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Thalidomide and pentoxifylline block the renal effects of supernatants of macrophages activated with *Crotalus durissus cascavella* venom

A.M.C. Martins[1], A.C.L. Nobre[2], A.C. Almeida[2], G. Bezerra[2], A.A.M. Lima[2], M.C. Fonteles[3] and H.S.A. Monteiro[2]

Departamentos de [1]Análises Clínicas e Toxicológicas, and [2]Fisiologia e Farmacologia, Instituto de Biomedicina e Unidade de Pesquisas Clínicas, Universidade Federal do Ceará, Fortaleza, CE, Brasil
[3]Departamento de Fisiologia, Universidade Estadual do Ceará, Fortaleza, CE, Brasil

Correspondence
H.S.A. Monteiro
Unidade de Pesquisas Clínicas
Departamento de Fisiologia e Farmacologia
Faculdade de Medicina, UFC
Caixa Postal 3229
60420-970 Fortaleza, CE
Brasil
Fax: +55-85-281-5212
E-mail: martinsalice@hotmail.com

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Abstract

Because thalidomide and pentoxifylline inhibit the synthesis and release of tumor necrosis factor-α (TNF-α), we determined the effect of these drugs on the renal damage induced by supernatants of macrophages activated with *Crotalus durissus cascavella* venom in order to identify the role of TNF-α in the process. Rat peritoneal macrophages were collected with RPMI medium and stimulated *in vitro* with *C.d. cascavella* venom (10 µg/ml) in the absence and presence of thalidomide (15 µM) or pentoxifylline (500 µM) for 1 h and washed and kept in culture for 2 h. Supernatant (1 ml) was tested on an isolated perfused rat kidney (N = 6 for each group). The first 30 min of each experiment were used as control. The supernatant was added to the perfusion system. All experiments lasted 120 min. The toxic effect of the preparation of venom-stimulated macrophages on renal parameters was determined. At 120 min, thalidomide (Thalid) and pentoxifylline (Ptx) inhibited ($P < 0.05$) the increase in perfusion pressure caused by the venom (control = 114.0 ± 1.3; venom = 137.1 ± 1.5; Thalid = 121.0 ± 2.5; Ptx = 121.4 ± 4.0 mmHg), renal vascular resistance (control = 4.5 ± 0.2; venom = 7.3 ± 0.6; Thalid = 4.5 ± 0.9; Ptx = 4.8 ± 0.6 mmHg/ml g^{-1} min^{-1}), urinary flow (control = 0.23 ± 0.001; venom = 0.44 ± 0.01; Thalid = 0.22 ± 0.007; Ptx = 0.21 ± 0.009 ml g^{-1} min^{-1}), glomerular filtration rate (control = 0.72 ± 0.06; venom = 1.91 ± 0.11; Thalid = 0.75 ± 0.04; Ptx = 0.77 ± 0.05 ml g^{-1} min^{-1}) and the decrease in percent tubular sodium transport (control = 77.0 ± 0.9; venom = 73.9 ± 0.66; Thalid = 76.6 ± 1.1; Ptx = 81.8 ± 2.0%), percent tubular chloride transport (control = 77.1 ± 1.2; venom = 71.4 ± 1.1; Thalid = 77.6 ± 1.7; Ptx = 76.8 ± 1.2%), and percent tubular potassium transport (control = 72.7 ± 1.1; venom = 63.0 ± 1.1; Thalid = 72.6 ± 1.0; Ptx = 74.8 ± 1.0%), 30 min before and during the stimulation of macrophages with *C.d. cascavella* venom. These data suggest the participation of TNF-α in the renal effects induced by supernatant of macrophages activated with *C.d. cascavella* venom.

Key words
- Nephrotoxicity
- *Crotalus durissus cascavella*
- Pentoxifylline
- Thalidomide
- Macrophage

Introduction

Snakebites are an important public health problem in Brazil. The genus *Crotalus* contains several species of snakes responsible for high morbidity and mortality rates (1). *Crotalus durissus cascavella* is a snake usually found in the scrubland of the Brazilian Northeast (2). Crotalic venom causes neurotoxicity, systemic myotoxicity, edematogenic reactions, platelet aggregation, and acute renal failure. The most common complication observed in lethal snakebite victims in Brazil is acute renal failure (3), a process that can occur even after specific antivenom treatment. The pathogenesis of acute renal failure after snakebites appears to be multifactorial (4). Some evidence suggests the possible existence of a direct nephrotoxic agent in the venom, but this does not exclude the release of mediators (5) or rhabdomyolysis as causative agents. Alternatively, these underlying causes can potentiate each other (6,7).

We demonstrated in isolated rat kidney that the venom of *C.d. cascavella* causes changes in renal function such as increase in perfusion pressure, urinary flow and percent sodium tubular transport (4). We have also reported that macrophages activated by *C.d. cascavella* venom release nephrotoxic mediators (8).

The participation of tumor necrosis factor-α (TNF-α) in renal injury has been recently demonstrated (9-11). The protective effect of pentoxifylline against the damage induced by ischemia-reperfusion has been demonstrated in several experimental models (12,13).

The aim of this study was to investigate the action of thalidomide and pentoxifylline on the renal effects induced by supernatants of macrophages activated by *C.d. cascavella* venom.

Material and Methods

Macrophage cultures

Rat peritoneal macrophages were collected with RPMI medium 4 days after an injection of 10 ml thioglycolate (3%, *ip*) and placed on plastic tissue culture dishes, as previously described (14). After incubation at 37ºC in a 5% CO_2 atmosphere for 1.5 h, the nonadherent cells were removed by washing the dishes three times with RPMI medium. The cell pattern was determined by cell morphology analysis with a light microscope. The total cells (95% macrophages) were incubated at 37ºC in a 5% CO_2 atmosphere for 2 h in fresh medium (control), and after this period in a medium containing 10 µg/ml *C.d. cascavella* venom. Thalidomide (15 µM) and pentoxifylline (500 µM) were added 30 min before the addition of venom and kept in the medium throughout the period of macrophage stimulation with *C.d. cascavella* venom (10 µg/ml). The supernatant was discarded and, after additional washing, the cells were incubated for 1 h with 1 ml RPMI medium without venom or drugs. Cell-free incubation medium was obtained by centrifugation for 5 min and 1 ml of supernatant was adjusted to 1.3 x 10^7 cells/ml and its effects were tested on an isolated rat kidney. Six rats were used in each group of perfusion. Macrophage viability was determined by Trypan blue exclusion as described elsewhere (15). Macrophage viability ranged from 89 to 97%.

Isolated rat kidney

Adult Wistar rats of both sexes (240-280 g) were fasted with free access to water 24 h before each experiment. The animals were anesthetized with sodium pentobarbital (50 mg/kg body weight). The perfusion fluid was a modified Krebs-Henseleit solution of the following composition: 147 mM Na^+, 5 mM K^+, 2.5 mM Ca^{2+}, 2 mM Mg^{2+}, 110 mM Cl^-, 2.5 mM HCO_3^-, 1 mM SO_4^{2-}, and 1 mM PO_4^{2-}. Bovine serum albumin (BSA, 6 g/100 ml; fraction V), 0.075 g urea, 0.075 g inulin, and 0.15 g glucose were added to the solution, resulting in a final perfusate volume of

100 ml. BSA was previously dialyzed for 48 h at 4ºC in 1.5 liter of Krebs solution, and the solution was changed after 24 h (16,17). The pH was adjusted to 7.4 and the perfusion system, based on Bowman's technique (18), was modified (19) by the addition of an artificial lung to improve oxygenation (20) and of a 1.2-mm Millipore filter (21). Flow calibration and the resistance of the system were determined before each experiment. Perfusion pressure was determined at the tip of the stainless steel cannulae. The right renal artery was cannulated through the upper mesenteric artery and the kidney was isolated (22-24) and submitted to uninterrupted perfusion. After an equilibration period of 15 to 20 min, the experiments were carried out over a total period of time of 120 min. The supernatants of macrophages stimulated with *C.d. cascavella* venom plus pharmacological inhibitors were added 30 min after the beginning of the experiment. Perfusion pressure was measured at 5-min intervals. Urine and perfusate samples were collected every 10 min for the determination of sodium, chloride, potassium, and inulin levels and osmolality. Sodium and potassium concentrations were determined by flame photometry using a model 445 flame photometer (Instrumentation Laboratory Inc., Lexington, MA, USA) and inulin levels were also determined (4,18). Chloride was determined with a LabTest kit (LabTest Diagnóstica, Lagoa Santa, MG, Brazil). The osmolality of the samples was measured with an Advanced Osmometer (Wescor 5100c, Needham Heights, MA, USA) at vapor pressure.

Drugs

C.d. cascavella venom was obtained from the regional ophiology laboratory of Fortaleza (LAROF-CE). Thalidomide was obtained from ICN Biomedical Inc., Aurora, OH, USA. Pentoxifylline, RPMI medium, albumin, inulin, and glucose were purchased from Sigma, St. Louis, MO, USA. Thioglycolate was obtained from Difco Laboratories, Detroit, MI, USA.

Statistical analysis

Data are reported as mean ± SEM and were analyzed statistically by analysis of variance (ANOVA) followed by the Bonferroni test. The level of significance was set at $P < 0.05$.

Results

Effect of tumor necrosis factor inhibitors

Previous results have shown that infusion of the supernatant of macrophages stimulated with *C.d. cascavella* venom (10 µg/ml) after 30 min of internal control caused alterations of renal function parameters (8). In the present study, thalidomide and pentoxifylline, TNF inhibitors, reversed all the renal changes promoted by the supernatant of macrophages stimulated with *C.d. cascavella* venom.

The data in Table 1 show that thalidomide (15 µM) and pentoxifylline (500 µM) inhibited the increase in perfusion pressure, renal vascular resistance, urinary flow, and glomerular filtration rate induced by supernatants of macrophages stimulated with *C.d. cascavella* venom.

Treatment with thalidomide and pentoxifylline also inhibited the decrease in percent tubular sodium, chloride and potassium transport caused by supernatants of macrophages stimulated with *C.d. cascavella* venom as described in Table 2.

Discussion

Acute renal failure has been frequently reported after bites of snakes from the *Viperidae* family (25). Some investigators (5) have reported three possible mechanisms involved in this phenomenon: hemodynamic

Table 1. Thalidomide and pentoxifylline inhibit the renal effects of the supernatant of macrophages stimulated with *Crotalus durissus cascavella* venom.

	RPMI	Venom	Thalidomide (15 µM) + venom	Pentoxifylline (500 µM) + venom
PP (mmHg)				
30	116.3 ± 1.8	117.5 ± 2.2	116.0 ± 2.2	115.4 ± 3.3
60	115.7 ± 0.8	124.6 ± 2.9*	119.0 ± 3.1	121.4 ± 3.1
90	117.6 ± 1.4	137.0 ± 1.3*	121.0 ± 4.0	122.0 ± 5.5
120	114.0 ± 1.3	137.1 ± 1.5*	121.0 ± 2.5	121.4 ± 4.0
RVR (mmHg/ml g^{-1} min^{-1})				
30	4.8 ± 0.3	4.7 ± 0.9	4.6 ± 0.5	4.5 ± 0.9
60	4.7 ± 0.1	6.6 ± 0.5*	4.6 ± 0.4	4.6 ± 1.0
90	4.4 ± 0.2	7.1 ± 0.8*	4.7 ± 0.6	4.5 ± 0.8
120	4.5 ± 0.2	7.3 ± 0.6*	4.5 ± 0.9	4.8 ± 0.6
UF (ml g^{-1} min^{-1})				
30	0.21 ± 0.05	0.18 ± 0.001	0.19 ± 0.009	0.20 ± 0.003
60	0.20 ± 0.01	0.26 ± 0.03*	0.20 ± 0.04	0.20 ± 0.06
90	0.22 ± 0.02	0.38 ± 0.02*	0.20 ± 0.006	0.19 ± 0.013
120	0.23 ± 0.01	0.44 ± 0.01*	0.22 ± 0.007	0.21 ± 0.009
GFR (ml g^{-1} min^{-1})				
30	0.76 ± 0.04	0.72 ± 0.06	0.77 ± 0.06	0.79 ± 0.05
60	0.79 ± 0.05	0.95 ± 0.05	0.73 ± 0.05	0.82 ± 0.06
90	0.70 ± 0.04	1.41 ± 0.17*	0.71 ± 0.05	0.78 ± 0.06
120	0.72 ± 0.06	1.91 ± 0.11*	0.75 ± 0.04	0.77 ± 0.05

Macrophages were inoculated with 10 µg/ml venom in the presence or absence of 15 µM thalidomide or 500 µM pentoxifylline for 2 h at 37ºC. The supernatant was tested on the isolated rat kidney. Data are reported as means ± SEM for six kidney perfusions carried out for each set of conditions. PP = pentoxyfylline; RVR = renal vascular resistance; UF = urinary flow; GFR = glomerular filtration rate.
*$P < 0.05$ compared to other values in the same time group (ANOVA followed by the Bonferroni test).

alterations, immunologic reactions and direct nephrotoxicity. Some investigators have emphasized the importance of rhabdomyolysis as a cause of acute renal failure after crotalid bites (7), while others have reported nephrotoxicity in the rat isolated kidney after administration of crotalid venom (4,26).

Perfusion of the isolated kidney has been extensively used as a model to study the vascular effect of biologically active substances, preventing the interference of blood-borne cells, hormones and other factors with renal function transported by blood (16).

We have recently demonstrated the renal effect of the supernatant of macrophages activated with *C.d. cascavella* venom (8). Acting as alarm cells, macrophages signal the presence of foreign bodies by elaborating and releasing several substances, including cytokines and arachidonic acid metabolites (27).

An increase in serum TNF-α levels has been reported to occur in mice injected with *Bothrops asper* and *B. jararaca* snake venom (28). It has been recently demonstrated that renal cells can produce and release TNF-α (29) and that the kidney is highly sensitive to this cytokine. Hence, it is plausible to relate this protein to the renal damage associated with envenomation or other inflammatory stimuli, as reported by other investigators (9,10).

It has been observed that pentoxifylline can regulate calcium homeostasis (30) and thalidomide can affect the proliferation of endothelial cells and the expression of alpha-beta 3 integrin on the surface of endothelial cells (31). Thalidomide inhibits TNF-α synthesis by degrading the mRNA for this

Table 2. Thalidomide and pentoxifylline inhibit the renal tubular effects of the supernatant of macrophages stimulated with *Crotalus durissus cascavella* venom.

	RPMI	Venom	Thalidomide (15 µM) + venom	Pentoxifylline (500 µM) + venom
%TNa+				
30	80.0 ± 1.8	80.2 ± 0.8	79.5 ± 2.5	83.6 ± 2.0
60	79.0 ± 1.2	74.4 ± 2.5*	76.6 ± 1.7	85.9 ± 1.0
90	77.0 ± 1.0	73.6 ± 1.0*	77.4 ± 0.9	83.6 ± 1.0
120	77.0 ± 0.9	73.9 ± 0.6*	76.6 ± 1.1	81.8 ± 2.0
%TCl-				
30	79.5 ± 1.2	78.1 ± 1.3	78.4 ± 2.5	77.6 ± 1.0
60	77.9 ± 1.5	73.2 ± 2.1*	76.7 ± 1.8	75.9 ± 1.5
90	76.9 ± 1.5	72.6 ± 1.5*	76.9 ± 1.9	75.6 ± 1.8
120	77.1 ± 1.2	71.4 ± 1.1*	77.6 ± 1.7	76.8 ± 1.2
%TK+				
30	73.2 ± 1.3	74.1 ± 1.3	75.4 ± 1.3	74.6 ± 1.2
60	72.9 ± 1.4	73.2 ± 2.1	74.7 ± 1.2	74.9 ± 1.4
90	71.5 ± 1.2	64.8 ± 1.5*	73.9 ± 1.5	73.6 ± 1.5
120	72.7 ± 1.1	63.0 ± 1.1*	72.6 ± 1.0	74.8 ± 1.0

Macrophages were inoculated with 10 µg/ml venom in the presence or absence of 15 µM thalidomide or 500 µM pentoxifylline for 2 h at 37ºC. The supernatant was tested on the isolated rat kidney. Data are reported as means ± SEM for six kidney perfusions carried out for each set of conditions. %TNa+ = percent tubular sodium transport; %TCl- = percent tubular chloride transport; %TK+ = percent tubular potassium transport. *P < 0.05 compared to other values in the same time group (ANOVA followed by the Bonferroni test).

cytokine (29). Thalidomide and pentoxifylline blocked the synthesis and release of TNF-α from macrophages in culture after the same period of incubation and concentration as used in our experiments (12). Using a similar approach, it was demonstrated that thalidomide and pentoxifylline alone did not modify the functional parameters of the kidney (32).

In some situations, receptor-mediated events induced by TNF-α or Fas (apoptosis-mediating surface antigen fas; CD95) may play a role in apoptosis in acute renal failure (33). Recently, it was demonstrated that pentoxifylline may exert a protective effect against ischemic acute renal failure by inhibiting TNF-α production in rabbits (34).

In the present study, thalidomide and pentoxifylline reversed all renal alterations induced by the supernatants of macrophages stimulated with *C.d. cascavella* venom. Our findings suggest that thalidomide and pentoxifylline inhibit the release of renal cytotoxic mediators by macrophages activated with *C.d. cascavella* venom, and indicate the need for investigation of the possible participation of TNF-α in this process. The results also support the need for investigations of the possible use of these drugs in the treatment of snakebites.

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