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Role of apoptotic protease-activating factors (factors: 1, 2, 3) in L-arginine-induced programmed cell death of hepatocytes

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Summary

The aim of the study was an immunhistochemical evaluation of the expression of Apaf 1, Apaf 2 and Apaf 3 (apoptotic protease activating factors) in L-arginine, a substrate of nitric oxide (NO) inducing apoptosis of rats' hepatocytes.

The rats used in this experiment were divided into 2 equal groups. Experimental rats received per os L-arginine 40 mg/kg body weight every other day for 2 weeks and were decapitated after 3 weeks of the experiment. Control rats received per os 2 ml of distilled water every other day for 2 weeks and were decapitated after 3 weeks of the experiment.

Specimens of the liver taken after decapitation were examined immunohistochemically, using the standard three-step method to detect the immunolocalization of Apaf 1, Apaf 2, Apaf 3. The results of immunohistochemical examinations were subjected to qualitative evaluation based on the intensity of a colour reaction at the antigen-antibody site in the rat liver examined in individual groups. The quantitative evaluation was conducted with the Analysis-pro software. The surface area of cells with a positive colour reaction (+) of antigen-antibody was calculated.

The results of the study show that L-arginine, as a donor of exogenous nitric oxide, did not have an apoptotic effect leading by the intrinsic pathway, through the formation of the apoptosome and the activation of caspase 9 in the hepatocytes of rats.

Keywords: nitric oxide, apoptosis, Apaf 1, 2, 3, liver

The term "programmed cell death" is used in reference to physiological cell death during development, whereas cell death caused by external factors is described as "apoptosis" (10). In the present study we examined L-arginine-induced apoptosis in the hepatocytes of rats.

L-arginine is exogenous nitric oxide (NO). In an organism L-arginine is changed into NO. Endogenous NO is produced by enodthelial cells (11), macrophages (6), nerve endings (5), neutrophils, monocytes, mastocytes, and blood platelets (14), as well as by hepatocytes (7), examined in the present study. Nitric oxide (NO) is a modulator of many processes in human and animal organisms. Its positive and negative influence depends on the dose and the kind of cells.

Apoptosis consists of 3 phases: decisive phase, executive phase and degradation phase (3). The main elements of the executive phase are cysteine proteases from the interleukin-1-beta-converting enzyme (ICE) family called the caspase family (9). Caspases digest proteins behind asparaginian residue using one of their cysteine residues (15) – hence their name: cysteine--dependent asparaginian specific proteases. To date, 14 caspases have been discovered and described (8). Scientists distinguish initiating, effector and proinflammatory caspases.

Initiating caspases are activated in protein complexes. In response to the binding of death receptors (extrinsic pathway) or signals from the cell interior (intrinsic pathway), initiating caspases are activated as a result of dimerization in DISC (extrinsic pathway) and in the apoptosome consisting of Apaf 1, 2, and 3 (intrinsic pathway) (14, 18).

Apaf-1, apoptotic protease activating factor 1 occurs in every cell of an organism. Its highest concentration is observed in cells of highly apoptotic organs (the spleen, brain and lungs of a foetus) (15). Apaf-1 needs the ATP-derived energy to be able to act.

In intact mitochondria Apaf-2/cytochrome C (apoptotic protease-activating factor 2) is involved in the respiratory chain and ATP synthesis. Apaf-3/caspase 9/ICE-LAP6/Mch6 (apoptotic protease activating factor 3/ICE-like apoptotic protease 6/Mammalian CED-3 homologue 6) is activated thanks to apoptosome activation.

The present study examined L-arginine-induced apoptosis in the hepatocytes of rats. The process of the formation of a protein complex, the apoptosome, and of its product, active caspase 9, was analyzed.

Material and methods

The 16 rats (Wistar female) used in present study were divided into 2 equal groups. The animals received L-arginine at a dose of 40 mg/kg body weight (Argininum, Curtis Healthcare, Poznań, Poland) (experimental group) or 2 ml of distilled water (control group); every other day for 2 weeks and were decapitated after 3 weeks of the experiment.

The study was approved by the First Local Ethics Committee of the Medical University in Lublin.

After decapitation the hepatic specimens were collected and fixed in 10% formalin, dehydrated in an alcohol series and embedded in paraffin blocks. The blocks were cut into 5 µm sections, which were placed on silanized glasses. Two specimens from the liver collected from each animal were used. Next, paraffin was removed in xylene and in a graded alcohol series. The specimens were subjected to thermal preparation in an acid medium (10 mM citrate buffer pH 6.0). Next, endogenous peroxidase was blocked by incubation in H_2O_2. After that the specimens were incubated with rabbit primary antibody caspase 9 (Lab Vision RB-1205--PO) in 1% TBS/BSA, and with rabbit primary antibody Apaf 1 (Lab Vision RB-9263-P0) in 1% TBS/BSA. Then the DakoCytomation kit was used for immunohistochemical reactions, which included biotinylated secondary antibody, streptavidin conjugated with horse-radish peroxidase and AEC substrate. After chromatogen staining, the specimens were placed in haematoxylin solution and rehydrated. Photographic documentation was prepared using a computer--guided Colour Video Camera CCD-IRIS (Sony).

The results of immunohistochemical examinations were subjected to qualitative evaluation and quantitative evalua-tion, using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany). Microscopic images, magnification ×125, were analysed to assess protein expression in 3 randomly chosen areas, 781193.35 µm^2 each. The surface area of cells with positive reaction (+) was calculated.

The results were presented as means and the standard deviation of the mean using MANOVA, ANOVA, post hoc Scheffe tests. An error risk of 5% and statistical significance at $p \leq 0.05$ were accepted.

Results and discussion

The immunohistochemical reaction for caspase 9 and protein Apaf 1 in all groups subjected to qualitative evaluation was focal (fig. 1, 2).

The quantitative evaluation of the immunohistochemical reaction showed a statistically insignificantly decreased caspase 9 and Apaf 1 reaction in the experimental group compared to the control one (tab. 1).

Previous studies demonstrated that the influence of exogenous nitric oxide (NO) on tissues depends on

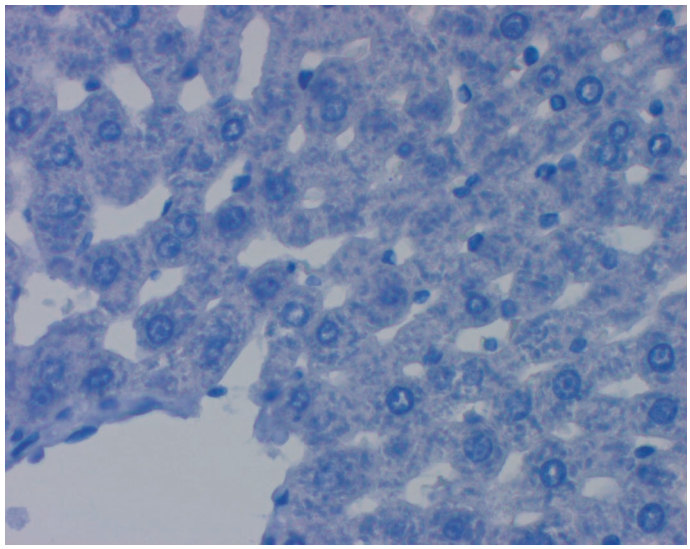

Fig. 1. Experimental group. The liver section of the L-ARG – treated rat showing caspase 9 reaction of low intensity. AEC + H staining. Magnification about × 280

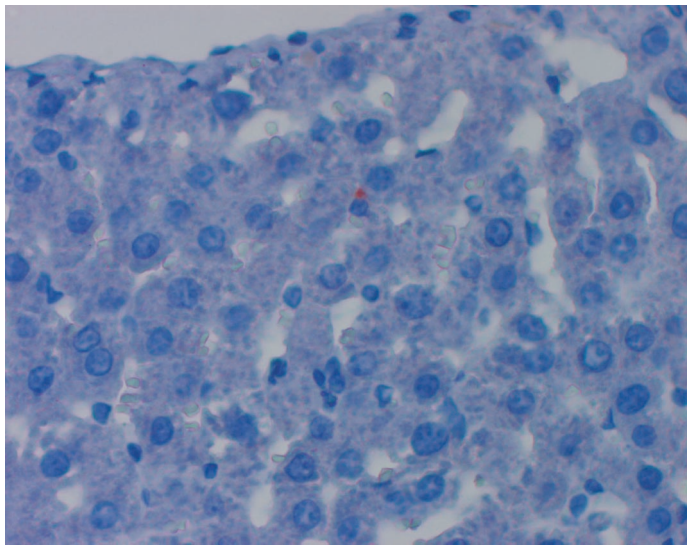

Fig. 2. Experimental group. The liver section of the L-ARG – treated rat. The rat liver section showing Apaf 1 reaction of low intensity. AEC + H staining. Magnification about × 280

Tab. 1. Mean area occupied by caspase 9 and Apaf-1 reaction in the rat liver in control and experimental groups (μm²) (n = 8; $\bar{x}$ ± SD)

Caspases	Control group	Experimental group
Caspase 9	1863.61 ± 118.69	1577.36 ± 299.35[a]
Apaf 1	2277.06 ± 431.13	1820.74 ± 376.90[b]

Explanations: a, b – means with different superscript letters differ significantly at p ≤ 0,005

the kind of cells and the dose of L-arginine. L-arginine, as a donor of exogenous nitric oxide, induced the apoptotic signal in normal renal tubular cells of rats and did not affect hepatocytes. In the rats' hepatocytes, a low expression of effector caspase 3 was observed (13). This caspase (besides caspases 6 and 7) is involved in or initiates the destruction of cellular DNA, which leads to cell destruction. Caspases are relevant elements of the executive, irreversible phase of apoptosis (9).

Another study analyzed the intrinsic, mitochondrial pathway in the L-arginine-induced apoptosis of rats' hepatocytes (12). We detected immunohistochemically the location and expression of Bax and Bcl-2 proteins from the Bcl-2 family of proteins. This experiment showed that L-arginine, as a donor of exogenous nitric oxide, did not induce the mitochondrial way of apoptosis by a direct or indirect damage of hepatocytes' mitochondria and the activation of proapoptotic Bax protein. Proteins from the Bcl-2 family are involved in the decisive phase of apoptosis. They regulate apoptosis by affecting essential cell processes.

Bcl-2 family proteins are located on the internal mitochondrial membrane. One can distinguish antiapoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Brag) and proapoptotic proteins (Bax, Bcl-xS, Bak, Bik, Bad, Bin). The anti/proapoptotic protein ratio determines the death or survival of a cell (4). Proapoptotic Bax protein, among others, regulates the outflow of proapoptotic factors from the intermembranous space of mitochondria (cytochrome C, apoptosis-inducing factor (AIF) (16).

Mitochondria-derived cytochrome C is one of the components of a protein complex called the apoptosome. This is the activation mechanism of caspase 9, an important initiator caspase in apoptosis (17). Cytochrome C is known as apoptotic protease-activating factor 2 (Apaf 2). This factor needs apoptotic protease-activating factor 1(Apaf-1) to activate procaspase 9 (1). Active caspase 9, which is known as apoptotic protease-activating factor 3, activates caspases 3, 6, 7 (2).

The present and previous studies showed that L-arginine administered to rats in a dose of 40 mg/kg body weight every other day for 2 weeks does not induce the apoptosis of liver hepatocytes by the intrinsic mitochondrial pathway. The authors did not observe increasing expression of the components of the apoptosome (Apaf 1), the proapoptotic member of the Bcl-2 family group Bax, and the initiating caspase 9. Besides mitochondria, the intrinsic pathway leads through endoplasmic reticulum. Apart from the intrinsic pathway, apoptosis is induced via the extrinsic pathway. The analysis of the reticular and extrinsic pathways of apoptosis in rats' hepatocytes after L-arginine treatment will be the subject of the authors' next studies.

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