Ostreolysin induces sustained contraction of porcine coronary arteries and endothelial dysfunction in middle- and large-sized vessels

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Abstract

Ostreolysin (Oly), a cytolytic and cardiotoxic protein from the oyster mushroom (Pleurotus ostreatus), is lethal for mice with an LD50 of 1170 μg/kg following intravenous application. Its cardiotoxicity is associated with hyperkalemia, which is probably a consequence of potassium released from the lysed cells. Moreover, sub-micromolar concentrations of Oly induce a concentration-dependent increase in rat aortic ring tension, suggesting that ischaemia, and consequent hypoxic injury of cardiomyocytes, could also derive from vasospasm induced by this toxic protein.

The purpose of the present study was to demonstrate histopathological lesions caused by Oly after parenteral application to rats, and to define the mechanisms of Oly-induced vasoconstriction using inhibitors verapamil, lanthanum chloride, and selective endothelin receptor antagonist TBC3214, which have different molecular targets, in vitro on porcine coronary artery rings. We found that Oly causes endothelial injury with perivascular oedema in the heart and lungs, as well as myocardial haemorrhages in rats. Treatment of porcine coronary artery rings with Oly causes concentration-dependent vasoconstriction and prevents endothelium-mediated relaxation. Using TBC3214 as a selective blocker of the endothelin A receptor, we showed that vasoconstriction induced by Oly was independent of endothelin release and its effects. Verapamil (1 μM) greatly reduced Oly-evoked contractions of porcine coronary artery rings, while lanthanum abolished them completely. These results provide evidence that the contraction of coronary arteries by Oly is due mainly to the increased influx of Ca2+ from the extracellular space through voltage-dependent L-type Ca2+ channels and cation non-selective channels. Experiments suggest that Oly damages endothelial cells both in vitro and in vivo, and probably exhibits direct contractile effects on coronary smooth muscle cells.

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1. Introduction

Ostreolysin (Oly) is a 15 kDa cytolytic protein isolated from the oyster mushroom (Pleurotus ostreatus) (Berne et al., 2002). It belongs to the aegerolysin protein family (PF06355; IPR 009413) that comprises a few isolated and sequenced proteins, and several genomic transcripts and sequences predicted from expressed sequence tags of fungi, bacteria and plants (Berne et al., 2005).

Although the edible oyster mushroom is a widely cultivated species appreciated from the gastronomical point of view, sporadic local intoxications have been described in humans and animals. These intoxications developed after ingestion of large quantities of the fresh mushrooms. Al-Deen et al. (1987) have suggested that the...
toxicity is associated with thermolabile proteinaceous molecule(s). They also showed that the P. ostreatus water extract provokes haemorrhages in the intestine, liver, lung and kidney, and degenerative changes in the liver of the tested mice, with an LD_{50} higher than 3000 µg/kg.

Later investigations of the potentially lethal effects of Oly revealed that this protein is responsible for cardiorespiratory and toxic effects in rodents and their death. In vivo functional effects of Oly were first studied in male mice, in which it exhibited an LD_{50} of 1170 µg/kg (Zuzek et al., 2006). Cardiorespiratory effects were shown to predominate and to be the probable cause of death. The ability of Oly to induce vasospasm was further studied in vitro on isolated rat aortas (Rebolj et al., 2007). Oly caused contraction of aorta rings and reduced endothelium-mediated relaxation of aortic rings pre-contracted with norepinephrine, but the mechanism of Oly-induced vasoconstriction is not known. It was speculated that the toxin could act either by stimulating the release of endothelins from the endothelial cells, or directly by increasing Ca^{2+} flux into the vascular smooth muscle. Although the latter effect was not shown directly on myocardial cells, it was demonstrated that Oly can permeabilize cell membranes and form discrete pores with a functional radius of about 2 nm, allowing calcium and other ions to pass through the membrane (Sepcic et al., 2003). The change in ionic composition of cytoplasm may be responsible for the disruption of cellular membranes and cytolytic effects of the toxin (Berne et al., 2005).

Till now, Asp-haemolysin (Sakaguchi et al., 1975; Ebina et al., 1994) has been the only aegerolysin whose histopathological effects in mice after i.v. administration were studied in detail (Ebina et al., 1983, 1984). This lethal and cardiotoxic component of the pathogenic mould Aspergillus fumigatus has an LD_{50} of 750 and 350 µg/kg for mice and chicken, respectively, following the intravenous administration (Sakaguchi et al., 1975). In this study, the effects of Oly on the heart, kidney, liver, spleen, thymus, brain and lungs were studied in rats treated with 1 mouse LD_{50}. The results were evaluated by histopathology and by immunolocalization of Oly within the tissue. Further, with the aim of revealing the mechanism(s) by which Oly causes contraction of blood vessels, the effect of Oly on pig coronary artery ring tension was studied using TBC3214, a selective endothelin receptor antagonist (ET_{A}); verapamil, a well-known L-type Ca^{2+} channel blocker; and lanthanum chloride, a non-specific cation channels’ blocker. The selected compounds are inhibitors of different molecular targets which are involved in the vascular smooth muscle contraction physiology.

2. Materials and methods

2.1. Ostreolysin isolation and preparation

Oly was purified from the fruiting bodies of freshly collected oyster mushrooms (strain Plo 5) as described (Berne et al., 2002). The mushroom mycelium was taken from the ZIM collection of the Biotechnical Faculty, University of Ljubljana, Slovenia (Raspor et al., 1995). The protein solution was desalted and kept in aliquots at −20 °C. Before i.v. injection, sodium chloride was added to a final concentration of 0.9%.

2.2. Animals

For the study of cardiorespiratory effects and immunolocalization, 20 male Wistar albino rats, weighing 230–270 g, were obtained from the Medical Faculty, University of Ljubljana. The experiments were approved by the Veterinary Administration of the Republic of Slovenia (Permit no. 323-318/2003).

2.3. Drugs

The following drugs were used: substance P (SP), lanthanum chloride (LaCl_{3}) and verapamil (Sigma Chemical Co., St Louis, USA). ET_{A} antagonist TBC3214 was generously provided by Biological Encysive Pharmaceuticals (Uxbridge, UK).

2.4. Tissue collection, storage and histological examination

Oly, dissolved in 0.9% saline, was administered i.v. through a polyethilene heparinised cannula inserted into the left Vena jugularis at a dose of one mouse LD_{50} (1170 µg/kg) and at a volume of 200 µl as a bolus (n = 6). The same volume of saline was injected similarly into control rats (n = 3). Liver, heart, lung, kidney, spleen, thymus and brain were removed immediately after death from control (n = 3) and from Oly-treated (n = 6) animals, fixed in 10% neutral buffered formalin and paraffin-embedded after fixation. Control rats were sacrificed using carbon dioxide. Tissue blocks selected for histopathology comprised: cross-sections of the left and right lateral liver lobe, the longitudinal section through the heart that contains the left and right atria and ventricle walls as well as septum, cross-section blocks through the central part of the left and right kidney, two transversal sections of the left caudal lobe of the lung, transversal sections through the spleen, and random sections of thymus. Half the brain was available for histopathology. It was fixed in one block and cut to coronal sections after completed fixation.

Histopathological examination was performed under a light microscope on 4 µm thick haematoxylin–eosin (HE) stained tissue sections. HE staining was performed according to the standard protocol used in the laboratory comprising: deparaffinisation and rehydration (xylene – two changes, series of decreasing concentrations of ethanol – 100% – two changes, 96% – two changes, 70%, distilled water), staining with Meyer’s haematoxylin, washing and bluing in running tap water, staining with eosin, dehydration in increasing concentration of ethanol (70%, 96% – two changes, 100% – two changes), clearing in xylene (two changes) and mounting with synthetic resin and cover glass. Lesions were evaluated and graded by an experienced pathologist according to standardized grade scales (0–5) that were constructed on the basis of (A) grading of lesions in a slide or in organ cross-section and (B) grading of circumscribed, focal or multifocal lesions. Grading of lesions in a slide or in organ cross-section (A) was as follows: 0 = no lesions; 1 = minimal (area of tissue with lesions is less than 10% of tissue section); 2 = mild (area of tissue with lesions is 10–25% of tissue section); 3 = medium (area of tissue with lesions is 25% to 45%
of tissue section); 4 = severe (area of tissue with lesions is 45–75% of tissue section); and 5 = very severe (area of tissue with lesions is more than 75% of tissue section).

Grading of circumscribed, focal or multifocal lesions (B) was performed as follows: 0 = no lesions; 1 = minimal (barely visible deviation from normal tissue or only slightly higher number or amount of individual structures, evaluated subjectively as up to 10%); 2 = mild (mild deviation from normal tissue or mildly higher number or amount of individual structures, evaluated subjectively as 10–25%); 3 = medium (medium deviation from normal tissue or moderately higher number or amount of individual structures, evaluated subjectively as 25–45%); 4 = severe (large deviation from normal tissue or much higher number or amount of individual structures, evaluated subjectively as 45–75%); 5 = very severe (very strong deviation from normal tissue or very high number or amount of individual structures, evaluated subjectively as 75–100%).

2.5. Immunostaining for Oly

Immunostaining was made on paraffin, and on frozen tissue sections (lung and heart of six and five animals injected with Oly, respectively). Paraffin tissue blocks were prepared as described for histopathology, except that tissue sections for immunohistochemistry were mounted on slides coated with 3-aminopropyltriethoxysilane (APTES). Organs used for the frozen sections were removed immediately after death, and tissue blocks were frozen in liquid nitrogen and stored at −80 °C until immunostaining. Frozen tissue was cut into 10 µm tissue sections on a freezing microtome (Leica) before use. Two procedures were applied for immunostaining: Dako REALEnVision Detection System (Peroxidase/DAB+, Rabbit/Mouse), and immunofluorescence (IF). Primary antibodies used in both procedures were rabbit polyclonal antibodies raised against Oly (IgG, H+L, purified, 2 mg/mL), produced as described in Berne et al., 2002.

Dako REAL™ EnVision™ Detection System (Peroxidase/DAB+, Rabbit/Mouse) was used for immunohistochemistry on paraffin tissue sections, with and without antigen retrieval (citrate buffer pH 6.0, microwave 10 min) according to the instruction manual of the producer. IF with AlexaFluor® 546 Goat anti-rabbit IgG (H+L) secondary antibodies (Invitrogen, Carlsbad, CA, USA, 2 mg/mL) in 0.1 M sodium phosphate with 0.1 M sodium chloride, pH 7.5 was made on paraffin and frozen tissue sections of the lung and heart and additionally on the frozen sections of the liver, kidney and brain. Primary and secondary antibodies were diluted in 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS) pH 7.2. Dilutions of primary antibodies used with paraffin tissue sections and Dako REAL™ EnVision™ Detection System were 1:500, 1:1000, 1:1500 and 1:2500; dilution of primary antibodies for IF was 1:2500, and dilution of secondary antibodies used in IF was 1:50. Paraffin and frozen sections were stained for IF using the same protocol, except that no antigen retrieval was used on frozen sections. Slides were soaked for 10 min in PBS with 1% of bovine serum albumin (BSA), washed in PBS, incubated in solution of primary antibodies for 60 min, washed in PBS, incubated with secondary antibodies for 60 min, washed in PBS and mounted. Immunostained paraffin tissue sections were dehydrated and mounted in resin, while frozen tissue sections were mounted with water based mounting medium.

The following controls were included in all staining procedures: a) tissue sections of organs obtained from animals that were not treated with Oly and b) tissue sections from organs of treated animals incubated in 1% BSA in PBS pH 7.2 instead of primary antibodies (negative control).

2.6. Preparation of porcine coronary arterial rings

Porcine hearts, collected daily from the local slaughterhouse, were immersed in ice-cooled Krebs–Henseleit solution and transported to the laboratory within 45 min. The left anterior descending coronary artery was isolated 2–3 cm from its origin, and fat and connective tissue were removed. Isolated vessels were cut into rings about 4 mm long. The arterial ring preparations were mounted between two stainless steel hooks and immersed in the 10 ml baths with Krebs–Henseleit solution of the following composition (mM): NaCl, 118; KCl, 4.7; CaCl2 2.5; MgSO4, 1.6; KH2PO4, 1.2; NaHCO3, 24; d-glucose, 10; bubbled with 95% O2 and 5% CO2, and maintained at 37 ± 0.1 °C. During the 60–90 min of equilibration period, the basal tension was set to 5 g before starting the experiments (Kuzner et al., 2004).

In the first set of experiments, contraction dose–responses to increased potassium concentrations (10, 20, 30, 40, 50, 60 and 70 mM) were determined. Maximal increases in contractile responses were measured with 60 mM potassium in the bath solution. 40 mM potassium provoked 95% of the maximal increase, and was further used to produce two successive stable contractions, as shown in Fig. 5A. The last contraction was taken as a reference value (100%) against which the Oly-induced contractions were compared. The presence of intact endothelium was assessed by 20 mM SP, applied on potassium-pre-contracted rings. SP was used at 5, 10, 20, 40 in 60 mM concentrations, and maximal relaxant response on potassium-pre-contracted coronary ring preparations was achieved using 20 mM SP, as shown in Fig. 5A. Only coronary rings which responded with at least 30% relaxation were used. In the second set of experiments, contraction dose–responses to Oly were determined using final concentrations of the protein of 5, 10, 20 and 40 µg/mL. 10 µg/ml of Oly were used to determine the mechanism of arterial ring contraction after their treatment with various inhibitors. Before treatment with Oly, the arterial rings were incubated for 15 min with the following inhibitors: verapamil (10 µM), lanthanum chloride (La3+, 5 mM), or TBC3214 (1 µM). After inhibitor- and Oly-washout, the sequence of 40 mM potassium chloride (3 ×) and SP (1 ×) was applied again to check the contractility response of the arterial rings.

2.7. Data analysis and statistics

Histopathology data were analysed by Student’s t-test, P-values lower than 0.05 being considered as significant. In cases of failed normality test, Mann–Whitney Rank Sum test for grading of lesions, and Fisher exact test for statistical significance between control and treated groups were used. The percentage of tension produced by Oly was...
calculated based on the maximum tension level obtained by high KCl (40 mM). Results were expressed as the mean ± standard deviation (SD). Statistical significance was tested using Student’s t-test, and probabilities less than 5% (P < 0.05) were considered to be significant.

3. Results

3.1. Histopathological changes induced by Oly

Histopathology revealed severe lesions in lung and heart of treated rats, which developed within 10 min after application of Oly. The most prominent lesions comprised significant myocardial haemorrhages (5/6 treated rats, 0/3 controls); significant development of peracute perivascular oedema in the heart (5/6 treated rats, 0/3 control rats) and lung (6/6 treated rats, 1/3 control rats) with non-significant detachment of endothelial cells (3/6 treated rats, 0/3 control rats) and uneven, statistically non-significant (P > 0.05) myocardial congestion (6/6 treated rats, 1/3 controls) (Figs. 1 and 2). Detachment of endothelium was more apparent in larger veins. Oedema (Fig. 1B) and haemorrhages (Fig. 1F) were most prominent around small- and middle-sized pulmonary and myocardial vessels, and were not observed in other organs (liver, kidney, spleen, brain, and thymus).

The average grade of these lesions also differed between control and treated groups. Myocardial congestion was 2.7 ± 1.0 in treated rats and 0.7 ± 1.1 in controls. Detachment
of endothelial cells was 2.0 ± 2.0 in treated rats and 0 ± 0.0 in controls. In both parameters described above the difference was statistically not significant (\( P > 0.05 \)). Significant differences were observed for myocardial haemorrhages (treated rats 1.7 ± 1.0, controls 0 ± 0.0), peracute perivascular oedema in the heart (treated rats 1.7 ± 1.0, controls 0.0 ± 0.0) and for peracute perivascular oedema in the lung (treated rats 3.3 ± 1.2, controls 0.7 ± 1.1).

Other organs were free of observable lesions – only accidental lesions were observed.

### 3.2. Tissue localization of Oly

Immunostaining on paraffin tissue sections gave only non-specific background staining (data not shown) and IF had to be applied on frozen sections to reveal the localization of Oly bound to the tissue as an intense red signal. The intensity and distribution of the fluorescence signal were compared to negative controls incubated without primary antibodies (Fig. 3A, C and E). IF gave the most intense signal in the endothelial cell layer of all examined vessels in lung and heart (Fig. 3B, D and F). Some diffuse IF was observed in other parts of the tissues but was considerably weaker and so evaluated as non-specific background staining.

### 3.3. Effect of Oly on coronary artery ring tension

To determine the effects of Oly on vascular smooth muscle tension, experiments were performed on an isolated pig coronary ring vessel preparation with preserved endothelium. No spontaneous contractile responses were observed under control conditions. Addition of Oly (5–40 \( \mu \)g/ml) to the bathing medium induced a dose-dependent increase in tension (Fig. 4). The increase in contractile response of coronary rings was maximal at 40 mM potassium in the bath solution – \( 18.44 \pm 1.16 \) g (n = 7). After washout of the preparation, the tension returned smoothly to the basal level (Fig. 5A). The contractile responses were the largest (17.52 ± 1.75 g, n = 2) at the highest Oly concentration tested (40 \( \mu \)g/ml), and muscle contracture was stable before washing the preparation, the latter being followed by complete return to its resting value (data not shown).

Pre-treatment of isolated aorta ring preparations with Oly did not prevent subsequent depolarization induced by a modified Krebs–Henseleit solution containing 40 mM potassium (equimolar substitution for Na\(^+\)). Expressed as percentage of the maximal pig coronary ring tension following the depolarization evoked by 40 mM potassium, Oly provoked a response of 8% at 5 \( \mu \)g/ml and reached a maximal contractile response of 95% at 40 \( \mu \)g/ml (Fig. 4). The final concentrations of Oly (10–40 \( \mu \)g/ml), that produced significant increase in coronary ring tension in vitro, are comparable to the maximal calculated concentrations of Oly in the blood in vivo after injection of one LD\(_{50}\), which produced cardiorespiratory arrest. The most noticeable effect was the ability of Oly to diminish the endothelium-mediated relaxation of coronary ring preparations pre-contracted with high potassium (Fig. 5A).

### 3.4. Effect of Oly on coronary ring tension in the presence of contractile response inhibitors

Endothelium-dependent indirect vasoconstriction triggered by Oly and mediated by endothelin was studied. For this purpose, arterial ring preparations were preincubated with a very selective endothelin A receptor (ET\(_A\) antagonist, TBC3214. As shown on the representative recordings in Fig. 5B, TBC3214 (1 \( \mu \)M) decreased the amplitude of the contractile response, but the effect was not statistically significant (\( P > 0.05 \)). Rings pre-contracted with 40 mM potassium, with at least 30% of relaxant response to SP, were considered as endothelium-preserved. At concentration of 10 \( \mu \)g/ml, Oly diminished endothelium-mediated relaxation of arterial rings pre-contracted with 40 mM potassium (Fig. 5B).

To assess the involvement of voltage-gated L-type Ca\(^{2+}\) channels in the increase of coronary ring tension provoked by Oly (10 \( \mu \)g/ml), a series of experiments were performed with verapamil (1 \( \mu \)M), a well-known L-type Ca\(^{2+}\) channel blocker. As shown in a representative experiment (Fig. 5C), verapamil significantly reduced the Oly-induced increase in coronary ring tension (from 6.08 ± 2.02 g to 1.84 ± 1.29 g (\( P < 0.05 \))). After washout of the coronary ring preparation,
the maximal contractile response to 40 mM potassium took much longer (not shown). Relaxant response to SP was only partly preserved.

Another pathway of Ca$^{2+}$ entry contributing to the contractile response of Oly, was assessed by studying the effects of La$^{3+}$, a known inhibitor of some types of Ca$^{2+}$ influx (Kuriyama et al., 1998). As shown in a representative experiment (Fig. 5D), La$^{3+}$ (5 mM) abolished vasoconstriction of the arterial rings. After the washout of coronary ring preparation, the maximal contractile response to 40 mM potassium and the relaxant response to SP were completely preserved.

Fig. 3. Immunolocalization of ostreolysin in the heart and lung. Left panels (A, C, E) are negative control tissue sections of myocardium and arteriolar endothelium (A), arteriolar endothelium in the lung (C) and vein in the lung (E). Right panels (B, D, F) represent sections of the same tissues from Oly-treated rats. Arrows indicate intense fluorescence signal that arises from Oly at the arteriolar endothelium in the heart (B), arteriolar endothelium in the lung (D), as well as in the vein in the lung (F). Staining procedure was performed as described in details in the Materials and methods section. Scale bars, 72 μm.
The contractile responses of Oly were significantly decreased \((P < 0.05)\) by verapamil, abolished by \(\text{La}^{3+}\), and not significantly \((P > 0.05)\) affected by TBC3214, as summarised in Fig. 6.

4. Discussion

The results of the experiments obtained with Oly are similar to those obtained with another highly homologous aegerolysin-like protein, Asp-haemolysin from the mould \(A.\ fumigatus\), which causes a wide spectrum of serious diseases including invasive aspergillosis (Rementeria et al., 2005). However, histopathological findings in mice after the i.v. administration of Asp-haemolysin showed perivascular lesions of various degrees not only in the heart and lungs, but also in the kidney, liver and brain. The authors ascribed the origin of these lesions to the increased capillary permeability and cytotoxicity accompanied by the appropriate biochemical alteration of serum (Ebina et al., 1983, 1984). Histopathological lesions, especially endothelial detachment and perivascular oedema in the heart and lungs, supported by immunolocalization of Oly on endothelial vascular layer, show similar toxicity effects of Oly and Asp-haemolysin after parenteral application. In the present study, the effects of Oly on contractile responses of isolated pig coronary arteries were examined for the first time. We showed that Oly can bind to (Fig. 3) and damage endothelium \((P < 0.05)\) (Figs. 1 and 5). Oly was also observed to have a significant effect \((P < 0.05)\) on pig coronary ring smooth muscle contractility, increasing the muscle tension in a concentration-dependent manner (Fig. 4). The sustained contractions of pig coronary preparation induced by depolarization with high \((40 \text{ mM})\) concentrations of KCl (Fig. 5) are associated with \(\text{Ca}^{2+}\)-entry through voltage-gated \(\text{L}\)-type \(\text{Ca}^{2+}\) channels (Nakayama et al., 1996; Tanaka et al., 2000). This fact is supported by the experiments using verapamil, where blockade of the \(\text{L}\)-type voltage-gated calcium channels caused a significant decrease of the maximal contractile response to Oly (Figs. 5C and 6). The main trigger of contraction in vascular

![Fig. 4. Concentration–response curve of porcine coronary ring preparations: maximal contractions obtained as a function of Oly concentration. Ring tension is expressed as % of the maximal artery tension evoked by 40 mM potassium. The data represent means ± SEM from six to eight experiments.](image)

![Fig. 5. Effects of Oly on smooth muscle contractility in pig coronary ring preparations. (A) Representative contractile response of coronary ring preparations to the high external \(\text{K}^{+}\) \((40 \text{ mM})\), Oly \((10 \mu\text{g/ml})\), and relaxation response of high potassium-pre-contracted coronary ring to SP \((20 \text{ nM})\). (B) Contractile response of coronary ring preparation to Oly \((10 \mu\text{g/ml})\) in the presence of TBC3214 ETA blocker (1 \(\mu\text{M}\)). Note a smaller contractile response to Oly in the presence of TBC and a diminished relaxation response to SP. (C) Representative isometric contractions evoked by 10 \(\mu\text{g/ml}\) Oly in the presence of verapamil \((10 \mu\text{M})\). Note the significant decrease in coronary ring tension evoked by Oly \((10 \mu\text{g/ml})\) and the preserved relaxation response to SP. (D) Contractile response of coronary ring preparation to Oly \((10 \mu\text{g/ml})\) in the presence of lanthanum \((5 \text{ mM})\). Note the absence of contractile response to Oly in the presence of \(\text{La}^{3+}\) and a completely preserved relaxation response to SP. Abbreviations used: W – washout, Oly – ostreolysin, TBC – TBC3214 ETa blocker and SP – substance P.)
smooth muscle is the influx of Ca$^{2+}$ from the extracellular space, cytoplasm, and sarcoplasmic reticulum (Hill et al., 2001). Oly increases coronary ring tension in a dose-dependent manner, which is consistent with the previous studies on rat aorta rings (Rebolj et al., 2007). However, the amplitude of the muscle tension response in coronary vessels to the same final Oly concentrations was approximately two-fold higher than that reported in rat (Rebolj et al., 2007).

The increase in coronary ring tension induced by Oly may be related to substances released from the endothelium or induced by direct action of Oly on smooth muscle cells in the arterial vessel wall. Endothelium regulates the underlying smooth muscle vascular tone by release of different endothelium-derived factors such as endothelium-derived hyperpolarizing factor (EDHF) and endothelin-1 (ET-1). ET-1 is one of the most potent and important in vitro vasoconstrictors known, and produces contraction after binding to endothelin ET$_A$ receptors on vascular smooth muscle (Maguire and Davenport, 1995). The fact that diltiazem, a selective L-type Ca$^{2+}$ channel blocker, does not block the increased activity of [Ca$^{2+}$]i in pig coronary smooth muscle cells provoked by endothelin (Wagner-Mann and Sturek, 1991), suggests a completely different pathway of contractile response. In vascular smooth muscles, endothelin (ET) binds to the predominant ET-A receptor and produces vasoconstriction by ultimately accelerating Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels (Miyauchi et al., 1990).

In order to reveal the contribution of endothelins to Oly-induced contraction, experiments were conducted in coronary rings preincubated with the ET$_A$ receptor inhibitor TBC3214. Although the contractile response of Oly in the presence of endothelin receptor antagonist decreased, the effect was not significant ($P > 0.05$) (Fig. 5B). We can conclude that Oly does not increase the coronary arterial tension via the endothelin-dependent pathway.

Oly binds to and permeabilizes cholesterol-rich artificial membranes and several types of cells (Sepčič et al., 2003; Sepčič et al., 2004; Chowdhury et al., 2008). Because Oly is able to form pores in lipid membranes (Sepčič et al., 2003), increase in cytosolic Ca$^{2+}$ activity may derive from the opening of voltage-gated calcium channels or from cation non-selective channels formed by Oly. To investigate the role that voltage-gated Ca$^{2+}$ channels and influx of external Ca$^{2+}$ might play in a contractile response of coronary artery, preparations were preincubated with the Ca$^{2+}$ channel blocker verapamil before being exposed to Oly. Verapamil significantly reduced the contractile response (Fig. 5C) and is therefore likely that Oly increases intracellular Ca$^{2+}$ by depolarization and activation of voltage-gated Ca$^{2+}$ channels. On the other hand, the pores formed by Oly in smooth muscle cells might be strongly selective for Ca$^{2+}$ and cause Ca$^{2+}$ influx not due to depolarization and opening of L-type Ca$^{2+}$ channels. Less probably, the pore formed by Oly could be partially blocked by the action of verapamil.

Since La$^{3+}$ inhibits both voltage-dependent L-type and non-selective cation channels (Hescheler and Schultz, 1993; Krautwurst et al., 1994), our results suggest that L-type voltage-dependent and non-selective calcium entry into the smooth muscle cells may contribute to the contractile effect of Oly. At concentrations above 10 µg/ml, Oly diminished the endothelium-mediated relaxation of arterial rings pre-contracted with 40 mM potassium, suggesting injury of endothelial cells as the mechanism responsible for endothelium-mediated relaxation. La$^{3+}$ completely protects the relaxant response of coronary arteries to SP, probably due to the blockade of cation non-selective conductance in smooth muscle cells. SP is an endothelium-dependent vasodilator of the pig coronary artery that functions via the production of nitric oxide (NO) and EDHF as relaxing agents (Beny et al., 1986; Bény et al., 1989; Pacicca et al., 1992).

We conclude that the Oly-induced increase of pig coronary ring tension is mainly associated with Ca$^{2+}$ influx from the extracellular space through L-type voltage-dependent calcium channels. Cation non-selective conductance in coronary smooth muscle cells that are La$^{3+}$-sensitive also contributes significantly to the contractile response of Oly. Another possibility, that cannot be excluded, is that Oly induces new cation-permeable pores in smooth muscle cells. Moreover, besides the contractile response, Oly can also damage endothelial cells and cause in vivo interstitial and alveolar oedema which, together with vasospasm, further extravagates tissue hypoxia. All these mechanisms play an important role in the cardiopulmonary toxicity of Oly.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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