IMMUNOHISTOCHEMICAL EVALUATION OF CASPASE 3 EXPRESSION IN RATS’ HEPATOCYTES AFTER L-ARGININE THERAPY

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Abstract

The apoptotical effect of nitric oxide on effector apoptotical caspase 3 in rats’ hepatocytes was examined. The experiment was performed on 16 white Wistar female rats divided into two equal groups. The rats from the experimental group received orally L-arginine in a dose of 40 mg/kg b.w. every other day for 2 weeks. The rats from the control group received orally 2 ml of distilled water in the same manner as the experimental group. All the rats were decapitated after 3 weeks of the experiment. After decapitation, specimens from the liver were collected, fixed in 10% formalin, and then embedded in paraffin blocks. Protein caspase 3 on slides was detected using the standard three-step immunohistochemical method. The quantitative evaluation of caspase 3 expression showed that the area occupied by positive caspase 3 reaction in the liver of the experimental group (128.11 µm² ± 96.54) was comparable to that in the control group (212.18 µm² ± 116.59) (P=0.25). The dose of L-arginine used was similar to that applied in pregnant women treated for gestosis. The study shows that L-arginine as a donor of exogenous nitric oxide has no an apoptotic effect on rats’ hepatocytes.

Key words: liver, apoptosis, L-arginine, caspase 3, nitric oxide.

Nitric oxide (NO) is a wide-range modulator of many processes occurring in human and animal organisms. It was discovered by Priestley (the discoverer of another eight gases, including oxygen) in 1770 (16, 19, 20, 21). In the organism, NO is produced from L-arginine with the help of nitric oxide synthase (NOS) (10). Excessive endogenous NO is involved in the pathogenesis of many diseases, e.g. atherosclerosis, degenerative diseases of the nervous system, inflammations, autoimmune diseases, and cancers. Deficient endogenous NO is implicated in the development of hypertension, pre-eclampsia, arteriosclerosis, and hypercholesterolaemia (18). Exogenous NO is administered to patients with venous atherosclerosis of the lower limbs, coronary disease, and pregnancy-induced hypertension.

The effects of NO on cell death are not fully known. It was demonstrated that the proapoptotic influence of NO was caused by oxidative stress induced in the cell. The so-called “nitrosative stress” was described in the rat’s macrophages, where NO-induced apoptosis was observed (8).

In this experiment, the apoptotical effect of NO on the effector apoptotical caspase–3 in rats’ hepatocytes was investigated.

Material and Methods

The experiment was performed on 16 white Wistar female rats with a baseline body weight of 200-250 g and ages of 3.5-4 months, divided randomly into two equal groups: control and experimental. The animals received standard feed and water ad libitum.

The rats from the experimental group received L-arginine (Argininum, Curtis Healthcare, Poland) through the stomach tube every other day in the amount of 40 mg/kg b.w. (5 mg of L-arginine in 1 ml of distilled water) for 2 weeks. The rats in control group received 2 ml of distilled water in the same manner as the experimental group. All the rats were decapitated after 3 weeks of the experiment.

Two hepatic specimens from each animal collected for immunohistochemical examinations were fixed in 10% formalin, dehydrated in alcohol series, cleared in xylene, and embedded in paraffin blocks. The blocks were cut into 5 µm-sections, which were placed on the silanised glasses. After the removal of paraffin in xylene and hydration in graded alcohol series, the sections were subjected to thermal preparation in the acid medium for antibodies against caspase 3 protein. After cooling for 20 min, the preparations were rinsed with distilled water and placed in the tris buffered saline
Next, endogenous peroxidase was blocked by incubation in 0.3% H2O2 solution (99 ml of TBS, 0.1g of NaN3, 1 ml of 30% H2O2) for 20 min. The sections were rinsed in TBS for about 10 min and incubated with rabbit primary antibody at room temperature for 60 min. (Lab Vision Ab-4-RB-1197-PO) in 1% TBS/BSA diluted 1:100. Then the DakoCytomation kit was used for immunohistochemical reactions, which included biotinylated secondary antibody against rabbit antibodies (Biotinylated Link Universal) (15 min incubation); streptavidin conjugated with horse-radish peroxidase (Streptavidin-HRP) (15 min incubation) and AEC substrate - HRP reaction dye (AEC substrate chromogen) (15 minutes of incubation). After the use of each reagent the specimens were rinsed in TBS for 10 min. After chromatogen staining the specimens were placed in haematoxylin solution for about 1 min and thoroughly rinsed with distilled water. The specimens were covered with coverslip using Aquatex fluid. For each section its negative control without primary antibody was prepared.

The results of immunohistochemical examinations were subjected to qualitative evaluation, taking into account the intensity of colour reaction at the antigen-antibody site. The quantitative evaluation was analysed using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany). The microscopic images, magnified 125x were analysed assessing the protein expression in 3 randomly-chosen areas, 781,193.35µm² each. The surface area of cells with a positive reaction (+) was calculated.

The results were presented as means and standard deviation of the mean using the One Way ANOVA test. 5% error risk and statistical significance at P≤0.05 were accepted. The statistical analysis involved the differences in mean area with positive immunohistochemical reaction for antibody examined in the individual groups.

The photographic documentation was prepared using the computer-guided Colour Video Camera CCD-IRIS (Sony).

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

Results

Qualitative evaluation showed focal caspase 3 reaction in the slides prepared from control and experimental animals (Figs 1 and 2). The colour reaction was bright, pale pink, and filled evenly the whole hepatocyte cytoplasm. Medium-intensive reaction was found in the L-ARG group (Fig. 2). The intensivity of the positive reaction was almost the same in the control and experimental groups.

Quantitative evaluation showed the area occupied by increased positive caspase 3 reaction in the L-ARG group compared to the control group (Table 2). However, there was no statistical significance (P=0.25).

![Fig. 1. Control group. Slightly increased caspase 3 reaction in the rat liver. AEC+H staining. About 280x](image1)

![Fig. 2. Experimental group. The liver section of the L-ARG-treated rat showing caspase 3 reaction of low intensity. AEC+H staining. About 140x](image2)

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<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Experimental</th>
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<tr>
<td>Caspase 3</td>
<td>212.18µm²</td>
<td>128.11µm²</td>
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<tr>
<td>± 116.59</td>
<td>± 96.54</td>
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Table 1

Mean area occupied by caspase 3 reaction in the rat liver in all groups. Statistical significance of differences. One Way ANOVA test

Discussion

The process of apoptosis is divided into three phases: decisive, executive, and degradation (4). The executive phase is irreversible. Its relevant elements are cysteine proteases from the interleukin -1-β-converting enzyme (ICE) family called the caspase family (12). These enzymes are present in the cell in their inactive form (proenzymes, zymogenes, pro-caspases) and are activated during apoptosis. They activate one another and other enzymes (6). Their action results in damage to protein cytoskeleton, topoisomerases, kinases, nuclear
membrane, and poly ADP-ribose polymerase (PARD— responsible for DNA repair) proteins, and the damage to nuclear enzymes, DNA fragmentation between individual nucleosomes.

Caspases are also involved in the activation of transglutaminase and kinases responsible for biochemical modifications in cellular membranes; thanks to that, the apoptotic body has a dense membrane, which prevents the outflow of the content and is simultaneously recognised by the adjacent cells and is phagocytosed by them (9).

To date, 14 caspases have been described and described (11). They were divided into initiating, effector, and proinflammatory caspases. The effector caspases are involved in/or initiate the destruction of cellular DNA, which leads to cell destruction (3, 6, 7). Caspase 3/CPP32/Yama/apopain (cysteinyl aspartic acid-protease-3) activates CAD endonuclease (A caspase-activated DNase in mice and rats) (7, 15), inactivates DNA-repairing enzymes (5), and cleaves the cytoskeleton proteins (actin, spectrin, lamine) (13). It is capable of inducing cell death by itself (1).

In the present study the effects of exogenous NO on the hepatocytes were examined by administering L-arginine as a substrate of NO (2, 3). Nitric oxide is one of the most effective “free radical scavengers”. It is estimated that it is about 10,000-100,000 times more effective than vitamin E. It reacts with peroxide radicals, forming harmless compounds. However, its too high concentrations make it harmful. It inhibits glutathione peroxidase so it cannot neutralise peroxides, converting them into alcohols. NO also combines with O₂ and converts into pernitrate, which initiates the process of lipid peroxidation.

Such a connection was noted by Kronon et al. (14), who described the so-called nitric oxide paradox, i.e. small doses of L-arginine, a precursor of NO (L-arginine solution in the concentration of 4 mmol/L), exerted protective effects on the myocardium of newborns by reducing oxygen-free radicals while its high doses (10 mmol/L) caused an increase in the number of oxygen free radicals, which resulted in damage to vessels and myocardium.

In the present study, the dose of L-arginine was similar to that used in pregnant women treated for gestosis. This dose should be safe for a mother and a foetus (the so-called dose scavenging free radicals) (17). The study showed, that L-arginine as a donor of exogenous NO did not induce an apoptotic effect in rats’ hepatocytes.

References