Mechanisms of cell death induction by L-amino acid oxidase, a major component of ophidian venom

Sudharsana Rao Ande · Phaneeswara Rao Kommoju ·
Sigrid Draxl · Michael Murkovic · Peter Macheroux ·
Sandro Ghisla · Elisa Ferrando-May

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Abstract L-amino acid oxidase (LAAO) from the Malayan pit viper induces both necrosis and apoptosis in Jurkat cells. Cell death by necrosis is attributed to H_2O_2 produced by oxidation of α -amino acids. In the presence of catalase that effectively scavenges H_2O_2 , a switch to apoptosis is observed. The major factors contributing to apoptosis are proposed to be: (i) generation of toxic intermediates from fetal calf serum (ii) binding and internalization of LAAO. The latter process appears to be mediated by the glycan moiety of the enzyme as desialylation reduces cytotoxicity. D-amino acid oxidase (DAAO), which catalyzes the same reaction as LAAO but lacks glycosylation, triggers necrosis as a consequence of H_2O_2 production but not apoptosis in the presence of catalase. Thus induction of cell death by LAAO appears to involve both the generation of H_2O_2 and the molecular interaction of the glycan moiety of the enzyme with structures at the cell surface.

Keywords Amino acids · Apoptosis · Flavin · Glycosylation · Necrosis

Abbreviations

AIP	Apoptosis-inducing protein
DAAO	D-Amino acid oxidase
LAAO	L-Amino acid oxidase
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-CH ₂ OC(<i>O</i>)-2,6 dichloro benzene, fluoro methyl ketone
DEVD-AFC	Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin
FCS	Fetal calf serum
Lpt-medium	LAAO pretreated-medium

Introduction

Apoptosis is a controlled and regulated form of cell death that plays an important role in the development and maintenance of higher organisms. It is defined by several morphological and biochemical hallmarks, like the exposure of phosphatidylserine to the outer leaflet of the plasma membrane, nuclear condensation, and chromatin cleavage into oligonucleosomal fragments. By contrast, necrosis represents an accidental form of cell demise resulting in early cell lysis, spillage of intracellular contents into the surrounding tissue, and inflammation. Experiments performed in cell culture show that one and the same toxic insult, e.g. the exposure to a prooxidant, can trigger either apoptosis or necrosis depending on its dose and duration of exposure [1, 2]. Intracellular energy levels and mitochondrial function are primarily involved in the determination of the shape of cell death [3]. In fact, ATP and NAD^+ were shown to be rapidly depleted in necrotic but not apoptotic cell death [4]. Moreover, under low ATP conditions typical apoptotic

S. R. Ande, P. R. Kommoju contributed equally to this work.

S. R. Ande · P. R. Kommoju · S. Ghisla
Biochemistry Research Group, University of Konstanz,
D-78464 Konstanz, Germany

E. Ferrando-May (✉)
Molecular Toxicology Group, Department of Biology,
University of Konstanz, D-78464, Konstanz, Germany
e-mail: elisa.may@uni-konstanz.de

S. Draxl · M. Murkovic
Institute of Food Chemistry and Technology,
Graz University of Technology, Petersgasse 12, A-8010 Graz,
Austria

P. Macheroux
Institute of Biochemistry, Graz University of Technology,
Petersgasse 12, A-8010 Graz, Austria

stimuli may induce necrosis [5, 6]. Recently, an elevation of the cytosolic ATP level was shown to occur upon apoptosis induction and to be required for the manifestation of apoptotic hallmarks [7].

One of the major regulators of cell death by apoptosis is the Bcl-2 protein, which exhibits a protective function against a wide array of stimuli and treatments, including chemotherapeutic agents, oxidative stress, growth factor withdrawal, or neurotoxins. Several mechanisms have been proposed for Bcl-2's antiapoptotic effects, the best characterized being the control of mitochondrial integrity and the prevention of the release of apoptogenic molecules from the mitochondrial intermembrane space (reviewed in [8]). More recently Bcl-2 has been shown to operate upstream of mitochondria in the regulation of pathways that emanate from the endoplasmic reticulum. This involves the control of calcium homeostasis and of calcium fluxes between the ER and the mitochondria (see e.g. [9]). Further, Bcl-2 has been shown to protect from oxidative stress via upregulation of antioxidant enzymes and by elevating the concentration of cellular thiols [10–14].

It has been reported that L-amino acid oxidases (LAAOs), present in the venom of various snakes, such as crotalids, elapids and viperids, possess antibacterial properties [15] as well as an anti-HIV activity [16] and can induce cell death [17–20]. These enzymes are members of the well-studied family of flavin (FAD) dependent oxidases. They catalyze the dehydrogenation of L-amino acids and the concomitant generation of H_2O_2 according to the sequences shown in Scheme 1.

In some snake species LAAO constitutes up to 30% of the total venom proteins [21]. Several related proteins with LAAO activity have been described that exhibit antimicrobial, antineoplastic or apoptosis-inducing activity [22]. The sources of these proteins are quite diverse ranging from the giant African snail (achacin from *Achatina fulica* Férussac [22]), to sea hares (dolabellin A and aplysianin A from *Dolabella auricularia* and *Aplysia kurodai*, respectively [23]), and parasite infected fish (Apoptosis-Inducing Protein, AIP from *Chub mackerel* [18]). Interestingly the substrate preference of these enzymes is distinct from that of the snake venom LAAO, which is selective for aromatic (Phe, Tyr) and hydrophobic (Ile, Leu, Val) amino acids. Both aplysianin A and AIP are highly specific for the basic amino acids Lys and Arg, whereas achacin and dolabellin A have a broader substrate specificity that includes aromatic, hydrophobic and basic amino acids [18].

Despite these differences in substrate specificity, it is assumed that the general mechanism of toxicity of these enzymes is based on the generation of cytotoxic amounts of H_2O_2 [24]. This hypothesis is reinforced by the protective effects of catalase, a scavenger of H_2O_2 . However, D-amino acid oxidase (DAAO), which generates the same products

from the oxidation of D-amino acids, was reported to lack antibacterial activity [15]. On the other hand the apoptosis-inducing activity of achacin and AIP have also been ascribed to the consumption of the amino acid substrates in the medium, e.g. Lys in the case of AIP [18] and aromatic and basic amino acids in the case of achacin [17]. A further factor that might play a crucial role in induction of cell death could be specific interactions of LAAO at the cell surface, as first suggested by Suhr and Kim [19]. Since the publication of that report, we have solved the 3D-structure of LAAO [25]. It shows a ≈ 20 Å long and narrow channel that connects the active center to the “outside” of the protein, and provides a route for substrate entry and product release [25]. In addition LAAO was found to be N-glycosylated at Asn361 and Asn172. The two glycan residues are remarkably homogeneous, consisting of a bis-sialylated, biantennary, core-fucosylated dodecasaccharide [26]. Intriguingly, the glycans are located in the vicinity of the channel [26]. This observation led us to posit the hypothesis that the proapoptotic effect of LAAO may involve interaction with the cell surface via sialic acid binding immunoglobulin-like lectins (siglecs). The effect could be twofold. Firstly, binding of LAAO to siglecs could target the exit of the active site channel to the plasma membrane resulting in high local concentrations of H_2O_2 that would be directly delivered to the cell and thus escape detoxification by catalase present in the medium. Secondly, LAAO may act via crosslinking of siglecs, a process that has been shown to trigger apoptosis in eosinophils and neutrophils [27].

Here, we report on the effects of LAAO from the venom of the Malayan pit viper (*Calloselasma rhodostoma*) on the viability of Jurkat cells and the influence of catalase, of LAAO's glycan moiety, and of growth medium composition. Our results have important implications for understanding the mechanisms of cell death triggered by this family of enzymes.

Materials and methods

General chemicals, reagents and enzymes

DMSO, *o*-dianisidine and propidium iodide were from Sigma. Annexin-V Alexa Fluor 488, Sytox, Hoechst 33342, TO-PRO3, DEVD-AFC and AFC were from Molecular Probes, Göttingen, Germany. Catalase, mucin, neuraminic acid and neuraminidase were from Sigma, Ehrlich's solution from Fluka and horseradish peroxidase from Roche Diagnostics, Mannheim, Germany. L-amino acid oxidase (LAAO) from the venom of the Malayan pit viper (*Calloselasma rhodostoma*) was obtained as described [26, 28]. D-amino acid oxidase (DAAO) was either from the

yeast *Rhodotorula gracilis* or from pig kidney and was a kind gift from Prof. L. Pollegioni (Univ. Insubria, Varese, Italy). Human medium chain acyl CoA dehydrogenase (hM-CAD) was obtained as described [29]. Chemicals used for derivatization of the amino acids were from Agilent (USA).

Cell culture and incubation conditions

Jurkat cells (clone E6) were a kind gift of T. Brunner, University of Bern, Switzerland. Jurkat cells stably expressing the Bcl-2 gene were obtained from S.J. Korsmeyer, Dana Farber Cancer Institute, Boston, USA. Cells were grown in RPMI-1640 medium supplemented with 10% FCS in a humidified atmosphere at 37°C. For toxicity experiments 3×10^4 cells/ml were plated in a 96-well microtiter plate and incubated in the presence or absence of 1.5 μ M catalase for 30 min and treated subsequently with varying concentrations of LAAO for 12 h or 24 h. Where indicated catalase (1.5 μ M) and zVAD-fmk (20 μ M) were added 30 min prior to LAAO treatment. Samples for Western blot analysis were prepared as follows: 2×10^5 cells were seeded in 12-well plates and preincubated with catalase (1.5 μ M) and zVAD-fmk (20 μ M) for 30 min. The cells were then treated with 0.03 μ M LAAO for 15, 30, and 45 min, followed by three washing steps with PBS and lysis in SDS sample buffer.

Measurement of H₂O₂ production

LAAO (0.03 μ M) or DAAO (0.03 μ M) in combination with 2 mM D-Ala were added to the cell culture medium either in absence or presence of catalase (1.5 μ M). 100 μ l aliquots were recovered at various time points and filtered through 0.22 μ filters (Millipore). The amount of H₂O₂ in the filtrate was determined by a peroxidase-coupled assay [28]. The assay mixture contained 0.25 μ M horseradish peroxidase and 0.4 mM *o*-dianisidine.

Viability assays and membrane alterations

For apoptosis and necrosis analysis, cell nuclei were stained with a mixture of the membrane-impermeant dye Sytox (0.4 μ M) and the membrane permeant dye Hoechst 33342 (250 μ g/ml) for 10 min at 37°C and subsequently examined at the fluorescence microscope. Cells with condensed nuclei were scored as apoptotic, cells with damaged plasma membrane and noncondensed nuclei were scored as necrotic. For the detection of phosphatidylserine exposure, cells were stained with Alexa488-labeled Annexin V (1:20 dilution) at 25°C for 15 min and with the DNA counterstain TO-PRO3 (1 μ g/ml) for 1 min and subsequently analyzed by FACS.

LAAO and DAAO activity assay

Enzyme activity was determined in 0.1 M Tris/phosphate buffer, pH 7.8 at 25°C using the horseradish peroxidase/*o*-dianisidine assay [28]. 1 ml assay mixture contained horseradish peroxidase (0.25 μ M), *o*-dianisidine (0.4 mM) and L-leucine (1 mM) or D-alanine (2 mM). The reaction was started by addition of 5–10 μ l of the L- or D-AAO solution corresponding to a concentration $\approx$ 0.4 μ M.

Amino acid analysis

200 μ l of RPMI medium were incubated with 0.03 μ M LAAO and 1.5 μ M catalase at 37°C and samples were collected after 0, 5, 10, 20, 40 min, and 1, 1.5, 2, 4, 8 and 24 h. The samples were quenched with 200 μ l 0.1 N HCl and filtered (UFC5 BCC filter, Millipore). The filtrate was lyophilized and samples were dissolved in 500 μ l of eluent A (see below) for HPLC analysis. 1 μ l of the sample was applied to a reversed phase column. (HPLC equipment: Agilent HP1100 liquid chromatograph, Agilent, Waldbronn, Germany; column: Hypersil AA-ODS, 5 μ m 200 $\times$ 2.1 mm, Agilent, USA). Eluent A contained 108 mM sodium acetate, 1.3 μ M triethylamine and 59 mM tetrahydrofuran in water. The pH was adjusted to 7.3 with acetic acid. Eluent B contained 20% v/v of 33 mM sodium acetate in water, pH 7.3, 40% v/v methanol and 40% v/v acetonitrile. The gradient was 100 to 40% of eluent A for 17 min with a flow of 0.45 ml/min at 40°C. The derivatization was carried out online using orthophthaldialdehyde and 0.4 N borate, pH 10.2. Primary amino acids were detected at 338 nm with 390 nm as a reference wavelength. Amino acids were identified by their retention times with respect to standard elution profiles. Quantification was performed by an external standard method using a mixture of amino acids with a concentration of 1 mg/ml each. Standard solutions were prepared daily from stocks that were kept protected from light at -20°C .

Preparation of medium depleted of L-amino acids (Lpt-medium)

RPMI-1640 medium containing 10% FCS was incubated in 12-well plates (1 mL each) with 0.03 μ M LAAO and 1.5 μ M catalase (final concentrations) for 24 h at 37°C. The enzymes were then heat-inactivated (30 min at 80°C) and removed by filtration through a 0.2 μ m filter (Spartan 13/0, 2 RC filter unit, Schleicher & Schuell Biosciences, Dassel, Germany). Alternatively LAAO was inactivated by shifting the pH of the medium to 9.5 followed by shock freezing and storage at -20°C for 24 h [30]. Before addition to the cells the pH of the medium was lowered again to

7.4. For the experiments shown in Fig. 5, panel b, “Lpt-medium” was prepared by heat inactivation in the absence of FCS.

Measurement of caspase activity

Caspase activity was assessed by measuring the cleavage of the fluorogenic substrate DEVD-AFC. Cells were lysed in 25 mM HEPES pH 7.5, 5 mM MgCl_2 , 1 mM EGTA and 0.5% Triton X100. The fluorimetric assay was performed in microtiter plates with sample volumes corresponding to a total protein amount of 5 μg in a final volume of 100 μl . DEVD-AFC cleavage was followed in reaction buffer (50 mM HEPES, 10 mM DTT, 1% sucrose, 0.1% CHAPS) at a substrate concentration of 40 μM . The release of AFC was detected over a period of 30 min at 37°C with $\lambda_{\text{exc.}} = 390 \text{ nm}$ and $\lambda_{\text{emiss.}} = 505 \text{ nm}$. Absolute activity was calibrated with AFC standard solutions and the formation of 1 pmol AFC/min was defined as one micro unit (μU). The amount of protein in cell lysates was determined using the Pierce BCA (bicinchoninic acid) protein assay according to the manufacturers protocol (Bender and Hobein GmbH, Heidelberg, Germany). Calibration was performed using BSA as a standard.

Desialylation of LAAO

Desialylation of native LAAO was carried out by treatment with neuraminidase following the procedure of the manufacturer (Sigma). 1 ml reaction mixture containing 1 mg LAAO and 0.1 U of neuraminidase in 0.1 M potassium phosphate buffer pH 6.0, was incubated for 24 h at 37°C. A colorless protein precipitate was removed by centrifugation (13000 rpm for 10 min) and the yellow supernatant was loaded onto a Sephadex G-200 gel filtration column. Fractions containing LAAO were collected and identified by their UV/Visible spectra and activity. These were concentrated using Millipore filters (30,000 MW cutoff, Centriprep YM-30). Desialylation was assessed by SDS PAGE and by measuring the amount of released N-acetylneuraminic acid using Ehrlich's reagent [31]. To this end an aliquot of the desialylation reaction was passed through a 5,000 MW cutoff filter (Centriprep, YM5). Volumes of the filtrate corresponding to the amounts of LAAO indicated in Fig. 6(a) were made up to 1 ml with 0.1 M borate buffer, pH 8.0 and incubated for 40 min at 95°C. After cooling to 25°C, the samples were mixed with 3 ml of ethanol and 1 ml of Ehrlich's reagent [31]. Following incubation for 20 min at 70°C the absorbance at 558 nm was measured photometrically. The amount of sialic acid present in the samples was determined from a standard curve obtained with commercial N-acetyl neuraminic acid. As a positive control the glycoprotein mucin from bovine submaxillary tissue [32] was employed. The percentage of

total sialic acid released was $\% \text{ NAN} = (\text{OD}_{558} \times 100) / (\text{mg protein} \times \text{slope of standard curve})$.

Generation of antibodies specific for LAAO

Antigen was prepared by heat denaturation of 1 mg purified LAAO at 90°C for 5 min in presence of β -mercaptoethanol. Rabbits were immunized by multiple subcutaneous injections of 500 μl of LAAO (300 $\mu\text{g}/\text{ml}$ in phosphate buffered saline pH 7.2) plus 500 μl Freund's complete adjuvant for the first immunization and with Freund's incomplete adjuvant for the booster immunizations. Injections were repeated biweekly and serum was obtained after the third injection. 5 ml of antiserum was concentrated to 2 ml by ultrafiltration and loaded onto a 1 ml Hi-Trap Protein G column (Amersham Biosciences); the IgG-fraction was purified following the manufacturers instructions. The buffer was exchanged for PBS by dialysis and samples were concentrated to 1 ml volume.

Western blotting

Jurkat cells were lysed in cell lysis buffer (10% SDS, 0.1 M Tris-HCl, pH 7.5) in the presence of protease inhibitors (Complete Inhibitor, Roche) and lysates were resuspended in SDS sample buffer. Samples were separated on 12% SDS PAGE, electroblotted to a nitrocellulose membrane (Schleicher & Schüll Biosciences, Dassel, Germany) and blocked in blocking buffer containing 5% milk powder in PBST (phosphate buffered saline with 0.1% Tween 20) for 2 h. The membrane was then incubated with α -LAAO antibodies for 2 h followed by incubation with a peroxidase-conjugated secondary antibody (1:10000, Sigma) for 1.5 h. The LAAO-specific signals were visualized by chemiluminescence using a commercially available kit (Super Signal, Pierce).

Results

L-amino acid oxidase (LAAO) induces different modes of cell death in Jurkat cells

Incubation of Jurkat cells with LAAO induced necrosis in a concentration dependent fashion, as revealed by morphological changes of treated cultures (Fig. 1(a)). In the presence of catalase, a scavenger of H_2O_2 , the extent of necrotic cell death was greatly reduced. This decrease was accompanied by a corresponding increase in the number of cells with apoptotic morphology. The conversion from a necrotic to an apoptotic mode of cell death was dependent on catalase concentration, 0.15 μM catalase being sufficient to completely suppress necrosis induced by 0.03 μM LAAO (Fig. 1(b)). In

Fig. 1 LAAO induces apoptosis and necrosis in Jurkat cells. (a) Catalase-dependent switch from necrosis to apoptosis and effects of zVAD and Bcl2. Jurkat cells clone E3, Jurkat cells stably overexpressing Bcl-2 and cells stably transfected with a control vector (Neo) were incubated with the indicated concentrations of LAAO in the absence or presence of catalase (1.5 μM). After incubation cells were stained with a mixture of the dyes Sytox and Hoechst and counted using fluorescence microscopy. Cells with condensed or fragmented nuclei were scored as apoptotic and lysed cells with non-condensed nuclei were scored as necrotic. 200–250 cells were counted for each concentration in three independent experiments. For caspase inhibition, Jurkat cells were preincubated for 30 min in the presence of zVAD-fmk (20 μM) before exposure to LAAO (0.03 μM) and catalase (1.5 μM). The bars

indicate the results after 24 h of incubation and the horizontal mark within the bar (■) the results at 12 h. (A): apoptosis; (N): necrosis; (C): catalase; (L): LAAO (b) Necrosis – apoptosis switch depends on catalase concentration. Jurkat cells clone E3 were incubated with LAAO (0.03 μM) in the presence of the indicated amounts of catalase. Cell death was assessed as in (a). (c) Assessment of caspase activity. Cells were treated as in (a) with 0.03 μM LAAO in the presence or absence of catalase and of zVAD-fmk for 24 h. Caspase 3/7-like activity was determined by fluorimetric detection of DEVD-afc cleavage. (d) Exposure of phosphatidylserine. Upon incubation under the indicated conditions, cells were stained with Annexin-V/To-Pro 3 and then analyzed by flow cytometry

all subsequent experiments, a 10-fold higher concentration of catalase was routinely used in order to ensure that cells exposed to LAAO would die by apoptosis.

Apoptosis induction by the combined action of LAAO and catalase was corroborated, first by measuring caspase 3/7-like activity. As shown in Fig. 1(c), executor caspases were only activated in the presence of both LAAO and catalase, while LAAO alone had no effect. Secondly, we monitored the integrity of the plasma membrane and the exposure of phosphatidylserine in treated cultures by FACS analysis (Fig. 1(d)). Incubation with LAAO and catalase resulted in the appearance of annexin V-positive cells with intact plasma membrane, a typical hallmark of apoptosis, while in the absence of catalase all cells were positive for propidium iodide, indicating massive cell lysis. Nuclear condensation, caspase activation and phosphatidylserine exposure induced by LAAO and catalase were completely abolished by the pan-caspase inhibitor zVAD-fmk (Fig. 1(a), (c) and (d)). By contrast, this inhibitor had no effect on the toxicity mediated by LAAO alone, suggesting that caspases are not involved in this necrotic type of cell death.

In order to assess whether cell death induced by LAAO involves the Bcl-2-family of apoptosis regulators we have investigated the effects of LAAO in Jurkat cells overexpressing Bcl-2. As shown in Fig. 1(a), these cells were completely protected from cell death induced by the combined treatment with LAAO and catalase, while they were as sensitive as wild-type cells with respect to the necrotic type of death induced by LAAO alone.

One potential mediator of the cytotoxic effect of LAAO is H_2O_2 generated by the oxidation of α -amino acids present in the culture medium (see Scheme 1). We have therefore assessed the time course of H_2O_2 production by LAAO under our specific incubation conditions. The results depicted in Fig. 2 show that upon addition of $0.03 \mu\text{M}$ LAAO the concentration of H_2O_2 increased rapidly, reaching a maximum value of $\sim 220 \mu\text{M}$ after 0.5–1 h and subsequently declining monophasically with a half time of about 4 h. No bulk H_2O_2 was detectable when incubations were performed in the presence of catalase, indicating that scavenging of H_2O_2 in the medium occurred very efficiently under our experimental conditions.

Scheme 1 LAAO-catalyzed dehydrogenation of L-amino acids. The products of this reaction, the α -imino acids, hydrolyze spontaneously to the α -keto acid and ammonia. The reducing equivalents derived from

Fig. 2 Time course of H_2O_2 production. H_2O_2 concentrations were measured in medium containing either LAAO or DAAO (both at $0.03 \mu\text{M}$) in the presence or absence of catalase as indicated. In the case of DAAO, D-Ala (2 mM) was supplemented as a substrate. Readings are the average of three independent experiments. The curves through the data points were generated using a biexponential algorithm. The estimated rates for the ascending and descending slopes are $\approx 2.5 \text{ min}^{-1}$ and $\approx 0.25 \text{ min}^{-1}$ for LAAO, and $\approx 4 \text{ min}^{-1}$ and $\approx 1.5 \text{ min}^{-1}$ for DAAO. (L): LAAO; (D): DAAO; (C): catalase

Taken together these results indicate that LAAO elicits two different modes of cell death depending on the presence of a scavenger of H_2O_2 : at high H_2O_2 concentrations cells die by necrosis. When H_2O_2 is removed from the culture medium, cells die nonetheless but display apoptotic morphology. In the latter case, cell demise is mediated by caspases and is susceptible to inhibition by Bcl-2.

Apoptosis is induced by LAAO but not by the related amino acid oxidase DAAO

To assess whether the switch from necrotic to apoptotic cell death observed in the presence of catalase is specific for LAAO, a related amino acid oxidase, DAAO was used as an alternative source of H_2O_2 . DAAO catalyzes basically the same chemical reaction as LAAO, the only difference being the utilization of D- instead of L-amino acids

(Scheme 1). Moreover, DAAO is not glycosylated, i.e. it lacks the two glycan moieties linked to Asn 172 and Asn 361 on the surface of LAAO [26]. Since D-amino acids are absent from the normal growth medium, H_2O_2 production catalyzed by DAAO requires supplementation of D-amino acids to the medium. A concentration of 2.0 mM D-Ala was selected as it leads to the production of approximately the same H_2O_2 quantities as in the experiments using LAAO. This was confirmed by measuring the H_2O_2 concentration in DAAO-treated culture medium (Fig. 2). Similarly to LAAO, DAAO rapidly generated high H_2O_2 levels which decayed monophasically with a half time of ~ 1 h. The longer persistence of H_2O_2 in the case LAAO ($t_{1/2} \sim 4$ h) is due to the presence of several amino acid substrates that are oxidized at different rates (see Fig. 4). Again, addition of catalase efficiently eliminated H_2O_2 from the culture medium incubated with DAAO.

The effects of the addition of varying concentrations of DAAO on Jurkat cell cultures are reported in Fig. 3. Both necrosis and apoptosis were induced at concentrations between 0.003 and 0.03 μM . At concentrations above 0.03 μM cells died predominantly by necrosis. In contrast to LAAO, however, addition of catalase to the cultures did not shift this necrotic cell death to apoptosis, but resulted in an overall reduction of toxicity. This finding suggests that DAAO exerts its toxic effect mainly via the production of H_2O_2 whereas LAAO exhibits further, H_2O_2 -independent effects that can result in cell damage.

Fig. 3 DAAO induces necrosis in Jurkat cells. Cells were incubated with the indicated concentrations of DAAO in the presence of 2 mM D-Ala as a substrate, in the absence or presence of catalase (1.5 μM). Cell death was assessed as in Fig. 1(a). The bars indicate the results after 24 h of incubation and the horizontal mark within the bar (■) the results at 12 h. (A): apoptosis; (N): necrosis; (C): catalase; (D): DAAO

One mode of apoptosis induction by LAAO is via alteration of the chemical composition of the culture medium

A plausible mechanism that may contribute to the apoptosis-inducing effects of LAAO is the depletion of L-amino acids contained in the cell culture medium. Indeed, it has been reported that achacin from the body surface mucus of the giant African snail *Achatina fulica* Férussac [17] and apoptosis inducing protein (AIP) from parasite-infected fish [18] deplete media of specific amino acids, which may contribute to their toxicity. It is therefore important to determine whether the addition of LAAO and catalase to the culture medium under conditions that induce apoptosis also results in the consumption of specific essential amino acids. To this end, the concentration of several relevant amino acids in medium containing LAAO (0.03 μM) and catalase (1.5 μM) was monitored over time by HPLC. The concentrations of some specific L-amino acids decreased rapidly in the presence of LAAO and catalase (Fig. 4(a)). For individual amino acids this occurred with substantially different half times and probably reflected the substrate selectivity of the enzyme under conditions close to those encountered *in vivo*. Thus the aromatic (Tyr, Phe, $t_{1/2} \approx 20$ min) and the hydrophobic amino acids Val and Leu ($t_{1/2} \approx 40$ min) were oxidized rapidly (Fig. 4(a)) resulting in complete depletion within a few hours. The hydrophobic amino acid Ile appeared not to be affected under the same conditions, while the concentration of basic amino acids was slightly altered. It is important to note that depletion of Tyr, Phe, Val and Leu preceded the onset of apoptosis, which was observed after 4 h (Fig. 4(b)).

In order to substantiate the putative proapoptotic effect of L-amino acid depletion by LAAO, Jurkat cells were grown in medium pretreated with LAAO (LAAO pre-treated medium = Lpt-medium) to remove relevant amino acids and tested for their viability. To this end the medium was incubated with LAAO and catalase for 24 h, followed by inactivation of the LAAO. This was achieved alternatively by either heating the samples to 95°C or by the pH/freeze method reported by Coles et al. [30] (see Materials and methods). In both cases complete inactivation of the enzyme in the Lpt-medium was attained as verified with the conventional activity assay using L-leucine ([28] and data not shown). As shown in Fig. 4(b) incubation of Jurkat cells with Lpt-medium that had been obtained by heat inactivation resulted in apoptosis, although to a 10–20% lower level as compared to treatment with LAAO and catalase in normal medium. Again, apoptosis was completely prevented by zVAD-fmk (not shown). Surprisingly, however, supplementation of the L-amino acids Tyr, Phe, Leu and Val to the Lpt-medium at concentrations corresponding to those present in the original medium did not result in

Fig. 4 LAAO mediates depletion of essential amino acids in the culture medium. (a) Time course of amino acid depletion. The concentration of amino acids in medium treated with LAAO ($0.03 \mu\text{M}$) and catalase ($1.5 \mu\text{M}$) was determined by HPLC at the indicated time points. The curves through the data points are fits based on an equation for mono-

exponential decay. (b) Rate of apoptosis induction in cultures incubated with Lpt-medium. Cells were treated either with LAAO ($0.03 \mu\text{M}$) and catalase ($1.5 \mu\text{M}$) or incubated in Lpt-medium. Cell death was assessed as in Fig. 1(a) and (b). (A): apoptosis; (N): necrosis; (L): LAAO; (C): catalase

protection from apoptosis (Fig. 5(a)). Similar results were obtained when LAAO was inactivated using the pH/freeze method, i.e. in the absence of heat treatment (Fig. 5(d)). This suggests that changes in the medium composition other than the selective loss of hydrophobic amino acids may be responsible for the apoptosis promoting effect of Lpt-medium and consequently of LAAO in combination with catalase.

Fetal calf serum (FCS) which contains various growth and survival factors is required for cell survival and proliferation in culture. Removal of FCS has been reported to sensitize mammalian cells to a variety of apoptosis-inducing agents as well as to trigger apoptotic cell death [33, 34]. We therefore investigated whether FCS might play a role in LAAO-induced apoptosis. In contrast to the repletion of amino acids, addition of FCS rescued Jurkat cells from apoptosis induced by Lpt-medium (Fig. 5(a) and (d)), indicating that LAAO might exert its toxic effect via alteration of essential FCS components. Intriguingly, a lower percentage of apoptotic cells was observed when Lpt-medium was prepared in the complete absence of FCS (Fig. 5(b)), suggesting that LAAO might induce apoptosis not only by destroying essential FCS components but also via the generation of toxic oxidation products from FCS constituents.

LAAO consumes L-amino acids present in the medium and produces H_2O_2 , ammonia and α -keto acids. Based on the results described above (Fig. 4), hydrophobic and aromatic amino acids are rapidly depleted in the medium and converted to the respective keto acid compounds. In order to assess their potential toxic effect, the keto acids derived from Tyr, Phe, Leu, and Val were added in different combinations and at the same concentration of their parent amino acids to Jurkat cell cultures. The data shown in

Fig. 5(d) show that among the substances analyzed only the keto acid derived from tyrosine had an apoptosis promoting activity, leading to $\approx 15\%$ apoptotic cells 24 h after treatment.

Desialylation counteracts the proapoptotic activity of LAAO

In order to substantiate our hypothesis presented in the introduction that the glycan moiety of LAAO might be involved in cell death, we prepared LAAO lacking the sialic acid moieties and studied some of its properties with regard to the induction of apoptosis. Desialylation was assessed by SDS-PAGE and by quantification of released neuraminic acid [31] (see Material and methods for details). The inset in Fig. 6(b) shows that the electrophoretic mobility of LAAO is increased as expected after treatment with neuraminidase. The estimation of released N-acetylneuraminic acid is shown in Fig. 6(a). In each monomer of LAAO (MW ≈ 62000), 4 molecules of sialic acid (MW: $4 \times 306 = 1224 \text{ D}$) are present. This corresponds to $\approx 2\%$ of the total mass, [26] which is in good agreement with the value of $\approx 2.5\%$ that we determined experimentally (Fig. 6(a)). As a reference, we determined the sialic acid content of mucin that contains $\approx 12\%$ sialic acid by mass [32]. From these data we conclude that the desialylation of LAAO was essentially complete. Desialylation of LAAO did not affect its enzymatic activity (data not shown).

Native and desialylated LAAO were added to Jurkat cell cultures at concentrations corresponding to equal enzymatic activity. Under these conditions both native and desialylated LAAO caused apoptosis in the presence of catalase (Fig. 6(b)). However two differences became apparent: (i) the final extent of apoptosis obtained with desialylated

Fig. 5 FCS but not essential amino acids protect from apoptosis triggered by Lpt-medium. (a) Jurkat cells were incubated for 24 h in Lpt (LAAO-pretreated)-medium which was obtained by preincubation with LAAO followed by heat inactivation of the enzyme (see Material and methods) either without additions (–) or supplemented with either amino acids (+aa) or FCS (+FCS) or both (+FCS, +aa). Cell death was assessed as in Fig. 1(a). As a control, apoptosis was determined in normal medium (control) and in heat-treated normal medium (heat) (b) The same experiments as in (a) were performed with Lpt-medium lacking FCS. (c) The same experiments as in (a) were performed with Lpt-medium in which LAAO was inactivated by pH-shift and freezing (see Material and methods). As a control, apoptosis was determined in normal medium (control) and normal medium which had been subjected to the same inactivation procedure without prior incubation with LAAO. (d) Jurkat cells were incubated in normal medium supplemented with the keto acids derived from Tyr (Y), Leu (L), Val (V) and Phe (F) at the indicated concentrations

LAAO was reduced by $\approx 20\%$ compared to the native enzyme and (ii) the “threshold” concentration of desialylated LAAO required for 50% effect was increased ≈ 5 -fold compared to native LAAO. These results were obtained in three separate experiments using different preparations of desialylated LAAO. Thus, in addition to alterations of

Fig. 6 Effect of desialylation on LAAO-dependent apoptosis. (a) Estimation of sialic acid released from LAAO by neuraminidase treatment. LAAO or mucin were incubated with sialidase as described in Materials and methods. Aliquots corresponding to the indicated amounts of glycoprotein (LAAO or Mucin) were used for the estimation based on the Ehrlich's alkali method. (b) Effect of desialylated LAAO on induction of apoptosis. Jurkat cells were incubated for 24 h with the indicated concentrations of LAAO and desialylated LAAO in the presence of catalase (1.5 μM). Cell death was evaluated as in Fig. 1(a). Data points are the average of three individual readings. SDS-PAGE analysis of the desialylation reaction is shown in the inset. (A): apoptosis; (N): necrosis; (C): catalase; (L): LAAO (62 kDa); (ds-L): desialylated LAAO (61.5 kDa)

components of the medium, sialylation appears to be a further factor contributing to LAAO's apoptosis inducing activity.

Interaction of LAAO and desialylated LAAO with Jurkat cells

Since the above mentioned experiments suggested a potential interaction between LAAO and Jurkat cells, we performed immunoblot analysis of cells that had been exposed to the enzyme for various periods of time. Total lysates from these

Fig. 7 Fragmentation of LAAO but not other related enzymes upon incubation with Jurkat cells. (a) Native LAAO but not desialylated LAAO is fragmented upon incubation with Jurkat cells. Jurkat cells were incubated with LAAO and desialylated LAAO (dsLAAO) for the indicated time periods. Cells were harvested and washed three times with PBS. Cell lysates were analyzed by immunoblot with rabbit α -LAAO polyclonal antibodies. Native LAAO (LAAO) is shown as a

positive control. Untreated cells were used as negative control. (b) Interaction of glycan-free enzymes with Jurkat cells. Mammalian DAAO (mDAAO), yeast DAAO (yDAAO) and medium chain acyl Co-A dehydrogenase (MCAD) were incubated with Jurkat cells as described in (a). Western blot analysis was carried out using the respective polyclonal antibodies. The isolated enzymes are shown as a reference

cells where investigated using an α -LAAO antibody. As indicated in Fig. 7(a) cell lysates from LAAO treated cells displayed two major protein fragments that were recognized by LAAO antibodies. The amount of LAAO recovered in these lysates appeared to increase with time from 15 to 45 min. Since lysate preparation occurred in the presence of general protease inhibitors the fragmentation seems to reflect a specific proteolytic event occurring during incubation of LAAO with the cells. This observation may suggest that LAAO is taken up by the cells and subsequently cleaved by endogenous proteases. A specific signal was detected also in lysates from cells treated with desialylated LAAO, however no fragmentation of the enzyme was observed in this case. Control experiments were performed using three enzymes that do not carry glycans. These were (heterologously expressed) human medium chain acyl CoA dehydrogenase (hMCAD) [29], mammalian DAAO [35] and yeast DAAO [36]. Figure 7(b) shows that all three proteins were retained to various extents in cell lysates but did not undergo fragmentation, suggesting that intracellular proteolysis is a specific feature of LAAO.

Discussion

In cell culture, treatment with LAAO triggered necrosis in a dose-dependent fashion. This is a consequence of a pro-

tracted exposure to high levels of H_2O_2 . It is well established that under conditions of severe oxidative stress mitochondrial integrity is compromised leading to an opening of the permeability transition pore, mitochondrial calcium overload, loss of membrane potential, and the collapse of the cell's energy metabolism ultimately resulting in cell lysis. Interestingly, removal of H_2O_2 by addition of catalase to the medium did not inhibit cell death but resulted in an apoptotic type of cell demise. zVAD-fmk protected cells from this proapoptotic insult, indicating that under these conditions, caspases are the main death effectors. Overexpression of Bcl-2 also conferred full protection to the cells. This is consistent with the reported properties of Bcl-2 in upregulating the cell's antioxidant capacity as well as its ability to antagonize proapoptotic Bcl-2 family proteins [37, 38]. In some instances, Bcl-2 was shown to inhibit or at least attenuate cell death by necrosis [11, 39–41], however, in these systems necrosis resulted from transient stress and probably involved the integration of death and survival signals. In the present study, necrosis is the consequence of a massive oxidative insult that overwhelms the protective ability of Bcl-2 and bypasses critical control points of the cell death pathway.

The switch from apoptosis to necrosis observed in the presence of catalase could be possibly explained by the ability of LAAO to convey low residual amounts of H_2O_2 to the cells in a manner that is unaffected by the scavenger in

the medium. In fact, a correlation between the shape of cell death and the dose and duration of the oxidative insult has been already reported in the literature [2, 42]. This effect is specific for LAAO, since DAAO, an enzyme that catalyses the same reaction from D-amino acids becomes innocuous when combined with catalase (Fig. 3).

Three potential mechanisms that might contribute to the cytotoxicity of LAAO observed in the presence of H_2O_2 scavengers have to be considered:

1. Depletion of essential amino acids has been suggested to contribute to the cell killing activity of achacin from the body surface mucus of the giant African snail (*Achatina fulica* Férussac) and the apoptosis inducing protein (AIP) from parasite-infected fish, both members of the LAAO family. These enzymes deplete either arginine, lysine, tryptophane, and tyrosine, or only lysine from the culture medium [17]. Ophidian LAAO also appears to be very effective in the degradation of L-amino acids. Degradation is, however, very much dependent on the structure of the amino acid itself; e.g. only aromatic and hydrophobic amino acids are metabolized rapidly by LAAO, with Tyr, Phe, Val and Leu being essentially depleted within 4 h (Fig. 4). Deprivation of essential amino acids can act as apoptotic trigger, as shown in human A375 melanoma cells grown in tyrosine and phenylalanine-free medium, [43] however apoptosis is delayed compared to the effect induced by LAAO. In the latter case, 70% of the cells became apoptotic within 24 h while only 33% apoptosis was observed after 72 h incubation under tyrosine and phenylalanine-free conditions. This suggests that amino acid depletion may only partially, if at all, contribute to LAAO toxicity, an observation, that is supported by the failure to prevent cell death upon restoring normal amino acid levels in a medium that had been pretreated with LAAO (Lpt-medium) (Fig. 5).
2. Inhibition of cell death in our experimental setting could only be achieved by resupplementation of fetal calf serum. This suggests that LAAO activity leads to alterations of yet unidentified essential components of FCS. In fact, we have observed a partial activity of LAAO on small peptides (unpublished observations).
3. The third mechanism of LAAO toxicity addressed in this study regards the interaction of LAAO with the cell surface and its subsequent internalization. Binding of the enzyme to the cell surface may support the efficient delivery of low amounts of H_2O_2 to the cells. It is interesting to note in this regard, that the glycan residues of LAAO reside in the vicinity of the channel connecting the active center to the surface of the protein [25]. Suhr and Kim [19] have provided some evidence that LAAO from a Korean snake (*Agkistrodon hylas*) labeled with a fluorescent dye interacts with the cell membrane. We have

been able to reproduce these results qualitatively using fluorescently labeled LAAO. However, they are difficult to interpret in a straightforward manner since also fluorescently labeled mammalian DAAO exhibits some degree of aggregation to the surface of Jurkat cells (data not shown [44]). Supportive evidence for an interaction of LAAO with Jurkat cells emerges from our immunoblot analysis (Fig. 7). Here, specific proteolysis of LAAO, but not of DAAO or of other, unrelated enzymes was detected in lysates obtained from cells treated with the respective enzymes suggesting that LAAO may be taken up and proteolytically processed by Jurkat cells. The point should be reiterated that processing seemed to depend on the terminal structure of the N-glycan moiety, since desialylated LAAO was not fragmented upon incubation with the cells (Fig. 7(a)). Along these lines, the control proteins used which are not glycosylated, are not proteolytically degraded either (Fig. 7(b)). The requirement of glycosylation for the activity of apoxin I from the venom of the Western diamond rattlesnake (*Crotalus atrox*), a homologue of LAAO, has been mentioned by Torii et al. [45].

Our results are in accordance with the study of Suhr and Kim, who have provided evidence that the apoptotic effects of LAAO are different from those caused by the addition of exogenous H_2O_2 [19,46]. This contrasts sharply with the report by Torii et al. [24, 45] that apoxin I induces apoptosis in mammalian cell lines by a mechanism proposed to be completely dependent on the generation of H_2O_2 . Further results by Torii et al. [45] are also difficult to reconcile both with our and Suhr and Kim's findings [19, 46]. Torii et al. claim that apoxin I, which was expressed in a human embryonic kidney cell line (293 T) and was secreted into the medium, induces apoptosis [45]. Furthermore, and in contrast to their first report [24], apoxin I was not incorporated into the target cells [45]. In an attempt to reproduce these results we have not been able to detect LAAO activity in cell culture medium when LAAO was transiently expressed in 293 T-cells (unpublished results). It is most likely that the nature (sequence/structure) of the glycan moieties linked to the LAAO protein differs substantially between ophidian LAAOs and LAAOs expressed in mammalian or yeast cells. In the first case a high degree of homogeneity in terminal sialylation appears to predominate [26]. However, this is most probably not so for other expression systems, and this, in turn, might lead to absence of or to reduced internalization of LAAO into the cell. Indeed this would be consistent with our observation of absence of proteolytic processing of desialylated LAAO (Fig. 7(a)).

Internalization is a well described mechanism of action of bacterial toxins like diphtheria toxin and cholera toxin, which are bound to the plasma membrane, get internalized and

subsequently processed inside the cell [47–50]. This internalization occurs via receptor mediated endocytosis [49, 50]. Our results suggest that some of the toxic effects of ophidian LAAO may occur by similar mechanisms and might involve sialic acids. Candidate mediators of an interaction between LAAO and the cell surface might be siglec receptors [26, 51]. Indeed there is an abundance of reports on siglec receptors mediating cell related interactions [51–56]. Binding of LAAO to the cell surface might be a mechanism to potentiate the effects of H₂O₂ by enhancing its local concentration. Once being internalized LAAO might further exert toxic effects by generating peroxide and metabolizing amino acids and/or other factors important for cell survival. In this context a recent report by Zhang et al. [57] is of interest according to which both LAAO and DAAO exert antimicrobial activity and are bound to the surface of bacteria. This could point to hitherto unrecognized modes of interactions between proteins and cell surfaces, the elucidation of the underlying molecular factors being a challenging endeavor.

Conclusion

LAAO, a major component of snake venom, is a potent cytotoxin acting primarily via the production of high amounts of H₂O₂, which kill the cells by necrosis. However, LAAO retains its cytotoxicity also in the presence of antioxidants. In this case the mode of cell demise switches to apoptosis. Apoptosis induction by LAAO involves at least two different mechanisms, the first one being an alteration of the composition of the growth medium, in particular of its serum components. Secondly, immunoblot experiments show that LAAO is retained by Jurkat cells where it undergoes specific proteolysis. This is suggestive of an interaction of the enzyme with the cell surface, a process which seems to require its glycan moieties. The study of the mechanisms of action of animal toxins is an important step toward their potential application as anticancer agents.

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