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***In vitro* studies with renal proximal tubule cells show direct cytotoxicity of *Androctonus australis hector* scorpion venom triggered by oxidative stress, caspase activation and apoptosis**

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Abstract

Scorpion envenomation injures a number of organs, including the kidney. Mechanisms proposed to explain the renal tubule injury include direct effects of venom on tubule epithelial cells, as well as indirect effects of the autonomic nervous system, and inflammation. Here, we report direct effects of *Androctonus australis hector* (Aah) scorpion venom on the viability of Renal Proximal Tubule (RPT) cells *in vitro*, unlike distal tubule and collecting duct cells. Extensive NucGreen nuclear staining was observed in immortalized rabbit RPT cells following treatment with Aah venom, consistent with cytotoxicity. The involvement of oxidative stress is supported by the observations that 1) anti-oxidants mitigated the Aah venom-induced decrease in the number of viable RPT cells, and 2) Aah venom-treated RPT cells were intensively stained with the CellROX[®] Deep Red reagent, an indicator of Reactive Oxygen Species (ROS). Relevance to normal RPT cells is supported by the red fluorescence observed in Aah venom treated primary rabbit RPT cell cultures following their incubation with the Flica reagent (indicative of caspase activation and apoptosis), and the green fluorescence of Sytox Green (indicative of dead cells).

Keywords

Aah; scorpion venom; renal proximal tubule; apoptosis; oxidative stress

1. Introduction

More than a million cases of scorpion envenomation occur worldwide on an annual basis [1]. While many cases are minor, other cases cause serious medical problems, including

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those caused by scorpions in the Buthidae family. The Buthidae family is found worldwide, including North Africa, North and Central America, Asia, and South Africa [1]. Of particular interest, is *Androctonus australis hector* (Aah), one of the most toxic scorpions to humans, which is often implicated in fatal scorpion sting accidents in Algeria [2]. The venom of this scorpion contains numerous toxins, whose actions may have serious medical consequences following envenomation [1, 3]. Included amongst these toxins are the scorpion α toxins, which bind to voltage-gated sodium channels, causing prolonged depolarization, and neuronal excitation [4]. Both the sympathetic and parasympathetic nervous system is stimulated by these neurotoxins, resulting in the release of catecholamines and other vasoactive peptides, as well as hypertension, cardiac dysfunction and pulmonary edema, amongst other symptoms [1, 3]. An inflammatory response is observed, which causes metabolic derangements and organ damage [3].

Scorpion venom produces multiple adverse effects in various tissues, including the kidney, a highly vascularized organ which is vulnerable to toxin injury [3, 5, 6]. Clinical manifestations of renal injury include proteinuria, hematuria and hemoglobinuria. Inflammatory reactions and nephrotoxic effects of venoms are also observed [7, 8]. Renal failure may occur, particularly following scorpion envenomations in North Africa, the Middle East and Eastern Mediterranean region, as well as in South Asia. The underlying causes of the renal injury which occurs following envenomation are not clearly understood. However, renal vasoconstriction may be involved [3]. Renal vasoconstriction very likely occurs as a consequence of the catecholamines that are produced by the sympathetic nervous system following envenomation, and this renal vasoconstriction may be responsible for the renal ischemia, which may also occur following envenomation. In addition, inflammatory responses may contribute to the renal injury that occurs following envenomation [8]. Ultimately, the activation of the parasympathetic nervous system, and inflammatory responses may cause renal tubular necrosis. However, it is unclear whether the tubular necrosis observed in the renal cortex following envenomation is a direct effect of components of scorpion venom, or an indirect effect of the activated sympathetic nervous system and/or inflammatory response.

In this report, the effects of *Androctonus australis hector* (Aah) scorpion venom on renal tubule epithelial cells *in vitro* are examined. Immortalized renal proximal tubule (RPT) cells, as well as established renal tubule epithelial cell lines, and primary RPT cell cultures are examined with regards to their response to Aah venom. Our results indicate that Aah venom is cytotoxic to RPT cells, unlike the tubule epithelial cells derived from other nephron segments, and that underlying mechanisms include oxidative stress, as well as apoptosis.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DME), Ham's F12 Medium (F12), soybean trypsin inhibitor, trypsin, ReadyProbes® Cell Viability Imaging Kit, Image-iT™ LIVE Red Poly Caspases Detection Kit, and CellROX® Deep Red Reagent were from Life Technologies Corp (Carlsbad, CA). Collagenase Class IV was from Worthington (Freehold, NJ). Bovine insulin, human transferrin, triiodothyronine (T₃), hydrocortisone, PGE₁, Glutathione,

Ascorbic acid, α -tocopherol, Menadione, and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were from Sigma Aldrich Chemical Corp. (St Louis MO). Selenium was from Difco laboratories (Detroit, MI). The rabbit kidney proximal tubule cell line RPT clone 8 was immortalized as described previously [9]. The OK cell line, and the HK-2 cell line were obtained from the American Type Culture Collection. The MDCK cell line was obtained from Dr. Milton H. Saier, Jr. (UCSD, San Diego, Calif.), and the mouse M1 collecting duct cell line was obtained from Dr. Alejandro Bertorello (Stockholm, Sweden). Crude venom of *Androctonus australis hector* (Aah) scorpion was provided by the Pasteur Institute (Algiers), and kept at -20°C until use. The Prism 6 software was from GraphPad, Inc. (San Diego, CA).

2.2. Cell culture

Established cell lines were routinely maintained in Medium K-1, a hormonally defined serum free medium, as previously described [10]. Medium K-1 consists of a 50:50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 Medium containing 15 mM HEPES and 20 mM sodium bicarbonate (DME/F12) (pH 7.4), which is supplemented with 5 $\mu\text{g/ml}$ bovine insulin, 5 $\mu\text{g/ml}$ human transferrin, 5×10^{-12} M triiodothyronine (T_3), 5×10^{-8} M hydrocortisone, 25 ng/ml PGE_1 , and 5×10^{-8} M selenium. In addition, 92 U/ml penicillin and 0.2 mg/ml streptomycin are present. Water used for medium and growth factor preparations was purified using a Milli-Q deionization system. Cultures were maintained in a humidified 5% CO_2 /95% air mixture at 37°C .

Primary rabbit RPT cell cultures were initiated from rabbit kidneys, as previously described [11]. The New Zealand White rabbits (2–2.5 kg) used to obtain kidneys for primary cultures were euthanized following a procedure approved by the IACUC of the University at Buffalo, which is in compliance with the National Institutes of Health guide for the care and use of Laboratory animals. Rabbit kidneys were perfused via the renal artery, first with phosphate buffered saline (PBS), and subsequently with DME/F12 containing 0.5% iron oxide (w/v), until the kidney turned gray-black in color. Renal cortical slices, were homogenized with five strokes of a sterile glass homogenizer. The homogenate was poured, first through a 253 μm , and then through an 83 μm mesh nylon sieves. Tubules and glomeruli on the 83 μm sieve were transferred into a tube containing sterile DME/F12. Glomeruli (containing iron oxide) were removed with the magnetic stir bar. The remaining proximal tubules were incubated for 2 min at 23°C in DME/F12 containing 0.05mg/ml collagenase class IV and 0.5 mg/ml soybean trypsin inhibitor. The dissociated tubules were washed by centrifugation, resuspended in DME/F12, and plated into 35 mm cultures dishes (or 24 well plates) containing Medium RK-1 (i.e. DME/F12 supplemented with 5 $\mu\text{g/ml}$ bovine insulin, 5 $\mu\text{g/ml}$ human transferrin, 5×10^{-8} M hydrocortisone, 92 U/ml penicillin and 0.01% kanamycin (rather than streptomycin)). The cultures were then maintained at 37°C , in a 5% CO_2 -95% air, humidified environment. The medium was changed 1 day after plating, and every 3 days thereafter.

2.3. Venom treatment

The established and immortalized renal cells were plated into 96 well plates at 10^3 cells/well into Medium K-1. The cell number used for inoculation was determined using a Coulter

Counter. The following day, the venom, Aah, was added at varying concentrations, diluted in the supplemented medium.

2.4. Colorimetric MTT (tetrazolium) assay and viability examination

Cells cultured in 96-well plates were incubated with Aah in supplemented media (in Culture medium) for 5 days (or as specified). After the incubation with Aah, 10 μ l of 5mg/ml MTT solution was added to each well, followed by a 4 hr incubation at 37°C. Metabolically active cells convert MTT into purple formazan, permitting a spectrophotometric estimation of the cell number [12]. However, first, the purple formazan crystals that are produced must be dissolved in acid-isopropanol. Thus, 110 μ l of 0.04 N HCl in isopropanol was added to all wells, and mixed thoroughly to dissolve the purple formazan crystals. Then, the optical density was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (BioTek®, Winooski, VT). The relative number of viable cells was then calculated.

A ReadyProbes® Cell Viability Imaging Kit was used to detect cell viability after 5 days incubation with venom. While the blue dye (DAPI) was used to stain all living cells, the green dye was used to stain dead cells. The cells were visualized using a Zeiss Axio Observer fluorescence microscope, and photographed. Ten random fields were imaged for each group, and the number of total cells and dead cells were obtained by counting. The percentage of dead cells was calculated as follows: % Dead cells = (the number of dead cells)/(the total cell number)

2.4. Antioxidant assay and intracellular Reactive Oxygen Species detection

Immortalized RPT cells were plated in 96 well plates in either Medium K-1 further supplemented with an antioxidant mixture: GSH (0.064mM), Ascorbic acid (0.43mM), α -Tocopherol (1.13 μ M) and Selenium (4.76 μ M), or no further supplement. The following day, the venom was added at varying concentrations, and the cells were incubated for 5 days. In a portion of the cultures, the relative number of viable cells was evaluated using the MTT assay. In other cultures, the CellROX® Deep Red Reagent was used to assess the level of intracellular reactive oxygen species. The CellROX® Deep Red reagent (which is initially non-fluorescent) freely enters the cells, where it is cleaved by endogenous esterases. The reagent becomes highly fluorescent after being oxidized by ROS [13, 14], having an absorption/emission maxima of ~644/665nm. Immortalized RPT cell line were incubated at 37°C with CellROX Deep Red reagent (5 μ M) for 24h, either in the presence of Aah (100 μ g/ml), a positive control (50 μ M Menadione), or no further supplement. The cultures were then visualized under a fluorescence microscope.

2.5. Apoptosis and toxicity studies

After 5 days in culture, primary RPT cells were incubated with 100 μ g/ml Aah. Three days later, the cells were labeled with FLICA, Hoechst 33342 and SYTOX Green (the components of an Image-iT LIVE Red Poly Caspases Detection Kit). FLICA (excitation/emission maximum of 550/595 nm) stains cells with activated caspases (including caspase-1, -3, -4, -5, -6, -7, -8, and -9). SYTOX Green (excitation/emission maxima of 504/523 nm) stains only dead cells, while Hoechst 33342 (excitation/emission maxima of 350/461 nm) stains all cells.

Cells stained with FLICA, Hoechst 33342 and/or SYTOX Green were detected using a Zeiss Axio Observer inverted microscope. In each experimental condition, images of at least 25 microscope fields/culture in duplicate culture were acquired using Axiovision software. The number of cells stained with FLICA, Hoechst 33342 and SYTOX Green was determined in each microscope field using the NIH Image J Program. The proportion of apoptotic and dead cells relative to the total number of cells (stained with Hoechst 33342) was then calculated. In addition, the effect of a 3 day incubation with 100 µg/ml Aah on the relative number of viable primary RPT cells was determined using the MTT assay. The primary RPT cell cultures used for the MTT assay were in 24 well culture plates.

Statistical analysis

Results are expressed as Means $\pm$ the Standard Error of the Mean (SEM). Comparison within groups was made by student t tests using Prism 6 software. The difference was considered statistically significant when $p < 0.5$.

3. Results

3.1. Effect of Aah venom on the relative number of viable cells

In order to investigate Aah-induced nephrotoxicity *in vitro*, immortalized RPT cells were treated for 5 days with Aah venom (at concentrations ranging from 20–110 µg/ml). Subsequently, the relative number of viable cells was determined using the MTT assay. Fig. 1A shows the altered morphology and reduced number of immortalized RPT cells in the presence of 51 µg/ml Aah venom. Fig. 1B shows that the relative number of viable cells was reduced by more than 60% at Aah concentrations >20 µg/ml. The effects of lower concentrations of Aah venom (1–5 µg/ml) were also studied, and, as shown in Fig. 1C, a maximal response was still obtained at all concentrations tested. In order to determine whether this response to Aah is a characteristic of renal proximal tubule (RPT) cells, two other RPT cell lines were studied, including Opossum Kidney (OK) (Fig. 2A) and the human HK-2 cell line (Fig. 2B). The results indicated that Aah venom caused a reduction in the number of viable OK cells (Fig. 2A), and HK-2 cells (Fig. 2B), that was similar in magnitude, and occurred within the same concentration range (>20 µg/ml) as observed with MDCK cells.

In order to determine whether renal tubule epithelial cells derived from other nephron segments possessed a similar sensitivity to Aah venom, studies were conducted with the distal tubule cell line MDCK, derived from canine kidney, as well as the mouse M1 cortical collecting duct cell line. However, as illustrated in Fig. 3A, Aah venom did not significantly reduce the number of viable MDCK cells, and as illustrated in Fig. 3B, Aah venom was only slightly inhibitory to M1 cells at 25 µg/ml. A significantly higher Aah concentration (51 µg/ml) was required to obtain a maximal reduction in the number of viable cells, a 35% reduction, which was also lower than that obtained with the RPT cell lines.

3.2 Induction of Cell Death by Aah Venom

The reduction in the number of viable cells in immortalized RPT cells treated with Aah venom may be due to an increase in the frequency of dead cells. In order to examine this

hypothesis, immortalized RPT cells were treated with 51 µg/ml Aah venom for 5 days. The cultures were then loaded with both NucGreen dead stain, as well as NucBlue live stain. Fig. 4A shows that venom treated cultures predominantly stained green (indicative of dead cells), unlike control cells, which were predominantly blue. The effect of increasing concentrations of Aah venom on the number of dead cells was determined by NucBlue/Green staining (Fig. 4B). MTT assays were conducted in parallel cultures (Fig, 4C). The results indicate that at Aah concentrations >20 µg/ml more than 60% of the cells were dead (Fig 4B), and that the relative number of viable cells was reduced by > 90%. Thus, the reduction in the number of viable cells in the venom-treated cultures could primarily be explained by Aah venom-induced cell death, although other mechanisms (such as a decrease in growth rate) may also be involved.

3.3. Oxidative Stress in Aah venom treated cultures

The hypothesis was examined that the effect of Aah venom on viability can be attributed to oxidative stress. In order to examine this hypothesis initially, the effect of antioxidants on the Aah venom-induced decrease in the viability of immortalized RPT cells was examined. Fig. 5 shows that the inhibitory effect of Aah venom on the relative number of viable RPT cells was significantly reduced following the addition of an antioxidant mixture (containing glutathione (GSH), 2-phospho-ascorbate, α-tocopherol, selenium). These results may be explained if Aah venom causes an increase in reactive oxygen species (ROS). In order to evaluate this hypothesis, the influence of Aah venom on the intracellular oxidative stress, the CellROX® Deep Red Reagent was employed. This cell-permeant dye exhibits a bright red fluorescence when oxidized by ROS. Fig. 5 shows that after exposure to 100 µg/ml Aah venom for 24h, the immortalized RPT cells were uniformly bright red, unlike untreated control cells. Similarly, after treatment with 50 µM menadione (a positive control) the immortalized RPT cells were uniformly bright red. Menadione stimulates the formation of unstable semiquinones by mitochondrial NADH-ubiquinone oxidoreductase (complex I), which recycle to reform quinones and ROS [15].

3.4. Effect of Aah venom on the apoptosis and death of primary RPT Cells

Our studies above indicate that Aah venom causes oxidative stress, and the death of immortalized rabbit RPT cells. Oxidative stress has been closely linked to apoptosis. Thus, apoptosis was investigated as being the underlying mechanism of killing of RPT cells that occurs in response to Aah venom. Primary rabbit RPT cells were employed in these studies. The primary RPT cells were used to develop the immortalized rabbit RPT cell line, and very closely resemble normal cells, having just been removed from the animal.

The primary RPT cells were incubated for 3 days with 100µg/ml Aah venom. Subsequently, the cultures were examined by fluorescence microscopy for the presence of activated caspases, which are involved in executing the apoptotic process. Caspases are activated during apoptosis, and bind to FLICA reagent, which emits fluorescence. The upper panel of Fig. 6A shows the red fluorescence of the FLICA reagent in primary RPT cells treated with Aah venom, unlike untreated cells. , In addition, SYTOX green fluorescence was observed in Aah venom treated cultures (indicative of dead cells), which varied in quantity in different microscope fields. The number of apoptotic and dead cells in Aah venom treated and control

cultures was quantitated. As illustrated in the lower panel of Fig. 6A, the proportion of apoptotic cells in primary RPT cells with Aah was 5.8 fold higher than the control (i.e. $13.0 \pm 0.1\%$ and $2.3 \pm 0.4\%$, in the presence and absence of Aah, respectively). The number of dead cells in Aah treated cultures also increased 9.5%-fold vs. controls (i.e. $13.8\% \pm 1.5\%$, and $1.5 \pm 0.2\%$, in the presence and absence of Aah, respectively).

In order to determine whether the increase in apoptotic cells and dead cells was associated with a decrease in viable cells, the MTT assay was conducted. Fig. 6B shows that after a 3 day incubation with either $100 \mu\text{g/ml}$ or $200 \mu\text{g/ml}$ Aah, the relative number of viable cells decreased by $40 \pm 2\%$, and $61 \pm 7\%$, respectively. Thus, apoptosis was indeed associated with a decrease in the number of viable cells in Aah treated cultures, although we cannot rule out that other mechanisms, such as growth inhibitory affects, are involved.

4. Discussion

Scorpion venom rapidly redistributes from the blood to other organs after envenomation [6, 16, 17]. After 15 min, the kidneys have accumulated the highest levels, followed by the liver, lungs and heart, while the brain exhibits the lowest level [1, 6, 16–18]. Venom toxins leave the blood more rapidly than other venom components, and can accumulate in such tissues as the kidney at concentrations high enough to cause direct toxicity [19]. However, many of the symptoms that emerge following scorpion envenomation are indirect effects, resulting from inflammatory reactions, and an “autonomic storm,” of neurotransmitters [3, 16, 20]. The activity of ion channels may also be affected following envenomation, both due to direct affects of peptide toxins on the ion channels themselves, as well as indirect effects [1, 21]. The renal injury which may occur has been attributed to the catecholamine storm, the resultant renal vasoconstriction, and renal ischemia [3]. However, in addition, immunopathologic changes have been observed, with accompanying tubular necrosis in mice injected with *Androctonus australis hector* scorpion venom [8]. Tubular necrosis has also been observed following envenomation with *Hemiscorpius lepturus* scorpion venom [22, 23]. However, it is unclear whether the renal injury resulting from scorpion venoms is due to a direct effect of the venom components, or a whether renal injury depends upon the catecholamine storm and/or the inflammatory response.

In the present study, the ability of Aah venom to elicit direct cytotoxic effects on renal tubule epithelial cells was evaluated. Towards these ends, the sensitivity of different kidney tubule epithelial cell lines to Aah venom was examined by incubating the cultures with venom at concentrations ranging from $25 \mu\text{g/ml}$ to $110 \mu\text{g/ml}$ over a 5 day period. By this means, it was shown definitively, that kidney tubule epithelial cell lines derived from the distal tubule (MDCK) and the cortical collecting duct (M1) are not affected by Aah venom regardless of venom concentration. In contrast, our MTT assay results indicated that Aah venom caused a reduction in the number of viable primary RPT cells, as well as the number of viable immortalized RPT cells was similarly, the number of viable cells was reduced by incubation with Aah venom at levels as low as $25 \mu\text{g}$ in the RPT cell lines, OK and HK-2. A maximal cytotoxic effect was still obtained when immortalized RPT cells were treated with much lower Aah venom concentrations ($1\text{--}5 \mu\text{g/ml}$). The No Observed Effect Level (NOEL) was not within this range. Similarly, when immortalized RPT cells were treated with Aah venom

for a much shorter time interval (24 hours) substantial levels of ROS were generated. That the loss of cell viability could be attributed to this ROS generation was indicated by the protective effects of antioxidants against the Aah venom induced cytotoxicity.

In studies with experimental animals, the potency of Aah venom is evaluated by determining its median lethal dose, or LD₅₀ value (e.g. 260 µg Aah venom/kg for IV injection into a mouse) [24]. When experimental animals are injected with a lethal dose of scorpion venom, the venom quickly accumulates in the blood, where it may achieve levels as high as 740 ng/ml [6] (close to 1 µg/ml, an Aah venom concentration that is still maximally cytotoxic to immortalized RPT cells). In humans who have been stung either by *Androctonus australis garzonii* (Aag) or *Buthus occitanus tunetanus* (Bot), mean serum venom concentrations of 21.72 ± 6.51 ng/ml have been reported by Krifi et al. [25]. However, the actual venom concentration present in these patients immediately after the scorpion sting was probably considerably higher.

In fact, in experimental animals the level of venom in blood declines as much as 6 fold within 1 hour after administrating venom [26]. During this time, the venom is redistributed to other tissues [26]. Kidney tissue accumulates the largest concentration of venom after this redistribution, and because this is coupled with a slow removal rate of venom, the exposure of renal tissue to venom may be of a duration as prolonged as in the experiments described in this report [26–28]. Moreover, venom toxins can achieve very high tissue concentrations after their redistribution from the blood to other tissues, particularly the kidney, and thus have the potential to cause direct toxicity [19]. Little is known about the consequences of prolonged incubations with the individual venom toxins, which is one justification for long term tissue culture studies.

The differential susceptibility of different types of renal tubule epithelial cells to Aah venom can be attributed to the differential expression of cellular targets recognized by such scorpion venom toxins, including voltage-gated K⁺ channels [29]. Several types of voltage-gated K⁺ channels are specifically present in renal proximal tubules, including Kv1.10 and KVLQT1 (unlike other nephron segments), and may be blocked (or activated) by components in scorpion venom [29, 30]. Indeed, Kv1.10 is reported blocked by Charybdotoxin (ChTX) from *Leiurus quinquestriatus* [3]. Presumably, similar components of Aah scorpion venom may block voltage gated K⁺ channels in the RPT, resulting in the marked destruction, distortion and necrosis of proximal tubule cells in the renal cortex of animals treated with Aah venom [8].

The Aah venom-induced reduction in the relative number of viable RPT cells observed in our MTT assays could possibly be simply explained by an inhibitory effect of Aah venom on growth. However, our NucGreen staining results indicate that a large proportion of immortalized RPT cells treated with Aah venom are dead, unlike the control group. These results are consistent with the *in vivo* studies reporting necrosis in renal tubular epithelial cells treated with scorpion venom [5, 31–33]. These results support the hypothesis that the tubule necrosis observed *in vivo* following scorpion envenomation is due to direct effects of Aah venom on tubule epithelial cells. However, these results do not exclude the hypothesis that the “autonomic storm” contributes to the renal tubule necrosis in animals injected with

scorpion venom [34]. Although there is considerable evidence indicating the involvement of the “autonomic storm” and the inflammatory response in animals envenomated with scorpion venom [3, 8], scorpion venom derived from *Hemiscorpius lepturus* primarily acts via direct haemolytic, nephrotoxic and hepatotoxic actions [21, 31].

We observed that reduction in the number of viable RPT cells treated with Aah venom was significantly alleviated when RPT cells were treated with antioxidants, including glutathione (GSH), 2-phospho-ascorbate, α -tocopherol, as well as increased selenium. This latter observation suggests that oxidative stress is involved in Aah venom-mediated nephrotoxicity. Consistent with this hypothesis are the reports indicating that *in vivo* Aah venom-induced nephrotoxicity is associated with a decrease in the tissue’s antioxidant defense systems, including catalase and GSH [8]. Increased lipid peroxidation has also been reported in the kidneys and other organs of animals injected with Aah, as well as Egyptian *Scorpio maurus palmatus* venom [35–37]. Dousset et al. [37] have shown protective effects of the antioxidant N-acetylcysteine (NAC) after envenomation of mice with lethal doses of toxic fractions of Aah venom. The results of our study are in accordance with these reports, further demonstrating that oxidative stress plays a critical role in the cytotoxicity induced by Aah venom.

Our studies with the CellROX Deep Red Reagent indicated that ROS were generated during a 24h incubation with Aah venom. Based on this finding, and the protective effect of antioxidants, one may postulate that the renal damage induced by *Androctonus australis hector* scorpion venom is mediated by free radicals. Indeed, scorpion venoms have been observed to stimulate the production of ROS, which may serve as the source of renal damage following experimental envenomations [8, 37]. The ROS generated under these conditions may underlie several complex mechanisms, including membrane lipid peroxidation, protein denaturation, and DNA damage, which may in turn induce apoptosis in kidney proximal tubules and renal cell lines [37, 38].

In order to determine whether Aah venom-induced cell death is a consequence of apoptosis, primary RPT cells were stained with FLICA, so as to detect activated caspases (including caspase-1, -3, -4, -5, -6, -7, -8, and -9). Caspase activation initiates the sequence of events leading to apoptosis in animal cells [39]. Scorpion venom-induced apoptosis is not well documented *in vivo*; however, scorpion venom-induced apoptotic cell death has been reported in a number of different cancer cell lines. Diaz-Garcia et al. [40] observed that the mode of cell death induced by *Rhopalurus junceus* venom in Hela cells involved up-regulation of *bax*, a pro-apoptotic gene, and the down-regulation of *bcl-2*, an anti-apoptotic gene. Bcl-2 down-regulation occurred as a consequence of *p53* up-regulation, and was associated with the activation of caspases, chromatin condensation, and the formation of apoptotic nuclei. Gupta et al. [41] similarly obtained evidence that the inhibitory effect of *Heterometrus bengalensis* scorpion venom on the growth of the human leukemic cell lines U937 and K562 occurred through apoptosis. The apoptosis was characterized by membrane blebbing, chromatin condensation and DNA fragmentation. Similarly, Wang et al. (2005) [42] obtained evidence that *Buthus martensi* Karsch (BmK) venom induces the death of the malignant human glioma U251-MG cell line by apoptosis. Similarly, evidence for apoptosis was obtained when the human breast carcinoma cell line SKBR3 was treated with *Tityus*

discrepans scorpion venom [43]. Similar apoptotic effects, including nuclear fragmentation and a decrease in cell density, were observed when using two peptides, neopladine1 and neopladine2, purified from *Tityus discrepans* scorpion venom [43].

Oxidative stress and apoptosis can be initiated by a number of different components in Aah venom. Aah Venom, like other scorpion venoms, contains toxins, such as AaH II, that bind to voltage gated sodium channels (VGSCs), thereby inhibiting the inactivation of such VGSCs [44]. However, the RPT does not possess such VGSCs. Aah Venom also contains toxins in the kaliotoxin family that bind to Ca^{2+} -activated K^{+} channels [45]. Moreover primary rabbit RPT cells have been observed to possess Ca^{2+} -activated K^{+} channels (BKCa's) that are inhibited by kaliotoxin family members, including kaliotoxin itself, iberiotoxin and Charybdotoxin. However, our preliminary studies (Taub, unpublished) indicate only partial inhibition of immortalized rabbit RPT cell growth is obtained when these toxins are present at concentrations that completely inhibit these BKCa's in primary rabbit RPT cells). Thus, it seems likely that other components of Aah venom are responsible for the cytotoxicity.

To summarize, our results indicate that the apoptosis induced death of RPT cells treated with Aah venom can be explained by the generation of free radicals, and oxidative stress. The intensity of the oxidative stress may determine the cell death pathway, since exposure to high concentrations of H_2O_2 has been shown to result in necrosis, while lower concentrations result in apoptosis [46]. Further experiments are required to delineate the mechanisms involved in the cytotoxicity of Aah venom, and to understand how cell death regulatory proteins that control cell death pathways interact so as to modulate the death that occurs in RPT cells in response to Aah venom.

5. Conclusion

The clinical consequences of scorpion envenomations are extremely diverse in humans, depending upon the scorpion and quantity of venom inoculated, as well as the patient. However, immediately following a scorpion sting a number of responses can be observed rapidly, depending upon severity, including neurohormonal and hemodynamic responses, as well as pulmonary edema and shock [8]. In contrast, scorpion sting nephropathy does not present itself immediately, although the kidney can be affected by the adrenergic storm following a scorpion sting [28]. Indeed, even when the kidney is directly perfused for 2 hours with scorpion venom tubular transport is not affected, although there is an increase in vasculature resistance, a decrease in the glomerular filtration rate and a decrease in urinary flow [17]. This is not to say that renal tubule damage does not occur in the kidney over more prolonged periods following scorpion envenomation. Indeed, lipid peroxidation occurs, and renal tubule damage may even be observed 24 hours after a sublethal dose of Aah venom [8]. However, renal insufficiency generally takes a number of days to appear following scorpion envenomation, as exemplified by the case study with a 64 year old woman who developed edema and reduced urine output 7 days after a scorpion sting [28]. Thus, in vitro studies concerning the chronic as well as acute effects of scorpion venom on renal proximal tubule cells in vitro are important to study.

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Highlights

- Androctonus australis hector venom reduces renal proximal tubule cell viability
- Androctonus australis hector venom induces oxidative stress in proximal tubule cells
- Androctonus australis hector venom causes caspase activation in proximal tubule cells

Figure 1.

Effect of Aah on immortalized RPT cells. (A) Images of RPT cells after 5 days incubation +/- 66 µg/ml Aah (B) Effect of Aah concentration (25–110 µg/ml) on the relative number of viable cells as compared percentagewise with the untreated, control group. (c) Effect of Aah venom concentration (1–5 µg/ml) on the relative number of viable cells as compared percentagewise with the untreated, control group. The results represent means $\pm$ SEM of triplicate determinations. *Statistically different from control at $P < 0.01$

Figure 2.

Effect of Aah on the relative viability of A) OK and B) HK-2 cells, respectively. The upper frame of A and B shows photomicrographs of either OK or HK-2 cells in the presence or absence of 66 μg/ml Aah. The lower frame of A and B shows the relative number of viable cells as a function of increasing concentrations of Aah, after a 5 day incubation with increasing concentrations of Aah. The relative number of viable cells was determined by means of the MTT assay. The results are shown as a percentage of viable cells detected in the untreated, control group. Data is expressed as the mean ± SEM. *Statistical significance compared to untreated group, $P < 0.02$.

Figure 3.

Effect of Aah on the relative viability of A) MDCK and B) M1 cells. The upper frames of A and B show photomicrographs of MDCK and M1 cells in the presence or absence of 66 µg/ml Aah. The lower frames of A and B shows the relative numbers of viable cells as a function of increasing concentrations of Aah, after a 3 day incubation (OK cells), or 4 day incubation (HK-2 cells). MTT assay was performed after 5 days incubation of MDCK and M1 cells with Aah. Values are mean $\pm$ SEM (n=3). *Statistical significance compared to the control, $P < 0.01$. ns Not significant.

Figure 4.

Cytotoxic effect of Aah in immortalized RPT cells. Immortalized RPT cells were treated with 66 $\mu\text{g/ml}$ Aah for 5 days. Subsequently, (A) dead Cells (green) were visualized under a fluorescent microscope after the 5 day incubation with 21 $\mu\text{g/ml}$ Aah. Intact cells were stained with Dapi (blue). (B) The % dead cells (green) in the cell population (blue) was quantitated as a function of increasing Aah concentration, as described in Materials and Methods. (C) In parallel cultures treated with increasing concentrations of Aah (or untreated), viable cells were quantitated by the MTT assay. Values are the mean $\pm$ SEM of triplicate determinations. *Significant difference versus the control group, $P < 0.01$.

Figure 5.

Evidence of oxidative stress in immortalized RPT cells treated with Aah. (A) Protective effect of an antioxidant mixture (GSH (0.064mM), Ascorbic acid (0.43mM), α -Tocopherol (1.13 μ M) and Selenium (4.76 μ M)) against the cytotoxicity resulting from a 5 day incubation with increasing concentrations of Aah; values are the mean $\pm$ SEM of triplicate determinations. *Significant difference compared to the control group, $P < 0.02$. ns Not significant. (B) Immortalized RPT cells were incubated at 37°C with CellROX Deep Red reagent for 24h in the presence of either Aah (100 μ g/ml), Menadione (50 μ M) or no additive (Control), followed by an examination of the cultures under a fluorescent microscope. Representative microscope fields are illustrated.

Figure 6.

Effect of Aah on apoptosis the viability in primary RPT cells. A. Primary RPT cells were treated with 100 μg/ml Aah or untreated for 3 days. . Subsequently, the cultures were stained for activated Caspase, using an Image-iT LIVE Red Poly Caspases Detection Kit. Nuclei were counterstained with 1μM Hoe33342. SYTOX Green was employed to stain for dead cells, with altered plasma membrane integrity. The upper panel shows representative fluorescent images. The lower panel shows the percentage of apoptotic and dead cells in the cultures (as determined by scoring microscope fields using ImageJ). Values are averages $\pm$ SEM of determinations made in microscope fields in each of 3 culture dishes. (B) Effect of a 3 day incubation with Aah on the viability of primary RPT cells was determined using the MTT assay. Values are averages $\pm$ SEM *Statistical significance compared to the control, $P < 0.05$.