U-shaped Dose Response in Vasomotor Tone: A Mixed Result of Heterogenic Response of Multiple Cells to Xenobiatics

Ok-Nam Bae,* Kyung-Min Lim,* Jee-Yeon Han,* Byoung-In Jung,* Jin-Young Lee,* Ji-Yoon Noh,* Seung-Min Chung,* Moo-Yeol Lee,§ Joo-Young Lee,§ and Jin-Ho Chung*†

*College of Pharmacy, Seoul National University, Seoul 151-742, South Korea; †AMOREPACIFIC CO/R&D Center, Gyeonggi-do 446-729, South Korea; ‡College of Pharmacy, Chonnam National University, Gwangju 500-757, South Korea; and §Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, South Korea

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U-shaped response has been frequently encountered in various biological areas including epidemiology, toxicology, and oncology. Despite its frequent observation, the theory of U-shaped response has been crippled by the lack of a robust mechanism underlying and incomplete in vitro and in vivo correlation. In the present study, a novel mechanism is provided for a U-shaped response, based on the findings of agonist-induced vasomotor tone change affected by menadione (MEN) (synthetic vitamin K₃), a reactive oxygen species generator, and arsenic, an environmental pollutant, which showed typical U-shaped responses in both in vitro aortic contractile response and in vivo blood pressure. U-shaped responses by MEN and arsenic were a combined result from heterogenic susceptibilities and responses of multiple target cells composing blood vessels, that is, endothelium and smooth muscle. Notably, endothelium, a regulator of vasomotor tone, was primarily affected by low-dose stimuli, whereas smooth muscle, an effector of vascular contraction, was affected later by high-dose. The dysfunction of smooth muscle was produced by high-dose MEN-induced hydrogen peroxide, resulting in the attenuation of vascular contractile reactivity, whereas low-dose MEN-induced superoxide led to the quenching of vasodilatory nitric oxide in endothelial cells, resulting in the enhancement of vasoconstriction. This mechanistic theory, the difference in susceptibilities and responses to a common stimulus between regulator and effector components of a system, could give a new insight into the explanation of various U-shaped responses and provide a new evidence for the need of the risk assessment of toxicants with a wider dose range.

Key Words: U-shaped dose response; vasoconstriction; arsenic; menadione; endothelial cells; smooth muscle cells; heterogenic responses; risk assessment.

Biological dose–response to xenobiatics or obnoxious stimuli in general, has been explained fundamentally as linear with a threshold at low doses. Sometimes, a biphasic dose response or a reversal in response, for example, low-dose stimulation and high-dose inhibition or vice versa, has been encountered in vitro as well as in vivo in various biological fields such as endocrinology, toxicology, oncology, pharmacology and epidemiology (Celik et al., 2005; Marque et al., 2003; Salehi et al., 2006). The concept of this U-shaped response or hormesis revolutionizes the basic understanding of biological phenomena and especially, gave a substantial impact on the scientific and regulatory areas, where the traditional linear or threshold dose–response relationship has been generally accepted, such as drug evaluation and most importantly, risk-hazard assessments (Calabrese and Baldwin, 2003; Calabrese et al., 2006; Conolly and Lutz, 2004; Crump, 2007; Kaiser, 2003). Considering the recent reports on the wide and general involvement of U-shaped responses in almost full spectrum of biological areas, conventional way of risk assessment of low dose might not be accurate by the simple extrapolation based on the traditional theory of the threshold or linearity (Cook and Calabrese, 2006).

Despite the great excitement, any clear explanation enough to be extended to the most of the U-shaped responses has not been provided due to the complexity and heterogeneity of the specific experimental settings. Some reports suggest that U-shaped response appears as an overcompensatory response to maintain homeostasis (Cedazo-Minguez et al., 2003; Mothersill and Seymour, 2004). Others report that U-shaped dose responses take place by the combined contribution of more than two types of receptors with different characteristics such as affinity, accessibility or signaling cascade (Joels, 2006). These theories were, however, only applicable to limited experimental conditions and in most of the U-shaped dose response cases, underlying mechanism has not been fully understood yet (Jonas, 2001; Thayer et al., 2005). As advocated by Klaffen (2000), a sound mechanism is critical for U-shaped dose response to gain its credibility and scientific ground and generalize into the understanding of other biological phenomena. Beside the lack of a robust mechanism, the failure to correlate between in vitro and in vivo findings is another obstacle in the way to the generalization of U-shaped
responses. Although Brandes (2005) sought the possibilities with epidemiological data, demonstration of in vitro and in vivo correlation in controlled experimental setting has been very difficult to achieve yet.

U-shaped dose responses can be found in the responses of blood vessels to various chemicals or therapeutic drugs (Derkach et al., 2000; Didion and Faraci, 2002). Endothelium and smooth muscle cells make up blood vessel, maintaining vasomotor tone and vessel integrity. Endothelium modulates vasomotor tone by the release of vasodilator substances like nitric oxide (NO) or vasoconstrictor substances like endothelin (Cockcroft, 2005; Vanhoutte et al., 2005). Smooth muscle cells directly function as contractile machinery effectuating blood vessel contraction via calcium and myosin light chain (MLC) phosphorylation (Ogut and Brozovich, 2003; Somlyo and Somlyo, 1994). Although U-shaped dose response in vasomotor changes was explained in part as different contributions of one cell type, either endothelium or smooth muscle cells, but not on the level of the whole tissue, blood vessel in this case.

In the present study, we found similar typical U-shaped dose responses in the regulation of vascular contractile responses in vitro and in vivo with two xenobiotics with different toxic mechanisms; menadione (MEN), a synthetic vitamin K3 and representative reactive oxygen species (ROS) generator widely investigated for the role of ROS in vascular system (Lee et al., 1999; Rosen and Freeman, 1984), and arsenic, an environmental pollutant and another well-known vascular toxic chemical (Chen et al., 2007; Kumagai and Sumi, 2007). We elucidated the underlying mechanism for the U-shaped dose response, which was the result of the heterogeneity in the dose-dependent responses of endothelium and smooth muscle cells to a common stimulus. Based on these results, a novel mechanism is provided for the understanding of U-shaped dose relationship, which could be expanded to unveil other biological phenomena and provide the important evidence for the application of the U-shaped dose relationship into the risk assessment.

MATERIALS AND METHODS

Reagents. The following reagents were purchased from Sigma (St Louis, MO): acetylcholine, dimethyl sulfoxide (DMSO), diithiothreitol (DTT), catalase (CAT), ethylenediaminetetraacetic acid (EDTA), N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES), lucigenin, luminol, MEN, N-monomethyl-l-arginine (l-NMMA), 3-morpholinosydnonimine (SIN-1), phenylephrine (PE), serotonin creatinine sulfate, superoxide dismutase (SOD), trichloroacetic acid (TCA), Tris–HCl, Triton X-100, and Dowex AG50W-8 column. Anti-MLC antibody, anti-phospho MLC antibody, and horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-nitrotyrosine antibody was from Upstate (Lake Placid, NY). [3H]-l-arginine was purchased from Amersham (UK). All other reagents used were commercial products of the special grade.

Animals. All the animal protocols were approved by the Ethics Committee of Animal Service Center at Seoul National University. Male Sprague–Dawley rats (Dae Han Biolink Co., Chungbuk, Korea) weighing 300–400 g were used throughout all experiments. Prior to experiments, animals were acclimated for one week in the laboratory animal facility maintained at constant temperature and humidity with a 12-h light/dark cycle. Food and water were provided ad libitum.

Cell culture. Primary cultured human aortic endothelial cells (HAECs, third passage), endothelial cell growth media-2 (EGM-2) Bullet kit and subculture reagents were obtained from Clonetics Corp. (Walkersville, MD). Rat thoracic smooth muscle cells (A7r5) were purchased from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Gibco BRL (Rockville, MA). HAECs in EGM-2 or A7r5 in DMEM were seeded at density of 2500–5000 cells/cm² and incubated at 37°C under humidified 5% CO₂/95% air and grown to 80–90% confluency for experiments.

Preparation of blood vessels in organ bath. Isolation of rat thoracic aortic rings was performed as reported previously (Lee et al., 1999). Briefly, after animals were sacrificed by decapitation and exsanguination, the rat thoracic aorta was isolated carefully and cut into ring segments. The rings were mounted in organ baths filled with Krebs–Ringer solution (pH 7.4): 115.5 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.026 mM calcium–disodium EDTA and 11.1 mM glucose. Organ baths were continuously gassed with 95% O₂/5% CO₂ and maintained at 37°C. The viability of intact endothelium was determined by confirming the characteristic relaxation to acetylcholine (1 µM) in aortic rings precontracted by PE (1 µM). In some experiments, we removed endothelial cells from aortic rings by gently rubbing the intimal surface with a wooden stick. The removal of endothelium was confirmed by the absence of relaxation response to acetylcholine.

Blood vessel contraction. To determine the contraction of blood vessels, MEN or arsinite was added to the organ bath containing aortic rings for appropriate time, and an agonist (PE, serotonin [5-HT], or endothelin-1 [ET-1]) was cumulatively added. The change of tension was measured isometrically, using Grass FT03 force transducers (Grass Instrument Co., Quincy, MA) and was recorded using AcqKnowledge III computer program (BIOPAC Systems Inc., Goleta, CA). In experiments using SOD and CAT, these reagents were incubated for 5 min before addition of MEN.

Calcium increase in A7r5 cells. Measurement of intracellular calcium in A7r5 cells was carried out as previously described (Bkaily et al., 1999). After cells are loaded with Fluo-4/AM (6.5 µM) for 45 min, MEN or vehicle (DMSO) was treated for 5 min. Then, PE-induced calcium increase was monitored continuously using a confocal microscopy (Leica, Germany). The intracellular calcium increases was analyzed with the ratio of fluorescence intensity (F/F₀). F₀ is the average fluorescence value and F is the maximum fluorescence value measured before and after PE addition, respectively.

MLC phosphorylation. After aortic rings without endothelium were exposed to MEN for 30 min, 10⁻⁵ M of PE was added for 2 min. After reaction was terminated by ice-cold acetone containing 10% TCA and 10 mM DTT, the aortic rings were washed with acetone (10mM DTT) and then lyophilized in −80°C. The lyophilized samples were placed in sample buffer containing 8M urea, 2% sodium dodecyl sulfate (SDS), 0.01% bromphenol blue, 62.5 mM Tris–HCl and 5% β-mercaptoethanol for 3 h to extract protein. The protein extracts were subjected to SDS-polyacrylamide gel electrophoresis (15%). Phosphorylation of MLC is determined by Western blot using anti-phospho MLC (Sakurada et al., 1998). To confirm that samples were not different in the amount of MLC, immunoblotting of MLC was performed using anti-MLC.

Detection of chemiluminescence. Generation of ROS was measured using luminol- or lucigenin-induced chemiluminescence. Aortic rings without endothelium were placed in a Krebs–Ringer solution continuously gassed with 95% O₂/5% CO₂ and allowed to equilibrate for 30 min at 37°C. Luminometer tubes containing 2 ml of Krebs–Ringer solution with luminol (250µM) or lucigenin (250µM) were prepared and MEN was added. Aortic rings were
placed to each tube and chemiluminescence was measured for 30 min using luminometer (Berthold, Germany).

In experiment with HAEC, cells were detached using Trypsin/EDTA and suspended in HEPES buffer (pH 7.4): 25mM HEPES, 140mM NaCl, 5.4mM KCl, 1.8mM CaCl2, 1mM MgCl2 and 5mM glucose. HAEC count was adjusted to 2.5 × 10^5 cells/ml. Luminometer tubes containing 1ml of HAEC suspended HEPES buffer with lucigenin or luminol were prepared and MEN or DMSO was added. Chemiluminescence was measured for 30 min.

**Measurement of endothelial NO synthase activity.** HAEC were seeded to 12 well plate at density of 4 × 10^4 cells/well. After 48 h, MEN or DMSO was treated to HAEC for 30 min in HEPES buffer containing 3 μCi/ml of [3H]-L-arginine. t-NMMA (100 μM) was used as positive control to inhibit endothelial NO synthase (eNOS). Cells were washed two times with ice-cold Ca^{2+}-free PBS (pH 7.4): 137mM NaCl, 2.7mM KCl, 4.3mM NaHPO4, and 1.4mM KH₂PO₄. After addition of 0.3M perchloric acid, cells were scraped and centrifuged at 12,000 × g for 10 min. Supernatant was neutralized with 3M K₂CO₃ and centrifuged at 10,000 × g for 2 min. Collected supernatant was applied to Dowex AG50-8 columns and the eluted [3H]-l-citrulline was measured by scintillation counting.

**3-Nitrotyrosine formation.** Detection of 3-nitrotyrosine in HAECs was performed using anti-nitrotyrosine antibody. HAECs were seeded on glass coverslip (Nunc, Naperville, IL) at density of 1.6 × 10^4 cells/well and incubated in Hank’s balanced salt solution containing MEN for 2 h. After fixed with 3.7% formaldehyde, cells were permeablized using 0.5% Triton X-100. The formation of 3-nitrotyrosine was detected using anti-nitrotyrosine antibody (Upstate, Lake Placid, NY) and fluorescein isothiocyanate–conjugated secondary antibody (Sigma). The analysis of fluorescence intensity was carried out using confocal microscopy (Leica). SIN-1 (500 μM) was used as a positive control.

**Blood pressure measurement.** After rats were anesthetized with urethane (1,250 mg/kg, ip), left carotid artery was cannulated with polyethylene tubing filled with heparinized saline (20 U/ml) for the measurement of blood pressure, and left jugular vein was cannulated for iv injections. Arterial pressure was monitored with MLT 0380 transducer and Power Lab data acquisition system (ADI Instruments, New South Wales, Australia) interfaced to Chart 5 software. To determine the effects of arsenite on blood pressure, various concentration of arsenite in sterile saline was administered for 3 min by an iv infusion. Results are expressed as a change of mean arterial pressure (MAP) between baseline and final 30 sec out of 3-min arsenite administration.

**Statistical analysis.** The means and standard errors of means were calculated for all treatment groups. The data were subjected to one-way analysis of variance followed by Duncan’s multiple range test or t-test to determine which means were significantly different from each other or control. In all cases, a p-value of < 0.05 was used to determine significance. Immunoblotting and immunofluorescence data are representative figures of more than three experiments.

**RESULTS**

When gradual vessel contractions were induced by cumulative addition of PE, a contractile agonist, MEN enhanced vasoconstriction at low concentrations (1 and 10 μM), whereas higher concentrations (37 and 50 μM) resulted in the inhibition of vasoconstriction (Fig. 1A), displaying a typical U-shaped dose response. Without endothelium, enhancing effect of MEN at low concentration (10 μM) on vasoconstriction was abolished.
(Fig. 1B), suggesting that the potentiation of vasoconstriction by MEN is endothelium-dependent. In contrast to low-dose stimulatory effect, inhibitory effect by high-dose MEN was retained in the aortic rings without endothelium, implying that the suppression of vasoconstriction is not endothelium-dependent, but smooth muscle-dependent. These U-shaped dose responses could also be observed with other endogenous vasoconstrictor agonists such as 5-HT and ET-1 with similar patterns to PE, regardless of agonist types (Fig. 1C).

Agonist-induced intracellular calcium increase and subsequent phosphorylation of MLC play key roles in smooth muscle cell contraction (Ogut and Brozovich, 2003; Woodrum and Brophy, 2001). To determine whether high-dose MEN has effects on these key molecular pathways in smooth muscle, PE-induced calcium increase in a rodent smooth muscle cell line, A7r5, and MLC phosphorylation in the aortic rings without endothelium were evaluated using fluorescent confocal microscopy and western blot analysis, respectively. High-dose MEN (37μM) inhibited the PE-induced intracellular calcium increase and MLC phosphorylation significantly (Figs. 2A and 2B). Especially high-dose and low-dose effects in MLC phosphorylation (Fig. 2B) were in a good agreement with the functional observation of vasoconstriction (Fig. 1B). These results suggest that the suppression of vasoconstriction by MEN is mediated through the direct inhibition of smooth muscle contractile machineries. MEN did not induce cytotoxicity up to 50μM as measured by lactate dehydrogenase (LDH) leakage, suggesting these effects were not from nonspecific cytotoxicities (Fig. 2C).

It is well known that ROS can affect vascular functions such as calcium regulation resulting in modulation of the vasomotor tone in blood vessels (Grover et al., 1995; Lounsbury et al., 2000; Partridge et al., 2005). We evaluated if high-dose MEN could induce ROS production with lucigenin and luminol probe, which are specific for superoxide and hydrogen peroxide/hydroxyl radical, respectively. High-dose MEN increased both lucigenin- and luminol-induced chemiluminescence in aortic rings without endothelium. These signals were reduced by SOD and CAT, respectively (Fig. 3A), confirming that high-dose MEN could induce the production of superoxide and hydrogen peroxide in smooth muscle cells. To investigate the role of ROS in the inhibitory effects of high-dose MEN, effects of ROS inhibitors on PE-induced intracellular calcium increase was evaluated. As a result, CAT lessened the inhibitory effect of MEN on PE-induced calcium increase significantly (Fig. 3B), which matched well with the reversal of vasoconstriction inhibition by CAT (Fig. 3C). On the contrary, SOD did not affect calcium increase or vasoconstriction.

**FIG. 2.** Inhibition of calcium increase and MLC phosphorylation by high-dose MEN in smooth muscle. (A) Effect of MEN (37μM) on PE-induced calcium increase was determined in fluo-4 loaded A7r5 cells. F₀ and F are the fluorescence intensities before and after PE (10 -5M) stimulation. Shown on the right is the quantification of calcium increase. (B) Western blot analysis of MLC phosphorylation in aortic rings without endothelium (Representative out of three independent experiments). MLC-P, phosphorylated MLC. (C) Cytotoxicity was examined by LDH leakage, following treatment with MEN or a positive control, lysolecithin (LPC; 100μM). Values are mean ± SEM of three independent experiments. *Represent significant differences from corresponding control (p < 0.05). Scale bar, 10 μm.
Hydroxyl radical scavengers, mannitol, and DMSO did not reverse the inhibitory effects of MEN (Fig. S1, see Supplementary Data). These results suggest that hydrogen peroxide plays a key role in the suppression of vasoconstriction by high-dose MEN.

Chemicals can provoke hypercontraction by decreasing NO levels through inhibition of eNOS or depleting NO availability by ROS generation and subsequent formation of peroxynitrite (Drexler and Hornig, 1999; Thomas et al., 1996). Effects of low-dose MEN on NO synthesis and ROS generation were investigated using HAEC. Although eNOS activity was not affected (Fig. 4A), superoxide anion was generated by low-dose MEN (Fig. 4B). Hydrogen peroxide was not produced, a significant difference from the effect of high-dose MEN in smooth muscle cells. To confirm whether the superoxide anion produced by low-dose MEN could generate peroxynitrite resulting in the decrease of NO availability in effect. In accordance with these results, SOD treatment attenuated the vessel contraction induced by low-dose MEN (Fig. 4D).

Combining the results, effects of MEN on agonist-induced vasoconstriction can be summed up to a U-shaped dose response as shown in Figure 5A. The stimulation of vasoconstriction observed at low doses was from the dysfunction of endothelium, mediated by generation of superoxide anion and subsequent quench of NO through peroxynitrite formation. On the other hand, disruption of smooth muscle cell contraction was involved in the inhibition of vasoconstriction at high doses of MEN, resulting from the generation of hydrogen peroxide and decrease of agonist-induced calcium increase and phosphorylation of MLC (Fig. 5B). U-shaped dose response in confocal microscopy. The formation of 3-nitrotyrosine in HAEC was significantly increased by low-dose MEN with a similar extent to SIN-1, a peroxynitrite generator and at the same time reversed by SOD treatment (Fig. 4C), suggesting that superoxide anion produced by low-dose MEN could generate peroxynitrite resulting in the decrease of NO availability in effect. In accordance with these results, SOD treatment attenuated the vessel contraction induced by low-dose MEN (Fig. 4D).

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agonist-induced vasoconstriction has been often observed with other chemicals. Arsenite, an environmental contaminant, also showed a similar pattern to MEN in in vitro aortic ring systems, endothelium intact and endothelium denuded (Fig. 5C) possibly from the mixed effect of dysfunctions of endothelial cells (Lee et al., 2003) and smooth muscle cells. To demonstrate that these U-shaped dose responses can be reproduced in vivo, we evaluated the effects of arsenite or MEN on blood pressure changes in rats. Arsenite showed a strong trend of U-shaped response with a good correlation to in vitro data (Figs. 6A and 6B) confirming that this kind of U-shaped dose response can actually take place in the body. Enhancing effects of MEN on blood pressure could also be shown in vivo although suppressive effects could not be demonstrated due to the lethal toxicities of high-dose MEN (Fig. 6A). The patterns of the dose–responses in Figures 5A and 6B well matched the criteria of hormesis provided by previous report, low-dose stimulation reaching around 30–60% greater than control and its dose range to be less than 20-fold (Calabrese and Baldwin, 2003).

**DISCUSSION**

U-shaped dose response observed in this study was a manifestation of heterogenic susceptibilities of multiple target cells to a common stimulus. Endothelium, which serves as a regulator of vasomotor tone, was affected primarily by low-dose MEN, whereas smooth muscle cell, which acts as an effector of vascular contraction, was affected later by high-dose MEN. These heterogenic susceptibilities of endothelium and smooth muscle resulted in a paradoxical U-shaped dose response of the blood vessel on the whole, that is hyper-contraction at low-dose and hypocontraction at high-dose MEN. Notably, a similar U-shaped response could be found with arsenic, another environmental pollutant widely known to be involved in various vascular toxicities and also could be reproduced in in vivo PE-induced pressor response model.

ROS can induce cellular dysfunction in blood vessel (Archer et al., 1993; Pou et al., 1992), sometimes resulting in vasoconstriction (Heinle, 1984; Liu et al., 1998; Peters et al., 2000) while sometimes producing vasorelaxation (Davidson et al., 1997).
Depending on the species of reactive oxygen produced and the type of blood vessels affected, ROS differentially regulates vasomotor function. MEN could generate both superoxide anion and hydrogen peroxide in blood vessels and for its potent ROS generation potential, it has been widely employed as an experimental tool to investigate the role of ROS in vascular system (Lee et al., 1999; Rosen and Freeman, 1984). Our result suggests that hydrogen peroxide
generated by high-dose MEN plays a key role in the suppression of vasoconstriction in smooth muscle (Fig. 3), whereas superoxide by low-dose MEN contributed predominantly to the dysfunction of endothelium through NO attenuation (Fig. 4), resulting in enhanced vasoconstriction. Grover et al. (1995) reported that hydrogen peroxide interrupts sarcoplasmic reticulum Ca\(^{2+}\) pump in smooth muscle cells leading to the decrease of angiotensin II–induced vasoconstriction mediated through the disruption of Ca\(^{2+}\) homeostasis, which agrees well with our results suggesting high-dose MEN-induced hydrogen peroxide suppressed Ca\(^{2+}\) mobilization, MLC phosphorylation and smooth muscle contraction (Figs. 2 and 3). On the other hand, through the rapid reaction with NO at 4.3–19 \(\times 10^{-3}\) M/S, superoxide can limit the availability of NO to smooth muscle cells through decreasing the half-life of NO and increased formation of toxic peroxynitrite (Czapski and Goldstein 1995) which accounts for the increased 3-nitrotyrosine formation and vasoconstriction by MEN in the current study (Fig. 4).

Although eNOS inhibition is known to be the major mechanism of the arsenite effects on endothelium, ROS also plays important role in the deleterious effects of arsenite against endothelium and smooth muscle cells (Kumagai and Pi, 2004; Lee et al. 2003), supporting further that ROS generating chemicals might commonly produce U-shaped response in blood vessel. Arsenic is known to produce ROS through upregulation of nicotinamide adenine dinucleotide phosphate (reduced) oxidase in endothelial cells and smooth muscle cells (Qian et al., 2005; Lynn et al., 2000). Arsenite-induced ROS production causes DNA damage, lipid peroxidation, and protein modification as well as alterations of antioxidant defenses, which are associated with the alterations of cell signal transduction (Kumagai and Surni, 2007). Increased ROS generation was also implicated in part in the decreased eNOS activity through reducing cofactor tetrahydrobiopterin and resultant uncoupling of NO synthase. Considering that many examples of ROS generating chemicals could be easily found in therapeutic drugs or natural ingredients, it would be interesting to investigate if other vascular active chemicals exhibiting ROS generating potential could produce similar phenomena as shown in the present study.

The current study gives an important point of view that could be widely applicable to the other cases of U-shaped dose response. In a multicellular system working as a whole like tissues and organs, a component acting as a regulator is to be sensitive enough to detect even a minute change to maintain homeostasis of a system. Due to its sensitivity, it could be an easy target even by a slightest functional perturbation induced by chemicals or certain stimuli. A component working as an effector, on the other hand, is to be more durable and tolerable than the regulator component to sustain the system even in a harsh condition. Accordingly, it should not give way to a minute functional perturbation. Supporting this view in part, there are many cases of endothelial dysfunction (Davignon and Ganz, 2004; Heistad, 2006; Lee et al., 2003; Thomas et al., 1996; Wattanapitayakul et al., 2000), whereas reports on the disruption of smooth muscle function can be hardly found. Another important point derived from the current investigation, is that so-called overcompensatory or paradoxically beneficial effect at low dose in the U-shaped dose responses (Calabrese and Baldwin, 2003) might be a result of impaired regulatory function underlying the importance of detailed evaluation of the lower dose range in the risk assessment. Thayer et al. (2005) also proclaimed this opinion by strongly criticizing the negligence or overlook of lower dose effect of xenobiotic exposure. In this regard, it would be interesting to review the previous studies of U-shaped responses more closely to discriminate some possible involvement of regulatory dysfunction or heterogenic cellular components.

Previously, most of the mechanistic theories of U-shaped responses have been suggested, based on an isolated observation with a unique physiological agonist or a specific chemical, making them hard to be generalized into the explanation of other cases of U-shaped responses. In the present study, same patterns of U-shaped dose response of the vasomotor tone change were induced by two xenobiotics with distinct vascular toxic mechanisms, MEN and arsenic. Although endothelial dysfunction is mediated by different modes of action, that is, reactive oxygen generation by low-dose MEN (Fig. 4) and eNOS inhibition by arsenic (Lee et al., 2003), overall functional modulation displayed similarly as a hypercontraction of blood vessel. Furthermore, this phenomenon could be reproduced by three distinct vascular contractile agonists, PE, endothelin, and 5-HT (Fig. 1C), strongly supporting that U-shaped response from mixed heterogenic response of multiple cells, constituting and working as a tissue could be generally observed regardless of agonists or xenobiotics.

With this study, we believe that an important mechanical explanation and theoretical background have been provided for understanding and elucidation of various U-shaped dose responses by xenobiotics. More practically, our study gives the important evidence for the application of the U-shaped dose relationship into the risk assessment, strongly supporting the needs for the estimation of integrated systemic responses over wider dose ranges of toxicants.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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