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Research Article

Molecular basis of cardiotoxicity upon cobra envenomation

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Abstract. Various clinical manifestations leading to death have been documented in most cases of bites caused by venomous snakes. Cobra envenomation is an extremely variable process and known to cause profound neurological abnormalities. The complexity of cobra venom can induce multiple-organ failure, leading to death in case of severe envenomation. Intramuscular administration of Malayan spitting cobra (*Naja sputatrix*) crude venom at 1 μg/g dose caused death in mice in approximately 3 h. Analysis of gene expression profiles in the heart, brain, kidney, liver and lung revealed 203 genes whose expression was altered by at least 3-fold in response to venom treatment. Of these, 50% were differentially expressed in the heart and included genes involved in inflammation, apoptosis, ion transport and energy metabolism. Electrocardiogram recordings and serum troponin T measurements indicated declining cardiac function and myocardial damage. This not only sheds light on the cardiotoxicity of cobra venom but also reveals the molecular networks affected during envenomation.

Key words. Microarray; gene expression; venom; myocardial; cardiotoxicity; mouse.

Introduction

Snakebites are a significant medical problem, especially in rural agricultural regions of Africa and Asia. In the United States and other (European and Pacific) countries where cobras are naturally uncommon, envenomation results from human interactions in zoos, research laboratories and private homes from collections of exotic pets. With the rising trend for keeping exotic pets, snakebites have become more common in the United States and other parts of the world [1]. Approximately 30% of the 3000 species of snakes in the world are venomous and considered to be dangerous to humans [2]. In the United States, there are approximately 7000–8000 venomous snakebites annually [3]. Venomous snakebite incidents are much higher in Australia, home for some of the world's most poisonous snakes [4]. In Victoria, South Australia, alone, 1100 venomous bites are reported annually. In Asia the number of bites exceeds 200,000 annually [5], with cobra bites the most frequent among them [6]. Some species (e.g. king cobra) are capable of causing death within 10 min [7].

Snake venoms are a cornucopia of protein and non-protein components. The proteins, which comprise both enzymatic and non-enzymatic molecules, are the most biologically active constituents of crude venom [8]. Most of these enzymes are hydrolases, such as proteinases, phosphodiesterases and phospholipids. Hyaluronidase, a spreading factor, aids in the distribution of other venom components throughout the tissues of the prey. The non-enzymatic proteins include cardiotoxins, neurotoxins, proteinase inhibitors, myotoxins and acetylcholinesterase inhibitors [2]. Given the presence of numerous toxins in crude venoms, it is not surprising that any serious snakebite will show evidence of injury to several organs, leading to multi-system

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failure and death [9]. Indeed, liver and kidney tissues have been shown, histologically, to be damaged by sublethal doses of cobra venom administered intramuscularly in rabbits [10, 11], whilst neurotoxicity is a hallmark of most elapid and hydrophid envenomation [3]. Respiratory insuffiency, neurotoxicity or renal failure had been reported immediately upon cobra envenomation [12]. Cardiac complications have been reported as a consequence of viper bites [9, 13, 14]. Though it has been shown that snake venoms can affect multiple tissues, eliciting a plethora of clinical manifestations, how this occurs is unclear. Previous studies of snake envenomation provided clues to the physiology [9–11, 13, 14] but not to the underlying mechanisms. They were also mostly constrained to the study of one organ system at a time. In vitro studies using human umbilical vein endothelial cells treated with viper venoms followed by microarray analysis demonstrated that subtle functional differences existed between similar venoms [15].

In this report, we investigated the effects of cobra (*Naja sputatrix*) envenomation on the heart, brain, kidney, liver and lungs. Crude venom was injected intramuscularly (1 μg/g) into mice, and gene expression profiles of the organs (liver, lung, kidney, heart and brain) were studied using oligonucleotide microarrays. Interestingly, it was found that the heart exhibited the greatest changes in gene expression as compared to the other organs, whilst the brain and liver were least affected by the cobra venom. Genes that were affected in the heart included those that encode for mediators of the immune response, apoptosis-related proteins such as the tumour necrosis factor receptor and ion transporters. These observations provide a new perspective and possibly reveal another dimension to cobra envenomation. Taken together with physiological studies, they suggest that cobra venom is cardiotoxic, an aspect often overlooked [6]. This study also provides new molecular targets for the alternative treatment of cobra envenomation, which is necessary in cases where antivenom administration is unavailable, delayed or contraindicated.

Materials and methods

Snake venom

Lyophilized *N. sputatrix* crude venom (NS venom) obtained from the National Zoo, Malaysia, was reconstituted in sterile saline and stored at –20 °C. Dilutions from the stock venom for subsequent experiments were all carried out using sterile saline solution.

Experimental Animals

All animals were handled according to the guidelines [16] given by the Council for International Organization of Medical Sciences (CIOMS) on animal experimentation (WHO, Geneva, Switzerland). Male Swiss albino mice (16–20 g) were maintained on an ad libitum intake of standard laboratory chow and drinking water prior to and after treatments. NS venom (50 μl), at a concentration of 1 μg/g body weight (LD_{50} – 0.75 μg/g body weight), was injected intramuscularly into the left hind leg (n = 36). Control mice (n = 27) were treated similarly but with equal volumes of sterile saline solution. Mice were sacrificed after 3 h by cervical dislocation, and whole hearts, brains, kidneys, livers and lungs were rapidly removed, pooled and stored frozen in liquid nitrogen until use. The LD_{50} is a standardized measure for expressing and comparing the toxicity of chemicals. It is the lethal dose that kills half (50%) of the animals tested.

Microarray GeneChip analysis

Total RNA isolated from mouse organs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) were processed and hybridized to each array of the MG_U74Av2 GeneChip Array Set (Affymetrix, Santa Clara, CA, USA). Relative messenger RNA (mRNA) expression levels were scored as plus or minus fold changes as compared to the saline-treated controls using Microarray Suite 5.0 software (Affymetrix). The genes were subjected to hierarchical clustering and principle component analysis using Genesis 1.2.0 Software (Graz University of Technology, Austria) [17]. Genes whose expression was significantly altered in the heart by NS venom treatment and their concomitant expression in the other organs were classified according to their biological functions as described in the NetAffx Analysis Center [18] and PubMed (http:www.ncbi.nlm.nih.gov) databases.

Reverse transcription and real-time quantitative PCR

Total RNA (300 ng) was reverse transcribed in a 15-μl reaction mixture containing random hexamers (Alpha DNA, Montreal, Quebec, Canada) at 25 °C for 10 min, followed by 37 °C for 60 min and 95 °C for 5 min. Gene-specific (forward and reverse) primers used for subsequent polymerase chain reaction (PCR) amplification are as follows: *Kallikrein 5 gene*, 5′-CAAAAAGCCTGCTGACATCACA-3′ and 5′-CCAGCTTGGGCTCCTCAGT-3′; *chemokine ligand 25 gene*, 5′-CCAGCACAGGATCAAATGGAAT-3′ and 5′-AGGTTGCAGCTTCCACTCACTT-3′; *lymphotoxin B gene*, 5′-TCGGGTTGAGAAGATCATTGG-3′ and 5′-GGGTGAGGGCAAGATGCA-3′; *tumor necrosis factor receptor gene*, 5′-TCAGGCTCTAGCTATAAAGCACACTT-3′ and 5′-TTTCCACCCCTGGCATGTAG-3′; *suppressor of cytokine signaling gene*, 5’-CCGTGGGTCGCG- AGAAC-3′ and 5′-AAGGAACTCAGGTAGTCACGGAGTA-3’ *calbindin D9K gene*, 5’-GTTCCCCAGCCTCCTGAAG-3’ and 5’-CTCCATCGCCATTCTTATC-CA-3′; *serine protease 1-1 gene*, 5′-GCCTGCAGTT-CAACCTCACA-3′ and 5′-GGTTTGGAGGAGGTGTT- GGA-3′; *serine protease inhibitor 1-2 gene*, 5′-GGGTG-ACACTCACACGCA-

GAT-3′ and 5′-ATGTCAGCCTCCGATGTTTGT-3′ and *uteroglobin gene*, 5′-TGGCTCA- GACCTGCAAAATG-3′ and 5′-TCATGATGTTTATCC-TGGTCTCTTG-3′. Ribosomal RNA was used as the internal calibrator. The final PCR reactions contained complementary DNAs (cDNAs), primers (100 nM) and SYBRgreen Master Mix (Applied Biosystems, Foster City, CA, USA). Amplification was performed for 1 cycle at 50 °C for 2 min, followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min per cycle. A dissociation protocol was carried out at the end of each experiment to ensure that the amplifications were specific. All reactions were carried out in triplicate.

Surface ECG measurements

For the electrocardiogram (ECG) measurements, mice were anaesthesized intraperitoneally with a mixture (100 µl/100 g body weight) consisting of an equal volume of hypnorm (0.315 mg of fentanyl and 10 mg of fluanosome; Jansen Pharmaceutica, Beerse, Belgium) and midazolam (5 mg of dormicum and 2 ml of water, Roche Diagnostics). Needle probes (29-gauge, MLA1204) were inserted into the front and back left paw pads of each mouse, and signals were captured using the Animal BioAmp differential amplifier (ML136). Signals were digitized using an eight-channel Powerlab 8SP (ML785), and recordings were displayed with Chart 5 software. All probes and equipment for ECG measurements were obtained from ADInstruments, NSW, Australia. Recording was started pre-treatment 0 min, and the animal was monitored continuously for 3 h after intramuscular administration of 1 µg/g body weight NS venom or an equal volume of sterile saline solution (control).

Blood gas analysis

Carotid artery blood samples were obtained from anaesthesized NS venom-treated and control mice as described previously [19] and analyzed for pH, HCO_3, arterial pCO_2, arterial pO_2 and arterial O_2 saturation using an i-STAT portable clinical analyzer (i-STAT Corporation, NJ, USA).

Determination of serum troponin T

Serum was obtained from mouse whole blood 3 h after NS venom and saline treatment and stored at –20 °C before analysis. Blood concentrations of troponin T were measured using the first-generation Elecsys Troponin T STAT assay (Roche Diagnostics, Indianapolis, IN, USA) and analyzed by the Elecsys Systems 2010 according to manufacturer's instruction. The test was calibrated (Elecsys Troponin T CalSet), and the lower and upper limits of the assay were 0.01 ng/ml and 25.00 ng/ml, respectively. Mice put on artificial respiration were anaesthesized and mechanically ventilated (0.5 ml tidal volume and 120 breaths/min), via tracheotomy, with room air using a Harvard Rodent Ventilator Model 683 (Harvard Apparatus Holliston, MA, USA) immediately before either NS-venom or saline was administered. Ventilation was maintained throughout the treatment period.

Statistical analysis

Statistical analyses of data obtained from quantitative realtime PCR were performed using the unpaired Student's *t* test, and results were expressed as mean ± SD. $p < 0.05$ was considered statistically significant.

Results

Intramuscular injection of NS venom (1 µg/g body weight) into mice brought about observable paralysis and respiratory distress within 3 h (beginning approximately 150 min after venom administration), with death occurring within the next 15–30 min. The administration of higher doses of NS venom was observed in our laboratory to cause death in less than 30 min. Since fatality in victims of cobra bites often occurs after a few hours, the dosage of 1 µg/g body weight was deemed appropriate for further studies.

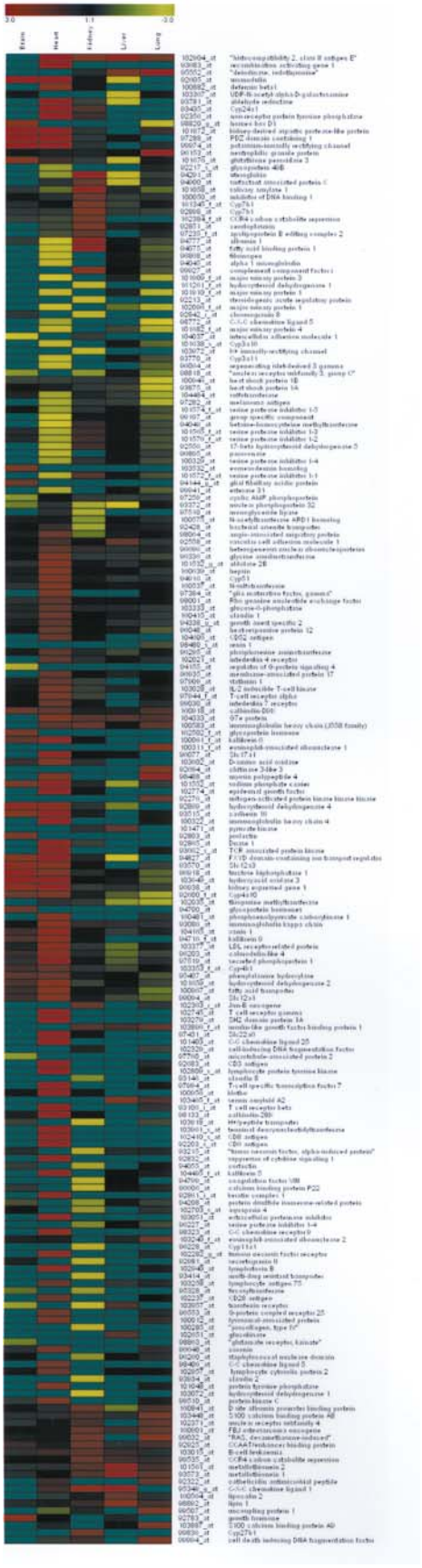
Hierarchical clustering and principal component analysis of expression data

In a bid to understand the mechanisms of toxicity and the pathways involved, a multiorgan gene expression profile was generated using oligonucleotide microarrays, involving five major organs in the body, namely the brain, heart, kidney, liver and lung.

RNA isolated from the venom-treated organs and their corresponding controls was pooled before microarray probe preparation and hybridization to minimize interindividual variation. Expression analysis was carried out using GeneChips from the MG_U74Av2 Array set, with each chip representing ~12,500 genes and expressed sequence tags (ESTs). All probe sets designated 'absent' in all five organs by the analysis software were discarded, and only genes whose expression changed by 3-fold or greater in at least one pairwise comparison (between each NS venom-treated organ and its corresponding saline-treated control) were deemed significant.

A total of 203 genes were found to be significantly and differentially expressed. These probe sets were subjected to hierarchical clustering [16, 20], where expression patterns between organs and amongst genes within each organ are represented as an expression image (fig. 1A). Interestingly and somewhat surprisingly, the heart showed significant expression changes in the greatest number of genes when compared to the other organs. A hundred and four genes were found to be differentially expressed in the heart after NS venom administration, of which the majority (87 genes) were upregulated. In contrast, the brain appeared to be much less affected by the envenomation, as only 29

A



B

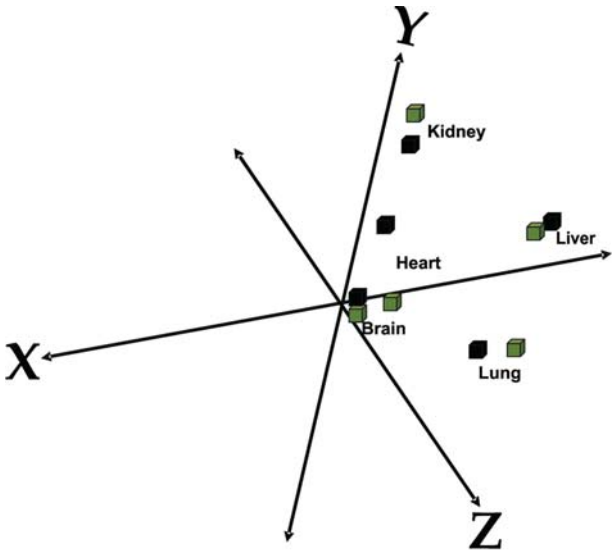


Figure 1. Hierarchical clustering and principal component analysis of gene expression in the mouse brain, heart, kidney, liver and lung. (*A*) Expression data for 203 genes that exhibited a 3-fold or greater change in expression in at least one organ after intramuscular administration of NS venom (1 μg/g body weight) for 3 h was analyzed by average linkage hierarachical clustering. All measurements are relative to the corresponding saline-treated controls for each organ and were expressed as log ratios by the microarray analysis software. The color scale ranges from saturated green for log ratios –3.0 and below (decreased expression) to saturated red for log ratios 3.0 and above (increased expression). Missing values are indicated by turquoise-colored boxes. Each gene is represented by a single row and identified by its Affymetrix probe set ID, whilst each organ is represented by a single column. (*B*) Principal component analysis clustered experiments in three-dimensional space according to differences in the overall expression of genes between venom-treated and control experiments. The largest difference in gene expression profile was seen in the heart, as determined by the spatial distance between the representative points. ■ control, ■ intramuscular injection of 1 μg/g body weight of NS venom for 3 h.

genes were significantly differentially expressed. This is not unusual given the presence of the blood brain barrier (BBB), a specialized structure composed of endothelial cells continuously linked by tight junctions, which serves to regulate the selective transport and metabolism of substances from the blood to the brain and vice versa. In this way, the BBB protects the brain from neurological insults [21]. Some of the genes that were differentially expressed include glutathione peroxidase 3 (upregulated 5.2-fold), an antioxidant enzyme involved in decreasing oxidative stress and components of metabolic pathways (e.g. phenylalanine hydroxylase, upregulated 2.8-fold; and fructose biphosphate, upregulated by 7.7-fold]). The observation that NS venom was ineffective in bringing about major changes in the gene expression profile of the brain also serves to validate this model system of envenomation such that most changes deemed significant are real. Fifty genes were differentially regulated by NS venom treatment in the kidney, of which 20 were downregulated and 30 showed increased expression. The lung was found to have 9 genes whose expression was depressed, whilst 18 transcripts were induced by the crude venom. The liver, the main detoxifying organ in the body, was also seemingly unaffected by NS venom in terms of gene expression (23 genes showing significant changes), though studies have reported that hepatocytes were severely damaged by intramuscular administration of cobra venom [10]. The genes that clustered together were mainly those involved in the immune response and whose expression was largely significantly changed in the heart. Little inter-organ clustering of genes was observed (fig. 1A). This is probably due to the presence of organ-specific gene expression, as indicated by the large number of undetectable transcripts, or the fact that the changes elicited by cobra envenomation varied greatly among the different systems.

Principal component analysis of experiments (fig. 1B) was performed to confirm the differential changes in gene expression profiles among the organs [16, 22]. Experiments were clustered in three-dimensional space according to the temporal gene expression profiles in each dataset, and spatially distant experiments are not similar to each other. The greatest distant existed between the NS venom-treated heart and its corresponding saline-treated control as compared to the other organ pairs (fig 1B), suggesting that these two experiments represent very different physiological conditions. Again, the venom-treated brain and liver were clustered most closely to their control in space, indicating little changes in gene expression in their respective organs. Being distinct organ systems, the heart, brain, kidney, liver and lung experiments were spatially distributed.

Functional clustering of genes in the heart

Since the heart demonstrated the greatest number of significant gene expression changes (~50% of the total number of genes differentially regulated in all the organs) in response to *N. sputatrix* envenomation, further categorization of these 104 probe sets according to their known functions in physiological pathways was employed to dissect the effects of NS venom. Results of the clustering are shown in table 1 and include the corresponding fold changes of the genes in brain, kidney, liver and lung.

Nine main biological groups were identified amongst the differentially expressed genes in the heart, and they include mediators of immune response (34 genes), apoptosis (7 genes), ion transport and binding (10 genes), signal transduction (10 genes), hypotension (3 genes), carbohydrate metabolism (6 genes), lipid metabolism (9 genes), amino acid metabolism (4 genes) and electron transport (6 genes). It is, perhaps, not unexpected that NS venom would induce the expression of genes involved in the immune response pathways, as it really is a complex mixture of potentially antigenic compounds. Genes that are amongst the most highly upregulated include the recombination activating gene 1 (RAG1, +415.9-fold), CD8 antigen (+52.3-fold) and T cell receptor-β (+27.9-fold). RAG1 is involved in lymphocyte activation, and its high level of induction suggests that the adaptive immune response plays an important role in cellular defense during envenomation. Two genes, tumour necrosis factor (TNF) receptor and lymphotoxin B, play dual roles in mediating immune response as well as apoptosis. CD28 antigen and secreted phosphoprotein 1 (upregulated 3.5- and 10.2-fold, respectively) are antiapoptotic, suggesting that there is a compensatory response to the upregulation of proapoptotic genes. Additionally, NS venom altered the expression of genes which code for proteins that induce hypotension (kallikreins 5, 6 and 9), bind and transport Ca^{2+}, K^+ and Na^+, and which are components of metabolic pathways.

The expression of a number of genes was significantly changed in at least three organs, and they encode proteins such as histocompatibility 2, immunoglobulin heavy chain (J558 family), uromodulin and uteroglobin. These proteins may have important functions in multiple organs and hence, were coordinately affected during envenomation.

Real-time quantitative PCR

The expression of nine genes that may have important roles in mediating physiological responses to NS venom in the heart was quantitated by realtime PCR (fig. 2). Specific primers were used for PCR amplification. The relative expression of each gene in the heart was obtained after normalizing against an internal control (18S ribosomal RNA) and a calibrator, in this case, the saline-treated control (relative gene expression = 1). The genes encoding for chemokine ligand 25, lymphotoxin B, TNFα receptor, suppressor of cytokine signaling, calbindin D9K and kallikrein 5 were all upregulated (15.7-, 9.1-, 12.0-, 11.5-, 3.4- and 8.9-fold respectively, $p < 0.05$). On the other hand, the serine protease inhibitors 1-1 and 1-2 and

Table 1. Functional clustering of 104 genes exhibiting a 3-fold or greater change in expression in the heart 3 h after intramuscular administration of NS venom. (+), increase; (–), decrease or undetected. Underlined genes participate in apoptosis as well. Unless italicized, the genes are described by the proteins expressed from them.

Category of genes	Description	Heart	Brain	Kidney	Liver	Lung
				Fold change		
Immune response	alpha-1 microglobulin	–12.9	–	+1.1	–	–1.5
	CD3 antigen	+17.0	–	–	–	+1.3
	CD6 antigen	+34.8	–	–	–	–
	CD8 antigen	+52.3	–	–	–	–
	CD52 antigen	+3.2	–	+1.1	–	–
	chemokine ligand 5 (C-C)	+3.9	–	–	–	–
	chemokine ligand 5 (C-X-C)	–3.6	–	–	–	–6.7
	chemokine ligand 25	+15.7	–	–	–	–
	chemokine receptor 9	+20.0	–	–	–	–
	coronin	+4.2	–	–	–	–1.2
	defensin beta 1	+5.3	–	–	–	–
	histocompatibility 2	+45.9	–	+4.4	+7.1	+3.5
	lg heavy chain 4	+10.6	–	–	–	+1.1
	lg heavy chain (J558 family)	+2.9	–	+3.3	+1.1	+2.8
	lg kappa chain	+6.3	–1.1	+1.7	+1.5	+1.5
	interleukin 4 (IL-4) receptor	+3.1	–	–1.2	–1.0	+1.3
	interleukin 7 (IL-7) receptor	+3.4	+1.1	+1.1	–	+1.3
	lymphotoxin B	+13.8	–	–	–	–1.1
	lymphocyte cytosolic protein 2	+3.4	+1.1	–	–	–
	lymphocyte antigen 75	+3.4	–	–	+1.3	+1.3
	regenerating islet-derived 3 gamma	–54.6	–	–	–	–1.9
	recombination activating gene 1, RAG1	+415.9	–	–	–	–
	serine protease inhibitor 1-1, Spi 1-1	–4.4	–	+2.6	+1.1	+1.7
	serine protease inhibitor 1-2, Spi 1-2	–3.8	+1.2	+2.0	+1.1	–1.6
	serine protease inhibitor 1-3, Spi 1-3	–4.0	+1.2	+2.0	+1.1	–1.5
	serine protease inhibitor 1-4, Spi 1-4	–3.6	–	+1.5	+1.1	–1.6
	serine protease inhibitor 1-5, Spi 1-5	–3.5	–	+1.3	+1.1	–1.6
	T cell receptor (TCR) alpha	+4.3	–1.0	–1.5	–	+1.1
	T cell receptor (TCR) beta	+27.9	–	–	–	+1.6
	T cell receptor (TCR) gamma	+19.2	–	–	–	–
	tumour necrosis factor (TNF) receptor	+7.5	–	–	–	+1.2
	uromodulin	+12.0	+46.5	+1.1	–19.0	–
	uteroglobin	–3.1	–	+14.5	–35.3	–1.4
Apoptosis	CD28 antigen	+3.5	–	–	–	+1.1
	claudin 1	+3.4	+1.3	+1.0	+1.1	–1.3
	Dnase 1	+7.7	–	–1.5	–	–
	growth arrest specific 2	+3.0	+1.2	+1.0	–1.2	–1.3
	secreted phosphoprotein 1	+10.2	+1.6	+1.1	–1.8	–1.2
Ion transport/binding	calbindin-D9K	+3.4	+3.0	+1.2	–1.1	+2.5
	calbindin-28K	+33.6	–1.1	+1.1	–	–
	calmodulin-like 4	+6.8	+1.8	–1.2	–2.1	–1.5
	FXYD domain-containing transport regulator	+11.8	+5.4	+1.1	–7.0	–
	potassium-inwardly rectifying channel	+30.9	+13.9	+1.4	–	+1.3
	solute carrier family 12-1	+5.2	+2.0	–1.1	–1.5	–2.0
	solute carrier family 12-3	+5.6	+6.9	+1.1	–	+1.4
	solute carrier family 17-1	+10.3	–	+1.0	+1.1	–
	solute carrier family 22-6	+15.1	–	–1.0	–	–
	sodium phosphate carrier	+14.7	+4.3	–1.14	–3.34	–1.4
Signal transduction	epidermal growth factor	+8.6	–	–1.0	–	–
	G-protein coupled receptor 25	+3.9	–	–	–	–
	IL-2 inducible T-cell kinase	+4.1	–1.1	–1.5	+1.1	+1.2
	lymphocyte tyrosine kinase	+17.8	–	–	–	–
	protein kinase C	+4.3	–1.3	–	–	+1.2
	protein tyrosine phosphatase	+4.8	–	–	–	+1.2
	non-receptor tyrosine phosphatase	+12.9	–	+1.8	–	–
	Rho	+3.0	+1.5	+1.1	–1.0	+1.1
	suppressor of cytokine signaling-1, SOC1	+11.5	–	–	–	–
	TCR-associated protein kinase	+7.6	–	–	–	+1.1
Hypotensive activity	kallikrein 5	+8.9	–	–	+1.0	+1.1
	kallikrein 6	+11.6	+3.0	+1.1	–	–
	kallikrein 9	+6.6	+2.5	+1.1	–	–

Table 1 (continued)

Category of genes	Description	Heart	Brain	Kidney	Liver	Lung
				Fold change		
Carbohydrate metabolism	aldehyde reductase	+10.2	–	–1.5	–20.8	–
	alolase 2B	+5.5	+1.4	+1.1	+1.4	–1.1
	fructose biphosphatase 1	+4.8	+7.7	–1.2	+1.1	–1.9
	glucose-6-phosphatase	+3.1	+1.2	+1.3	+1.3	–1.3
	Klotho	+23.4	+1.1	+1.1	–	–
	phosphoenolpyruvate carboxykinase 1	+6.0	–	–	–	–
Lipid metabolism	fatty acid binding protein 1	–9.5	–	+12.2	+1.0	–1.9
	fatty acid transporter	+5.7	+1.9	+1.1	–1.1	–2.3
	hydroxysteroid dehydrogenase 2	+6.5	+2.3	–1.5	–1.0	–1.4
	hydroxysteroid dehydrogenase 4	+7.1	+7.0	–1.2	–2.6	–
	kidney expressed gene 1	+8.51	+6.8	–1.3	+1.4	–1.4
	LDL receptor-related protein	+6.9	–1.2	–1.2	–2.2	–1.1
	N-sulfotransferase	+4.4	–1.4	+1.0	–1.1	+1.1
	paroxonase	–4.6	–	–	–1.1	–1.4
	sulfotransferase	–3.6	–1.1	+1.2	+1.3	+3.5
Amino acid metabolism	D-amino acid oxidase	+4.7	+1.8	+1.5	–	–
	glycine amidinotransferase	+4.5	+1.1	–1.3	+1.3	–1.5
	phosphoserine aminotransferase	+3.0	–1.1	+1.3	–1.5	–1.0
	phenylalanine hydroxylase	+3.2	+2.8	–1.1	–1.0	–1.7
Electron transport	cytochrome P450 (CYP) 3a11	–28.1	–	–	+1.1	–1.6
	cytochrome P450 (CYP) 3a16	–30.1	–	–	+1.1	–1.9
	cytochrome P450 (CYP) 4a10	+3.4	+4.3	–1.1	–1.9	–3.6
	cytochrome P450 (CYP) 4b1	+3.0	+2.1	–1.2	–1.5	–1.0
	cytochrome P450 (CYP) 24a1	+12.4	–	+2.5	–	–
	cytochrome P450 (CYP) 51	+4.9	+1.2	–1.1	–1.3	–1.2
Others	albumin 1	+7.1	+1.3	+9.4	+1.1	–1.6
	angio-associated migratory protein	+3.5	+1.2	–2.3	–1.1	+1.0
	betaine-homocysteine methyltrans-ferase	–6.4	–	+1.8	+1.1	–1.5
	cadherin 16	+8.3	+5.2	–1.1	–	–
	extracellular proteinase inhibitor	+21.4	–	–	–	–
	fibrinogen	–9.1	–	–	+1.1	+1.7
	glycoprotein 49B	–3.3	–	–	–	–
	G7e protein	+5.3	–	+2.8	+2.9	+2.5
	heat-responsive protein 12	+3.3	+1.1	–1.0	–1.1	–1.7
	hepsin	+4.9	+1.2	–1.09	–1.0	–1.3
	hydroxyacid oxidase 3	+11.4	+9.1	–1.0	+1.2	–1.9
	lysosomal-associated protein	+4.3	–	–	–	+1.8
	myosin polypeptide 4	+5.2	–	–	–	+8.1
	nuclear riboproteins	+3.1	+1.1	+1.65	+1.4	+1.1
	stathmin 1	+3.2	–1.1	–1.4	–	+1.0
	terminal deoxynucleotidyltransferase	+52.3	–	–	–	–
	T cell-specific transcription factor 7	+23.3	–	–	–	+1.0

uteroglobin genes were downregulated (4.4-, 3.8- and 3.1-fold, respectively, $p < 0.05$). Hence, the gene expression changes quantified by real-time PCR correlated well with the microarray data (table 1). Due to the different specificity and sensitivity of the two technologies, the absolute values for fold change did not necessarily correspond. However, the expression profiles obtained in both cases proved to be similar, thereby validating the results of microarray analysis.

ECG measurements

Since the intramuscular administration of NS venom significantly altered the expression of a large number of genes in the mouse heart, ECG measurements were carried out to determine whether the venom affected the electrical activity of cardiac muscles (fig. 3A). Bradycardia was observed 15 min post-injection of venom. Further observations were noted, e. g. an increase in the amplitude of the QRS complex, prolonged PR interval and slight arrhythmia after 60 min, which indicated that cardiac contraction and conduction time from atria to ventricle were altered. This was accompanied by irregularities in heartbeat. After 120 min, electrocardiographic abnormalities showed mild ST elevation and fibrillation, both of which became more significant after 150 min, suggesting the development of ischemia. Bradycardia became increasingly pronounced over time. These electrocardiographic abnormalities persisted to 180 min post-injection, while cardiac

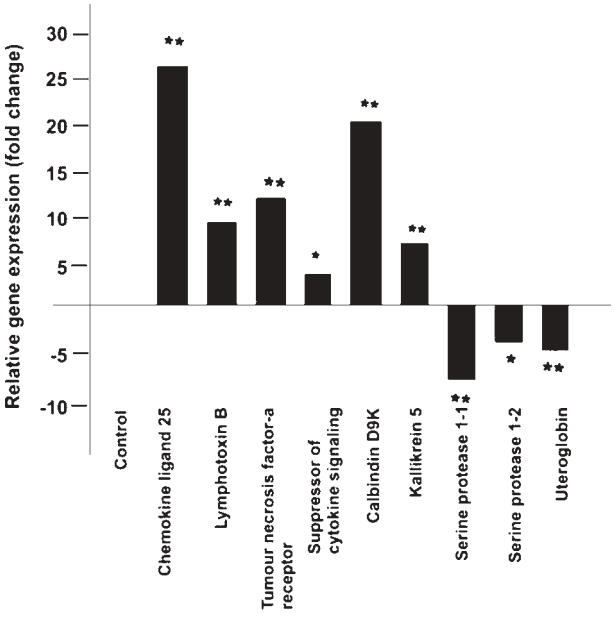


Figure 2. Quantitative realtime PCR analysis. Validation of microarray data for the expression of nine genes in the NS venom-treated heart was carried out by real-time PCR. Six genes were significantly upregulated by venom treatment, whilst four genes were downregulated. Each measurement was performed in triplicate, and the data are representative of duplicate experiments. Values are expressed as fold change ± SEM, $*p < 0.05$, $**p < 0.01$.

arrhythmias increased in frequency. There was no alteration in the electrocardiographic profile from saline-treated controls.

Cobra venoms contain neurotoxins which cause respiratory paralysis. To determine whether the changes in cardiac function were brought about by ensuing hypoxia, arterial pO_2 levels were measured (fig 3B). Arterial pO_2 was 94 ± 7.00 mmHg before venom administration (0min) and remained relatively unchanged up till 120 min after envenomation (94.0 ± 7.99 mmHg and 94.5 ± 2.5 mmHg after 60 min and 120 min, respectively). Moderate hypoxia developed when arterial pO_2 decreased by 32.4% to 63.5 mmHg and a further 5.3% to 58.5 ± 2.47 mmHg 150 min and 165 min, respectively, after NS venom treatment [23]. Hence, the abnormalities in ECG output up till 120 min were likely to have been due to the effects of NS venom and not a result of oxygen deprivation. Subsequently, gradual development of hypoxia likely contributed to increased ischemia and declining cardiac function on the whole.

Measurement of cardiac troponin T in NS venom-treated mice

It has been shown that cardiac troponin T is a sensitive and specific biomarker of cardiac injury in laboratory animals [24]. To determine whether the decline in cardiac function 3 h after NS venom treatment was accompanied

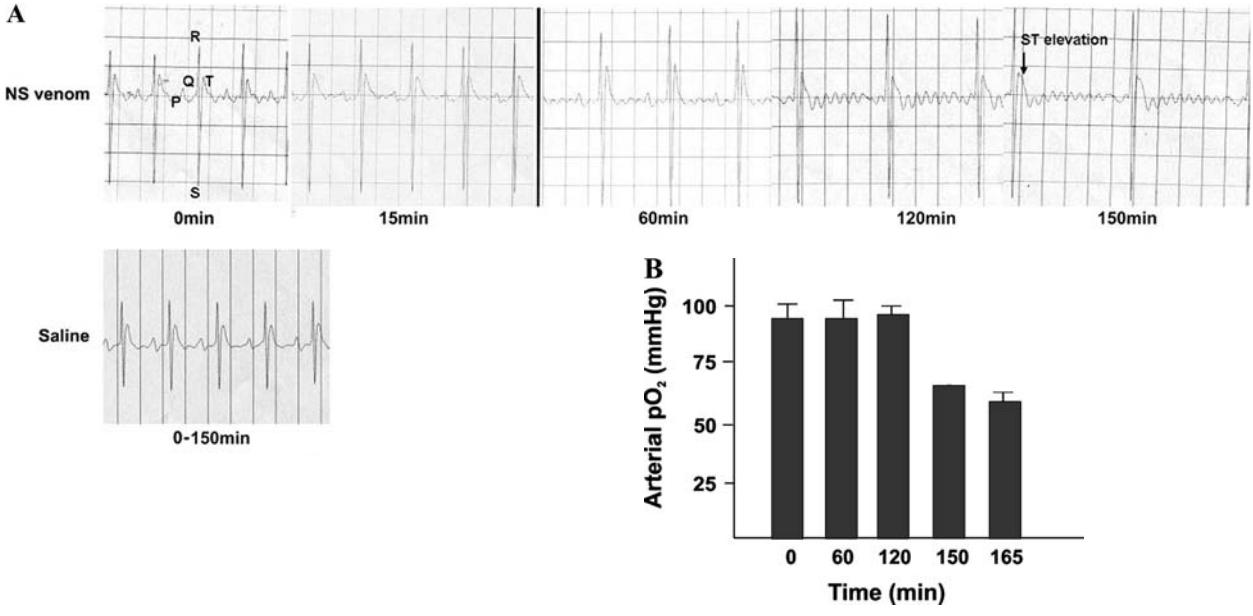


Figure 3. ECG and arterial pO_2 profile from NS venom-treated mice. (*A*) Recordings were started before the intramuscular injection of 1 µg/g body weight NS venom in anaesthesized mice (0 min). The animals were monitored continuously for 3 h after venom administration, and recordings are shown at different timepoints (15 min, 60 min, 120 min and 150 min postinjection). No changes in the ECG profile was observed in saline-treated controls during the whole period of observation. The QRS complex and PR interval are indicated. Cardiac arrhythmia developed gradually after the venom treatment, and it became irreversible after 150 min. ST elevation was more pronounced 150 min after envenomation. Results shown are representative of three independent experiments. (*B*) Arterial pO_2 in anaesthesized mice was measured before venom administration (0 min) and 60, 120, 150 and 165 min post-envenomation (n = 12). pO_2 was maintained at normoxic levels until the 150th min. Values are expressed as mean (mmHg) ± SEM.

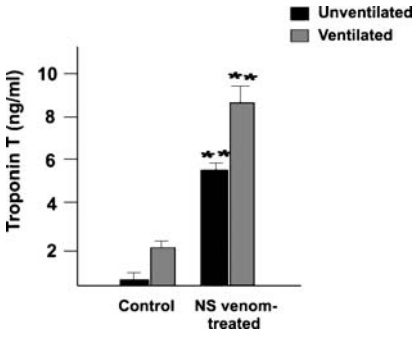


Figure 4. Elevated serum cardiac troponin T levels in NS venom-treated mice after 3 h. Serum was pooled from three mice, ventilated or unventilated, and the concentration of troponin T enzyme, which is released into blood by damaged myocytes, was determined using an assay specific for the cardiac isoform. Cardiac troponin T was significantly increased in mice injected with NS venom as compared to the saline-treated controls. Results shown are representative of three independent experiments. Values are expressed as mean ± SD. **, $p < 0.01$.

by myocardial damage, troponin T levels were determined in the serum of envenomated mice and their corresponding controls (fig. 4). Significantly elevated levels of cardiac troponin T were detected in NS-venom-treated mice as compared to saline-treated controls (5.65 ± 0.56 ng/ml compared to 0.40 ± 0.05 ng/ml, $p < 0.01$). Given that moderate hypoxia had been observed in the later stages of envenomation (fig. 3B), venom-treated mice were given respiratory assistance to see whether cardiac injury was solely an outcome of reduced oxygen levels in the heart. These animals also suffered from myocardial damage as demonstrated by the significant increase in troponin T levels as compared to similarly ventilated but saline-treated controls (8.49 ± 1.09 ng/ml versus 2.04 ± 0.24 ng/ml). The difference in serum troponin T levels between saline-treated ventilated and unventilated mice may have resulted from some mechanical stress.

Discussion

Snake venom contains a toxic mixture of enzymes, low molecular weight polypeptides, glycoproteins and metal ions capable of causing local tissue damage as well as multiplesystem failure. The clinical manifestation of snake envenomation is usually progressive, from initial signs limited to the bitten area to systemic effects that can be severe enough to lead to rapid death from multi-organ failure. In a bid to understand the possible underlying molecular mechanisms, we investigated the effects of cobra venom on global gene expression in the mouse brain, heart, kidney, liver and lung.

The results of this study identified a total of 203 genes that were significantly and differentially expressed in all five organs. Of these, the heart exhibited changes in the greatest number of genes whilst the brain and liver demonstrated relatively few changes (fig. 1). Genes encoding mediators of the immune response formed the majority of the genes whose expression was altered. The upregulation of T-cell-receptor (TCR) subunits α, β and γ (4.3-, 27.9- and 19.2-fold, respectively) implicates the involvement of T lymphocytes in the body's response to toxicants found in the venom. Indeed, mature T lymphocytes recognize pathogen-derived peptides on antigen-presenting cells by means of a multimeric protein ensemble termed the TCR-CD3 complex [25]. CD3, whose expression was induced 17-fold in the heart by NS venom, contributes to specialized structural and signaling functions which improve certain TCR-CD3 induced mature T cell responses, such as cytokine activation. In addition, the upregulation of immunoglobulin (Ig)κ- and Ig heavy-chain expression (6.3- and ~3.0-fold, respectively) is indicative of B cell response. B and T lymphocytes can generate specific responses to a tremendous number of antigens. The major source of antibody and TCR diversity in the immune system is attributed to the catalytic rearrangement of the variable (V), diversity (D) and joining (J) DNA elements of antigen receptor genes in lymphocytes by the recombination activating gene 1 (RAG 1; [26, 27]). The expression of RAG 1 was increased ~416-fold in the heart, which suggests the ongoing process of increasing lymphocyte repertoire in response to the various antigenic polypeptides found in NS venom. Lymphotoxin B (+13.8-fold) and TNF receptor (CD27; +7.5-fold), members of the TNF receptor family lacking a death domain, also perform key roles in T and B lymphocyte maturation and interaction. CD27 when bound to its ligand, CD70, enhances T cell proliferation and differentiation, Ig production and augments the secretion of TNFα by CD4+ lymphocytes [28, 29].

The establishment of immune surveillance, then immunity, is inadvertently coupled to inflammation. In the envenomed heart, genes encoding inflammatory mediators, interleukin (IL)-4 receptor (+3.1-fold), IL-7 receptor (+3.4-fold), uromodulin (+12.0-fold), chemokine (C-C) ligand 5 (+3.9-fold), chemokine receptor 9 (+20.0-fold) and chemokine ligand 25 (+15.7-fold) were all highly expressed compared to the saline-treated control. Chemokine ligand 25 binds to a high affinity receptor, CCX-CKR, in mouse and plays important roles in the trafficking of memory and effector T and B lymphocytes as well as in leukocyte homing to sites of injury [30]. Uromodulin triggers the activation of neutrophils and stimulates proliferation and cytokine production by mononuclear cells [31]. At the same time, anti-inflammatory genes were downregulated. Mouse serine protease inhibitors (SPis) are homologous to human α1-antitrypsin (AAT), an archetypal member of the serine proteinase inhibitor (SERPIN) gene family. Here, the expression of five isoforms of the mouse SPi family, 1-1 to 1-5, are downregu-

lated (4.4-, 3.8-, 4.0-, 3.6- and 3.5-fold, respectively). AAT is an acute-phase reactant regulated by IL-6 and possibly, nuclear factor-κB (NF-κB). It inhibits neutrophil superoxide oxidation, induces macrophage-derived IL-1 receptor antagonist release, reduces TNFα-induced apoptosis and lethality, and inhibits elastase-dependent inflammation [32–34]. Uteroglobin also possesses anti-inflammatory properties, inhibiting platelet aggregation, neutrophil chemotaxis and chemoinvasion. The increased expression of the gene encoding suppressor of cytokine signaling 1 (SOC1; 11.5-fold) is likely a compensatory response to repress inflammation by inhibiting cytokine activation pathways. Cytokines signal via the JAK-STAT pathway, leading to the expression of cytokine-responsive genes. SOC1 is a negative feedback inhibitor of JAK kinases, and insufficient induction results in autoimmunity [35].

In addition to taking part in lymphocyte development, lymphotoxin B and CD7 are also implicated in apoptosis. CD27 was shown to associate with a protein, Siva, in vitro, which contains a region homologous to death domains. FADD (Fas-associated death domain) and RIP (receptor-interactive protein) are adaptor proteins which then link Siva to caspases, excecutioners of the apoptotic pathway [29]. Expression of claudin 1 (+3.4- fold), a tight junction protein, was also shown to be increased in apoptotic cells [36]. In contrast, secreted phosphoprotein 1 promotes cell survival in response to toxicant injury, and its expression was increased in the NS venom-treated heart (10.2-fold). This increase in expression has been shown to be induced by activated protein kinase C, a signaling molecule whose expression was also upregulated in the heart (4.3-fold), demonstrating an adaptive response to venom-induced cell death.

Examination of genes whose expression was significantly altered in the heart by NS venom treatment indicates several that are involved in ion transport and binding, namely calbindin D9K and D28K (+3.4- and +33.6-fold, respectively), calmodulin-like 4 (+6.8-fold) and the potassium inwardly rectifying channel (Kir; +30.9-fold). The homeostasis of Ca^{2+}, K^+ and Na^+ concentrations in cardiac myocytes is of extreme importance, as these ions regulate cardiac contractility. The fundamental function of Ca^{2+} here is to enable excitation-contraction coupling, involving an increase in cytoplasmic Ca^{2+} levels via Ca^{2+} release channels on the sarcolemma as well as the sarcoplasmic membrane. Ca^{2+} then induces contractility by binding to troponin C. Rapid re-uptake of Ca^{2+} is required for relaxation. Alterations in any of the above steps may lead to heart failure. The significant increases in calbindin D9K and D28K, both Ca^{2+} buffers, suggest a possible elevation in Ca^{2+} concentration in cardiomyocytes during envenomation. Indeed, it has been shown that calbindin D28K is induced before or at the same time as increased Ca^{2+} transport and that increased expression correspondingly reduced Ca^{2+} mobilization [37]. Additionally, calbindins can alter Ca^{2+}-Mg^{2+}-ATPase/Ca^{2+}-pump activity. Calmodulin, an intracellular Ca^{2+} sensor, selectively activates specific downstream signaling pathways in response to local changes in Ca^{2+} concentration (3- to 5-fold increase in expression), leading to severe cardiac hypertrophy in transgenic mice [38]. Prolonged hypertrophy is accompanied by dilated cardiomyopathy, heart failure and sudden death due to arrythmias. The cardiac inward rectifier K^+ current plays a major role in repolarization of the action potential and stabilization of the resting potential [39]. Increased expression of the gene that encodes Kir possibly results in enhanced inward conductance and may result in hyperpolarization following action potential.

The upregulation of kallikrein 5, 6 and 9 gene expression suggests that NS venom could induce hypotension in the heart. Kallikreins cleave kininogens to release kinins, one of which is converted to bradykinin. Bradykinin, upon binding to its receptor, induces nitric oxide release from endothelial cells, resulting in vasodilation. This has been reported to be cardioprotective during cardiac failure and ischemia by opposing the vasoconstrictor activity of angiotensin II, thereby increasing coronary blood flow and improving myocardial metabolism [40]. However, in normotensive animals, as were used here, hypotension can contribute to cardiac instability. Indeed, hypotension is one of the clinical signs of severe snake envenomation [41]. Myocardial function depends on ATP supplied by the oxidation of several substrates. Under normal circumstances, this is achieved by β-oxidation of fatty acids. Diminished ATP generation due to derangement of fatty acid delivery causes cardiac diseases such as ischemia and dilated cardiomyopathy, heart failure and hypertrophy [42]. Here, the expression of fatty acid binding protein 1, which binds fatty acids intracellularly and transports them to their site of action in the cardiomyocyte for oxidation in the mitochondria, was markedly reduced (9.5-fold) in the NS venom-treated heart. The expression of other genes encoding important components of glucose, fatty acid and amino acid metabolism (table 1) was also significantly altered, suggesting disruptions in cardiomyocyte energy metabolism and possible compensatory mechanisms (e.g. increase in ATP production by glycolysis, glycogen mobilization and amino acid metabolism). The expression of genes encoding cytochrome (CYP) P450 enzymes, which are important in maintaining vascular tone and homeostasis by metabolizing arachidonic acid, is increased in the heart. 20-HETE, a product of ω- hydroxylation of arachidonic acid catalyzed by CYP450 enzymes, increases smooth muscle tone by inhibiting large-conductance K^+ channels and further increasing intracellular Ca^{2+} concentration [43]. This compounds the disturbances in cardiomyocyte ion fluxes described earlier. Taken together, it is clear that NS

venom has profound effects in the heart at the level of gene expression.

In contrast, the brain, kidney, liver and lungs were seemingly affected to a much lesser extent by the cobra envenomation. Understandably, the brain is protected by the BBB, preventing the entry of exogenous molecules and hence experiencing few changes in gene expression. The liver, kidney and lung, on the other hand, are the main detoxifying organs in the body, and it is somewhat surprising to see relatively few alterations in gene expression. A number of genes, including histocompatibility 2, Igκ and heavy chain, Spis, uromodulin and uteroglobin showed differential expression to varying extents in the liver, kidney and lungs, indicating the induction of immune response in these organs. The expression of cell death-inducing DNA fragmentation factor was increased in the lung (~4.0-fold; fig. 1A). It belongs to a family of cell death activators and is homologous to the 45-kDa subunit of the DNA fragmentation factor that is cleaved by caspases during apoptosis. Its role in promoting cell death has been reported [44]; thus, its expression in the lung is indicative of venom-induced apoptosis. The water channel, aquaporin (AQP) 4, is downregulated (~3.4-fold; fig. 1A) in the kidney in response to NS venom. AQP4 is expressed in the basolateral plasma membranes of collecting duct principal cells and is the main exit pathway for water reabsorbed by AQP2 [45], and its decreased expression may result in polyuria during envenomation. The kidney showed the second greatest changes in gene expression amongst the organs (50 genes differentially regulated). However, it is unclear whether the observed gene expression changes would translate into renal damage.

In fact, indistinct groups of genes were found to be differentially expressed in the brain, liver, kidney and lung, indicating that the effects of NS venom were not pronounced in these organs and possibly do not contribute to the eventual death of the animal. Histochemical and histological damage have been reported in the liver and kidneys of rabbits after intramuscular injections of sublethal doses of Egyptian cobra venom [10, 11]. The effects were time-dependent, with cells demonstrating nucleic acid loss, irregular nuclei and protein depletion after 12 h. Taken together with the results described here, it is plausible to think that multi-organ failure occurs in the event of a prolonged or delayed response to envenomation. Though venom concentration attains a maximal level after 20 min via intramuscular injection [46], the intrinsic toxicity of the venom and the amount injected are crucial determinants of clinical manifestation. Since it can take from anywhere between minutes to 24 h for victims to succumb to envenomation following snakebite, it is possible that a systemic failure occurs at later stages of the process. In this study of envenomation, 1 µg/g body weight of NS venom, when injected intramuscularly, resulted in death slightly after 3 h, and results of gene expression studies indicated that amongst the five organs investigated, the heart was very likely either the first organ targeted or the one that was most susceptible to NS venom.

Cardiac damage can be attributed to the known actions of the various components of crude venom. Phospholipase A_2 (PLA_2) and histamines can activate the body's immune system and cause inflammation. PLA_2 enzymes hydrolyze phospholipids of cellular membranes at the sn-2 position to generate free fatty acids and lysolipids. Arachidonic acid (AA), a product of PLA_2-mediated lipid hydrolysis, is a substrate of cyclooxygenase-2 and lipooxygenase-5. Both these enzymes metabolize AA via distinct pathways to generate eicosanoids, namely prostaglandins and leukotrienes, which are potent mediators of inflammation. AA is also the substrate for CYP 450 enzymes, as described earlier. PLA_2 from NS venom possesess hypotensive properties and has been shown in our laboratory to upregulate the expression of kallikreins [data unpublished]. Hypotension induced by envenomation is also due to the various factors in the venom that increase capillary membrane permeability (CPI), besides bradykinin. Histamine release has been reported to be responsible, whilst the CPI factor apparently exhibits cardiotoxic activity [47]. In addition, PLA_2 induces apoptosis via a mechanism involving ceramide that is independent of its hydrolytic products [48]. Cardiotoxin (CTX), which comprises ~60% of cobra venom by weight, is a cytolytic agent and can cause cell death. It has often been reported to produce myonecrosis [6], which could have accounted for the increase in cytokine and chemokine expression here [49]. The effects of CTX on the electrophysiology of the heart have been well studied, though the mechanisms of action of CTX are still unclear. CTX induces the contracture of isolated cardiac muscle cells, leading to systolic arrest and an irreversible decrease of the resting potential [50]. CTX was postulated to increase membrane permeability by causing pore formation, thus allowing Ca^{2+} influx and causing membrane depolarization. This is in agreement with the increase in intracellular Ca^{2+} concentration that was inferred from the observation of increased expression of Ca^{2+} binding and transport genes. Electrophysiological studies carried out here (fig. 3A) and by others [13, 14, 51] proved that snake venoms could indeed cause cardiac perturbations, which eventually led to the development of ischemia. At the same time, myocardial injury was observed (fig. 4), suggesting that CTX-induced Ca^{2+} influx may have caused cell damage resulting from ischemia. Moderate hypoxia in the later stages of envenomation very likely played a role in altering ECG parameters, especially in the development of ischemia, and even in causing myocardial damage. However, the occurrence of ECG abnormalities during normoxia and the presence of cardiac injury despite assisted respiration show that NS venom has car-

diotoxic effects. In addition, CTX, being cytolytic, may act directly on heart cells and along with other toxic components of the venom thus contribute to muscle degeneration. Neurotoxins (NTXs), believed to be the most lethal component in cobra venom, caused the eventual death of the animal by blocking neuromuscular transmission, resulting in respiratory paralysis. It was observed that the hearts continued to beat for a short period after cessation of respiration and when the mice were mechanically ventilated, life could be prolonged for up to 7 h, and the heart continued to beat for a short period of time upon cessation of respiration.

Given the complexity of snake venom, it is difficult to attribute the effects seen here to any one or few toxins. Microarray analysis carried out on heart tissue treated with varying doses of purified CTX, NTX and PLA_2 did not conclusively pinpoint any toxin as the main effector of whole venom toxicity (results not shown), clearly proving that the effects observed here really are the result of a collective toxicity and lethality of all major as well as minor venom components. Susceptibility of the heart to cobra venom could be due to the existence of specific target proteins on cell membranes which are recognized by venom components such as PLA_2 [52]. Besides being relatively specific for cardiac tissue, the ability of cardiotoxins to alter ionic fluxes is also manifested prominently in the heart, which requires a fine electrolyte balance for contractility.

The results of this investigation clearly indicate that NS venom is cardiotoxic, and this is likely true of cobra venoms in general, given their similarities in composition [7]. This is interesting because cobra venoms, as used here, are not mainly known for their cardiotoxic activity, though studies have been carried out over the years to suggest this [9, 53, 54]. It has been argued that most cases of cardiac instability by cobra envenomation are actually caused by hypoxia or acidosis secondary to respiratory paralysis. Though possible, the corresponding gene expression changes shown in the heart suggest that the venom can act directly on the heart. Hypoxia has been shown to stimulate the expression of cytoglobin [55] in all tissues and activate hypoxia-inducible factor-1, which in turn transcriptionally regulates the expression of a myriad of genes involved in oxygen regulation [56]. None of these genes have been found to be differentially expressed here. As a functional class, genes involved in glycolysis may have been upregulated in the heart as a result of hypoxia-induced ischemia, though it is unclear whether the extent and duration of hypoxia observed will actually lead to such changes. Indeed, many of the studies conducted involved chronic or severe and acute hypoxia. In this study, moderate hypoxia was observed up to 15 min before sacrifice and although respiratory failure resulted in death of the mice, it is doubtful that hypoxia had a major impact on gene expression in the organs.

Though neurotoxicity appears to be the main cause of death here, the cardiotoxicity of cobra venoms as contributory causes in cases of envenomation should not be overlooked. This is particularly important when victims of snakebites exhibit distress in the absence of signs or symptoms of neurotoxicity [7]. Changes in ECG profiles as a result of multi-systemic toxicity were unlikely to have occurred, as bradycardia was observed from 15 min post-envenomation. This is too short a time for the venom (administered at the dose of 1 μg/g body weight) to damage all organs. Hence, this study has presented evidence for the cardiotoxicity of cobra venoms, provided a molecular fingerprint of the events taking place in the major organs of the body during envenomation, especially so in the heart, and has possibly revealed potential targets for the treatment of snakebites at the molecular as well as tissue levels. Additionally, it demonstrates that gene expression is a useful approach for studying the effects of venoms in vivo and may prove to be especially meaningful in the study of individual toxins.

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