Doxorubicin-induced vasomotion and $[\text{Ca}^{2+}]_i$ elevation in vascular smooth muscle cells from C57BL/6 mice

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Aim: To explore the action of doxorubicin on vascular smooth muscle cells.

Methods: Isometric tension of denuded or intact thoracic aortic vessels was recorded and $[\text{Ca}^{2+}]_i$ in isolated aortic smooth muscle cells was measured by using Fluo-3.

Results: Doxorubicin induced phasic and tonic contractions in denuded vessels and increased levels of $[\text{Ca}^{2+}]_i$ in single muscle cells. Treatment with 10 µmol/L ryanodine had no effect on basal tension, but it did abolish doxorubicin-induced phasic contraction. Treatment with 10 mmol/L caffeine induced a transient phasic contraction only, and the effect was not significantly altered by ryanodine, the omission of extracellular Ca$^{2+}$ or both. Phenylephrine induced rhythmic contraction (RC) in intact vessels. Treatment with 100 µmol/L doxorubicin enhanced RC amplitude, but 1 mmol/L doxorubicin abolished RC, with an increase in maximal tension. Caffeine at 100 µmol/L increased the frequency of the RC only. In the presence of 100 µmol/L caffeine, however, 100 µmol/L doxorubicin abolished the RC and decreased its maximal tension. Treatment with 10 µmol/L ryanodine abolished the RC, with an increase in maximal tension. In Ca$^{2+}$-free solution, doxorubicin induced a transient $[\text{Ca}^{2+}]_i$ increase that could be abolished by ryanodine pretreatment in single muscle cells. The doxorubicin-induced increase in $[\text{Ca}^{2+}]_i$ was suppressed by nifedipine and potentiated by ryanodine and charybdotoxin.

Conclusion: Doxorubicin not only releases Ca$^{2+}$ from the sarcoplasmic reticulum but also promotes the entry of extracellular Ca$^{2+}$ into vascular smooth muscle cells.

Keywords: doxorubicin; rhythmic contraction; sarcoplasmic reticulum; vascular smooth muscle
or species[12–15]. In a previous study, Jiang et al found that an α-adrenergic receptor agonist, phenylephrine (PE), can evoke spontaneous RC in isolated intact aortas of C57BL/6 mice, and that this PE-induced RC is related to RyR of the SR of vascular smooth muscle cells[10].

In the present study, we used fresh isolated aortic vessel rings and single cells to investigate the effects of doxorubicin on vessel tension and [Ca²⁺], elevation. PE-induced RC was also compared to address the effects of doxorubicin on RyR of the SR of vascular smooth muscle cells.

Materials and methods
Animals and tissue preparation
Male C57BL/6 mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg) together with sodium heparin (100 IU/kg, ip), to prevent intravascular coagulation. The thoracic aorta was quickly dissected free and placed in Krebs Henseleit solution, which consisted of (in mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ (7 H₂O) 1.2, NaHCO₃ 25.2 and glucose 11.1. By means of a dissecting microscope, adhering perivascular tissue was carefully removed, and the descending thoracic aorta was cut into 2 mm-long rings. All experiments complied with national guidelines and were approved by the Institutional Animal Care and Use Committee.

Tension measurement
The vessel rings with or without endothelium were mounted onto two thin stainless steel holders, one of which was connected to a force displacement transducer and the other to a movable device that allowed the application of passive tension from 550 to 600 mg, a range that was determined to be the optimal resting tension for obtaining maximal active tension induced by a 60 mmol/L K⁺ solution. The mounted rings were kept in 2-mL organ baths containing the Krebs Henseleit solution, kept at 37 °C and continuously bubbled with a gas mixture of 95% O₂ and 5% CO₂ to maintain a pH of 7.4. The isometric tension was recorded and analyzed by a data acquisition and analysis system (BL-420E+, Chengdu Technology & Market Corp). After an equilibration period of 60 min, the contractile function of the vessel was tested twice by replacing the Krebs Henseleit solution with Ca²⁺-free PSS containing type IIA collagenase 2 g/L, papain 0.5 g/L, ovalbumin 5 g/L, and 1,4-dithiothreitol 1.75 g/L, at 37 °C for 35 min. After being washed twice in Ca²⁺-free PSS for 10 min, the tissue masses were dispersed with a Pasteur pipette using gentle up-down force, and the cell suspension was kept at 4 °C.

Measurement of [Ca²⁺]
In order to measure [Ca²⁺], the cells were seeded onto the coverslip of the 2-mL chamber, and 20 min later they were incubated in Ringer’s solution (in mmol/L) NaCl 135, KCl 5.6, MgCl₂ 1, CaCl₂ 2, HEPES 10, and glucose 10, pH 7.4 with NaOH containing 5 µmol/L acevolymethyl ester of Fluo-3 and 2.5 mg/L pluronic acid for 20 min[59]. Consequently, the fluorescence was scanned with a Leica TCS SP2 laser-scanning confocal microscope (Leica Microsystems AG, Germany), and the average fractional fluorescence (F/F₀) of the acquisition area (5 s) was measured ratiometrically (488:520 nm) using an LCS quantitative fluorescence measurement program (Leica Confocal Software, Germany) at room temperature (22–23 °C). Only the spiral-shaped and well-attached cells were selected for the experiment.

Drugs
Type IIA collagenase, papain, ovalbumin, phenylephrine, doxorubicin, caffeine, nifedipine, charybdotoxin (Sigma Chemical, St Louis, MO, USA), ryanodine (Wako Pure Chemical Industries, Japan), acetoxymethyl ester of Fluo-3, and pluronic acid (Molecular Probes, Eugene, USA) were dissolved in distilled water or Ca²⁺-free PSS. All subsequent dilutions were made with Krebs Henseleit solution. Similar dilutions of the solvents into the Krebs Henseleit solution were used as controls and had no effect on either the basal tension or the evoked tension of the vessel. All concentrations given are the final molar concentrations in the organ chambers.

Statistics
Data were expressed as means±SEM. The two-tailed paired-samples t-test was used to compare results in treated and untreated aortas, and the two-tailed independent samples t-test was used to compare the results in different groups. Differences were considered significant with a value of P<0.05. In [Ca²⁺], measurements, n represents the number of experiments.

Results
Effects of doxorubicin, ryanodine, and caffeine on vasomotion in denuded aortic vessels
Doxorubicin 10 µmol/L induced a small contraction (8±1.0 mg, n=4 from 4 mice), but 100 µmol/L doxorubicin induced a large two-phase contraction, i.e., an initial phasic contraction (21.2±2.1 mg, n=6 from 5 mice) and a later tonic contraction (40±5.6 mg, n=6 from 5 mice, Figure 1B) in denuded aortic
vessels. In a different experimental group, in which the denuded vessels were pretreated with 10 µmol/L ryanodine for 10 min, there was no significant induction of contraction per se; the initial phasic contraction was abolished but the later tonic contraction that was induced by 100 µmol/L doxorubicin increased robustly (406±38 mg, n=6 from 6 mice, P>0.01 vs 100 µmol/L doxorubicin alone, Figure 1C). However, replacement of the physiological bath solution with Ca²⁺-free bath solution had no significant effect on the initial phasic contraction (19±2.4 mg, n=6 from 5 mice, P>0.05), but it did abolish the later tonic contraction induced by 100 µmol/L doxorubicin (Figure 1D). Furthermore, pretreatment of the denuded vessels with 10 µmol/L ryanodine in Ca²⁺-free bath solution abolished both the phasic contraction and the tonic contraction induced by 100 µmol/L doxorubicin (Figure 1E).

Treatment with 10 mmol/L caffeine induced a transient contraction only (223±18 mg, n=6 from 6 mice), and this effect was not significantly affected by removal of Ca²⁺ from the bath solution (230±21 mg, n=6 from 6 mice), pretreatment of the denuded vessels with 10 µmol/L ryanodine (236±24 mg, n=6 from 6 mice), or both (225±19 mg, n=6 from 5 mice; Figure 2).

**Effects of doxorubicin, caffeine, and ryanodine on PE-induced RC in intact aortic vessels**

Spontaneous RC occurred 3–5 min after exposure to 10 µmol/L PE in intact aortas. Approximately 10 min after PE-induced RC, administration of 100 µmol/L doxorubicin significantly enhanced the amplitude of PE-induced RC and also resulted in a transient decrease in maximal tension (Figure 3A and Table 1). Furthermore, this increased amplitude of PE-induced RC was below the maximal tension before doxorubicin treatment. Therefore, the vessel tension spent more time below the maximal tension. In other words, doxorubicin at a 100 µmol/L concentration just enhanced vessel relaxation. In the caffeine group experiments, approximately 10 min after the occurrence of PE-induced RC in the intact aortic vessels, administration of 100 µmol/L caffeine did not significantly affect the amplitude of PE-induced RC, and it slightly increased the maximal tension, possibly due to the release of Ca²⁺ from the SR; however, it increased its frequency nota-
bly (from 1.01±0.17 to 1.58±0.15 per minute, n=6 from 6 mice; Figure 4A). Thirty minutes after washing out the caffeine, repeated application of PE elicited RC similar to that before the administration of caffeine (Figure 4B). In another experimental group, pretreatment of the intact vessels with 100 µmol/L caffeine for 10 min accelerated the occurrence of PE-induced RC, and subsequent administration of 100 µmol/L doxorubicin abolished PE-induced RC and significantly diminished its maximal tension (Figure 4C, Table 1).

In the ryanodine group experiments, approximately 10 min after the occurrence of PE-induced RC in the intact aortic vessels, administration of 10 µmol/L ryanodine induced dual effects on PE-induced RC: the amplitude of PE-induced RC, a measure of capacity to improve vessel relaxation, was temporarily enhanced at first (from 11.6±1.2 to 17.3±2, n=6 from 6 mice, P<0.05), and then PE-induced RC was abolished (Figure 5A and Table 1). Furthermore, in another group, pretreatment with 100 µmol/L ryanodine also abolished PE-induced RC (dashed line shown in Figure 5C). Thirty minutes after washout, the vessel tension returned to baseline, and repeated application of PE significantly augmented the amplitude of PE-induced RC; however, this enhanced RC was not found in the parallel control group (Figure 5B). This enhanced RC resulted in the vessel tension spending more time below maximal tension, therefore enhancing vessel relaxation (Figure 5C).

Doxorubicin increases cell [Ca^{2+}]

To further investigate the effect of doxorubicin on vascular smooth muscle cells, we employed [Ca^{2+}]i measurement with fast Ca^{2+} indicator Fluo-3/AM in freshly isolated aortic smooth muscle cells of mice. Figures 6A and B show that 10 µmol/L doxorubicin induced a small [Ca^{2+}]i elevation, and 100 µmol/L doxorubicin significantly enhanced [Ca^{2+}]i in the aortic smooth muscle cells. Exposure of the cells to 10 µmol/L ryanodine for 10 min strongly enhanced doxorubicin-induced Ca^{2+} influx (Figure 6C). When the cells were presented in Ca^{2+}-free solution, doxorubicin only induced a transient elevation of [Ca^{2+}]i (Figure 6D). With pretreatment of the cells with 10 µmol/L ryanodine in Ca^{2+}-free solution, the doxorubicin-induced increase in [Ca^{2+}]i was abolished (Figure 6E).

Considering the linkage between RyR, Ca^{2+}-activated K+(K_{Ca}) channels, and voltage-dependent Ca^{2+} channels, the corresponding inhibitors were used to verify involvement in the doxorubicin-induced increase in [Ca^{2+}]i. The cells were pretreated with the voltage-dependent Ca^{2+} channel inhibitor nifedipine (1 µmol/L) for 10 min. Treatment with 100 µmol/L doxorubicin was still capable of inducing an initial transient and later sustained increases in [Ca^{2+}]i, but the later maximal [Ca^{2+}]i increase was notably suppressed compared with the increase with doxorubicin treatment alone (Figure 7A). More interestingly, in another group, pretreatment with K_{Ca} chan-

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**Table 1.** Effects of doxorubicin, caffeine and ryanodine on the maximal tension and the amplitude of PE-induced rhythmic contraction (RC). The rhythmic contraction was induced by 10 mol/L phenylephrine (PE). n=6–8. Values are mean±SEM of n experiments from different mice. *P<0.05, **P<0.01 vs control.

| Groups | n | Maximal tension (MT, mg) | Amplitude of RC (% of control MT) |
|--------|---|-------------------------|----------------------------------|
| Control | 6 | 577±28                  | 9.9±0.9                          |
| Doxorubicin (100 μmol/L) | 6 | 515±26b                 | 14±1.1b                          |
| Control | 6 | 585±42                  | 9.7±1.3                          |
| Doxorubicin (1 mmol/L) | 6 | 774±27b                 | 0±0b                             |
| Control | 6 | 589±25                  | 10.2±1.5                         |
| Caffeine (100 μmol/L) + doxorubicin (100 μmol/L) | 6 | 210±29j                 | 0±0c                             |
| Control | 8 | 557±18                  | 9.6±1.1                          |
| Ryanodine (10 μmol/L) | 8 | 676±45s                 | 0±0c                             |
| Washout of ryanodine (10 μmol/L) | 8 | 780±78c                 | 23.5±2.1c                        |
nel inhibitor charybdotoxin (ChTX, 100 nmol/L) markedly enhanced the doxorubicin-induced increase in [Ca\textsuperscript{2+}] in vascular smooth muscle cells (Figure 7B).

**Discussion**

[Ca\textsuperscript{2+}] concentration is an important factor for the initiation and control of the contraction of vascular smooth muscle, and it is regulated by both the entry of extracellular Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+}-permeable non-selective cation channels such as transient receptor potential (TRP) channels on the cell membrane and the release of Ca\textsuperscript{2+} from SR. The Ca\textsuperscript{2+} released from SR seems to be mainly contributed to initial phasic contraction, while the Ca\textsuperscript{2+} that enters through voltage-dependent Ca\textsuperscript{2+} channels and/or Ca\textsuperscript{2+}-permeable non-selective cation channels on the cell membrane contributes to later tonic contraction\cite{17–19}.

In the present study, 100 µmol/L doxorubicin was shown to induce both phasic and tonic contractions in the denuded vessels. Treatment with 100 µmol/L doxorubicin was also shown to increase the [Ca\textsuperscript{2+}], of the aortic smooth muscle cells as measured by the application of Fluo-3, which is consistent with the results from the vasomotion experiments. Furthermore, doxorubicin-induced two-phase contractions and [Ca\textsuperscript{2+}], elevation were notably blocked by ryanodine and omission of Ca\textsuperscript{2+} from the bath solution, respectively. In addition, doxorubicin-induced [Ca\textsuperscript{2+}], elevation was also suppressed by a voltage-dependent Ca\textsuperscript{2+} channel inhibitor and reversely potentiated by blocking RyR and K\textsubscript{Ca} channels. Taken together, these results indicate that doxorubicin is capable of inducing Ca\textsuperscript{2+} release from SR and promoting extracellular Ca\textsuperscript{2+} influx. Interestingly, the later effect of doxorubicin has also been observed in myocardium\cite{17–19}.

Unlike treatment with doxorubicin (100 µmol/L), 10 mmol/L caffeine induced only a transient contraction in the denuded vessels, either in normal bath solution or in Ca\textsuperscript{2+}-free bath solution, and its effects were not significantly affected by
ryanodine (10 µmol/L). These phenomena imply that caffeine does not directly influence extracellular Ca^{2+} influx.

Among the three agents examined in the present study, 10 µmol/L ryanodine failed to induce both vessel contraction and an increase in the transient [Ca^{2+}]; of the cells. The data from previous studies indicate that the effects of ryanodine on vessel contraction and transient [Ca^{2+}], are determined by at least three factors, ie, the type of vessel, the basal tension of the vessel, and the concentration of ryanodine. Significant ryanodine-induced vessel contraction and/or increase in transient [Ca^{2+}], have been observed in dog cerebral arteries with basal tone at 1–100 µmol/L \[20\], in fetal rabbit distal (but not proximal) pulmonary artery smooth muscle cells at 50 µmol/L \[21\], and in rat femoral arteries at 30 nmol/L–30 µmol/L \[22\]; the absence of an effect of ryanodine on vessel contraction and/or transient [Ca^{2+}], has been shown in the ferret portal vein at a dose of 10 µmol/L \[17\], in the aorta of C57BL/6 mice at 10 µmol/L \[23\], and in rat aortas at 10 µmol/L \[23\]. Therefore, one should be very cautious to compare the effects of ryanodine on the vasomotion and/or transient [Ca^{2+}], of cells and to explain the interaction between ryanodine and other inhibitors of RyRs in different vascular smooth muscles.

In vascular smooth muscle cells, three types of RyR isoforms, RyR1, RyR2, and RyR3, have been identified so far \[24\], and experimental data show that RyR1 and RyR2 contribute to Ca^{2+} release underlying a single spark, whereas RyR3 down-regulates the activity of RyR1 and RyR2 by controlling the basal Ca^{2+} frequency \[24, 25\]. Moreover, growing evidence demonstrates that [Ca^{2+}], released from the SR of vascular smooth muscle cells contributes not only to the regulation of contraction but also to the regulation of relaxation. Ca^{2+}-dependent relaxation is mediated by discretely localized Ca^{2+} release events through RyR channels in the SR of the cells. These local increases in Ca^{2+} concentration in close proximity to the cell membrane, termed sparks, stimulate nearby Kv1 channels, causing spontaneous transient outward K+ currents, the hyperpolarizing currents that close voltage-dependent Ca^{2+} channels and decrease global [Ca^{2+}], and vasoconstriction \[26, 27\].

An earlier study demonstrates that PE-induced RC is a good model for indirect observation of Ca^{2+}-dependent relaxation \[10\]. Therefore, in this study, we compared the effects of doxorubicin on PE-induced RC with those of ryanodine and caffeine.

Depending on concentration, ryanodine exhibits two opposite effects on RyR channels. In the range of nanomolar to micromolar, ryanodine activates RyR channels, inducing Ca^{2+} release from SR, however, at higher concentrations, (micromolar to millimolar), ryanodine may block RyR channels, inhibiting Ca^{2+} release \[28\]. The data from this study showed that 10 µmol/L ryanodine abolished PE-induced RC, as observed in earlier studies \[10, 13\]. Surprisingly, after washout of ryanodine, repeated administration of PE elicited much greater RC to enhance vessel relaxation than that of the previous application of PE in the absence of ryanodine. These phenomena imply that: (1) ryanodine, at very high concentrations of 10 µmol/L, blocks all subtypes of RyRs equally so that the Ca^{2+} sparks in the cells and RC of the vessel do not occur in the presence of ryanodine \[24\], (2) after washout of ryanodine with normal solution, a residual effect of ryanodine modifies RyR1–2 to be more sensitive to Ca^{2+}. Repeated application of PE after washout of ryanodine therefore induces more Ca^{2+} release from the SR and subsequently enhances the opening of Kv1 channels and relaxation of the vessel. A recent study has demonstrated that ryanodine is able to increase the sensitivity of RyR2 to Ca^{2+} activation by approximately 1000-fold \[28\].

As observed in rat aortas \[13\], our results showed that 100 µmol/L caffeine did not influence the amplitude of PE-induced RC but increased its frequency and that, after washing out the caffeine, repeated administration of PE elicited RC similar to that of the previous application of PE in the absence of caffeine; this suggests that caffeine, unlike ryanodine, may only activate RyR1, but does not change their sensitivity to Ca^{2+}. An earlier study targeting RyR3 by the application of the antisense oligonucleotides has demonstrated that the deletion of RyR3 does not affect caffeine-induced responses in the vascular smooth muscle cells \[29\].

Because PE-induced RC is directly related to Ca^{2+} release from SR, the finding that 100 µmol/L doxorubicin enhanced the amplitude of PE-induced RC to improve vessel relaxation in association with a transient decrease in the maximal tension suggests that 100 µmol/L doxorubicin mainly promotes Ca^{2+} release from SR to regulate PE-induced RC. By contrast, 1 mmol/L doxorubicin abolished PE-induced RC, with an increase in the maximal tension, implying that 1 mmol/L doxorubicin perhaps induced extracellular Ca^{2+} entry strongly and inhibited PE-induced RC owing to a higher vessel contraction effect. Furthermore, the finding that 100 µmol/L doxorubicin abolished PE-induced RC and inhibited the maximal tension in the presence of 100 µmol/L caffeine indicates that doxorubicin has a synergic effect with caffeine to impair/deplete Ca^{2+} stores. Together, these data imply that doxorubicin may only activate RyR1–2 with caffeine. It is definitely necessary to probe the differences in receptor type and affinity for the three agents in future studies.

The results of our study show that the mechanism by which doxorubicin moderates [Ca^{2+}], is complicated. Even when voltage-dependent Ca^{2+} channels were inhibited with nifedipine, 100 µmol/L doxorubicin was still able to stimulate an initial transient and to sustain later increases in [Ca^{2+}]. Moreover, if the Ca^{2+} store was depleted by thapsigargin, an inhibitor of SR Ca^{2+} ATPase, doxorubicin could still induce an increase in [Ca^{2+}], (data not shown). These findings indicate that doxorubicin can also induce Ca^{2+} influx by activating Ca^{2+}-permeable non-selective cation channels besides voltage-dependent Ca^{2+} channels. On the one hand, it is well known that non-selective cation channels can also permeate monovalent Na+ ions to depolarize the membrane. On the other hand, to date, there is no evidence showing that doxorubicin can directly activate voltage-dependent Ca^{2+} channels. Thus, it is possible that doxorubicin indirectly activates voltage-dependent Ca^{2+} channels by enhancing non-selective cation channels, leading to membrane depolarization. Our data also show that doxorubicin-induced [Ca^{2+}], increases were markedly enhanced if the
cells were pretreated with 10 µmol/L ryanodine or ChTX. The results suggest that 100 µmol/L doxorubicin may stimulate RyR channels to activate K简单 channels, inducing membrane repolarization via close voltage-dependent Ca简单 channels. This pathway forms a negative feedback loop to avoid higher [Ca简单]i loading. However, at very high concentrations of doxorubicin, Ca简单 influx will have a dominant effect, leading to a huge increase in [Ca简单]i. Indeed, further work is needed to clarify the mechanism of doxorubicin’s effects on [Ca简单]i, in vascular smooth muscle cells.

In conclusion, 100 µmol/L doxorubicin induced both phasic and tonic contractions of the vessels by enhancing initial and later [Ca简单]i; these effects were abolished by ryanodine and the depletion of Ca简单 from the bath solution, respectively. These results indicate that doxorubicin is able to induce Ca简单 release from the SR of vascular smooth muscle cells and promote the entry of extracellular Ca简单 via voltage-dependent Ca简单 channels and non-selective cation channels. The mechanism by which doxorubicin triggers Ca简单 release from the SR of vascular smooth muscle cells is completely different from that of ryanodine or caffeine because both ryanodine and caffeine only act on the SR, without inducing Ca简单 entry, and ryanodine has dual effects on RyR channels.

In addition, the present study indicates that vasoconstrictor-induced RC is a useful model for the indirect observation of changes in the activity of the SR.

Acknowledgements
This project was supported by the National Natural Science Foundation of China (No 30070873).

Author contribution
Bing SHEN, Kai-he YE, Chun-ling YE, and Jia-hua JIANG designed the study; Bing SHEN, Kai-he YE, and Lan ZHUANG performed the experiments; Bing SHEN, Kai-he YE, Chun-ling YE analyzed the data; Bing SHEN and Jia-hua JIANG wrote the manuscript.

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