ASSESSMENT OF L-ARGININE AS AN EFFECT OF EXOGENOUS NITRIC OXIDE (NO) ON EXPRESSION OF MARKERS OF CELLULAR STRESS IN RATS’ HEPATOCYTES

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Received: October 25, 2011   Accepted: January 24, 2012

Abstract

The aim of this study was immunohistochemical evaluation of heat shock protein (Hsp70) and p-53 proteins in the L-arginine–induced cellular stress in hepatocytes of rats. Sixteen white Wistar female rats were divided into two equal groups. The rats from the experimental group received per os 40 mg/kg b.w. of L-arginine every day for 2 weeks and were decapitated after 3 weeks of the experiment. The rats from the control group received in the same manner 2 ml of distilled water and 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. Proteins Hsp70 and p-53 were detected on slides using the standard three step immunohistochemical method. The quantitative evaluation of Hsp70 and p-53 expression showed that the area of positive staining in the liver of the experimental rats (Hsp70 305,763.00 µm² +/-58,289.66, p-53 9,551.42 µm² +/-1,078.86) was comparable to that in the control groups (Hsp70 291,636.80 µm² +/-34,492.31, p-53 14,104.67 µm² +/-3,571.35). Our experiment showed, that L-arginine as a precursor of exogenous nitric oxide given to rats in dose similar to that used in pregnant women treated for hypertension did not exhibit an influence on hepatocytes.

Key words: rat, liver, L-arginine, HSP70, p-53, immunohistochemistry.

Nitric oxide (NO) is one of the most effective “free radical scavengers”. It reacts with peroxide radicals forming harmless compounds. However, too high concentrations of this free radical make it harmful. NO combines with O₂ and converts into pernitrate, which initiates the process of lipid peroxidation. Kronon et al. (11) showed a relation described as the nitric oxide paradox. Small doses of L-arginine, a precursor of NO, had a protective effect on tissues examined in their experiment. However, high doses caused an increase in the number of oxygen free radicals.

Free radicals are atoms, molecules, or ions with unpaired electrons. Many studies dealt with oxygen free radicals, which are also physiologically produced in the organism (2). Moreover, some free radicals originating from the outside, and not produced physiologically in the organism are dangerous for the organism (2). One of them is exogenous NO. It has proapoptotic influence on cells acting via oxidative stress (nitrosative stress) (6).

Heat shock protein 70 (Hsp70) is a cellular biomarker of every environmental stress. Oxygen stress and nitrosative stress belong to this group (19). p-53 is a protein, which plays a crucial role in coordination of the cell response to various factors, such as hypoxia, DNA damage, heat shock, or oxygen stress (10, 18). During the action of harmful factors, the concentration of Hsp70 and p-53 increases.

The presented study aimed at immunolocalisation of these proteins in the cytoplasm of hepatocytes of rats after L-arginine administration.

Material and Methods

The experiment was performed on 16 white Wistar female rats with a baseline body weight of 200-250 g and age of 3.5-4 months. The animals received standard feed and water ad libitum. The rats were randomly divided into two equal groups: control and
The rats from the experimental group received 40 mg/kg b.w. of L-arginine (Curtis Healthcare, Poland) through a stomach tube every day for 2 weeks. The rats in control group received in the same manner 2 ml of distilled water. All rats were decapitated after 3 weeks of the experiment.

The hepatic specimens 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. Then the sections were subjected to thermal preparation in the acid medium for Hsp70 antibody and base medium for p-53 protein antibody. After cooling, the preparations were rinsed with distilled water and placed in tris-buffered saline (TBS). Next, endogenous peroxidase was blocked by incubation in 0.3% H2O2 solution (99 ml of TBS, 0.1g NaN3, 1 ml of 30% H2O2). The specimens were incubated with rabbit primary Hsp70 antibody (Lab Vision Ab-3, RB-080-A0) and mouse primary p-53 anybody (Lab Vision P53 Ab-1;MS-104-PO) at room temperature in 1% TBS/BSA, 1/100 dilution (Hsp70) or 1/50 (p-53). Then the DakoCytomation kit was used for immunohistochemical reactions, which included: biotinylated secondary antibody against rabbit antibodies (Biotinylated Link Universal); streptavidin conjugated with horse-radish peroxidase (Streptavidin-HRP), and AEC substrate - HRP reaction dye (AEC substrate chromagen). After chromatogen staining, the specimens were placed in the haematoxylin solution and thoroughly rinsed with distilled water. The specimens were covered with coverslips using the Aquatex fluid.

The photographic documentation was prepared using the computer-guided Colour Video Camera CCD-IRIS(Sony). The results of immunohistochemical examinations were subjected to qualitative evaluation taking into account the intensity of colour reaction at the antigen-antibody site in the liver in particular groups.

The quantitative evaluation was performed using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany). The microscopic images, magnified 125x, were analysed assessing the protein expression in three randomly chosen areas, 781,193.35 µm², each. The surface area of cells with positive reaction (+) was calculated. The results were presented as means and standard deviation of the mean using the ONE WAY ANOVA test. Statistical significance at P≤0.05 was accepted.

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

### Results

The colour reaction in the liver at the site of Hsp70 antibody binding was focal in all examined groups and filled almost the whole area of the hepatocyte cytoplasm. Staining was bright pink and evenly distributed (Figs 3, 4). Quantitative evaluation showed similar Hsp70 reaction in the L-arginine-treated and control groups (Table 1).

The positive p-53 reaction was observed in both groups (Figs 1, 2). The staining was bright pink, and finely granular. It filled the whole cytoplasm of the cells. Groups of cells showed various intensity of the reaction, expressed as differences in the colour intensity. Quantitative evaluation showed that the area of the p-53-positive staining was comparable in control and L-arginine-treated groups (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>Experimental</th>
<th>ONE WAY ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-53</td>
<td>14,104.67 ± 3,571.35 µm²</td>
<td>9,551.42 ± 1,078.86 µm²</td>
<td>P=0.07</td>
</tr>
<tr>
<td>HSP70</td>
<td>291,636.80 ± 34,492.31 µm²</td>
<td>305,763.00 ± 58,289.66 µm²</td>
<td>P=0.73</td>
</tr>
</tbody>
</table>

**Fig. 1.** Control group. The liver section showing p-53 reaction of low intensity. AEC+H staining. About 140x.

**Fig. 2.** Experimental group. The liver section showing p-53 reaction of low intensity. AEC+H staining. About 280x.
Fig. 3. Control group. Quite high intensity of Hsp70 reaction in the rat liver. AEC+H staining. About 280x.

Fig. 4. Experimental group. Quite high intensity of Hsp70 reaction in the rat liver. AEC+H staining. About 140x.

Discussion

In presented study L-arginine was used as a substrate of exogenous NO (1, 4). In the organism, the endotheliocytes immediately regenerate L-arginine from L-citrulline after formation of a portion of NO (15), so the endogenous NO is useless to examine the effects of NO on the life processes in cells. The increase in cGMP in blood after L-arginine administration is the proof of synthesis of exogenous NO (8).

A lot of donors of exogenous NO were used in the studies on the effects of NO on various tissues and organs in experimental animals and humans (5, 9, 14). Huguenin et al. (9) used nonsteroidal anti-inflammatory drugs - donors of nitric oxide (NO-NSAID). This group contains: NO-inbutrofen, NO-aspirin (14), and nitrosulindac (9). Chae et al. (3) used sodium nitroprusside. In our study we used L-argininie as a substrate of nitric oxide.

Excessive concentration of NO, as well as its deficiency in the organism are the reasons of many diseases, such as atherosclerosis, inflammations, cancers, hypertension, or hypercholesterolaemia (16). Exogenous NO is used to treat patients with venous atherosclerosis of the lower limbs, coronary disease, and pregnancy-induced hypertension.

The L-arginine in the presented study was administered to rats in similar dose to that used in pregnant women treated for gestosis (15). This dose should be safe for mother’s and a foetus’s organs.

Hsp70 is a sensitive biomarker of cell stress caused by a lot of toxins. Farzaneh et al. (7) noticed an increase in expression of Hsp70 after menadione, CdCl$_2$ and NaAsO$_2$ administration. Other scientists working on a Drosophila model, reported that Hsp70 could be used as a sensitive biomarker in evaluation of the risk of exposure to environmental pollution (12). Hsp70 is also a known factor of endogenous adaptation of cells to stress in many tissues (17).

Our experiment showed, that L-arginine as a precursor of exogenous NO, administered to rats in dose similar to that used in pregnant women treated for hypertension, did not exert an influence on hepatocytes.

No significant differences were noted between the experimental and control groups in terms of the positive immunohistochemical reaction for both analysed proteins involved in the process of cellular stress. The results are consistent with our previous examinations, showing that L-arginine as a donor of exogenous NO did not have an apoptotic effect on hepatocytes in the rats (13).

References

inhibits proliferation and induces apoptosis in human prostatic epithelial cell lines. Prostate 2004, 61, 132-141.


