrated graphically in Figure 2.The numbers of GST-P-positive foci (per cm^2 liver tissue), considered as a preneoplastic lesion for assessment of carcinogenic activity,[16] were significant by increased in rats treated with MMA (2.43 ± 1.10/cm^2), DMA (2.26 ± 1.44/cm^2) and TMAO (2.48 ± 1.60/cm^2) compared with the controls (1.38 ± 0.79/cm^2). Similarly, the areas of GST-P-positive foci were also significantly increased in rats treated with MMA (0.30 ± 0.21 mm^2/cm^2), DMA (0.29 ± 0.24 mm^2/cm^2) and TMAO (0.27 ± 0.19 mm^2/cm^2) compared with the controls (0.18 ± 0.14 mm^2/cm^2).

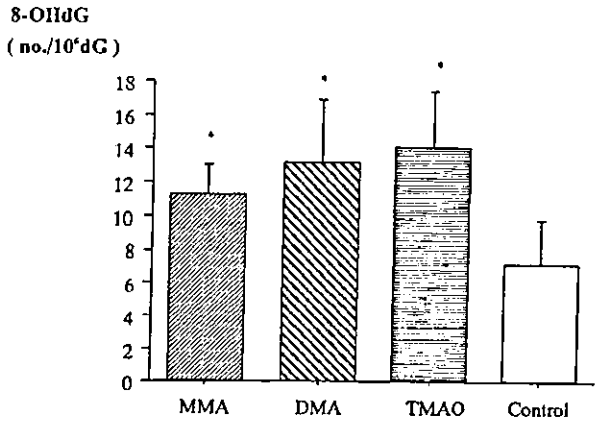

FIGURE 3 – Level of 8-OHdG formation in the liver. *, $p < 0.05$ *vs.* control.

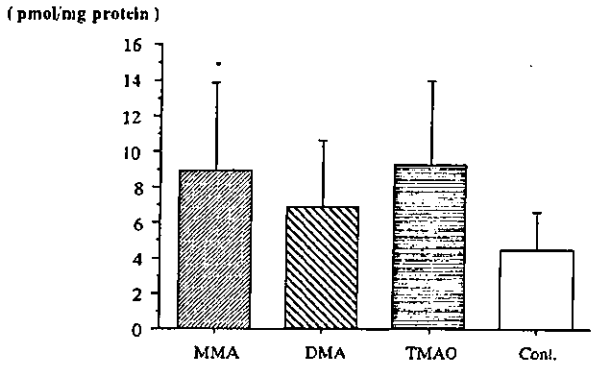

FIGURE 4 – Western blotting of CYP 2B1 protein level in the liver. *, $p < 0.05$ *vs.* control.

The 8-OHdG level, a biomarker of cellular oxidative stress, was found to be significantly enhanced in the livers of rats receiving any of the treatments compared with the controls. Arranged in order of decreasing potency, the levels were: TMAO, DMA and MMA (Fig. 3).

Hepatic microsome cytochrome P-450 content was increased with all 3 arsenic treatments, although there were no significant differences from the control group at $p < 0.05$ (data not shown). CYP 2E1 protein levels did not show significant variation between the 4 groups (data not shown), but CYP 2B1 was significantly increased by MMA, DMA or TMAO administration, and to a marked degree in the case of MMA (Fig. 4). Elevated *CYP 2B1/2* mRNA levels were observed in all arsenic treatment groups, although no significant differences in any arsenic group were observed compared with the control group (Fig. 5).

DISCUSSION

The 3 organic arsenicals MMA, DMA and TMAO all increased the development of GST-P-positive foci in the present rat liver medium-term bioassay for carcinogens. The degrees of enhancement in terms of numbers and areas were almost the same in all 3 cases. Numbers and areas of GST-P-positive foci induction in the liver were earlier found to be closely correlated with eventual hepatocellular carcinoma incidence after 2-year continual test chemical administration.[26] Thus GST-P-positive foci represent appropriate end-point markers[16] for preneoplastic lesions. The data therefore indicate that MMA and TMAO as well as DMA can exert a promoting potential on rat liver carcinogenesis.

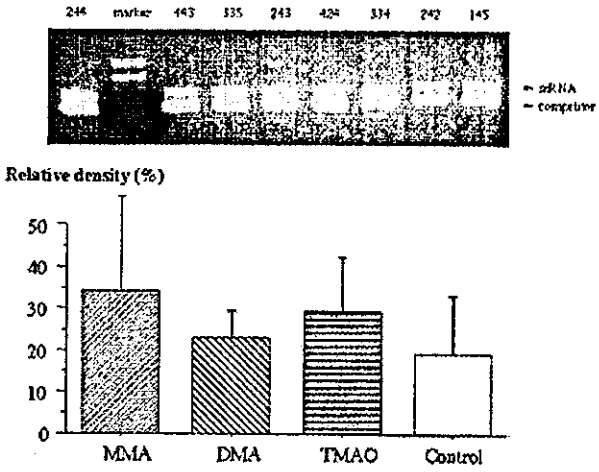

FIGURE 5 – Illustration of *CYP* 2B1/2 gene expression analysis by RT-PCR. The intensity of the *CYP* 2B1/2 RT-PCR product peak was compared with a standard RNA competitor. Enhancement was found with MMA (lane 145), DMA (lanes 244, 243 and 242) and TMAO (lanes 335 and 334) compared with the control group (lanes 443 and 424). Lane 2 was the inner marker.

Rat hepatic 8-OHdG levels were increased in rats treated with 1 of the 3 organic arsenicals. Kasai *et al.*[24] earlier reported that cellular 8-OHdG increased after exposure to agents that produced oxygen radicals. Klaunig *et al.*[27] proposed that oxidative stress can play important roles in "nongenotoxic" hepatic carcinogenesis, and in previous studies from our laboratory, we obtained evidence that the active metabolite of DMA causes DNA damage via oxygen radicals.[11,28,29] The present study shows that methylated arsenicals form oxygen radicals and generate 8-OHdG. The numbers and areas of GST-P-positive foci in the livers treated with arsenicals are higher compared with the control group and demonstrate the same tendency in 8-OHdG levels, in line with the reactive oxygen species theory of arsenic carcinogenesis. Again, this is consistent with the conclusion that arsenicals impact on hepatocarcinogenesis by oxygen radical-induced oxidative stress causing DNA damage and eventually mutation and carcinogenesis.

We also detected an increase in total hepatic cytochrome P-450 content in the liver after treatment with MMA, DMA and TMAO. Previously, arsenite treatment was found to decrease CYP 2B enzyme activity in primary cultures of rat hepatocytes.[25,30] In our study CYP 2E1 protein levels demonstrated no significant intergroup difference, whereas CYP 2B1 appeared to increase. *CYP 2B1/2* mRNA levels also exhibited a similar trend. The mammalian cytochrome P-450 isoenzyme system plays an important role in detoxifying drugs and toxins, mostly by oxygen insertion reactions. Cytochrome P-450 can also generate reactive oxygen species. Brown *et al.*[14] reported that methylated organic forms of arsenicals such as MMA and DMA show similar patterns of biochemical effect, with increases in hepatic P-450 levels and decreases in glutathione content being a sign of oxidative stress, along with elevated serum alanine transferase. Therefore we can speculate that the 3 organic arsenicals are metabolized by P-450, mainly CYP 2B1 in the liver. This metabolism may generate hydroxy radicals. Klaunig *et al.*[31] noted that in the promotion step of the cancer process, reactive oxygen species generation due to oxidative stress is related to P-450 enzyme activity. Thus we may speculate that this P-450 function can affect promotion by causing clonal expansion of initiated cells that are more neoplastic.

It was recently reported that the methylated trivalent arsenicals methyloxoarsine [MMA(III)] and iododimethylarsine [DMA(III)] were 77 and 376 times more potent, respectively, in terms of induction of DNA damage than inorganic arsenite.[32] Monometh-