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Enhanced vasocontraction of rat tail arteries by toxoflavin

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1 It has been suggested that the toxic effect of toxoflavin (TXF) produced by *Pseudomonas cocovenenas* is mainly due to the impairment of electron transfer of the mitochondrial respiratory chain. However, the cardiovascular effect of TXF is unknown. In the present study, the effect of TXF on the isometric contraction of rat isolated tail artery strips and the underlying mechanisms were investigated.

2 The basal force of the tissues was not affected by the toxin. However, the application of TXF before or during KCl (60 mM) stimulation potentiated KCl-induced vasocontraction, specifically the tonic phase of the contraction.

3 When the vessel strips were precontracted with phenylephrine (Phe), TXF further enhanced the tonic contraction of the tissue. Pretreatment of tissues with TXF also potentiated subsequent vasocontraction induced by Phe. The vasocontractor effects of TXF and Phe, however, were not additive.

4 The vascular effect of TXF was not mediated by oxygen-derived free radicals since catalase and SOD did not affect TXF-enhanced vasocontraction. In contrast, the vasocontractor effect of TXF was dependent on extracellular Ca^{2+} and abolished by nifedipine (a Ca^{2+} antagonist). TXF also had no effect on caffeine- or U46619-induced vasocontraction.

5 It is suggested that TXF may potentially contract blood vessels via its effect on Ca^{2+} channels. This effect of TXF depends on the contractile status of the vascular tissues.

Keywords: Toxoflavin; vascular smooth muscle; contraction; phenylephrine; calcium channel; oxygen-derived free radicals

Introduction

Consumption of fermented corn meal, banana, and coconut often causes food poisoning due to contamination with *Pseudomonas cocovenenas* (Zhao *et al.*, 1990). Toxoflavin (TXF) and bongkrekic acid are two toxins produced by *Pseudomonas cocovenenas* and responsible for the mortality and morbidity of *Pseudomonas cocovenenas* poisoning (van Damme *et al.*, 1960). TXF, known as xanthothricin, can also be produced by a culture of the genus *Streptomyces* (Machlowitz *et al.*, 1954). That the same toxin has two different biological sources signifies a biological function of TXF. It has been suggested that the toxic effect of TXF may result from the formation of hydrogen peroxide (Latuasan & Berends, 1961). Particularly, TXF may act on iron-sulphur cluster in NADH-Q oxidoreductase, participating or promoting the Fenton reaction (Koppenol, 1993; Giulivi *et al.*, 1995) to generate H_2O_2 (Xu *et al.*, 1990). Due to the lack of knowledge on the spectrum of biological effects of TXF as well as the relative mechanisms, no effective detoxicating method is yet available for TXF poisoning.

An increased generation of oxygen-derived free radicals (OFR) and a simultaneously decreased production of antioxidants, such as superoxide dismutase (SOD) and vitamin E, have been suggested as being involved in human essential hypertension (Kumar & Das, 1993). OFR may act directly on vascular endothelium (Wu *et al.*, 1992) and smooth muscle cells (SMC). In the former case, OFR may inactivate endothelium-generated nitric oxide (NO) (Ikeda *et al.*, 1994) or impair the production of NO (Seccombe *et al.*, 1994). In the latter case, it has been shown that hydroxyl radicals generated by metal ions plus hydrogen peroxide contracted single SMC isolated from the basilar artery of the rat (Steele *et al.*, 1991). Assuming that the biological effects of TXF were due to the generation of OFR, TXF should be able to induce vasocontraction and to increase blood pressure. However, the vascular effect of TXF and the underlying mechanisms are unknown to date. In the present study, the vascular effect of TXF was studied in rat isolated tail artery strips, precontracted with either KCl or phenylephrine. It was found that TXF enhanced the vasocontraction in an extracellular calcium-dependent manner, irrespective of the production of oxygen-derived free radicals.

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Methods

Measurement of tension development of rat tail artery strips

Tail arteries were isolated from male Sprague-Dawley (SD) rats (150–200 g) (6–8 weeks old). Segments of tail artery of approximately 1.5 cm in length were cut into helical strips. The strips were then mounted in a 10 ml organ bath chamber filled with a Krebs bicarbonate saline (bubbled with 95%O_2/5%CO_2) and were mechanically stretched to achieve a basal force of approximately 0.7 g. Tissues were routinely allowed to equilibrate for 1 h before the start of experiments. Indomethacin (1 μM) was routinely added to the Krebs saline which was composed of (in mM): NaCl 115, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, and $CaCl_2$ 1.8. The endothelium was removed by a rubbing procedure and the lack of endothelium was confirmed by the failure of acetylcholine (1 μM) to relax the tissue. The tension development was measured with an FT 03 force displacement transducer (Grass Ins. Co., Quincy). Data acquisition and analysis were accomplished using a Biopac system (Biopac Systems, Inc., Golata), including the MP 100 WS acquisition units, TCI 100 amplifiers, an AcqKnowledge software (3.01), universal modules and a Macintosh computer.

Chemicals and data analysis

TXF was prepared in Weifang Medical College, P.R. China, according to the method of van Damme *et al.* (1960). Briefly, strains of *Pseudomonas cocovenenas* (T7707-b) were cultured at

28°C under continuous rotation (180 turns min^{-1}) for 48 h (Zhao *et al.*, 1990). Consequently, the culture medium was saturated with $(NH_4)_2SO_4$ to remove bacterial and proteins. TXF was repeatedly extracted from the filtered medium with chloroform and petroleum ether. The content and the purity of TXF were determined with two ultraviolet spectrophotometers (Model 751 and Model UV-210A), respectively. Figure 1 shows the chemical structure of TXF. The present study was performed with two batches of TXF of which the stock concentrations were 362 $\mu g\ ml^{-1}$ and 500 $\mu g\ ml^{-1}$, respectively. Phenylephrine (Phe), acetylcholine, caffeine, U-46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$), indomethacin, and other chemicals were purchased from Sigma.

Data are expressed as X ± s.e. unless otherwise specified. Student's *t* test or analysis of variance in conjunction with the Newman-Keul's test were used where applicable. Group differences were considered statistically significant at the level of $P<0.05$.

Results

Effect of TXF on KCl-induced vasocontraction

KCl (60 mM) induced a biphasic contraction of vascular strips isolated from rat tail artery. In the presence of TXF (3 $\mu g\ ml^{-1}$), the phasic contraction (peak contraction) induced by KCl did not change whereas the tonic contraction was enhanced (left panel of Figure 2a). The total contraction of the tissue within 10 min of the application of stimuli was calculated as the integrated contraction, shown as the shaded area under the contraction curve in Figure 2a. KCl-induced integrated contraction was greater (14.04 ± 1.80 g.min) in the presence of TXF than in the absence of TXF (11.15 ± 1.52 g.min, $n=7$) ($P<0.05$). TXF (0.3–1 $\mu g\ ml^{-1}$) alone had no effect on the basal force of rat tail artery strips. The basal force was 0.68 ± 0.06 g and 0.68 ± 0.06 g before and 10 min after addition of TXF 1 $\mu g\ ml^{-1}$, respectively ($n=12$, $P>0.05$). Even 30 min after the application of TXF, the basal force still remained unchanged (data not shown). To determine whether the vasocontractor effect of TXF depended on the contractile status of the tissue, TXF was applied to the tissue immediately after the KCl-induced contraction reached its peak. The right panel of Figure 2a shows that, in the presence of KCl, TXF (3 $\mu g\ ml^{-1}$) further enhanced the tonic contraction of the tissue. In this group of experiments, KCl-induced integrated contraction was greater (15.62 ± 1.43 g.min) with the addition of TXF than that without TXF (9.79 ± 1.04 g.min, $n=8$) ($P<0.01$). TXF was also applied to the tissue at the middle of the tonic contraction induced by KCl (Figure 3). In this case, TXF (3 $\mu g\ ml^{-1}$) still effectively enhanced the tonic contraction ($n=3$).

Effect of TXF on phenylephrine-induced vasocontraction

In the presence of TXF, phenylephrine (Phe)-induced contraction of tail artery strips was significantly enhanced (Figure 4). The concentration-dependent contraction curve of tissues to PHE was shifted to the left after the tissues were pretreated with TXF (1 $\mu g\ ml^{-1}$) (Figure 5). In the next group of experiments, the tissue contraction was firstly induced by Phe at a fixed concentration (0.3 or 1 μM). When the contraction of the tissues reached the plateau phase, TXF was added at accumulated concentrations. At a concentration of 0.01 $\mu g\ ml^{-1}$, TXF did not modify Phe-induced vasocontraction. When the concentration of TXF was further increased, Phe-induced vasocontraction was significantly potentiated (Figure 6). However, when the concentration of TXF was higher than 10 $\mu g\ ml^{-1}$, vasorelaxation occurred irreversibly, possibly due to the tissue poisoning (data not shown). To test further whether the effects of TXF and Phe were additive, TXF at a single concentration (1 $\mu g\ ml^{-1}$) was added to the bath when Phe (0.3 to 30 μM)-induced vasocontraction reached the plateau phase as shown in Figure 7a. Irrespective of the concentrations of Phe, TXF always induced a similar amount of enhancement of isometric contraction of the tail artery strips (Figure 7b).

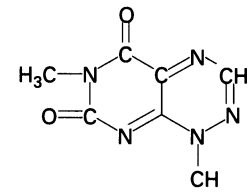

Figure 1 The chemical structure of toxoflavin.

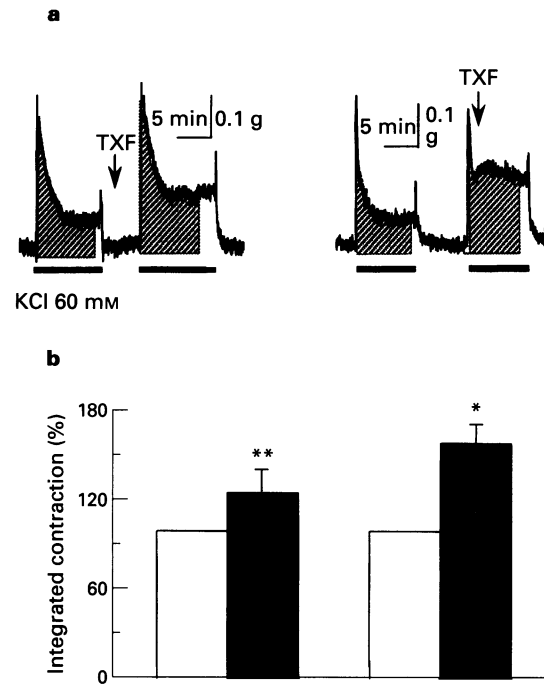

Figure 2 Effect of TXF on rat isolated tail artery strips precontracted with KCl (60 mM). Pretreatment of the tissues with TXF (1 $\mu g\ ml^{-1}$) enhanced KCl-induced integrated contraction, shown at the left of panel (a) and columns at left of (b) ($n=7$). Application of TXF following KCl stimulation, shown at the right of panel (a) and columns at right of (b) ($n=8$), also significantly enhanced KCl-induced integrated contraction. In (b) TXF enhanced KCl-induced integrated contraction was compared with that in the absence of TXF. Open column: 60 mM KCl; solid columns: 60 mM KCl plus TXF 1 $\mu g\ ml^{-1}$. Shaded areas in (a) indicate the calculated area as the integrated contraction. KCl-induced integrated contraction in the absence of TXF was taken as the control (100%). *$P<0.01$; **$P<0.05$.

Extracellular calcium-dependency of the vasocontracting effect of TXF

Without calcium in the bath solution, Phe (1 μM) had no effect on the isometric tension of tail artery strips (Figure 8a). In the presence of Phe, the stepwise addition of calcium to the bath solution induced a graded vasocontraction maximally developed around 1 mM calcium. Similarly, Phe and TXF together in the absence of extracellular calcium did not affect the isometric tension of tail artery strips. However, the extracellular calcium-dependent Phe-induced contraction of the tissues was significantly enhanced in the presence of TXF (Figure 8b). Furthermore, the extracellular calcium-dependent and Phe/TXF-induced contraction of the tissues was inhibited by nifedipine (1 μM), a classical calcium channel blocker. One example of the effect of nifedipine is shown in Figure 8a.

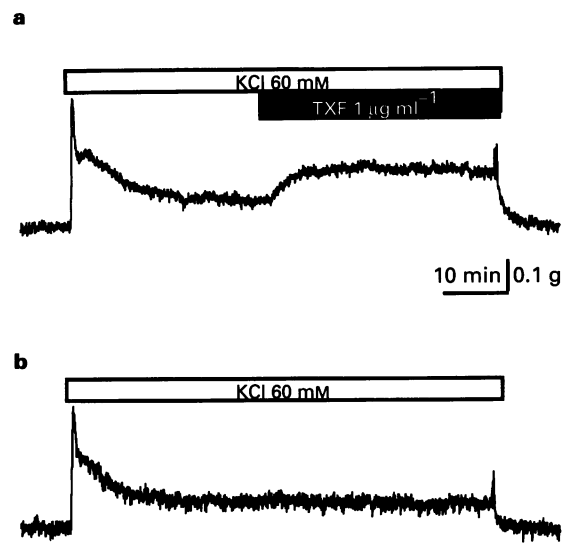

Figure 3 Representative records showing that TXF in the presence of KCl further increased the tonic contraction of the rat tail artery tissues.

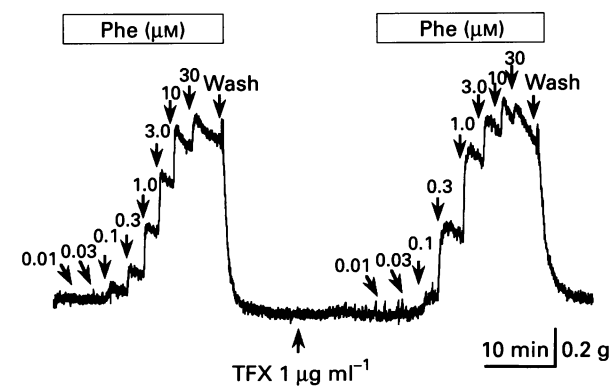

Figure 4 Representative records showing that pretreatment of the rat isolated tail artery strips with TXF enhanced the isometric contraction of the tissues induced by Phe at accumulated concentrations.

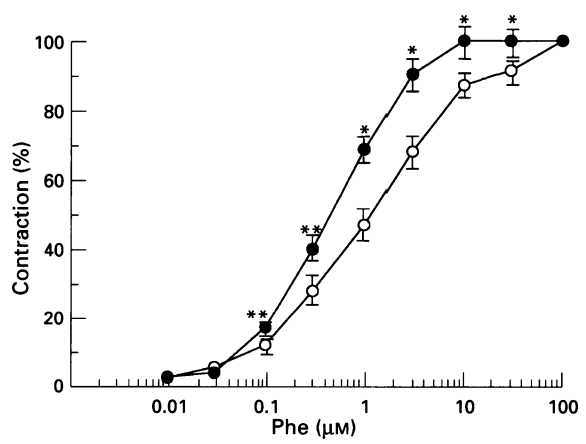

Figure 5 Pretreatment of the rat isolated tail artery strips with TXF (1 $\mu g ml^{-1}$, ●) enhanced the isometric contraction of the tissues induced by Phe (○) at accumulated concentrations. $n=8$. $^{*}P<0.01$; $^{**}P<0.05$.

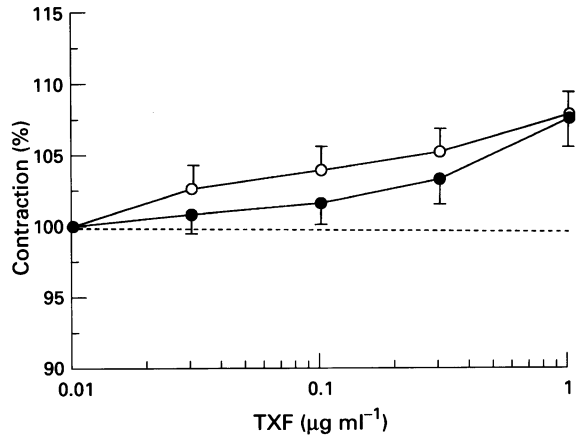

Figure 6 The enhancement of Phe-induced isometric contraction of the rat isolated tail artery strips by TXF at accumulated concentrations: (○) Phe 0.3 μM; (●) Phe 1.0 μM. $n=6-8$.

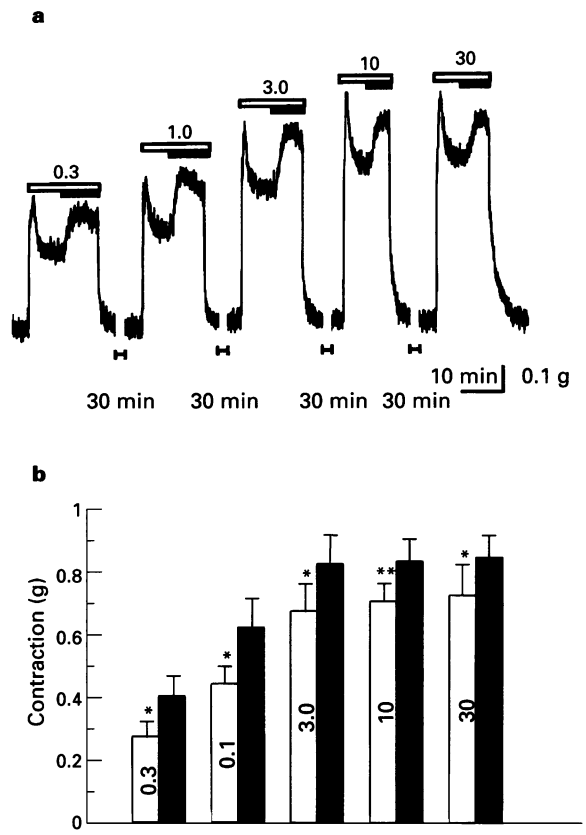

Figure 7 Application of TXF following Phe stimulation significantly enhanced Phe-induced tonic contractions. The representative records and experimental protocols are shown in (a) and the summary of the results in (b). In (a) open bar, Phe (μM); solid bar, TXF (1 $\mu g ml^{-1}$). In (b) open columns, Phe (μM); solid columns, TXF (1 $\mu g ml^{-1}$). The amplitude of the force development at the plateau phase of Phe-induced contraction was taken as control (100%) and compared with that after the addition of TXF. $^{*}P<0.01$, $^{**}P<0.05$. $n=8-12$.

Interaction of TXF and scavengers of oxygen-derived free radicals (OFR) on vasocontraction

To test whether TXF-induced enhancement of vasocontraction was due to the generation of OFR, two OFR scavengers, catalase (Cat) and SOD, were used. After the tissues were contracted with Phe, TXF was first added to the bath solution and then Cat and SOD were added (the left panel of Figure 9).

In the next group of experiments, the order of the addition of TXF and Cat/SOD was reversed (the right panel of Figure 9). In either case, TXF-induced enhancement of vasocontraction was not modified by Cat and SOD. Cat and SOD by themselves had no effect on the isometric tension of the tissues.

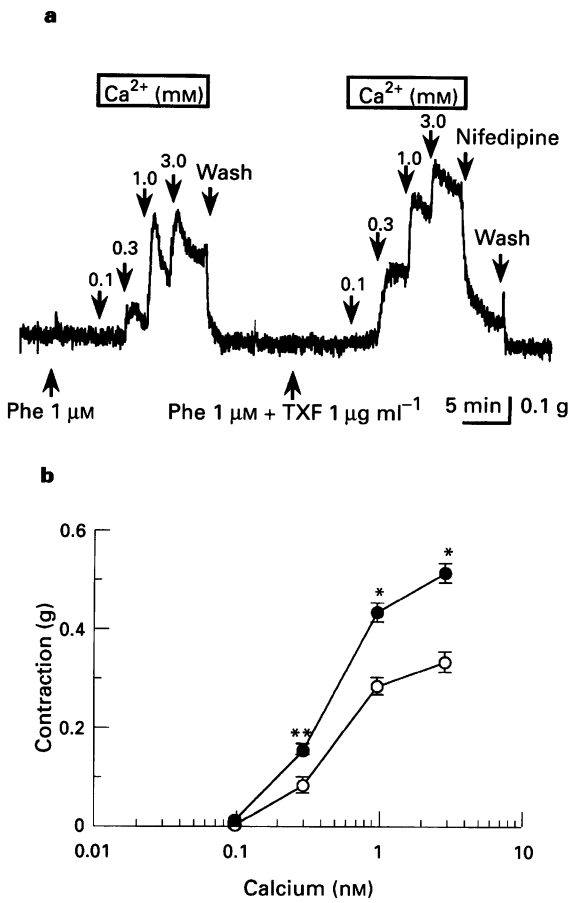

Figure 8 Calcium-dependence of Phe-induced and TXF-enhanced vasocontraction. The representative records and experimental protocols are shown in (a) and the summary of the results in (b). In (b) (○) Phe 1 μM; (●) Phe 1 μM plus TXF 1 μg ml^{-1}. The absolute amplitudes of the force development at the plateau phase of Phe-induced contraction was used and compared in the absence and then presence of TXF. *$P<0.01$, **$P<0.05$. $n=8$.

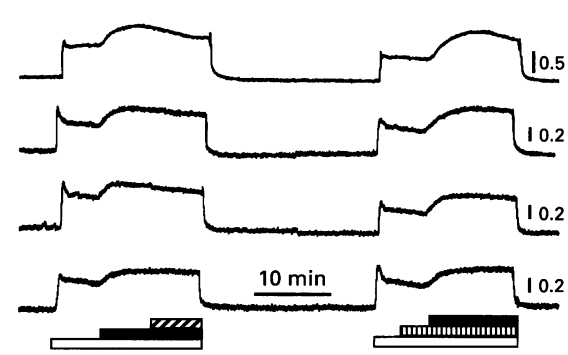

Figure 9 The interaction of TXF and scavengers of oxygen-derived free radicals on the isometric contraction of rat isolated tail artery strips. Cat, catalase; SOD, superoxide dismutase. Open bars, Phe 1 μM; hatched bars, Cat 40 u plus SOD 60 u; vertically lined bars, Cat 80 u plus SOD 120 u; solid bars, TXF 1 ug ml^{-1}.

Effects of TXF on the vasocontraction induced by caffeine or U-46619

The isolated vascular strips were also contracted with caffeine or U-46619 since these two agents mainly induce intracellular calcium-dependent vasocontraction. In the absence of extracellular calcium, caffeine (10 μM) induced a phasic vasocontraction, which was not affected by TXF (Figure 10a) ($n=4$). In another set of experiments, U46619-induced concentration-dependent vasocontraction was firstly recorded. After washing out U46619 for 1 h, TXF (1 μg ml^{-1}) was added to the bath solution and U46619-induced vasocontraction was examined again. It was clear that the presence of TXF had no influence on the vasocontractor effect of U46619 (Figure 10b). Furthermore, when the tissues were contracted to approximately 30% of the maximum active tension level by U46619, subsequent application of TXF still had no effect on the isometric tension of the tissues (Figure 10c).

Discussion

In addition to its toxicity related to food poisoning (Buckle & Kartadarma, 1990), TXF has been noted for its strong antibiotic activities (Gwynn *et al.*, 1988; Nagamatsu *et al.*, 1993).

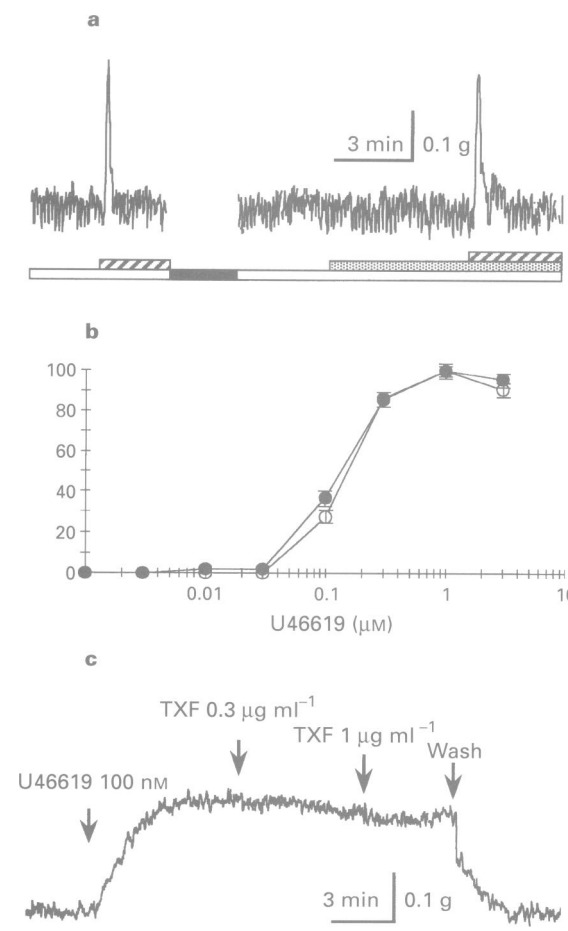

Figure 10 The interaction of TXF and caffeine or U46619 on the isometric contraction of the rat isolated tail artery strips. (a) Pretreatment of tissues with TXF (1 μg ml^{-1}) did not affect caffeine (10 mM)-induced phasic vasocontraction; open bars Ca^{2+} 0 mM; solid bars Ca^{2+} 1.8 mM/30 min; stippled bar TXF; hatched bar, caffeine. (b) The presence of TXF (●, 1 μg ml^{-1}) did not affect U46619-induced concentration-dependent vasocontractions (○). $n=4$. (c) TXF had no vasoactive effect on rat tail artery strips precontracted with U46619. Similar results were obtained in four tissue preparations.

Furthermore, TXF (>0.5 μg ml^{-1}) was reported to suppress the growth of lymphocytes and other blood cells (Yue, 1992) and to induce mutation of both plant and animal cells (Yue, 1989). The present study was the first to show the vascular effect of TXF. TXF potentiated the vasocontraction induced by either KCl or Phe. The vascular effect of TXF is not mediated by endothelium since in our preparations endothelium has been effectively removed. Moreover, indomethacin was routinely included in the tissue bath solution, indicating that the vascular effect of TXF could not be mediated by prostaglandin generation. Together, our results suggest that TXF potentiates the vasocontraction via a direct effect on vascular muscles.

In the present study, it was found that TXF did not by itself induce vasocontraction yet it potentiated vascular contractions to Phe and KCl. A possible explanation for this is that TXF may bring the vascular tissue to a threshold level of activation at which the active forces generated by other vasoconstrictors are potentiated. The increased intracellular calcium concentration is one of the underlying mechanisms for this threshold level of activation (Cocks *et al.*, 1993). If pretreatment of tissues with TXF elevated intracellular free calcium concentration closer to a threshold, the further increase in intracellular free calcium induced by other agonists through either intracellular calcium release or extracellular calcium influx can then cause stronger contraction. This hypothesis was not, however, supported by the finding that the vasocontraction induced by U46619 was not potentiated by TXF. It has been shown that the constrictor responses to U46619, a stable thromboxane-mimetic, in dog isolated coronary arteries (Angus & Brazenor, 1983) and in human coronary artery (Cocks *et al.*, 1993) were not altered by the calcium entry blockers including nifedipine, indicating that U46619 contracts vascular tissues mainly through the release of intracellular calcium. More interestingly, we found that TXF had no effect on caffeine-induced vasocontraction, which relies almost solely on calcium release from intracellular caffeine-sensitive pools. Together, these results indicate that TXF effect may not be due to a non-specific pre-activation (or conditioning) of vascular tissues on intracellular calcium pools by the toxin. More likely, TXF may act on the modulation of the opening of voltage-dependent calcium channels in the cell membrane, dependent on membrane potential or the functional status of these channels. At resting state, calcium channels are closed and may not be accessible to TXF. In the presence of KCl which contracts vascular muscle via the opening of voltage-dependent calcium channels, TXF may prolong the opening or enhance the opening probability of calcium channels. Similarly, it has been observed that Bay K-8644, a classical calcium channel agonist, had no direct effect on the basal force of rat tail artery strips. When the tissue was first treated with a low concentration of KCl to open calcium channels, subsequent application of Bay K-8644 significantly enhanced the isometric tension (Pang *et al.*, 1988). If opened voltage-dependent calcium channels were the target of TXF, how can one explain the fact that TXF also enhanced Phe-induced vasocontraction? The activation of α_1-adrenoceptors by Phe induces vasocontraction through various mechanisms. One of them is the modulation of L-type voltage-dependent calcium channels. In rat portal vein myocytes Phe has been demonstrated to stimulate L-type calcium channels (Lepretre *et al.*, 1994) coupled to a pertussis toxin-insensitive G protein. As described by other studies and ours (see Figure 8a), the application of blockers of L-type calcium channels inhibited both KCl and Phe-induced vasocontraction (Satake *et al.*, 1994). By the same argument, the opening of L-type calcium channels by TXF would potentiate both KCl- and Phe-induced vasocontraction. The involvement of L-type calcium channels in TXF effect is also indicated by the extracellular calcium-dependence of the TXF effect. Moreover, the effects of TXF and Phe were not additive. When Phe maximally contracted the tissue at concentrations greater than 10 μM, TXF still enhanced the isometric tension of the vascular strips. These results suggest that the enhanced activity of L-type calcium channels may not be the sole mechanism responsible for the effect of either TXF or Phe. Nevertheless, functional status-dependent activation of extracellular calcium entry in vascular smooth muscle cells may play a major role in TXF-enhanced vasocontraction. The patch-clamp experiments are needed to investigate the direct effect of TXF on L-type calcium channel currents.

It has been suggested that the toxic effect of TXF is mediated by the generation of OFR. However, our study clearly shows that the vasocontractor effect of TXF is not related to the production of free radicals since SOD and catalase did not inhibit the vasocontraction enhanced by TXF. It is possible that TXF-induced toxicity and vasocontraction may have different structural bases. Or else at low concentrations TXF may open calcium channels to contract SMC whereas at high concentrations (>10 μg ml^{-1}) TXF may mainly generate OFR which induced irreversible vasorelaxation. This is in agreement with other observations that as the production was enhanced, OFR may sequentially cause the contraction and death of smooth muscle cells (Steele *et al.*, 1991).

Since the chemical structure of TXF is known and its extracellular calcium-dependent vascular action is defined, the possibility of using TXF as a new opener of calcium channels to study the function and structure of voltage-dependent calcium channels in vascular SMC should not be overlooked. A better understanding of the vascular effect of TXF may also provide clues to the clinical treatment of TXF poisoning.

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