Effects of Benzo(a)pyrene on the Contractile Function of the Thoracic Aorta of Sprague-dawley Rats

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Abstract

Objective  To evaluate the possible vascular effects of an environment carcinogen benzo(a)pyrene (BaP).

Methods  The cytotoxicity of BaP and rat liver S9 (0.25 mg/mL)-activated BaP were examined by MTT assay. Thoracic aortic rings were dissected from Sprague-Dawley rats. Contraction of aortic rings was induced by 60 mmol/L KCl or 10⁻⁶ mol/L phenylephrine (PE) in an ex-vivo perfusion system after BaP (100 μmol/L) incubation for 6 h. [Ca²⁺] was measured using Fluo-4/AM. For in-vivo treatment, rats were injected with BaP for 4 weeks (10 mg/kg, weekly, i.p.).

Results  BaP (1-500 μm) did not significantly affect cell viability; S9-activated BaP stimulated cell proliferation. BaP did not affect the contractile function of endothelium-intact or -denuded aortic rings. BaP did not affect ATP-induced ([Ca²⁺]) increases in human umbilical vein endothelial cells. In BaP-treated rats, heart rate and the number of circulating inflammatory cells were not affected. Body weight decreased while blood pressure increased significantly. The maximum aortic contractile responses to PE and KCl and the maximum aortic relaxation response to acetylcholine were significantly decreased by 25.0%, 34.2%, and 10.4%, respectively.

Conclusion  These results suggest, in accordance with its DNA-damaging properties, that metabolic activation is a prerequisite for BaP-induced cardiovascular toxicity.

Key words: DNA damage; Benzo(a)pyrene; Cardiovascular toxicity; Vascular contraction

INTRODUCTION

Agents that damage deoxyribonucleic acid (DNA) can produce alterations in the genetic material of the host. Such agents can be subdivided into direct-acting agents and indirect-acting agents. Direct-acting agents are intrinsically reactive and do not require metabolic activation by cellular enzymes to interact with DNA. Indirect-acting agents require metabolic activation

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by cellular enzymes to form DNA-reactive metabolites. DNA-damaging agents include chemotherapeutic agents and environmental pollutants\textsuperscript{4}.

Benzo(a)pyrene (BaP) is an indirect-acting agent. It has been studied because it is a human carcinogen and because it is distributed widely in the environment. It is one of the most important representatives of polycyclic aromatic hydrocarbons (PAHs). BaP must be metabolically processed by aryl hydrocarbon receptor (AhR)-induced enzymes, such as cytochrome P4501A1 (CYP1A1) and epoxide hydrolase, to form carcinogenic BaP-diol epoxides (BPDEs)\textsuperscript{2}. The binding of BPDEs to DNA leads to the formation of mutations and may initiate carcinogenesis\textsuperscript{1-4}. Besides its DNA-damaging property, BPDE can also activate several signaling pathways, including the phosphatidylinositol 3-kinase (PI-3K)/Akt/mitogen-activated protein kinases (MAPKs)-dependent pathway, leading to the activation of transcription factors such as AF-1 and the increased expression of cyclin D1\textsuperscript{15-16}. As a result, BPDE treatment could induce cell proliferation and the passage of the G1/S checkpoint in the cell cycle, thereby contributing to its oncogenic transformation and tumorigenesis property\textsuperscript{15-16}. Although BaP is metabolized in the liver, BaP exposure does not result in hepatocarcinogenicity, but does cause lung cancer\textsuperscript{17}. Interestingly, studies have shown that chronic exposure of BaP could promote atherogenesis in animal models, possibly due to damage to blood vessels and enhancement of inflammation in vessel walls\textsuperscript{8,11}.

The calcium ion (\(Ca^{2+}\)) has an important role in the regulation of vascular tone, which is, in general, fairly constant. The initiation of contraction in vascular smooth muscle (VSM) is due to a rise in the concentration of free \(Ca^{2+}\) in the cytosol, which is caused by \(Ca^{2+}\) entry via adenosine triphosphate (ATP)-dependent ion channels\textsuperscript{12}. Aortic tension also plays an important role in the regulation of the entire cardiovascular system. Large arteries are involved in the regulation of hydrostatic capillary pressure\textsuperscript{13-14} and blood pressure through alteration of pulsatile pressure\textsuperscript{15}. In addition, arteries show a prominent “cushioning” effect in addition to their conducting function, and this effect is associated with the viscoelastic properties of the arterial wall\textsuperscript{14}. In particular, vasomotor reactions have been reported to be related to plasma volume and helping the maintenance of cardiovascular homeostasis\textsuperscript{13,14}. Although it has been suggested that PAHs are associated with a higher incidence of cardiovascular diseases (including atherogenesis\textsuperscript{17}), the effect of BaP on vascular tone is unclear. Therefore, in the present study, we aimed to evaluate the cardiovascular toxicity of BaP on the contractile function of rat thoracic aortas, and tried to elucidate the possible underlying mechanisms.

\section*{MATERIAL AND METHODS}

\subsection*{Ethical Approval of the Study Protocol}

All procedures were approved by the Ethics Committee for the Use of Experimental Animals of Zhejiang University (Hangzhou, China).

\subsection*{Reagents}

Phenylephrine (PE), acetylcholine (ACH), BaP, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Fluo-4/AM, ATP, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All other chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagents (Shanghai, China). BaP was dissolved in DMSO. The final volume of DMSO was <0.1% of the total experimental volume. All vehicle controls and treated cultures contained the same amount of DMSO. BaP was also dissolved in corn oil at 2 mg/mL for the in-vivo experiment. Current guidelines for use of biohazardous materials in Zhejiang University were followed when using BaP.

\subsection*{Cell Culture}

Human umbilical vein endothelial cells (HUVECs) were grown in 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 37 °C under humidified air (5% CO\textsubscript{2}), and used between passages 2 to 16.

\subsection*{Cytotoxicity Assay}

The toxicity of BaP was examined using the MTT assay. Briefly, HUVECs were seeded into a 96-well culture plate at a density of 1×10\textsuperscript{4} cells/well. 24 h later, the medium was discarded, and replaced with fresh medium containing the indicated concentrations of chemicals. Cells were treated with BaP (1, 5, 10, 100, 200, and 500 \(\mu\)mol/L) for 6 h. By the end of each time point, 20 \(\mu\)L of MTT (5 mg/mL in PBS) was added to each well and incubated for a further 3 h. The solution was then discarded and 150 \(\mu\)L of isopropanol added. After shaking for 10 min to ensure that the formazan had dissolved,
absorbance at 570 nm was read on a Microtiter Plate Reader (BioTek, Winoski, VT, USA). Relative survival was represented as the absorbance of treated sample/absorbance of the control group.

Verify the Metabolic Activation of BaP

BaP was first mixed with rat liver S9 (0.25 mg/mL; Targin Tech, Beijing, China), and then added to HUVECs for 6 h. The MTT assay was conducted as described above.

Animals and Treatment with BaP

Male Sprague-Dawley (SD) rats (230-260 g) were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). Rats were housed in an air-conditioned colony room at 20±2 °C, and food and water were available ad libitum. To detect the effect of BaP on thoracic aortas, rats were injected with BaP (10 mg/kg BW, i.p.) or an equivalent volume of corn oil (i.p.) once a week for 4 weeks (corn oil was the delivery vehicle)\(^9\)\(^{11}\). Peripheral blood was counted by a Hematology Analyzer (ADVIA2120, Bayer, Leverkusen, Germany). Blood pressure and heart rate were measured non-invasively in conscious animals by a tail-cuff non-invasive blood pressure apparatus (BP98A, Softron, Tokyo, Japan). At the end of experiments, rats were sacrificed and thoracic aortic rings prepared as described below.

Preparation of Thoracic Aortic Rings

Rats were anesthetized with 10% chloral hydrate (0.4 g/kg, i.p.) and killed by decapitation. Chests were opened and thoracic aortas rapidly removed. They were placed in a 4 °C Krebs-Henseleit (K-H) solution [mmol/L: NaCl 120.0, KCl 4.5, CaCl\(_2\) 1.25, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, NaHCO\(_3\) 25.0, and glucose 10.0 (pH 7.4)]. After the removal of superficial connective tissue, aortas were cut into rings of length 3-4 mm and subjected to studies on contractile functional.

Measurement of the Contractile Function of Thoracic Aortic Rings

Fresh isolated rings were mounted in 5.0-mL organ baths containing the K-H solution. The bath solution was maintained at 37 °C and bubbled continuously with a mixture of 95% O\(_2\) and 5% CO\(_2\). Aortic rings were equilibrated for 60 min at a resting tension of 2 g. Isometric tension was measured with a Force Isometric Transducer connected to a data-acquisition system (MedLab; Nanjing Medease, Nanjing, China). In some aortic rings, the endothelium was removed mechanically by gentle rubbing with moistened cotton. Removal of endothelial cells was confirmed by the loss of ACh-induced relaxation\(^{19-21}\). After 6 h of pre-incubation with BaP (100 μmol/L), contraction of endothelium-intact and endothelium-denuded aortic rings was induced by KCl (60 mmol/L) or PE (10\(^6\) mol/L). The vascular response was expressed as a ratio of the plateau contraction evoked by KCl or PE\(^{22}\). For in-vivo experiments, cumulative concentration response curves to PE (10\(^{-6}\) mol/L to 10\(^{-4}\) mol/L) and KCl (10 mmol/L to 60 mmol/L) were created in vehicle- and BaP-treated aortic rings. Relaxation responses for Ach (10\(^{-8}\) mol/L to 10\(^{-7}\) mol/L) were expressed as a percentage decrease of the maximum contractile response induced by PE\(^{23}\).

Measurement of Intracellular Ca\(^{2+}\) Concentration

HUVECs grown on coverslips were treated with vehicle or BaP (100 μmol/L) for 6 h. Cells were loaded with the Ca\(^{2+}\) sensitive dye Fluo-4/AM (1 nmol/L) for 30 min at 37 °C in serum-free 1640 medium. After loading, cells were washed thrice with serum-free 1640 medium. Loaded cells were maintained at 37 °C for 15 min before measurement of [Ca\(^{2+}\)]\(_i\), to allow the fluo-4/AM in the cytosol to de-esterify. Cells were placed on the stage of a Nikon Inverted Microscope equipped with a SFX-1 Microfluorimeter (Solamere Technology Group, Salt Lake City, UT, USA). During the experiments, cells were superfused in a 0.3-mL bath chamber at 35 °C under a constant flow (1 mL/min) of HEPES-buffered saline (135 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L CaCl\(_2\), 1 mmol/L MgCl\(_2\), 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.4)\(^{24}\). Cells were stimulated with ATP (10 μmol/L) for 1 min for studying the action of the BaP on ATP-induced increased [Ca\(^{2+}\)]\(_i\). After that, ATP was washed away twice using HEPES-buffered saline. Fluo-4 fluorescence was excited at 470 nm. Emitted flou-4 fluorescence was measured at 520 nm. Changes in [Ca\(^{2+}\)]\(_i\) were reported as the flou-4/AM ratio ΔF/ΔF\(_0\) (where ΔF is the difference between baseline fluorescence intensity and maximal fluorescence intensity after ATP stimulation, and ΔF\(_0\) is the difference between baseline fluorescence intensity and the minimal fluorescence intensity before ATP stimulation).

Statistical Analyses

Data are the mean±SEM. Statistical analyses
were carried out using the Student’s paired t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s t test. Differences were considered significant at \( P<0.05 \).

RESULTS

Effects of BaP on the Contractile Function of the Thoracic Aorta in vitro and [Ca\(^{2+}\)]\text{ii} in HUVECs

The cytotoxicity of BaP was examined by the MTT assay using HUVECs to determine the appropriate dosage for subsequent experimentation. BaP (1-500 \( \mu \)mol/L) did not induce significant cytotoxicity at 6 h (Figure 1). This finding was consistent with our previous reports that the cytotoxicity of BaP is relatively low in various cell types\(^{[25-27]}\).

![Figure 1. Cytotoxicity of BaP and rat liver S9-activated BaP on HUVECs. HUVECs were treated with various doses of chemicals for 6 h, and cell viability measured by the MTT assay. Data are the mean±SEM. Note. * \( P<0.05 \), ** \( P<0.01 \), BaP vs BaP+S9.](image)

We also examined the cytotoxic effect of rat liver S9-incubated BaP on HUVECs. The relative survival increased significantly after S9 activation of BaP at \( >5 \mu \)mol/L (Figure 1). This finding suggested that once BaP was metabolically activated to form BPDE, BPDE could then activate cellular signaling pathways which could stimulate cell proliferation\(^{[5-6,28]}\).

Using an ex-vivo perfusion system, the effects of BaP on the contraction of aortic rings were examined. At 10-200 \( \mu \)mol/L, BaP had no direct effect on the baseline tension of endothelium-intact aortic rings (Table 1). Thus, combined with the cytotoxicity data, 100 \( \mu \)mol/L was chosen as the concentration for further experimentation. In endothelium-intact vehicle aortas, KCl (60 \( \mu \)mol/L) provoked significant contraction with the relative tension as 1 at time 0, and gradually decreased to 0.61±0.08 after 6 h. BaP (100 \( \mu \)mol/L) did not significantly affect KCl (60 \( \mu \)mol/L)-provoked contraction during the 6-h period (Figure 2A). PE (10\(^{-6}\) mol/L) increased the contraction tension to a similar level as that seen with KCl (0.59±0.12) at 6 h, and BaP did not significantly affect PE-induced vascular tension either (Figure 2B).

The endothelium plays an important part in the regulation of vascular tone by releasing factors involved in relaxation and contraction\(^{[29]}\). Therefore, we also tested the effects of BaP on endothelium-denuded aortic rings. In such rings, KCl and PE also increased vascular tension to 0.65±0.07 and 0.36±0.03 at 6 h, respectively (Figure 2A and B). Similar to the endothelium-intact situation, BaP did not change KCl- and PE-induced contraction in endothelium-denuded aortic rings (Figure 2A and B).

<table>
<thead>
<tr>
<th>Table 1. Baseline Tension (g) of Aortic Rings Treated with BaP</th>
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<tr>
<td><strong>Control (n)</strong></td>
</tr>
<tr>
<td>Endothelium-intact</td>
</tr>
<tr>
<td>0.018±0.036(6)</td>
</tr>
<tr>
<td>Endothelium-denuded</td>
</tr>
</tbody>
</table>

*Note. Thoracic aortic rings were incubated in various concentrations of BaP (\( \mu \)mol/L) for 6 h. Baseline tensions (g) of aortic rings were evaluated by ex-vivo perfusion. Data are the mean±SEM.*

[Ca\(^{2+}\)]\text{ii} is also an important factor in the regulation of vascular tone, so the effects of BaP on [Ca\(^{2+}\)]\text{ii} were examined in HUVECs. BaP (100 \( \mu \)mol/L) did not change the [Ca\(^{2+}\)]\text{ii} in HUVECs induced by ATP (3.68±0.16 vs 4.42±0.33) (Figure 2C).

Weight, Blood Pressure, and Heart Rate in BaP-Treated Rats

Direct exposure to BaP for a short period did not affect the contractile function of the thoracic aorta, so we wondered if long-term exposure would show any effects. Therefore, 26 male SD rats received BaP at 10 mg/kg per week or an identical volume of corn oil (i.p.) for 4 weeks. First it was observed that BaP caused a slight decrease in weight (335.00±7.51 g vs 352.20±3.05 g, \( P<0.05 \)) at the end of treatment (4 weeks). There were no significant differences in heart rates between the two groups (data not shown). The
Figure 2. Effects of BaP on the relative tension of the thoracic aorta and [Ca²⁺], in HUVECs. (A) BaP did not affect the relative tension of endothelium-intact (+E) and endothelium-denuded (−E) rat thoracic aortic rings pre-contracted with KCl at a resting tension of 2 g in an ex-vivo perfusion system. (B) BaP did not inhibit the contraction induced by PE in +E and -E rat thoracic aortic rings at 6 h. (C) Effects BaP on ATP-induced changes of [Ca²⁺], in HUVECs. Data are the mean±SEM. The effect of BaP on blood pressure was not significant at 2 weeks of treatment (data not shown). BaP treatment, however, significantly increased blood pressure at the end of 4 weeks. Systolic blood pressure (SBP) was increased in BaP-treated rats by 12.0%, diastolic blood pressure (DBP) by 13.5%, and mean blood pressure (MBP) by 12.9% (P<0.05, compared with the corn oil-treated group) (Figure 3).

To assess possible BaP-induced systemic inflammatory processes we evaluated the numbers of peripheral blood cells. Except for a small decrease in BaP-treated rats with respect to lymphocyte number (7.685±0.88 vs 10.212±0.59 10⁹/L, P<0.05), no significant differences were found in other circulating inflammatory cells between vehicle- and BaP-exposed animals (Table 2).

Effects of Chronic Exposure of BaP on the Contractile Function of the Thoracic Aorta in vivo

After BaP treatment for 4 weeks, the rats were killed and the ex-vivo perfusion experiment done to measure the contractile function of thoracic aortic rings. PE (10⁻⁶ to 10⁻⁴ mol/L) (Figure 4A) and KCl (10-60 mmol/L) (Figure 4B) resulted in concentration-dependent contractions in the aortas of BaP-treated and vehicle-treated rats. In endothelium-intact aortic rings, the maximum contractile responses to PE (10⁻⁵ mol/L) and KCl (60 mmol/L) in control aortas reached 0.89±0.08 g and 1.22±0.06 g, whereas the vascular tension in BaP-treated aortas reached only 0.69±0.06 g (22.4% inhibition) and 1.19±0.06 g (29% inhibition), respectively. However, these differences were not statistically significant. In endothelium-denuded aortic

![Figure 3](image-url)  
Figure 3. Effects of BaP on systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial blood pressure (MBP) in SD rats after 4 weeks of treatment. Data are the mean±SEM. Note. *P<0.05 vs control.

Table 2. Analyses of Peripheral Blood Cells (10⁹/L) in SD Rats by BaP Treatment

<table>
<thead>
<tr>
<th></th>
<th>WBC</th>
<th>LYMPH</th>
<th>MONO</th>
<th>NEUT</th>
<th>RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=4)</td>
<td>10.955±0.95</td>
<td>7.685±0.88</td>
<td>0.395±0.19</td>
<td>2.77±1.16</td>
<td>8.4±0.22</td>
</tr>
<tr>
<td>BaP (n=5)</td>
<td>12.872±0.74</td>
<td>10.212±0.59</td>
<td>0.256±0.04</td>
<td>2.244±0.32</td>
<td>8.232±0.17</td>
</tr>
</tbody>
</table>

Note. Data are expressed as mean±SEM. *P<0.05 vs control. WBC: white blood cells; LYMPH: lymphocytes; MONO: monocytes; NEUT: neutrophils; RBC: red blood cells.
rings, the maximum contractile responses to PE (10⁻⁵ mol/L) and KCl (60 mmol/L) were reduced by 25.0% (1.11±0.13 g vs 1.48±0.08 g, P<0.05) and by 34.3% (0.87±0.10 g vs 1.33±0.10 g, P<0.05) after BaP-treatment.

In addition, ACh resulted in concentration-dependent relaxations in all aortic rings pre-contracted with PE (Figure 4C), but endothelium-dependent relaxation responses were inhibited in BaP-treated rats. The difference was statistically significant at high concentrations of 10⁻⁴ (0.78±0.01 vs 0.87±0.02, P<0.05) and 10⁻³ mol/L (0.82±0.01 vs 0.91±0.02, P<0.05).

**Figure 4.** Effects of long-term exposure to BaP on the contractile function of aortic rings. (A) Cumulative concentration-response curves of endothelial-intact (+E) or endothelial-denuded (-E) aortic rings to PE in BaP-treated and control SD rats. (B) Cumulative concentration-response curves of endothelial-intact +E and endothelial-denuded-E aortic rings to KCl in BaP-treated and control SD rats. (C) BaP inhibited the endothelium-dependent relaxation of ACh on thoracic aortic rings. Data are the mean±SEM. Note. P<0.05 compared with control.

**DISCUSSION**

In the present study, by measuring the contractile function of thoracic aortas in an ex-vivo perfusion system, we examined the cardiovascular effects of the DNA-damaging agent BaP, a ubiquitous environmental pollutant known to cause many diseases and which is implicated in atherosclerosis[8,9,17]. The major findings of the present study were: (i) that BaP did not have a significant effect on the contractile function of the thoracic aorta in vitro; and (ii) in-vivo exposure of BaP affected the contractile function of the thoracic aorta.

First it was shown that BaP had no direct effects on the contraction of thoracic aortas induced by KCl and PE, nor on the [Ca²⁺] in HUVECs induced by ATP (Figures 2A-C). This could be because BaP functions as an indirect-acting agent, requiring metabolic activation by cytochrome P₄₅₀ 1A and 1B enzymes to exert its deleterious effects in the organism[17]. The S9 experiment also supported this notion because it showed that cell proliferation was increased only after the metabolic activation of BaP (Figure 1). To further test this possibility, BaP was administered once a week for 4 weeks in SD rats. As predicted, the in-vivo experiments demonstrated that BaP not only caused a decrease in tensions of the aortic rings induced by KCl and PE, but also affected the endothelium-dependent diastolic function of aortas in their response to Ach (Figure 4). This finding indicated that, after in-vivo metabolic activation, BaP induced cardiovascular toxicity and affected the normal mediation of vascular tone.

Furthermore, the elevation in blood pressure coincided with the induction of cardiovascular toxicity in BaP-treated rats. Blood pressure is regulated in part through changes in vascular tone. Therefore, increased blood pressure with a normal heart rate further illustrates BaP-induced injury in the vascular wall. Our in-vivo experiments also revealed that the deleterious effects of BaP on thoracic aortas were not primarily the consequences of systemic inflammation because BaP showed only a minimal effect on the number of circulating inflammatory cells (Table 2).

Based on the information provided above, it is clear that BaP must be metabolized in vivo for it to cause cardiovascular toxicity, just as is the case for the induction of DNA damage in tissues/cells. Although cigarette smoke contains >4 000 toxic agents, PAHs (as represented by BaP) are probably...
the leading cause of the formation of DNA adducts by cigarette smoke.[30] Studies have demonstrated that whole-body exposure of SD rats to cigarette smoke for 28 consecutive days induced DNA adducts in the entire thoracic aorta.[31] Bulky DNA adducts have also been found in atherosclerotic lesions in cigarette-smoking humans.[32-34] Therefore, the formation of DNA adducts in the thoracic aorta may be one of the reasons why BaP affects the contractile function of aortic rings. Unfortunately, we did not examine the formation of BaP-DNA adducts, and further experiments are required to prove the presence of such adducts. Other mechanisms might also be involved. For example, it has been shown that BaP significantly suppresses the expression of prolyl 4-hydroxylase (P4Ha), an enzyme responsible for the formation of 4-hydroxyproline in mature functional collagen, thus playing a major role in remodeling of the arterial wall and tension regulation.[35] Another possible reason is that BaP can repress T-cadherin expression in VSM cells, which relates to changes in phenotypic status in smooth muscle cells,[36], and therefore could lead to changes in the contractile function of the thoracic aorta. However, these possible mechanisms require the support of experimental evidences, and thus are the subject of new studies in our research team.

In conclusion, we showed that the DNA-damaging agent BaP can affect the cardiovascular system, as evidenced by the impairment of the contractile function of the thoracic aorta. In addition, the mode of action of BaP (i.e., requirement for metabolic activation), probably contributes to the effects on the thoracic aorta. Recently, we also examined the cardiovascular effects of cisplatin (a first-line chemotherapeutic drug for many types of cancer), which directly damaged DNA without the perquisite of metabolic activation.[37]. Preliminary results showed that, unlike BaP, cisplatin directly affected the contractile function of the thoracic aorta. Therefore, it seems that some DNA-damaging agents can cause potential cardiovascular complications, and thus the cardiovascular toxicity of DNA-damaging agents should be considered in future studies.

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