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Short communication

Effect of the synthetic coumarin, ethyl 2-oxo-2H-chromene-3-carboxylate, on activity of *Crotalus durissus ruruima* sPLA2 as well as on edema and platelet aggregation induced by this factor

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ABSTRACT

We show that ethyl 2-oxo-2H-chromene-3-carboxylate (EOCC), a synthetic coumarin, irreversibly inhibits phospholipase A_2 (sPLA2) from *Crotalus durissus ruruima* venom (sPLA2r) with an IC_{50} of 3.1 ± 0.06 nmol. EOCC strongly decreased the V_{max} and K_m, and it virtually abolished the enzyme activity of sPLA2r as well as sPLA2s from other sources. The edema induced by sPLA2r + EOCC was less than that induced by sPLA2r treated with *p*-bromophenacyl bromide, which was more efficient at neutralizing the platelet aggregation activity of native sPLA2r. Native sPLA2r induced platelet aggregation of 91.54 ± 9.3%, and sPLA2r + EOCC induced a platelet aggregation of 18.56 ± 6.5%. EOCC treatment also decreased the myotoxic effect of sPLA2r. Mass spectrometry showed that EOCC formed a stable complex with sPLA2r, which increased the mass of native sPLA2r from 14,299.34 Da to 14,736.22 Da. Moreover, the formation of this complex appeared to be involved in the loss of sPLA2r activity. Our results strongly suggest that EOCC can be used as a pharmacological agent against the sPLA2 in *Crotalus durissus sp.* venom as well as other sPLA2s.

2H-1-benzopyrans (2H-chromenes) are important intermediates in the synthesis of many natural products and medicinal agents (Ashwood et al., 1986; Kim and Lee, 2002) including flavonoids, coumarin, and derivatives of these compounds. 2-H chromenes have been used experimentally as anti-inflammatory and anti-thrombotic therapeutics. Related to their anti-inflammatory activity, synthetic coumarins are known to inhibit arachidonic acid metabolism. *Crotalus durissus sp.* venom has several pharmacological effects that appear dependent, at least in part, on the enzymatic activity of secretory phospholipase A_2 (sPLA2). The aim of this work was to evaluate the effect of ethyl 2-oxo-2H-chromene-3-carboxylate (EOCC), a synthetic coumarin derivative, on the enzymatic activity of sPLA2 from *Crotalus durissus ruruima* venom (sPLA2r) as well as on sPLA2r-induced edema, myotoxicity, and platelet aggregation.

Secretory PLA2r was fractionated in two steps as described by Diz Filho et al. (2009). Whole venom (45 mg)

Abbreviations: CK, creatine kinase; EOCC, ethyl 2-oxo-2H-chromene-3-carboxylate; 7-HOC, 7-hydroxycoumarin; pBPB, *p*-bromophenacyl bromide; PLA2, phospholipase A_2; sPLA2, secretory phosholipase A_2; sPLA2r, secretory phospholipase A_2 from *Crotalus durissus ruruima*.

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was first fractionated by size-exclusion HPLC (Superdex 75, 1 × 60 cm, GE Healthcare), and the crotoxin fraction was purified by monitoring phospholipase A_2 (PLA2) activity (Fig. 1a). Crotoxin was then subjected to reverse-phase HPLC, which led to the identification of one main sPLA2 isoform (Fig. 1b). The molecular mass of purified sPLA2r was 14,299.34 Da, measured as described by Toyama et al. (2005).

Purified sPLA2r was chemically modified with EOCC using the procedure described by Iglesias et al. (2005). EOCC (100 nmol in 10 μL dimethyl sulfoxide) was incubated with sPLA2r (100 nmol in 1000 μL water) for 60 min at 37 °C. The products were fractionated by analytical reverse-phase HPLC (C5 large pore column, Supelco). The resulting sPLA2r had a molecular mass of 14,736.22 Da. This, taken with the 14,299.34 Da mass of native sPLA2r, suggests that two molecules of EOCC (218.21 Da) were complexed to sPLA2r.

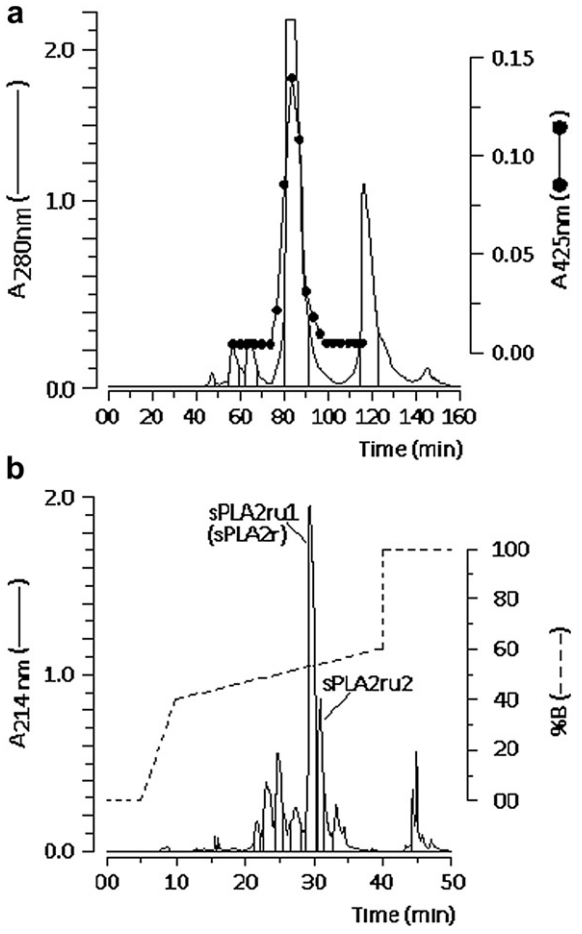

Fig. 1. (a) Chromatographic profile of *Crotalus durissus ruruima* whole-venom fractionation by size-exclusion chromatography (Superdex 75, 1 × 60 cm). Clarified, dried venom (45 mg) was dissolved in the mobile phase (0.2 M ammonium bicarbonate buffer, pH 8.0) and separated using a flow rate of 0.2 mL/min. Fractions were eluted by monitoring absorbance at 280 nm. Enzyme activity was monitored in 10–20 μL samples from each fraction corresponding to crotacetin, convulxin, giroxin, crotoxin, and crotamine. PLA2 activity was detected in the crotoxin fraction and monitored spectrophotometrically at 425 nm. (b) Fractionation of crotoxin from *Crotalus durissus ruruima* by reverse-phase HPLC (C18 μ-Bondapack) using a non-linear gradient of acetonitrile (66% in 0.1% of TFA) and monitoring at $A_{214\ nm}$. The purity of the resulting fractions, termed sPLA2ru1 and sPLA2ru2, was evaluated by tricine SDS-PAGE and mass spectrometry on a MALDI-TOF instrument. The main sPLA2 fraction used in this work was designated sPLA2r.

Amino acid analysis of native or treated sPLA2r samples was performed using the PICO-TAG system (Waters). Samples were hydrolyzed with 6N HCl in the presence of 1% of phenol over 24 h and derivatized with PITC. PTC-amino acids were then analyzed by reverse-phase HPLC. The global amino acid analysis revealed no significant differences between native sPLA2r and EOCC-treated sPLA2r (sPLA2 + EOCC).

PLA2 activity was measured using a chromogenic substrate (4-nitro-3-octanoyloxy-benzoic acid, BIOMOL, USA) as described by Lima et al. (2008). The enzymatic activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance at 425 nm after 20 min. Absorbance was measured using a Spectramax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA). Native sPLA2r had a Vo of 2.51 ± 0.34 nmol/min ($n = 12$). The chemical modification of sPLA2r with pBPB was done as described by de Casto et al. (2000). The Vo decreased to 0.48 ± 0.13 nmol/min ($n = 12$, $p < 0.05$) in the presence of the sPLA2 inhibitor, *p*-bromophenacyl bromide (pBPB). Similar to pBPB, EOCC decreased the Vo of sPLA2r to 0.38 ± 0.08 nmol/min ($n = 12$, $p < 0.05$). These results strongly suggest that EOCC and pBPB have comparable inhibitory potency against sPLA2r. To compare the half maximal inhibitory concentration (IC_{50}) of EOCC and pBPB, we used a substrate concentration of 10 mM and incubated sPLA2r with increasing amounts of each inhibitor (0–14 nmol in 10 μL) for 60 min prior to enzymatic evaluation. EOCC inhibited enzymatic activity in a dose-dependent manner, with maximal inhibition occurring in the presence of 8 nmol and no significant further inhibitory effect being seen with doses of 10–14 nmol (Fig. 2a). The inhibitory effect of EOCC was significantly higher than that observed for pBPB at lower doses (2–8 nmol), but both showed similar inhibitory effects above 10 nmol. Next, we evaluated the effect of the substrate concentration on sPLA2r activity following the protocol described by Toyama et al. (2003). At different substrate concentrations, sPLA2r exhibited moderate allosteric behaviour. The addition of EOCC or pBPB to the enzyme strongly and irreversibly decreased the V_{max} and K_m (Fig. 2b). The enzymatic assay was performed as already described and in all cases, 1 mg/mL sPLA2 solution was incubated with 10 nmol EOCC for 30 min before the enzymatic assay. A comparison EOCC-induced inhibition of sPLA2r and sPLA2 from other sources produced the following results (listed without and with EOCC): 7.83 ± 0.56 and 0.98 ± 0.32 nmol/min for bovine sPLA2; 6.53 ± 0.28 and 2.32 ± 0.17 nmol/min for honey bee sPLA2; 11.43 ± 0.47 and 1.83 ± 0.53 nmol/min for *Naja mossambica mossambica* sPLA2 (Sigma–Aldrich, V1627); 8.78 ± 0.62 and 0.56 ± 0.12 nmol/min for PrTx-III sPLA2 from *Bothrops pirajai* venom; 10.4 ± 0.31 and 1.83 ± 0.35 nmol/min for sPLA2r ($n = 12$, Fig. 2c).

Next, we analyzed the effect of EOCC on sPLA2r-induced edema. Male Wistar rats (120–150 g) were anaesthetized with inhaled halothane, and hind paw edema was induced by a single subplantar injection of native or modified sPLA2 (10 μg dissolved in sterile 0.9% saline solution per paw). Paw volume was measured using a hydroplethysmometer (model 7150, Ugo Basile, Italy) immediately before the