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Review

Ostreolysin A/Pleurotolysin B and Equinatoxins: Structure, Function and Pathophysiological Effects of These Pore-Forming Proteins

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Abstract: Acidic ostreolysin A/pleurotolysin B (OlyA/PlyB, formerly known as ostreolysin (Oly), and basic 20 kDa equinatoxins (EqTs) are cytolytic proteins isolated from the edible mushroom *Pleurotus ostreatus* and the sea anemone *Actinia equine*, respectively. Both toxins, although from different sources, share many similar biological activities: (i) colloid-osmotic shock by forming pores in cellular and artificial membranes enriched in cholesterol and sphingomyelin, (ii) increased vascular endothelial wall permeability *in vivo* and perivascular oedema, (iii) dose-dependent contraction of coronary vessels, (iv) haemolysis with pronounced hyperkalaemia *in vivo*, (v) bradycardia, myocardial ischemia and ventricular extrasystoles accompanied by progressive fall of arterial blood pressure and respiratory arrest in rodents. Both types of toxins are haemolytic within nanomolar range concentrations, and it seems that hyperkalaemia plays an important role in toxin cardiotoxicity. However, it was observed that the haemolytically more active EqT III is less toxic than EqT I, the most toxic and least haemolytic EqT. In mice, EqT II is more than 30 times more toxic than OlyA/PlyB when applied intravenously. These observations imply that haemolysis with hyperkalaemia is not the sole cause of the lethal activity of both toxins. Additional mechanisms responsible for lethal action of the two toxins are direct effects on heart, coronary vasoconstriction and related myocardial hypoxia. In this review, we appraise the pathophysiological mechanisms related to the chemical structure of OlyA/PlyB and EqTs, as well as their toxicity.

Keywords: ostreolysin A/pleurotolysin B; equinatoxins; pore-forming proteins; biological effects

1. Introduction

Ostreolysin (Oly) and equinatoxins (EqTs) belong to the group of pore-forming proteins with a defined native conformation which, upon an environmental trigger, is changed spontaneously to enable formation of a transmembrane pore consisting of several protein monomers [1]. Recently, Oly was found to consist of two proteins, ostreolysin A (OlyA) and pleurotolysin B (PlyB) in a 9:1 molar ratio, respectively, and with respective ~15 and ~59 kDa molecular masses [2]. Both OlyA/PlyB and EqT II are characterized structurally by a dominant β-structure scaffold that keeps a constant global domain tertiary structure, while the polypeptide elements needed for membrane penetration are provided by minor local conformational changes [3,4]. Like other pore-forming toxins, both Oly and EqTs bind to the cell membrane by recognising a specific membrane component. EqT II recognises the sphingomyelin head group [5], while Oly specifically binds to membrane domains containing both sphingomyelin and cholesterol [6]. This binding results in protein oligomerization at the membrane surface, and the formation of transmembrane pores, leading to colloid-osmotic mechanisms resulting in cell lysis. The aim of this review is to summarise the pathophysiological

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mechanisms related to the chemical structure of both proteins, and to discuss their toxicity on various levels of biological organization.

2. Ostreolysin A/pleurotolysin B

2.1. Origin

Oly was reported to be an acidic, cytolytic 15 kDa protein that was first isolated from the edible oyster mushroom *Pleurotus ostreatus* [7]. This protein was shown to be highly similar to Aa-Pri1, a predicted protein from *Agrocybe aegerita* [8] and similar to Asp-haemolysin from *Aspergillus fumigatus* [9] and the non-haemolytic proteins P16 and P18 from the bacterium *Clostridium bifermentans* [10]. Recently, Oly was reported to consist of two proteins, ostreolysin A (OlyA) and pleurotolysin B (PlyB) with membrane-attack complex/perforin (MACPF) domain [11], in a respective 9:1 molar ratio and with molecular masses of ~15 and ~59 kDa, respectively [2]. These proteins form a 13-meric rosette-like structure which has a central hydrophilic pore of about 4-5 nm diameter. The opened transmembrane pore is non-selectively permeable to ions and smaller neutral solutes, and a colloid-osmotic type mechanism is responsible for its cytolytic effect [11].

2.2. Chemical structure and biological properties

OlyA/PlyB is a pore-forming cytolysin whose primary role in the producing organism is not yet clarified. It belongs to the larger group of highly homologous proteins called aegerolysins (Pfam PF06355, InterPro IPR009413), currently comprising more than 350 entries in the NCBI databases. The structure, putative functional characteristics and occurrence of aegerolysins across the tree of life have been reviewed in details elsewhere [12,13]. OlyA/PlyB is specifically expressed during the formation of mushroom fruit bodies [7,14]. The complete structure of OlyA/PlyB is not yet solved. However, the protein characterisation by means of circular dichroism, UV-absorption and fluorescence spectroscopy revealed that, under physiological conditions, OlyA/PlyB adopts a monomeric and thermodynamically stable native-like conformation characterized by rigid tertiary and predominantly β-sheet secondary structures. This compact native state is necessary for the protein binding to cholesterol/sphingomyelin membrane domains [4].

Binding to natural and artificial lipid membranes followed by permeabilisation is a common feature of many aegerolysins [12]. In line with this, OlyA/PlyB has been reported to induce erythrocyte lysis and to be cytotoxic for various cell lines at sub-micromolar concentrations [15]. The lytic process starts by recognition of distinctive raft-like membrane microdomains enriched in cholesterol and sphingomyelin [6,16]. Due to their important functions in living cells and cell-cell interactions [17-19], there is an increasing need for new techniques to study lipid rafts. OlyA/PlyB, which recognises specifically the combination of the two main lipid raft components, sphingomyelin and cholesterol, is in this regard a very good candidate for a new marker of raft-like membrane microdomains [20].

2.3. Main pathophysiological effects

OlyA/PlyB was shown to be toxic and lethal to rodents, with an estimated LD_{50} of 1170 μg/kg when administered by intravenous (*i.v.*) way [21]. Following *i.v.* administration of 1 mouse LD_{50}, scratching, jumping, respiratory distress, cyanosis, paralysis and death of experimental mice generally occur within 3-5 minutes. After *i.v.* administration of 1 mouse LD_{50} into rats, OlyA/PlyB produced bradycardia, myocardial ischemia and ventricular extrasystoles accompanied by progressive fall of arterial blood pressure to the mid-circulatory pressure, leading to death of experimental animals [21]. Similar arrhythmias and time-course of arterial blood pressure produced by OlyA/PlyB were observed in pharmacologically vagotomised and artificially ventilated rats, indicating that vagotomy and hypoxia due to respiratory arrest after administration of this toxin are not primarily responsible for its cardiotoxicity [21]. OlyA/PlyB is lytic to human, bovine, sheep and rat erythrocytes at nanomolar range concentrations [21,22]. The mechanism responsible for the

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haemolytic effect of OlyA/PlyB is colloid-osmotic shock due to pore formation in cell membranes [22]. In addition, this toxin induces lysis of rat erythrocytes *in vivo*, as indicated by a significant increase in potassium concentration in serum (higher than 10 mM) and the red-coloured appearance of blood serum due to release of haemoglobin from damaged erythrocytes [21]. This observation was later confirmed by measuring the haemolytic activity on rat erythrocytes using a turbidimetric method [21]. Since such high blood potassium concentrations may cause cardiac arrest [23-25], it was concluded that hyperkalaemia probably plays an important role in the cardiotoxicity of OlyA/PlyB.

Respiratory arrest develops within few seconds after OlyA/PlyB administration, and hypoxia is a well-known cause of severe arrhythmias. However, arrhythmias also developed in artificially ventilated animals after *i.v.* administration of OlyA/PlyB, suggesting that mechanisms other than respiratory arrest are responsible for the cardiotoxic effects [21]. Myocardial hypoxia and cardiac arrest may be produced by sudden and significantly reduced blood flow through the coronary arteries. Since OlyA/PlyB is able to form pores in biological membranes [22], direct effects on porcine coronary arteries were studied. OlyA/PlyB induced a dose-dependent contraction of porcine coronary artery rings, as well as of rat aortas [26], and prevented the endothelium-mediated relaxation [27]. Contractile effects of OlyA/PlyB are probably due to direct effects on pig coronary smooth muscle cells. As revealed by fluorometric measurements, OlyA/PlyB increases the intracellular calcium concentration ($[Ca^{2+}]_i$) in smooth muscle A10 and NG108-15 cells, and alter their morphology which underlay its cardio- and neuro-toxic effects [28,29]. These effects can cause coronary vasoconstriction leading to myocardial ischemia accompanied by arrhythmias and heart failure. Pathological examination of main body tissues revealed that OlyA/PlyB induced perivascular oedema in the heart and lungs, as well as focal myocardial haemorrhages in rats injected with 1 mouse LD_{50}. The mechanism underlying oedema and myocardial haemorrhages is a damage of endothelial cells, both *in vitro* and *in vivo*, as revealed by histological examination of tissues [27].

The biochemical properties and biological effects of OlyA/PlyB are summarised in Table 1.

2.4. Biological use

OlyA/PlyB binds with high affinity to cholesterol and cholesterol/sphingomyelin-rich membrane domains in human urothelial cancer cells, and produces necrotic cell death [30]. Selectivity of OlyA/PlyB is based on the cholesterol and cholesterol/sphingomyelin-rich membrane domains in urothelial cancer cells, in contrast to normal urothelial cells. Fluorescent-labelled OlyA-mCherry has been utilised as a highly specific probe to visualise cholesterol/sphingomyelin rich membrane microdomains in living and fixed cells, and to study membrane trafficking [31].

3. Equinatoxins

3.1. Origin

Isolation of equinatoxin (EqT), a lethal protein from *Actinia equina L.*, was first described by Ferlan and Lebez [32]. Then, in 1988, isolation of three isotoxins (EqT I, EqT II and EqT III) was reported with mouse LD_{50} of 23, 35 and 83 µg/kg, respectively [33].

3.2. Chemical structure and biological properties

EqTs are cytolytic water-soluble proteins that readily interact with cell and artificial lipid membranes [33]. It has been shown that, in bovine lactotrophs, EqT II induces a rapid increase in $[Ca^{2+}]_i$ by the formation of Ca^{2+} permeable ion channels in lipid bilayers [35]. The composition could be resolved only after eludication of the crystal structure of EqT II [3]. This was the first resolved structure of a eukaryote pore-forming protein. EqT II is a single-domain protein based on a 12 strand β-sandwich fold with a hydrophobic core and a pair of α-helices, each of which is associated with the face of a β-sheet. In biological and artificial membranes, three to four EqT II molecules oligomerize and create cation-selective pores [36]. A detailed mechanism of pore formation by EqTs was

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described and reviewed by Anderluh et al. [37,38]. The high affinity of EqT II for membrane sphingomyelin makes it a suitable molecule for sensing membrane microdomains [20] and for membrane reorganization [39]. Recently, a neutron reflection study reveals new insight related to the binding process of EqT II into the plasma membrane. Thus, it was reported that EqT II binds in several distinct orientations which depend on membrane lipid composition [40]. Interestingly, EqT II exists on the cell surface as a mixture of oligomeric species including monomers, dimers, tetramers and hexamers, instead of a unique oligomeric form [41].

Table 1. Biochemical properties and biological effects of OlyA/PlyB.

Biochemical properties		**Reference**
Molecular mass (kDa)	15 (OlyA) – 59 (PlyB) molar ratio 9:1	[11] [34]
pI	5.0	[7]
Molecular targets	Cholesterol and sphingomyelin	[6]
Activity	Pore formation	[22] [2] [34]
	Involvement in fructification of oyster mushroom	[7] [14]
Effects *in vivo*		
LD_{50} (μg/kg mouse)	1170	[21]
Circulation, heart (rat)	Progressive drop of arterial blood pressure Bradycardia, myocardial ischaemia Ventricular extrasystoles	[21]
Blood (rat)	Haemolysis Hyperkalaemia ($[K^+] > 10$ mM)	[21]
Effects on isolated organs		
Rat aorta Porcine coronary artery	Increase in aortic ring tension	[26] [27]
Cellular and subcellular effects		
Human, bovine, sheep erythrocytes	Haemolysis (64 nM Oly)	[22]
Human umbilical vein endothelial cells Chinese hamster lung fibroblasts	Toxicity (ED_{50} = 2.2 μg/mL Oly) Toxicity (ED_{50} = 1.3 μg/mL Oly)	[26]
A10 smooth muscle cells	Increase in $[Ca^{2+}]_i$ (≥ 14 nM OlyA/1.56 nM PlyB)	[29]
NG 108-15 cells	Increase in $[Ca^{2+}]_i$ (≥ 7 nM OlyA /0.78 nM PlyB) Cell swelling, plasma membrane blebbing (≥ 700 nM OlyA /78 nM PlyB)	[28]

3.3. Main pathophysiological effects

Cardiorespiratory arrest in rats caused by EqT was first described by Sket et al. [42] but, at that time, the underlying mechanism was not fully understood. *In vitro* studies revealed that EqT (80-200 ng/mL) increases the permeability and resistance of the lung vasculature and produces, at concentrations higher than 150 ng/mL, interstitial and alveolar pulmonary oedema [43]. At low concentrations (0.1-3 μg/mL), EqT induces a transient negative inotropic effect followed by a long-lasting positive inotropic effect in isolated guinea pig atrium. It was proposed that the formation of prostaglandin E2 is responsible for this effect, since it could be inhibited by indomethacin, a well-known inhibitor of prostaglandin synthesis [44]. The positive inotropic effect described by these authors was also seen in experiments performed on isolated guinea pig hearts using EqT II [45], but only when the toxin had been applied at low concentrations (picomolar range). Higher concentrations of EqT II caused a pronounced negative inotropic effect. Cardiorespiratory effects, similar to those produced by EqT [42], were later confirmed using EqT II [46] and EqT III [47]. EqT II causes negative inotropic and chronotropic effects such as bradycardia, action potential conduction disturbances and extrasystoles. Similarly, the lethal dose of EqT III also produces arrhythmias, a drop of arterial blood pressure and cardiac arrest. The two isotoxins are haemolytic,

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and it is well known that the hyperkalaemia caused by the lysis of erythrocytes can produce serious arrhythmias leading to cardiac failure. A detailed study of the role of haemolysis in EqT lethality revealed an only marginal role of hyperkalaemia in the cardiotoxic effects of these toxins [47]. This is in agreement with data showing that the more toxic EqT II is less haemolytic than EqT III. As EqT III is the least toxic but causes the most pronounced hyperkalaemia, it seems that the elevation of plasma K^+ concentration is not the primary cause of cardiorespiratory arrest. *In vivo*, EqT II and EqT III cause similar alterations of electrocardiogram (ECG), breathing and blood pressure, indicating that the same cardiotoxicity mechanism may be involved.

EqTs belong to the group of pore forming toxins that enable passage of cations, mainly Ca^{2+}, through phospholipid bilayers, including the plasma membrane. Therefore, another possible pathophysiological mechanism of the cardiotoxicity may be a decreased coronary perfusion due to vasoconstriction. Vasoconstrictor effects of EqT II may also include endothelin-dependent pathway [48]. EqT II-triggered endothelin release is probably one of the mechanisms involved in the lowering of coronary flow induced by this toxin [49], since endothelin is well known to activate L-type calcium channels in smooth muscle cells. As EqTs form cation selective pores in cellular membranes, a direct effect on smooth muscle cells in the vascular wall may also play a major role. To answer this question, porcine coronary arteries were exposed to EqT III (1-100 nM), and the resting tension of smooth muscle as well as the maximum force of contractions were measured. The results revealed that EqT III also directly triggers contraction of isolated porcine coronary arteries at nanomolar concentrations. This mechanism probably explains most of the EqT III cardiotoxic effects [47]. On Langendorff rat heart preparations, EqT II (0.1-10 nM) was reported to cause arrhythmia as well as decreased coronary perfusion rate and left ventricular pressure in a dose-dependent manner. At higher concentrations, EqT II produces cardiac arrest within a few seconds. As in the rat heart, EqT II also decreases coronary flow in the porcine heart. This effect could be abolished by an antagonist of L-type voltage-dependent calcium channels (*i.e.* $Ca_V1.2$ channels) such as nicardipine. Additionally, it was shown that EqT II increases the tension of spontaneous contractions and induced long-lasting contracture of guinea pig *taenia caeci* smooth muscle, accompanied by a marked increase in $[Ca^{2+}]_i$ [50]. After *i.v.* administration, EqT II first enters into the right atrium and then the right ventricle of the heart before reaching the pulmonary circulation. EqTs have high binding affinity for sphingomyelin-rich cell membranes [5,51-53] and thus rapidly bind to blood cells and endothelium. In order to assess the possibility that a sufficient concentration of unbound EqT II is still present in the arterial blood and in the systemic circulation to produce direct cardiotoxic effects, perfusion experiments were performed on isolated rat lungs. After *in vitro* perfusion of the lung with a solution containing 100 nM EqT II, the toxin concentration in perfusates ranged between 0.8 and 5 nM. Effluent from the lungs contained enough EqT II to produce cardiotoxic effects on isolated Langendorff heart, as described previously [46]. This is in accordance with the findings that the lethal effects of EqT II are mainly attributed to its vasoconstrictor effects and direct cardiotoxicity. The mechanism of EqT II-induced respiratory arrest is not yet sufficiently explained. After *i.v.* administration of EqT I, EqT II or EqT III, the respiratory activity stops within a few seconds. It was shown, at least for EqT I, that electrical stimulation of the phrenic nerve triggers normal diaphragm muscle contraction indicating that neuromuscular transmission and function are unaffected by the toxin. Because respiratory arrest causes cardiac hypoxia, the alterations in blood pressure and electrical activity, similar to those observed after administration of one mouse LD_{50} of EqTs, could cause cardiac hypoxia. However, experiments performed on artificially ventilated animals, showed that artificial ventilation did not prevent the changes in ECG and blood pressure. Therefore, hypoxia was not confirmed as a primary cause for cardiotoxicity. Respiratory arrest may also be caused by respiratory reflexes activated through J-receptors in lung parenchyma, which are strongly stimulated under pathophysiological conditions like pulmonary oedema. EqTs are relatively large molecules (with molecular masses of around 20 kDa) and, due to their size, it is unlikely that they could pass the brain-blood barrier unless endothelial damage gives access to the neurons of the respiratory centre in the *medulla oblongata*. Recent preliminary results have shown that EqT II causes swelling and lysis of endothelial cells, an effect that may give toxin access to neuronal cells. Direct

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effects of EqTs on respiratory centres cannot be excluded since EqT II has been reported to produce swelling of differentiated neuroblastoma NG108-15 cells [54]. Moreover, axonal swelling at the node of Ranvier of myelinated nerve fibres has also been observed *in vitro* after application of EqT II [55].

3.4. Biological use

Despite the fact that low concentrations of EqTs are lethal, several studies have been reported on their effects on cancer cells [56-58]. Furthermore, they have been tested as immunotoxins targeting at *Giardia duodenalis* [59]. Their use as molecular probes for sphingomyelin and cholesterol-rich membrane domains seems to be more promising [20,60]. Finally, EqT II has been shown to inhibit endocytosis [39] and to cause membrane reorganization [39,61]. Development of immunotoxins targeting specific cells may be the most promising future use of equinatoxins.

The biochemical properties and biological effects of EqT II are summarised in Table 2.

Table 2. Biochemical properties and biological effects of EqT II.

Biochemical properties		**Reference**
Molecular mass (kDa)	20	[33]
pI	10.5	[33]
Molecular targets	Sphingomyelin	[36]
Activity	Pore formation	[36] [35]
Effects *in vivo*		
LD_{50} (μg/kg mouse)	35	[33]
Circulation, heart (rat)	Bradycardia, hypotension, extrasystoles	[62]
Blood (rat)	Platelet aggregation	[62]
Respiration (rat)	Respiratory arrest	[42]
Effects on isolated organs		
Rat skeletal muscle	Spontaneous twitches (10 nM)	[63]
Rat heart (Langendorff preparation)	Drop in perfusion rate, decreased left ventricular pressure, arrhythmia (0.1-10 nM)	[46]
Porcine coronary artery	Vasoconstriction (EC_{50} = 101.1 nM)	[48]
Cellular and subcellular effects		
Human erythrocytes	Haemolysis Pore formation / r=1.1 nm (50 ng/mL)	[36]
Rabbit Platelets	Aggregation (0.01 ng/mL)	[64]
V-79-379 A cell line	Toxicity (ED_{50} = 17 ng/mL)	[65]
Bovine lactotrophs Planar lipid bilayers	Toxicity (230 nM) Ion channel formation (650 nM) Increase in $[Ca^{2+}]_i$ (230 nM)	[35]
NG 108-15 cells	Cell swelling, increase in $[Ca^{2+}]_i$	[54]
ECV-304 cells	Cell swelling, lysis (1-10 nM)	[49]
Taenia caeci smooth muscle cells	Increase in $[Ca^{2+}]_i$, muscle contraction (10-500 nM)	[50]

4. Conclusions

The results of *in vivo* and *in vitro* studies indicate that hyperkalaemia that appears after the cytolytic action of OlyA/PlyB on blood and other exposed cells is mainly responsible for its cardiotoxic action. Coronary constriction and possible direct effect of OlyA/PlyB on cardiac tissue are additional mechanisms of cardiotoxicity. Besides direct cardiotoxic effects, OlyA/PlyB also damages endothelial cells and causes interstitial and alveolar oedema *in vivo*. This, together with coronary spasm, further amplifies tissue hypoxia and probably contributes to the respiratory arrest. All these mechanisms play an important role in the cardiorespiratory toxicity of OlyA/PlyB. On the other hand, EqTs are haemolytic, and the most studied EqT II also produces hyperkalaemia, but its effects are faster and hyperkalaemia seems to have a smaller effect on cardiac function than the direct action of the toxin. Both toxins have proven to be good tools to study cell membrane function

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as they bind to specific membrane domains. As potent cytotoxic agents they may be useful for designing new therapeutic substances. EqT II has already been considered as a potent immunotoxin.

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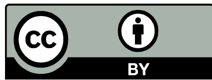

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