Initiation of Apoptosis in Hepatocytes During Prolonged Arterial Hypotension and Postresuscitation Period

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An increase in activity of Ca^{2+},Mg^{2+}-dependent endonucleases on the second hour of hypotension coincided with the presence of DNA fragments in agarose gel. A correlation was established between the duration of hypotension, Ca^{2+},Mg^{2+}-dependent endonuclease activity, and intensity of nuclear DNA fragmentation. Apoptosis of hepatocytes is triggered during ischemia and develops during reperfusion.

Key Words: *apoptosis; DNA fragmentation; Ca^{2+},Mg^{2+}-dependent endonucleases; hepatocyte nuclei; arterial hypotension*

Apoptosis is one of the mechanisms of cell death [9]. Biochemical marker of this process in a cell is activation of Ca^{2+}, Mg^{2+}-dependent endonucleases associated with chromatin DNA fragmentation [13]. Apoptosis activation is observed in ischemic damage to cells [2,5,10]. It is still not clear when this process is triggered: during hypoxia or reperfusion.

Our purpose was to investigate the time course of Ca^{2+}, Mg^{2+}-dependent endonuclease activities in cell nuclei of dog liver at different stages of ischemia and during postresuscitation period, including the early postreperfusion period.

MATERIALS AND METHODS

Experiments were carried out with 39 mongrel dogs of both sexes weighing 10-26 kg. After Promedol (8 mg/kg) and Nembutal (10-15 mg/kg) anesthesia and heparin treatment (500 U/kg), blood was let from the femoral artery so as to decrease arterial pressure to 40 mm Hg and maintain it at this level by fractional intra-arterial infusion of blood for 2, 4, and 6 h. The animals were resuscitated by intra-arterial infusion (during the first 5 min) of a mixture of blood with polyglucin (40 and 60% of blood loss volume) and heparin in microdoses. During the first hour of resuscitation, 20 ml/kg mixture of blood with plasma substitutes (blood-rheopolyglucin-lactasol in equal volumes) was infused intravenously.

For biochemical studies, the animals were sacrificed by electric current at 127 V under Nembutal narcosis after 2, 4, and 6 h of arterial hypotension and 1 h and 1-3 months after resuscitation. Control group consisted of anesthetized animals without massive blood loss. In all experiments, tissue for investigation was cut from the left lobe of the liver and placed on ice.

Cell nuclei were isolated by the detergent method. Tissue was put in cooled buffer A containing 50 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 10 mM $CaCl_2$, and 5 mM 2-mercaptoethanol. All procedures were carried out at 4°C. Tissue was crushed in a Teflon-glass homogenizer in buffer A.

The homogenate was filtered through 4 layers of gauze and centrifuged at 1500*g* for 10 min. The nuclei sediment was twice washed in buffer B consisting of 0.5% Triton X-100 in buffer A. For DNA measurements, cell nuclei were suspended in water,

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EDTA was added to a concentration of 20 mM and sodium dodecyl sulfate (SDS) to 1%, and light absorbance was measured at 260 nm (one optic unit corresponding to a DNA concentration of 40 μg/ml).

Nuclear extract DNA was precipitated by adding 0.5 volume 18% polyethylene glycol-6000 in lyzing buffer. The precipitate was separated by 15-min centrifugation at 10,000*g*. The extract was dialyzed for 12 h against 10 mM Tris-HCl (pH 8.0), 0.1 M EDTA, 0.5 M dithiotreitol, 50% glycerol, and stored at -20°C. For estimating endonuclease activity, plasmid DNA of strain rIS 19 [4] was used. Activities of Ca^{2+}, Mg^{2+}-dependent endonucleases were measured at 37°C in 20-μl incubation samples containing 2 μg DNA, 10 mM Tris-HCl, pH 7.4, 25 μg/ml bovine serum albumin fraction V, 0.5 mM dithiotreitol, 1 mM EDTA, nuclear extract, 2 mM $CaCl_2$, and 5 mM $MgCl_2$.

The samples were incubated for 30 min at 37°C. The reaction was stopped by cooling, and 5 μl mixture of 1% SDS, 0.2 M EDTA, pH 8.0, 0.25% bromophenol blue, and 50% glycerol was added. Electrophoresis was performed in 0.8% agarose with electrode buffer containing 0.04 M Tris-acetate (pH 8.3), 0.02 M Na acetate, 2 mM EDTA at 10 V/cm for 1 h. Gel was developed in ethidium bromide (0.5 μg/ml). The amount of endonuclease required for cleaving 1 μg DNA (50% DNA sample) for 30 min at 37°C was the unit of endonuclease activity. Protein was measured as described elsewhere [12].

To analyze the time course of hepatocyte chromatin DNA cleavage by Ca^{2+}, Mg^{2+}-dependent endonuclease, liver tissue homogenate was lyzed by adding 10% SDS to a concentration of 1% and 5.2 M NaCl to 1 M. DNA was extracted with an equal volume of a 24:1 chloroform — isoamyl ethanol mixture. Water phase was then depleted of proteins and precipitated by a double volume of 95% ethanol. DNA precipitate was dried at room temperature and dissolved in a buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, a 400-fold proteinase K solution in water was added to a final concentration of 40 μg/ml, and incubated for 2 h at 37°C. DNA was deproteinized with chloroformisoamyl alcohol mixture, precipitated with ethanol, and dissolved in buffer. DNA preparations were treated with RNAse A and TI (100 and 10 μg/ml, respectively). After 60-min incubation at 37°C, DNA was deproteinized and precipitated with 95% ethanol.

Dry precipitate was dissolved in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.1 M EDTA, and electrophoresed in 2% agarose blocks in a buffer containing 40 mM Trisacetate, pH 8.3, 2 mM EDTA, and 0.5 μg/ml ethidium bromide at 15 mA for 15 h. UV-exposed (254 nm) gels were photographed through an orange filter, and negatives were scanned in a Chromoscan-200 densitometer.

RESULTS

A direct relationship was revealed between the duration of arterial hypotension and the level of Ca^{2+}, Mg^{2+}-dependent endonucleases. The activity of the studied enzymes in hepatocyte nuclei by the second hour of hypotension was 3300±177 U/ml protein *vs.* 2636±187 U/ml protein in control ($p<0.05$). By the 4th hour of arterial hypotension, the activity of Ca^{2+}, Mg^{2+}-dependent endonucleases was 4966±385 U/ml protein ($p<0.001$), and by the 6th h it increased by 118% in comparison with control, being 5754±335 U/ml protein ($p<0.001$).

The causes and mechanisms underlying increased activity of DNA endonucleases are discussed. Activation of Ca^{2+}, Mg^{2+}-dependent endonucleases is believed to be associated with elevated intracellular Ca^{2+}. It was suggested that there is a high-molecular precursor of endoDNAses in a cell [1]. The quantity and activity of DNA-endonucleases formed from it may depend on the activity of high-molecular, apparently, Ca^{2+}-dependent proteases.

In addition to the the biochemical marker of apoptosis, its morphological marker — DNA disintegration yielding fragments of nucleosomal length — is observed in liver nuclei as early as by the second

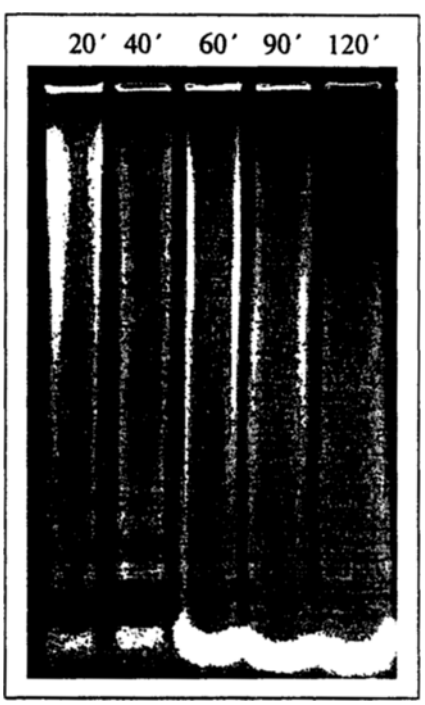

Fig. 1. Electrophoresis of autohydrolysis products of control dog hepatocyte nuclear chromatin DNA (2% agarose, ethidium bromide staining) in the presence of 5 mM $MgCl_2$ and 2 mM $CaCl_2$.

Fig. 2. Electrophoresis of autohydrolysis products of dog hepatocyte nuclear chromatin DNA (2% agarose, ethidium bromide staining) after 2-h hypovolemic hypotension in the presence of 5 mM $MgCl_2$ and 2 mM $CaCl_2$.

hour of hypotension. Electrophoresis of chromatin DNA isolated from hepatocyte nuclei of dogs exposed to 2-h arterial hypotension produced a band in the intact DNA zone in gel and up to 5 intact nucleosome bands. Interestingly, the intensity of

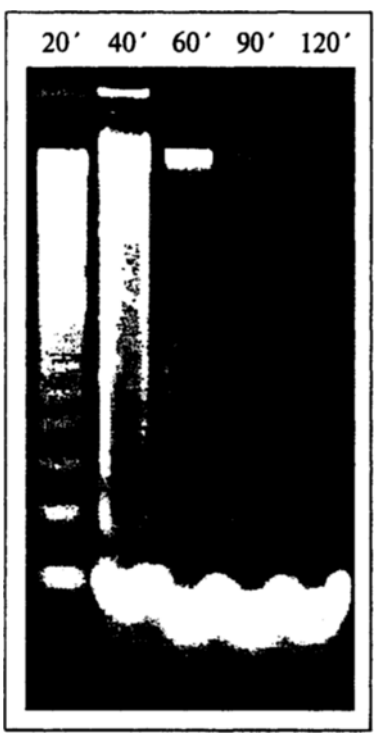

Fig. 3. Electrophoresis of autohydrolysis products of dog hepatocyte nuclear chromatin DNA (2% agarose, ethidium bromide staining) after 6-h hypovolemic hypotension in the presence of 5 mM $MgCl_2$ and 2 mM $CaCl_2$.

DNA fragmentation changes with duration of arterial hypotension. By the sixth hour of hypotension (Figs. 1-3), up to 7 nucleosome strips are detected in gel after a 20-min incubation.

Based on the increase in the activities of Ca^{2+}, Mg^{2+}-dependent endonucleases and on the time course of formation of nuclear DNA fragments, we have concluded that apoptosis in dog hepatocyte nuclei is triggered during the period of liver ischemia by the second hour of hypotension.

There is another viewpoint. DNA fragmentation characteristic of apoptosis was not observed in rabbit myocardial ischemia [7]. It was concluded that apoptosis is triggered during the reperfusion period. Such results can be explained by extremely low initial activity of Ca^{2+}, Mg^{2+}-dependent endonucleases in the myocardium [6]. Moreover, the time course of the activity of the most sensitive marker of apoptosis — Ca^{2+}, Mg^{2+}-dependent endonucleases was not presented [7]. Therefore, a specific pattern of chromatin DNA fragmentation, observed by the authors during reperfusion, is not proven. That is why we tried several approaches to solve the problem of apoptosis initiation: in addition to the biochemical marker of apoptosis, we observed the time course of DNA fragmentation in hepatocyte nuclei.

Our data are in line with and supplement previous reports demonstrating ruptures of chromatin DNA in rat cell nuclei in hypovolemic shock [11]. High activity of Ca^{2+}, Mg^{2+}-dependent endonucleases demonstrated in our experiments can explain the mechanism of formation of these ruptures.

It was demonstrated in a model of rabbit myocardial ischemia that Ca^{2+}, Mg^{2+}-dependent endonucleases are activated early, by the 40th min of ischemia, indicating the triggering of apoptosis mechanisms [1].

Our study showed that during the early (1 h) postresuscitation period, the activity of Ca^{2+}, Mg^{2+}-dependent endonucleases in dog hepatocyte nuclei had no evident trend to decrease and was 4702±245 U/mg protein, which is 78% higher than normal ($p<0.001$). Activation of DNA-endonucleases during the early postresuscitation period did not differ much from that at the end of the 4th hour of hypovolemic hypotension. Agarose gel electrophoresis of nuclear DNA 1 h after resuscitation revealed up to 5 nucleosome bands, in addition to a strip in the intact DNA zone. On the one hand, damage to hepatocyte nuclear chromatin DNA during reperfusion can be caused by generation of free radicals, whose main source is xanthine oxidase [8], and on the other, by decreased activity of antiradical and antiperoxide defense enzymes SOD, catalase, and glutathion peroxidase [3].

It is important that activation of Ca^{2+}, Mg^{2+}-dependent endonucleases in hepatocyte nuclei during the postresuscitation period is stable, indicating a destructive process in the liver. Two or three months after resuscitation, the activity of Ca^{2+}, Mg^{2+}-dependent endonucleases in hepatocyte nuclei is 5087± 509 U/mg protein *vs.* 2636±187 U/mg protein in the control ($p<0.001$). Our findings indicate that a remote postresuscitation period is characterized by lack of relationship between intensity of liver chromatin DNA fragmentation and duration of incubation. The latter fact implies developing changes in physicochemical characteristics of hepatocyte nuclear chromatin during this period.

Thus, a comprehensive approach to the investigation of the mechanisms of hepatocyte genome damage shows that apoptosis is triggered during ischemia and develops during reperfusion of the liver.

REFERENCES

1. S. S. Aleksandrova and A. G. Basnak'yan, *Vopr. Med. Khim.*, No. 4, 2-7 (1995).
2. V. L. Kozhura, Zh. V. Solov'eva, I. S. Novoderzhkina, and N. V. Nosova, *Anest. Reanimatol.*, No. 3, 24-28 (1994).
3. V. D. Konvai, *Purine Metabolism Disorders in the Liver during Postresuscitation Period and Its Prevention* [in Russian], Author's Synopsis of Doct. Med. Sci. Dissertation, Tomsk (1988).
4. T. Maniatis, E. Frich, and J. Sambrook, *Gene Engineering Methods. Molecular Cloning* [in Russian], Moscow (1984).
5. N. V. Nosova, *Changed Activity of Ca^{2+}, Mg^{2+}-Dependent Endonucleases in Dog Hepatocyte Nuclei in Prolonged Hypovolemic Hypotension and during the Postresuscitation Period* [in Russian], Author's Synopsis of Cand. Biol. Sci. Dissertation, Moscow (1992).
6. C. Giannakis, I. J. Forbes, and P. D. Zalewski, *Biochem. Biophys. Res. Commun.*, **181**, No. 2, 915-920 (1991).
7. R. A. Gottlieb, K. O. Burleson, R. A. Kloner, *et al.*, *J. Clin. Invest.*, **94**, No. 4, 1621-1628 (1994).
8. K. Ishii, S. Suita, and H. Sumimoto, *Res. Exp. Med.* (Berlin), **190**, 389-399 (1990).
9. J. F. R. Kerr, A. H. Wyllie, and A. R. Currie, *Brit. J. Cancer*, **26**, 239-257 (1972).
10. J. F. R. Kerr, C. M. Winterford, and B. V. Harmin, *Cancer*, **73**, No. 8, 2013-2026 (1994).
11. H. M. Lazarus and A. Hopfenbeck, *Surgery*, **85**, 297-302 (1979).
12. J. J. Sedmark and E. E. Crossberg, *Anal. Biochem.*, **79**, 344-352 (1977).
13. B. C. White, D. De Cracia, G. Krause, *et al.*, *Free Radic. Biol. Med.*, **10**, No. 2, 125-135 (1991).