IMMUNOHISTOCHEMICAL ASSESSMENT OF THE EFFECTS OF L-ARGININE AS A NITRIC OXIDE (NO) SUBSTRATE ON CASPASE 3 EXPRESSION IN RATS’ RENAL TUBULAR CELLS

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Abstract

The study was performed on 16 albino Wistar female rats divided into two equal groups: experimental and control. The rats from the experimental group received per os, every second day for 2 weeks 40 mg/kg b.w. of L-arginine. The rats from the control group received, in the same manner, 2 ml of distilled water. The animals were decapitated after 3 weeks of the experiment. After decapitation specimens from the kidneys were collected, fixed in 10% formalin, and then embedded in paraffin blocks. Protein caspase 3 was detected using the standard three step immunohistochemical method. Additionally, the apoptotic index was evaluated. The study shows that L-arginine, as a donor of exogenous nitric oxide, induced the apoptotic signal in normal renal tubular cells of the rats. The apoptotic index statistically significantly increased in the epithelial cells of the treated renal tubules compared to the control. The immunohistochemical reaction for the executing caspase 3 in the renal tubular cells, although increased in comparison with the control, was statistically insignificant.

Key words: rats, kidneys, apoptosis, L-arginine, caspase 3, immunohistochemistry.

In 1977, Murand (13) examining the muscular membrane of the vascular wall noticed that vasodilators such as nitroglycerin or sodium nitroprusside act through nitric oxide, which is their active metabolite. In 1980, Furchgott (6) named this active factor requiring the involvement of the endothelium– the endothelium-derived relaxing factor (EDRF). It was the nitric oxide.

Nitric oxide (NO) induces apoptosis via free radicals and oxidative stress caused in the cell. NO is also known to have anti-apoptotic effects. During the recent years, an increasing number of reports have been published concerning exogenous and endogenous NO, as well as its favourable action in many diseases. On the other hand, there are opinions that NO exerts numerous adverse effects. Exogenous NO is an easily available medicine. However, its influence on cell apoptosis has not been fully known.

In previous studies we examined rat’s hepatocytes after L-arginine therapy (14). The study showed that L-arginine as a donor of exogenous NO did not have an apoptotic effect on the hepatocytes.

In the presented study an influence of L-arginine on the expression of the apoptotic effector: caspase 3 in renal tubular epithelial cells was examined.

Material and Methods

The studied material consisted of 16 albino Wistar female rats with baseline body weight 200-250 g, aged 2.5-3 months. They were housed in cages (four rats per cage) with a natural light/dark cycle and received a standard feed and water ad libitum. The rats were divided into two equal groups: experimental and control. The rats from the experimental group received L-arginine through the stomach tube (Argininum, Curtis Healthcare, Poland) every second 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 of the control group received 2 ml of distilled water through the stomach tube every second day for 2 weeks. The rats were decapitated after 3 weeks of the experiment. The experimental protocols received the permission from the
Ethics committee for animal experimentation of the Medical University of Lublin.

The rat’s kidneys collected for immunohistochemical studies were fixed in 10% formalin buffered in phosphate buffer, pH 7.0, and after dehydration in ascending ethanol concentrations, and clearing in xylene, they were embedded in paraffin. Microtome sections were cut at 5 µm, and then adhered to the siliconised slides.

The protein expression level was evaluated with a standard three-step immunohistochemical procedure LSAB using DakoCytomation kits according to the manufacturer’s instructions. Rabbit caspase 3 (diluted 1:100, Lab Vision) antibodies were used as a primary antibody. Then a biotinylated secondary antibody was added, followed by horseradish peroxidase conjugated with streptavidin (DakoCytomation, Denmark). At the sites of immunolocalisation of the primary antibodies, a reddish colour appeared after adding a chromogen – AEC (DakoCytomation, Denmark). The colour reaction occurred because of the streptavidin’s strong affinity to biotin. For each preparation, a negative control (a slide without primary antibody) was used.

The expression of all proteins was evaluated in preparations from all rats from both groups (two preparations from every individual for each of the antibodies; for each antibody there were 16 control slides and 16 experimental slides). The slides were analysed using a light microscope. The photographic documentation was completed with a CCD-IRIS Colour Video Camera (Sony) connected with a computer.

The analysis of the microscopic images was performed at a magnification of 125x using the computer programme analySIS®Pro 3.0 (Soft Imaging System, Germany). From each slide, three randomly selected standard microscope fields of 781,193.35 µm² were assessed. The field of the sectioned surface of the kidney specimens with positive reactions was measured. The range of colours assessed by the computer as positive was set as: intensive red, red-pink, or pink, which was not assumed as a positive result.

The degree of apoptosis in the specimens was determined quantitatively using the apoptotic index (AI). Three specimens from each individual were examined. The nuclei stained dark with haematoxylin with reduced perimeter and diameter were accepted as the pyknotic ones, i.e. being one of possible manifestations of apoptosis. In the specimens magnified about 1,000x (linear areas: 985x, square spaces: about 1 mlm²), the number of pyknotic nuclei was counted in 100 cells of kidney specimens from experimental group, and compared with the respective control group. The projection microscope (MP3 PZO, Poland) was used. The examinations included only epithelial cells of renal tubules.

The results were analysed statistically using an ANOVA test and Student’s t-test. The averages, standard deviations, and the percentage of positive reactions in the examined tissue field were determined. The differences were considered statistically significant when P<0.05.

Results

The positive caspase 3 reaction was intensive in the experimental and control groups. It was focal and concerned the cytoplasm of the tubular epithelial cells. Characteristic location of the immunohistochemical reaction in the experimental specimens concerned these places in the tubular cells where the cytoplasm indented to the tubular lumen during the formation of apoptotic cells (“boiled” cells).

Quantitative evaluation showed an increased caspase 3 reaction in experimental group compared to control (Table 1), but this increase was statistically insignificant (P=0.07).

Evaluation of apoptotic index showed that the number of apoptotic cells statistically significantly increased in the experimental group in comparison with the control (P=0.00013) (Table 2).

| Table 1 |
| Area covered by caspase 3 reaction in the renal tubular cells |
| Control group | Experimental group | ONE WAY ANOVA |
| 541.9 µm² | 707.3 µm² | P=0.07 |
| +/-123.0 | +/-182.2 |

| Table 2 |
| Mean number of apoptotic cells in rat kidneys |
| Control group | Experimental group | ONE WAY ANOVA |
| Apoptotic index | 4.70 | 9.60 | P=0.00013 |
| +/-2.30 | +/-2.59 |

Discussion

Caspase 3 (cysteinyl aspartic acid-protease-3) is one of the effector caspases in executing phase of apoptosis. It is involved in, or initiates the destruction of cellular DNA, which leads to cell destruction. Caspase 3 activates CAD endonuclease (11), inactivates DNA-repairing enzymes (4), and cleaves the cytoskeleton proteins (9). It is capable of inducing cell death by itself (1).

It was demonstrated that L-arginine administered exogenously was converted into NO (7). Various exogenous precursors of NO were used in the studies on the effects of NO on various tissues and organs in experimental animals and humans (5, 8, 16), including non-steroidal anti-inflammatory drugs (NSAID) (16), so called donors of NO (NO-NSAID) (8), NO-ibuprofen (NCX 2111), NO-aspirin (NCX 4060) (16), and nitrosulindac (NCX 1102) (8). Sodium nitroprusside (SNP) (3) and S-nitroso-N-acethylpenicillamine (SNAP) (2, 12) belong also to other precursors of exogenous NO. According to Kwon et al.
SNAP was converted into NO already 24 h after its administration.

L-arginine is also a commonly applied precursor of NO (15). In the presented 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).

It was demonstrated that L-arginine, as a donor of exogenous NO, induced the apoptotic signal in normal renal tubular cells of the rats. The apoptotic index increased statistically significantly in the epithelial cells of renal tubules of the experimental group in comparison to the control.

The immunohistochemical reaction for the executing caspase 3 in the renal tubular cells, although increased in comparison with the control, was statistically insignificant. It is likely that other executing caspases, which were not examined in the present study, were activated (e.g. caspase 6). It will be a subject of the next study.

References