Measurement of apoptotic and necrotic cell death in primary hepatocyte cultures

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Summary

Hepatotoxicity, including drug-induced liver injury, is frequently accompanied by cell death. The latter is typically driven by apoptosis or necrosis, which substantially differ based upon biochemical and morphological criteria. The present chapter describes 2 commonly used methods to probe apoptotic and necrotic activities in adherent monolayer cultures of primary hepatocytes. The apoptosis assay uses a prototypical substrate of caspase 3, the main executor of apoptotic cell death, which can be cleaved, yielding a product that can be measured fluorimetrically. The second assay relies on the disruption of the cell plasma membrane, which typically occurs in necrotic cell death and that results in the extracellular release of cytoplasmic enzymes, such as lactate dehydrogenase. The latter can be indirectly assessed by spectrophotometrically measuring the consumption of reduced nicotinamide adenine dinucleotide.

Keywords

primary hepatocyte culture; apoptosis; caspase 3; necrosis; lactate dehydrogenase

1. Introduction

Drug-induced liver injury is a ubiquitous issue in clinical settings and pharmaceutical industry. Recently, a unifying mechanistic model of drug-induced liver injury has been introduced. According to this model, drug-induced hepatotoxicity relies on 3 consecutive steps, namely an initial cellular insult that leads to the occurrence of mitochondrial permeability transition, which in turn ultimately burgeons into the onset of cell death. The latter may be manifested as apoptosis or necrosis (1-3). Apoptosis is a genetically programmed and well-orchestrated process that can be triggered *via* 2 routes, namely through the mitochondria-mediated intrinsic cascade or through the death receptor-mediated extrinsic pathway. Both routes rely on the proteolytic activity of cystein proteases, the caspases, which are expressed as inactive pro-enzymes that become activated upon proteolytic cleavage. As such, 2 subsets of caspases can be distinguished, namely the

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initiator caspases and the effector caspases. The former, including caspase 8, caspase 9 and caspase 10, mediate the cleavage and thus the activation of effector caspases. Effector caspases, such as caspase 3, caspase 6 and caspase 7, subsequently cleave a large number of cellular proteins, like major cytoplasmic and nuclear elements, which forms the biochemical basis of the apoptotic morphological phenotype. The extrinsic apoptotic pathway is initiated by binding of a specific subset of ligands, such as Fas ligand, to their corresponding receptors at the cell plasma membrane surface, resulting in the proteolytic cleavage and auto-activation of procaspase 8. Activated caspase 8 induces caspase 3, which subsequently cleaves its cellular targets. In the intrinsic apoptotic pathway, cytochrome C is released from mitochondria, a process that is mediated by members of the B-cell lymphoma-2 protein family. Cytochrome C triggers caspase 9 activation, which then induces caspase 3 (4-9). Necrosis, as opposed to apoptosis, is a rather passive and unorganized process that is caused by a plethora of external stress factors, including extremely high concentrations of xenobiotics. It usually starts with the loss of ion homeostasis, which eventually evokes cell swelling, loss of cell plasma membrane integrity, and cell lysis (4, 5, 9-12).

The current chapter describes 2 methods to measure apoptotic and necrotic cell death in primary hepatocyte cultures at the activity level, using an established *in vitro* model of Fas-mediated cell death (13). The apoptosis activity method is based upon the use of a synthetic caspase 3 substrate called acetyl-aspartic acid-glutamic acid-valine-aspartic acid-7-aminotrifluoromethylcoumarin (Ac-DEVD-AFC) (Fig. 1) (*see* Note 1). When the AFC label is attached to the peptide substrate, it produces a blue fluorescence upon exposure to ultraviolet light. Caspase 3 cleaves this tetrapeptide between the second aspartic acid and the AFC moiety. Released AFC produces a yellow-green fluorescence at 505 nm when exposed to ultraviolet light, which can be measured fluorimetrically. The caspase 3 activity is proportional to the amount of free AFC produced (8, 14). The assay to assess necrotic cell death relies on the disruption of the cell plasma membrane, which results in the extracellular release of cytoplasmic enzymes. Among those is lactate dehydrogenase (LDH), a stable enzyme that leaks from the cell upon cell plasma membrane damage in relatively high amounts. LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of reduced (NADH) and oxidized nicotinamide adenine dinucleotide (Fig. 2). The principle of the assay described in this chapter is based upon this reaction. In particular, the consumption of NADH is spectrophotometrically assessed and serves as a measure that is proportional to LDH activity (15).

2. Materials

2.1. Establishment of the in vitro model of Fas-mediated hepatocellular cell death

1.   Hepatocyte seeding medium. William's medium E containing 7 ng/mL glucagon, 292 mg/mL *L*-glutamine, 7.33 IE/mL sodium benzyl penicillin, 50 μg/mL kanamycin monosulfate, 10 μg/mL sodium ampicillin, 50 μg/mL streptomycin sulfate and 10% fetal bovine serum. Prepare in a

[1]With respect to the specificity of the apoptosis assay, it should be kept in mind that Ac-DEVD-AFC not only is a substrate for caspase 3, but also for other caspases, including caspase 1, caspase 4, caspase 6, caspase 7, caspase 8 and caspase 10 (8). Hence, the enzymatic activity measured is not restricted to caspase 3 and thereby should be rather considered as caspase 3-like activity.

laminar air flow cabinet and store for maximum 7 days at 4°C. *Prior* to use, the hepatocyte seeding medium should be placed for 30 min in a thermostated bath at 37°C.

2. Hepatocyte culture medium. Serum-free hepatocyte seeding medium supplemented with 25 μg/mL hydrocortisone sodium hemisuccinate and 0.5 μg/mL insulin. Prepare in a laminar air flow cabinet and store for maximum 7 days at 4°C. *Prior* to use, the hepatocyte culture medium should be placed for 30 min in a thermostated bath at 37°C.

3. Hepatocyte cell death medium. Hepatocyte culture medium supplemented with 200 ng/mL Fas ligand (Alexis, Switzerland) and 2 μg/mL cycloheximide (Sigma-Aldrich, Belgium). Prepare *ex tempore* in a laminar air flow cabinet. *Prior* to use, the hepatocyte cell death medium should be placed for 30 min in a thermostated bath at 37°C.

4. Incubator ($37°C \pm 1°C$, $90\% \pm 5\%$ humidity, $5\% \pm 1\%$ CO_2).

5. Laminar air flow cabinet.

6. Thermostated bath.

2.2. Measurement of caspase 3 activity in cultured primary hepatocytes

1. Phosphate-buffered saline (PBS). 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 1.8 mM KH_2PO_4 in deionized water. Adjust to pH 7.4, sterilize by passing through a 0.22 μm filter and store for maximum 6 months at 4°C. *Prior* to use, PBS should be placed for 30 min at room temperature.

2. 25× concentrated reaction buffer. 250 mM 1,4-piperazinediethanesulfonic acid, 125 mM *DL*-dithiothreitol, 50 mM ethylenediaminetetraacetic acid tetrasodium salt dehydrate in double distilled water. Adjust pH of this solution to 7.4 (*see* Note 2) and store in aliquots of 2.5 mL for maximum 6 months at −20°C.

3. Reaction buffer. 1 volume part 25× concentrated reaction buffer and 24 volume parts double distilled water. Prepare this buffer *ex tempore* and place at least 10 min at 37°C *prior* to use.

4. *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] solution. 50 mM *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] in double distilled water. Sterilize this solution by passing through a 0.22 μm filter and store for maximum 6 months at 4°C.

5. Ethylenediaminetetraacetic acid tetrasodium salt dihydrate solution. 500 mM ethylenediaminetetraacetic acid tetrasodium salt dihydrate solution in double distilled water. Adjust the pH of this solution to 7.4. Sterilize by

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[2]The pH of the reaction buffer is a crucial parameter and should be checked regularly. Deviation of the pH can result in crystallization of the compounds in the reaction buffer, which in turn will affect the outcome of the assay (Table 2).

passing through a 0.22 μm filter and store for maximum 6 months at room temperature.

6. Phenylmethylsulfonyl fluoride solution. 100 mM phenylmethylsulfonyl fluoride in isopropanol. Protect this solution from light and store for maximum 2 weeks at −20°C.

7. Pepstatin A solution. 1.5 mM pepstatin A in dimethyl sulfoxide. Protect this solution from light and store for maximum 4 weeks at −20°C.

8. Aprotinin solution. 0.15 mM aprotinin in PBS. Protect this solution from light and store for maximum for 6 months at −20°C.

9. Leupeptin solution. 2.1 mM leupeptin in PBS. Protect this solution from light and store for maximum 4 weeks at −20°C.

10. Lysis buffer. 1 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DL-dithiothreitol, 20% *N*-[2-hydroxyethyl]piperazine-*N'*[2-ethanesulfonic acid] solution, 0.4% ethylenediaminetetraacetic acid tetrasodium salt dihydrate solution, 1% phenylmethylsulfonyl fluoride solution, 1% pepstatin A solution, 1% aprotinin solution, 2% leupeptin solution in double distilled water. Prepare this buffer *ex tempore* (*see* Note 3).

11. Ac-DEVD-AFC solution. 1 mg/mL Ac-DEVD-AFC in dimethyl sulfoxide. Protect this solution from light and store for maximum 6 months at −20°C.

12. AFC solution. 200 mM AFC in dimethyl sulfoxide. Protect this solution from light and store for maximum 2 weeks at room temperature.

13. Amber microcentrifuge tubes (VWR, Belgium).

14. Black 96-well plates (VWR, Belgium).

15. Multiplate reader.

2.3. Measurement of the extracellular release of lactate dehydrogenase in cultured primary hepatocytes

1. LDH reaction buffer (DiaSys, Germany). 4 volume parts of reagent 1 (*i.e.* NADH dissolved in 1 mM Good's buffer, pH 9.6) and 1 part of reagent 2 (*i.e.* pyruvate in 800 μM phosphate buffer, pH 7.5). Prepare this buffer *ex tempore* and place in a thermostated bath at 25°C before use.

2. Plastic microcuvettes.

3. Spectrophotometer, computer and software.

4. Ultrasonicator.

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[3]Dissolve the 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and the *DL*-dithiothreitol in 746 μL double distilled water when preparing a volume of 1 mL lysis buffer and subsequently add the different solutions.

3. Methods

3.1. Establishment of a monolayer culture of primary hepatocytes and induction of cell death

1.   Use freshly isolated primary rat hepatocytes (16).

2.   Evenly plate the hepatocytes on plastic culture dishes at a density of 0.56 $\times 10^5$ cells/cm^2 in hepatocyte seeding medium. Place the cell cultures in an incubator at 37°C and 5% CO_2 for 4 h.

3.   Remove the hepatocyte seeding medium and replace by identical volumes of hepatocyte culture medium. Place the cell cultures in an incubator at 37°C and 5% CO_2 for 24 h.

4.   Replace the hepatocyte culture medium. Place the cell cultures in an incubator at 37°C and 5% CO_2 for 20 h.

5.   Remove the hepatocyte culture medium and replace by identical volumes of hepatocyte cell death medium. Place the cell cultures in an incubator at 37°C and 5% CO_2.

6.   Sample at the start of cell death induction and 2, 4 and 6 h thereafter.

3.2. Measurement of caspase 3 activity in cultured primary hepatocytes

3.2.1. Sampling from cultured primary hepatocytes

1.   Remove the hepatocyte cell death medium from 10 cm diameter culture dishes and wash the cells twice with 10 mL PBS (*see* Note 4).

2.   Add 5.5 ml PBS to the culture dish and scrape the cells (*see* Note 5).

3.   Transfer the cell suspension to a 15 mL centrifuge tube on ice.

4.   Repeat steps 2 and 3.

5.   Centrifuge for 5 min at 4332*xg* and 4°C.

6.   Gently remove the supernatant and resuspend the pellet in 1 mL ice-cold PBS.

7.   Transfer the cell suspension to a 2 mL microcentrifuge tube on ice.

8.   Centrifuge for 5 min at 2040*xg* and 4°C.

9.   Remove the supernatant and directly merge the microcentrifuge tube into liquid nitrogen.

10.   Stock the microcentrifuge tubes, containing the dry pellets, at −80°C.

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[4]This washing step could remove dead cells and therefore may be omitted.
[5]The diameter of the culture dish used mainly depends on the purpose of the assay intended. For measurement of caspase 3 activity, it is strongly recommended to use 10 cm diameter culture dishes with hepatocytes plated at a density of 0.57×10^5 cells/cm^2 (*i.e.* a total of 4.4×10^6 hepatocytes). When using 6 cm diameter culture dishes (*i.e.* a total of 1.6×10^6 hepatocytes), harvesting of the cells and preparation of cell homogenates should be performed with 3 mL PBS and 50 μL lysis buffer, respectively.

3.2.2. Preparation of cell homogenates

1. Defrost the microcentrifuge tubes on ice.

2. Add 100 μL lysis buffer to the microcentrifuge tubes and resuspend by pipetting up and down (*see* Note 5).

3. Leave the microcentrifuge tubes on ice for 20 min.

4. Merge the microcentrifuge tubes in liquid nitrogen and subsequently thaw at 37°C in a thermostated bath for 2 min.

5. Repeat step 4 twice without mixing the microcentrifuge tube between the freeze-thaw cycles.

6. Centrifuge for 30 min at $16065 xg$ and 4°C.

7. Transfer 5 μL of the supernatant to another microcentrifuge tube, add 95 μL double distilled water and determine the protein concentration (*see* Note 6).

8. Transfer the remaining supernatant to a new microcentrifuge tube, merge into liquid nitrogen and store at −80°C.

3.2.3. Measurement of caspase 3 activity in the standards

1. Prepare the reaction buffer and the AFC solution.

2. Prepare a series of AFC standards (Table 1) in 2 mL amber microcentrifuge tubes protected from light.

3. Add 240 μL of each standard to a corresponding well in a black 96-well plate.

4. Repeat step 3 for the same standard.

5. Repeat steps 3 to 4 for the remaining standards.

6. Place the black 96-well plate in the multiplate reader and measure the fluorescence at 37°C with λ_{ex} 390 nm and λ_{em} 500 nm (*see* Note 7).

3.2.4. Measurement of caspase 3 activity in the samples

1. For each of the samples, calculate the volume that corresponds with 50 μg protein (x μL).

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[6]Among the different assays to assess protein concentration in biological samples, the Bradford method (17), is the most commonly used one. In this assay, which has been commercialized (Bio-Rad, Belgium), a protein sample is added to a solution of the dye Coomassie Brilliant Blue G-250 in phosphoric acid and ethanol. Under the acid conditions, the dye normally has a brownish colour, but upon binding to the protein, the blue form of the dye is produced. The optical absorbance of the solution is measured at a wavelength of 595 nm and is proportional to the amount of protein present and bound to the dye. It is a relative method, whereby bovine serum albumin is a typical standard used. Clearly, other assays, such as the bicinchoninic acid-based method (18) and the Lowry assay (19), can also be addressed to assess protein concentration.

[7]Temperature is a critical parameter in this assay, as it strongly influences protein stability and hence caspase 3 activity. Thus, it is of the utmost importance to ensure a constant temperature of 37°C in the cabinet of the multiplate reader before and during incubation of the black 96-well plate (Table 2).

2. Mix 230-x μL double distilled water with 10 μL 25× concentrated reaction buffer and × μL sample in a well of a black 96-well plate.

3. Repeat step 2 twice for the same sample.

4. Repeat steps 2 and 3 for the remaining samples.

5. In a well of the same black 96-well plate, mix 230 μL double distilled water with 10 μL 25× concentrated reaction buffer.

6. Repeat step 5 twice for the blank.

7. Incubate the black 96-well plate for 2 min at room temperature under mild shaking (*i.e.* 120 oscillations per minute).

8. Add 10 μL Ac-DEVD-AFC solution to each well using a dispenser pipette.

9. Slightly tap the black 96-well plate and place into the multiplate reader.

10. Place the black 96-well plate in the multiplate reader and perform 99 measurements of the fluorescence at 37°C with λ_{ex} 390 nm and λ_{em} 500 nm) over a total time frame of 60 min (*see* Note 7).

3.2.5. Processing of the results (Fig. 3) (*see* Note 8)

1. Collect the results of the measurement of the caspase 3 activity in the standards and calculate the mathematical equation of the best-fit line:

$$y = ax + b$$

2. Calculate the caspase 3 activity in the samples, expressed as nmol produced AFC/minute × μg protein, by completing the following equation:

$$[(\Delta F_{sample} - b) \times 250] / [a \times X \times P \times 60]$$

With parameters:

$F_{sample} = F_{sample} - F_{blank}$

b = intercept of the best-fit line

250 = total reaction volume (μL)/well

a = slope of best-fit line

X = volume (μL) sample added/well

P = protein concentration (μg/μL) of the sample

60 = reaction time (min)

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[8]From a statistical point of view, it is strongly recommended to perform the experiments on hepatocytes isolated from the livers of 3 different animals. Results can be processed and evaluated by 1-way analysis of variance followed by *post hoc* Bonferroni tests.

3.3. Measurement of the extracellular release of lactate dehydrogenase in cultured primary hepatocytes

3.3.1. Sampling from cultured primary hepatocytes

1. Take 500 μL cell culture medium from a 3.5 cm diameter culture dish and transfer into a recipient. Snapfreeze in liquid nitrogen and store at −20°C (*see* Note 9).

2. Scrape the cells in the remaining hepatocyte cell death medium using a policeman and transfer the cell suspension into a recipient. Snapfreeze in liquid nitrogen and store at −20°C.

3.3.2. Measurement of the lactate dehydrogenase activity

1. Defrost the microcentrifuge tubes on ice.

2. Homogenize the content of the cell culture medium and the cell suspension by ultrasonication with 20 pulses and 2×20 pulses with repeat duty cycle 0.5 s, respectively (*see* Note 10).

3. Mix 1 mL LDH reaction buffer with 20 μL homogenized cell suspension in a plastic microcuvette and place in the spectrophotometer (*see* Note 11).

4. After exactly 1 min, measure the decrease in absorption (ΔC/min) at 340 nm for a total of 1 min against an air blank (*see* Note 12).

5. Repeat steps 3 and 4 twice.

6. Repeat steps 3 to 5 for the corresponding homogenized cell culture medium.

3.3.3. Processing of the results (Fig. 4) (see Note 8)

1. Collect and average the 3 ΔC/min values for both the cell suspension (*i.e.* total LDH activity) and the cell culture medium (*i.e.* extracellular LDH activity).

2. Calculate the LDH index by assessing the relative extracellular release of LDH, using the following equation:

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[9]The diameter of the culture dish used mainly depends on the purpose of the assay intended. For measurement of the extracellular release of LDH, it is sufficient to use 3.5 cm diameter culture dishes, though culture dishes with a higher diameter can also be used.
[10]It is of the utmost importance to continuously keep samples on ice during ultrasonication, since this procedure produces heat, which may cause proteolysis and hence loss of enzyme activity.
[11]Temperature is a critical parameter in this assay, as it strongly influences enzyme activity. Therefore, it is important to continuously store the LDH reaction buffer at 25°C and to regularly check the temperature. The procedure may also be performed at 37°C, though in this case only 10 μL homogenized sample must be mixed with 1 mL LDH reaction buffer and a correction factor of 16030 must be handled during processing of the results (*see* Note 12).
[12]The assessment of the enzymatic activity relies on modifications in NADH concentration as a function of time (ΔC/min). Upon implementation of the Beer-Lambert law, this yields the equation $(\Delta A/min)/(\varepsilon \times l)$, whereby the parameters ΔA, ɛ and l represent the decrease in absorption, the molecular absorption coefficient ($cm^{-1}mol^{-1}l$) and the optical path (1 cm), respectively. When applied to LDH activity, this gives $[(\Delta A/min) \times 10^6]/(\varepsilon \times l)$. In this equation, LDH activity is expressed in units per liter, whereby 1 unit corresponds with the quantity of LDH that catalyzes the conversion of 1 μmol NADH/min at 25°C. This formula can be simplified by taken into account an established correction factor of 8095, as experimentally established by the manufacturer. Consequently, LDH activity can be calculated as $(\Delta A/min) \times 8095$. The LDH activity is automatically calculated by the computer software upon measurement of the samples (Table 3).

$$\text{LDH index} \, (\%) = [\,(\text{extracellular LDH activity}) \, / \, (\text{total LDH activity})\,] \times 100$$
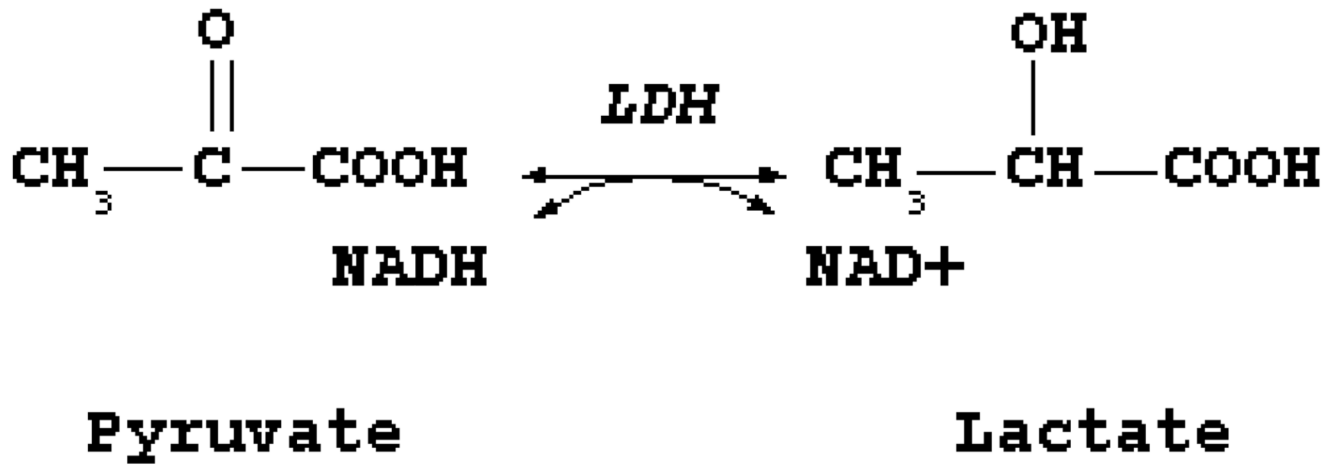
Acknowledgements

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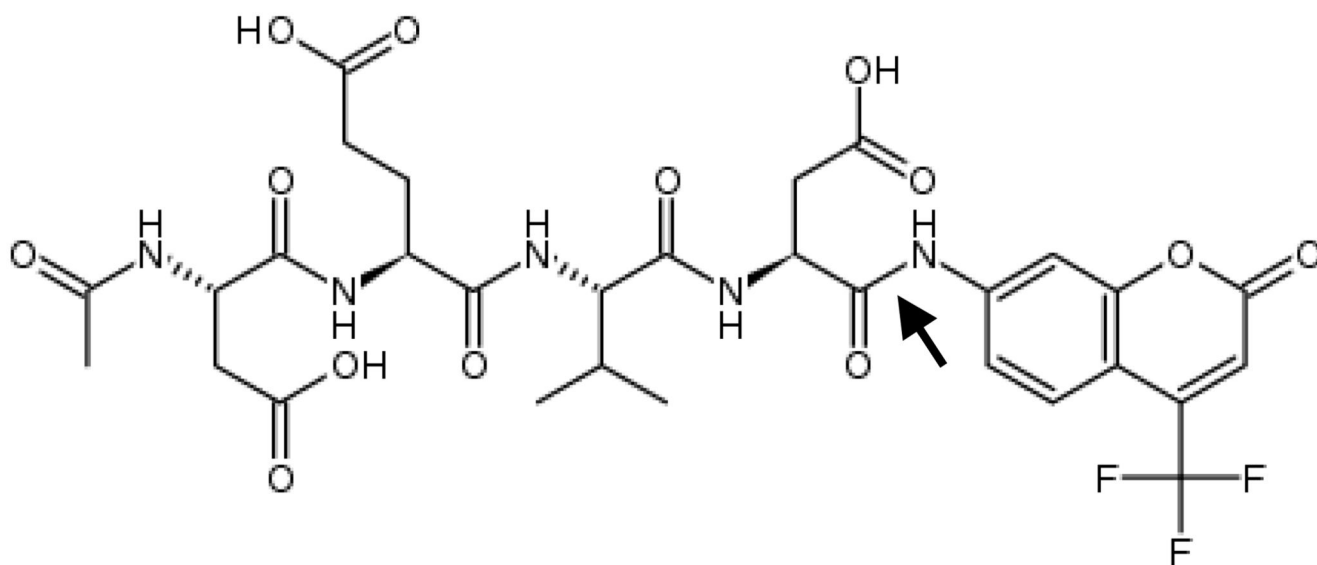
Figure 1. Chemical structure acetyl-aspartic acid-glutamic acid-valine-aspartic acid-7-aminotrifluoromethyl-coumarin (Ac-DEVD-AFC)
The arrow indicates the cleavage site for caspase 3.

$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-COOH \quad \underset{\underset{NADH}{\curvearrowright}}{\overset{LDH}{\rightleftharpoons}} \quad CH_3-\overset{\overset{\displaystyle OH}{|}}{CH}-COOH$$

NADH   NAD+

Pyruvate   **Lactate**

Figure 2. Catalytic function of lactate dehydrogenase (LDH)
LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of reduced and oxidized nicotinamide adenine dinucleotide.
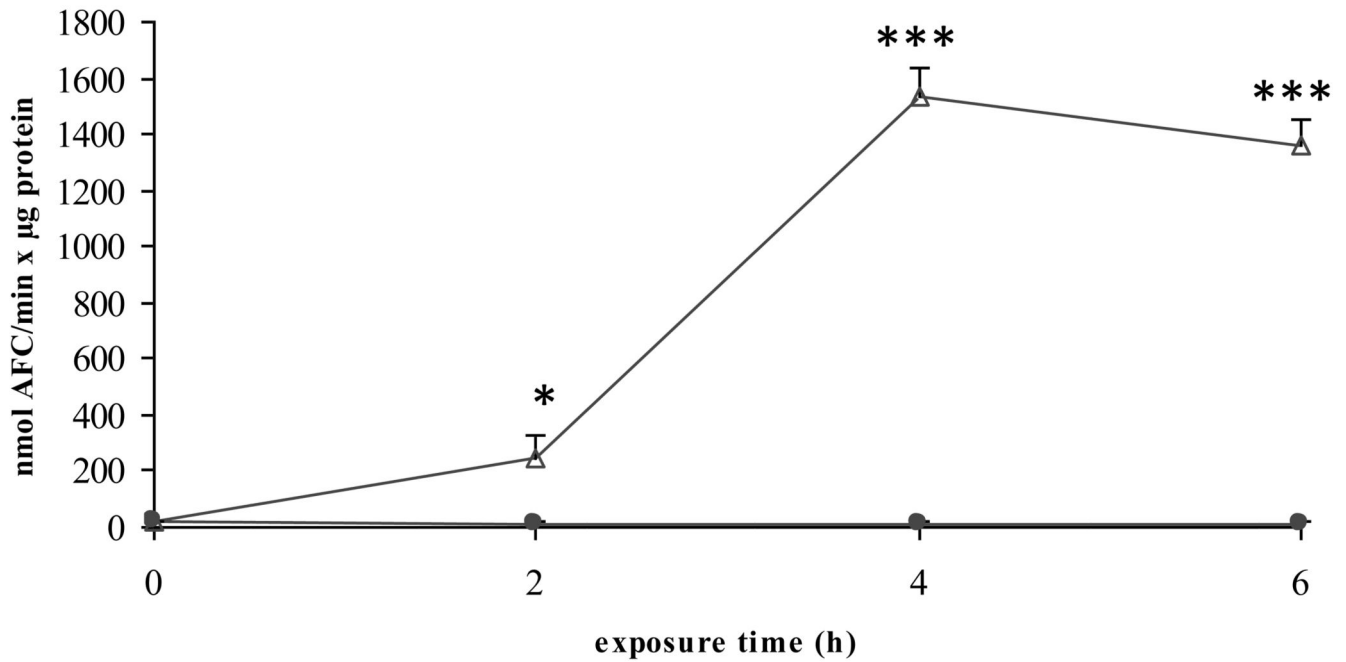
Figure 3. Measurement of caspase 3-like activity

Freshly isolated rat hepatocytes were cultivated in a monolayer configuration and were exposed to 200 ng/mL Fas ligand and 2 µg/mL cycloheximide, starting at 44 h postplating. Samples were taken at the start of the exposure (0 h), and after 2, 4 and 6 h. Caspase 3-like activity was determined and results are expressed as nmol aminotrifluoromethylcoumarin (AFC)/min × µg protein. Results represent the means ± standard deviation of 3 independent experiments. Results were evaluated by 1-way analysis of variance followed by *post hoc* Bonferroni tests. ● = control; = Fas ligand/cycloheximide; *$p < 0.05$; ***$p < 0.001$. Reproduced in modified form with permission from (13).
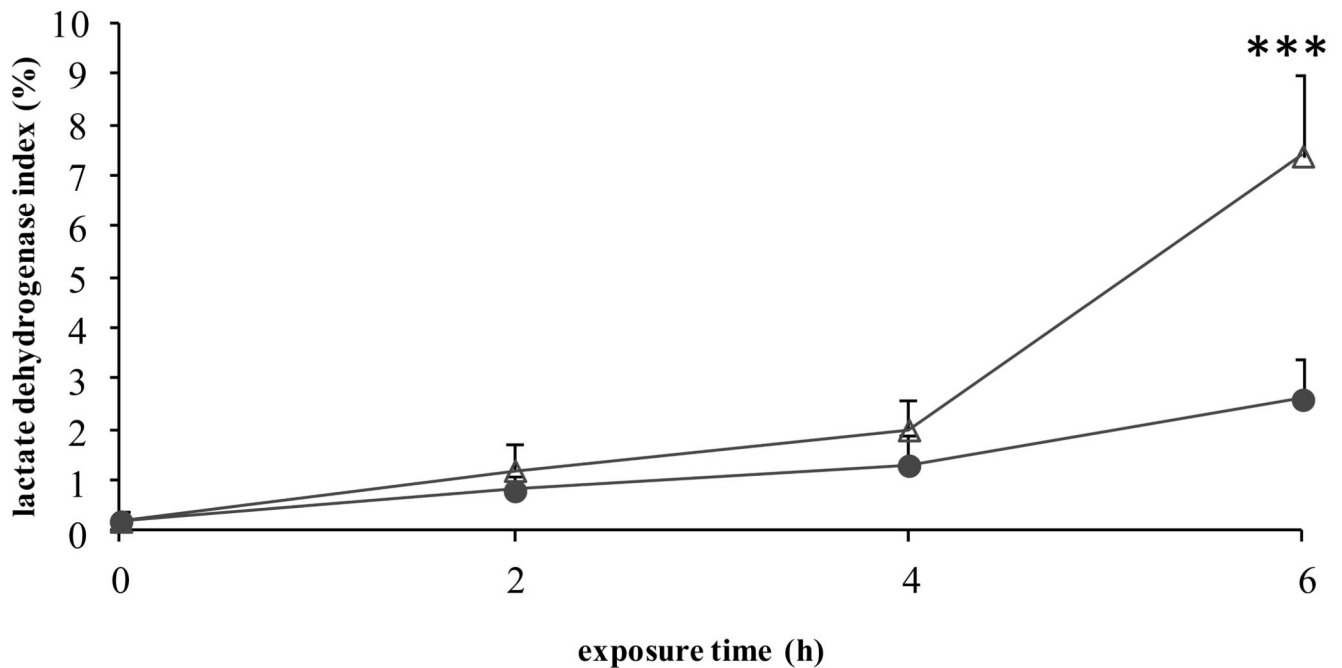
Figure 4. Measurement of lactate dehydrogenase leakage

Freshly isolated rat hepatocytes were cultivated in a monolayer configuration and were exposed to 200 ng/mL Fas ligand and 2 µg/mL cycloheximide, starting at 44 h postplating. Samples were taken at the start of the exposure (0 h), and after 2, 4 and 6 h. Lactate dehydrogenase activity was measured and the lactate dehydrogenase index was calculated as [(100 × lactate dehydrogenase activity in supernatant)/(lactate dehydrogenase activity in cell suspension)]. Results represent the means ± standard deviation of 3 independent experiments. Results were evaluated by 1-way analysis of variance followed by *post hoc* Bonferroni tests. ● = control; = Fas ligand/cycloheximide; ***$p < 0.001$. Reproduced in modified form with permission from (13).

Table 1

Preparation of the 7-amino-trifluoromethylcoumarin (AFC) standards

Standard number	Concentration AFC (nM)	Reaction buffer (µL)	Solution (µL)
0 (blank)	0	1000	0
1	1200	994	6 (AFC solution)
2	600	500	500 (standard 1)
3	300	500	500 (standard 2)
4	150	500	500 (standard 3)
5	75	500	500 (standard 4)
6	37.5	500	500 (standard 5)
7	18.75	500	500 (standard 6)
8	9.375	500	500 (standard 7)

Table 2

Troubleshooting of the caspase 3-like activity measurement procedure

Problem	Cause	Solution
Negative testing results	Decomposed reaction buffer and solutions	Prepare new reaction buffer and solutions and protect from light
	Air bubbles in the wells of the black 96-well plate	Tap the black 96-well plate before incubation in the multiplate reader
Divergent testing results	Incomplete lysis of the cells	Prepare new lysis buffer and strongly pipette up and down when lyzing cells
	Variation in temperature	Check temperature in the multiplate reader and ensure a constant temperature of 37°C
	Variation in pH and crystallization of the reaction buffer	Check the pH of the reaction buffer and adjust to 7.4

Table 3

Troubleshooting of the lactate dehydrogenase (LDH) assay

Problem	Cause	Solution
Negative testing results	Decomposed LDH reaction buffer	Prepare new LDH reaction buffer and store for a maximum of 5 days or 8 h at 4°C or 25°C, respectively
	Air bubbles in the microcuvette	Shake the microcuvette upon mixing the LDH reaction buffer and the sample
Divergent testing results	Incomplete homogenization of samples	Subject samples to an additional series of 20 ultrasonication pulses
	Variation in temperature	Check temperature and store the LDH reaction buffer at 25°C