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Atorvastatin and ethyl alcohol as the inductors of apoptosis in cardiomyocytes in rats

Atorwastatyna i alkohol etylowy jako induktory apoptozy w komórkach mięśnia sercowego u szczurów

INTRODUCTION

The incidence on cardiovascular diseases is the present civilization epidemic. This illness together with neoplastic diseases is the most frequent reason for hospitalization and inability to work. In the developed societies, it is the cause of about 50% of all deaths.

Atherosclerosis is the primary reason of a large majority of circulatory system diseases. It is characterized by the accumulation of lipids and fibrous elements in the large vessels. The conducted for years epidemiological studies have defined about 300 risk factors associated with atherosclerosis, which can be divided into environmental and genetic ones. The classic environmental factors are: high-fat diet, smoking, lack of exercises, and abuse of alcohol. Genetic component factors include: elevated levels of total serum cholesterol, elevated levels of low-density lipoprotein cholesterol (LDL-C), and reduced levels of high-density lipoprotein cholesterol (HDL-C). Moreover, it seems that higher risk of cardiovascular diseases concerns people with lower education level, and with the worst material condition [9,12,13].

This huge problem is a big challenge for health services. Health promotion is a significant element besides prevention, diagnostics, treatment, rehabilitation and protection. To estimate the health situation rightly, cultural, socio-economic aspects and all risk factors associated with atherosclerosis [12] should be taken into account. Most often SCORE algorithm is used to estimate these risk factors. SCORE algorithm is based on 5 main factors like: age, sex, smoking, systolic

pressure, and the level of total serum cholesterol. This model can define the risk of death associated with cardiovascular reasons in the next 10 years [9]. So the main direction of prophylactic activities should be focused on changing patients' life style. It should include the change of diet, giving up smoking, the increased physical activity, and also beginning the pharmacological treatment [12]. During the primary prevention 3 drugs are recommended to use: aspirin, statins, and angiotensin converting enzyme inhibitors (ACEI) [9].

Statins are included in group of drugs which give good results during cardiovascular diseases treatment. The effects are well documented. In the seventies of the XX century, first statin, Mevastatin was isolated from the mold Penicillium citrinum. Statins can be grouped into natural substances e.g. Lovastatin, Simvastatin, Pravastatin, and those that are synthetic like Fluvastatin and the investigated Atorvastatin. In this group the drugs dissoluble in water are the following: Pravastatin, and in lipids e.g. Atorvastatin. The discovery of these medicines made the progress in the multifactorial primary and secondary prevention settings in the coronary heart disease and strokes possible [2,6,16].

Statins are active inhibitors of 3-hydroxy-3-methyloglutaryl coenzyme A (HMG-CoA) reductase. There are hipolipemic drugs which decrease the endogenic cholesterol synthesis. As the result of this action the levels of total cholesterol, LDL cholesterol, and triglycerides are decreased, moreover HDL cholesterol levels get increased. Endogenic cholesterol comprises more than half of all cholesterol pool in human organism. HMG-CoA reductase catalyzes the conversion of 3-hydroxy-3-methyloglutaryl coenzyme A into mevalonic acid. This acid controls the rate of cholesterol synthesis in liver and other tissues. The reduced total cholesterol levels lead to a compensatory increase in the expression of high-affinity LDL receptors on hepatocyte membranes. This in turn results in an increased catabolism of LDL and its precursors in order to cover demand of cholesterol with is needed to cell membranes, vitamin D, and steroid hormones synthesis [5-7,10,16].

Inhibition of HMG-CoA reductase, leads to decrease in cholesterol synthesis and also to decrease in concentration of intermediary metabolites of this pathway. Among these metabolites we can distinguish isoprenyl radicals, which are responsible for post-transcription processing of proteins, i..e. prenylation. Slowness of proteins prenylation generates the pleiotropic effect of statins [2,6]. The most important pleiotropic effects in this group of drugs are: inhibition of lipids oxidation, and thrombus formation, slowing of inflammation processes, improvement of endothelin nitrogen oxide (NO) synthesis, inhibition of monocytes and leucocytes aggregation, suppression of endothelin and cytokines synthesis, and induction of apoptosis [7,11,16].

For the sake of this, the main effectiveness of statins during ischemic heart disease treatment is founded on the improvement of endothelial function, the slowing of inflammation processes and on vasodilatation action. Besides, statins stimulate angiogenesis, decrease blood clotting, and they influence an autonomic nervous system [6].

Statins, without Pravastatin, are metabolized in the liver by the cytochrome P-450 3A4 (CYP3A4) isoenzymes. Isoenzyme CYP3A4 metabolizes about 60% of all drugs, so it is necessary to monitor the undesirable effects, and drug-drug interactions [1,6,10].

Statins are relatively safe. The most serious undesirable effect is myopathy which is characterized by varying degree of complications, from tiredness to rabdomiolisis in extreme case. During the therapy, elevated concentration of creatine kinase (CK) in serum was observed in patients. This action can base on losing stability of myocytes membranes which is connected with reduced levels of cholesterol. Readers must be reminded that cholesterol is one of principal component

in bilaminar cytomembrane. Numerous investigations confirm that the greater dose of drug used during therapy, leads to increased probability of developing symptoms in muscular system. After stopping administration of statins, the concentration of creatine kinase was normal. Whereas the used drugs can increase concentration of transaminases in serum, and this is similar situation like in myopathy. The effect depends on dose, and after stopping therapy, regression of symptoms is observed. Another but less serious undesirable effects are: suffering of digestive system (nausea, bellyaches, diarrheas, and constipations), headaches, depressions, erythematous dermatitis, and sleep disorders. Statins are relatively safe for liver and kidneys, the recommended dose of Atorvastatin is small and its 10mg. Readers must be reminded that it is not necessary to give an additional dose of drug in patients with abnormal kidney activity and also in patients who are dialyzed [6,7,16]. The risk of undesirable effects is bigger during Lovastatin, Simvastatin, or Atorvastatin administration than during Pravastatin administration. This is probably connected with drug metabolism, because only Pravastatin is not decomposed by liver isoenzymes CYP3A4, which participates in biotransformation of other drugs used in clinical practice today [6].

The great number of advantages connected with statins therapeutic potential, made the authors undertake estimating of the expression of Fas receptor in rats' cardiac muscular tissue after Atorvastatin and ethanol administration. Initiation of apoptosis is one of multidirectional actions in this group of drugs. The examined Fas receptor which belongs to superfamilies of tumor necrosis factor (TNF) is a marker of the Extrinsic Pathway of apoptosis. Epidemiological studies have revealed that about 80% of men and 65% of women drink alcohol, so it is advisable to investigate the possible interactions between examined substances during therapy [7, 15].

MATERIAL AND METHODS

The experiment was carried on 30 female Wistar rats. The rats' body mass was 250-300g. The animals were placed into: 2 control groups (K and KA) and 4 experimental groups. Each group consisted of 5 animals. All rats were given standard granulated fodder and drinking water ad libitum. Moreover independently from groups they were drinking 20% aqueous solution of ethanol alcohol. Atorvastatin was administered by successive 4 week periods, in 1ml water suspension, once by day, by stomach tube. The medicines were given in 2 different doses: 1.14 mg/kg of body mass, this is maximal therapeutic dose using in humans and 11.4 mg/kg of body mass, tenfold greater dose than maximal therapeutic dose using in humans (Table 1).

Table 1. Comparison of examined groups

Examined groups					
Control groups		**Experimental groups**			
K	**KA**	**1**	**2**	**3**	**4**
Water (6 weeks)	Ethyl alcohol (6 weeks)	Atorvastatin 11,4mg/kg of body mass + ethyl alcohol (4 weeks)	Atorvastatin 11,4mg/kg of body mass + water (4 weeks)	Atorvastatin 1,14mg/kg of body mass + ethyl alcohol (4 weeks)	Atorvastatin 1,14mg/kg of body mass + water (4 weeks)

During the experiment the rats were kept in cages, in the same conditions. Relative air humidity was 60%, environmental temperature 21˚C, and the 24 hour cycle was kept (12h day, 12h night). The animals from all experimental groups were decapitated 24 hours following the last dose of the drug, the heart samples were taken for histological examination. The specimens were fixed in Baker's solution, then they were dehydrated in alcohol with increasing concentration, then placed in xylene and rinsed in 10% buffered formalin. Paraffin blocks were cut on the rotational microtome Leica RM 2135, in 5µm sections which were put onto slides and were stained with hematoxylin and eosin (H+E). The slides were closed with Canada balsam. Histological specimens were viewed and photographs were made in the light microscope produced by the Nikon Company.

Next immunohistochemistry examinations were made in order to estimate the expression of the Extrinsic Pathway apoptosis marker. The obtained material was incubated in thermostat in temperature 58˚C during 24h. In order to uncover antigen, the heart samples were heated in microwave in 0.01M citrate buffor pH 6.0. Next the slides were stippled by 0.03% H_2O_2 to block endogenic peroxidase. In the next step the slides were incubated by primary antibody FAS (C-20): Sc-715 (Santa Cruz Biotechnology Inc.) in dilution 1:1000. To detect primary antibody DAKO Envision+System/ HRP kit with catalogue number 4011 was used. The Ethical Committee of the Medical University of Lublin gave consent to this experiment.

RESULTS

H + E staining. On the samples, H+E stained from the control group, the heart parenchyma structure was correct. The striations were visible. The cells nuclei are basophilic and they were stained violet, the cytoplasm was stained by eosin in pink colour. We analyzed heart samples from the rats, which were given Atorvastatin in dose 11.4mg/kg of body mass/24h and ethanol (experimental group 1), the blood vessels were widened and filled by erythrocytes. It responded to local congestion. The cells nuclei were elongated and with low chromatin in comparison to the control group. On the samples from experimental group 3 (Atorvastatin 1.14 mg/kg of body mass/24h and ethyl alcohol), the tissue structure was more compacted in comparison to the control group, but morphological changes seemed to be smaller in comparison to the experimental group 1.

Immunohistochemistry results. Very weak expression of Fas receptor was observed in the single cardiomyocytes on the slides from control group K (Fig. 1). Similar results were visible on the specimens from experimental groups 2 and 4. The strongest expression of examined receptor was observed on the heart samples from experimental groups 1 (Fig. 2.) and 3 (Fig. 3) which were given Atorvastatin and ethanol (Table 2).

Table 2. The intensity of Fas receptor expression in examined groups

Examined groups					
Control groups		Experimental groups			
K	KA	1	2	3	4
+	++	++	+	++	+

+,++ the intensity of reaction

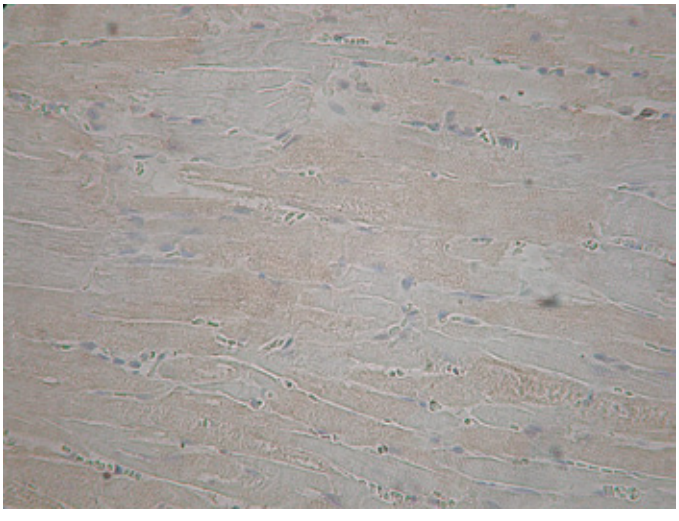

Fig. 1. Control group. Expression of Fas receptor in the single rat's cardiomyocytes.
Magn. ca. x400

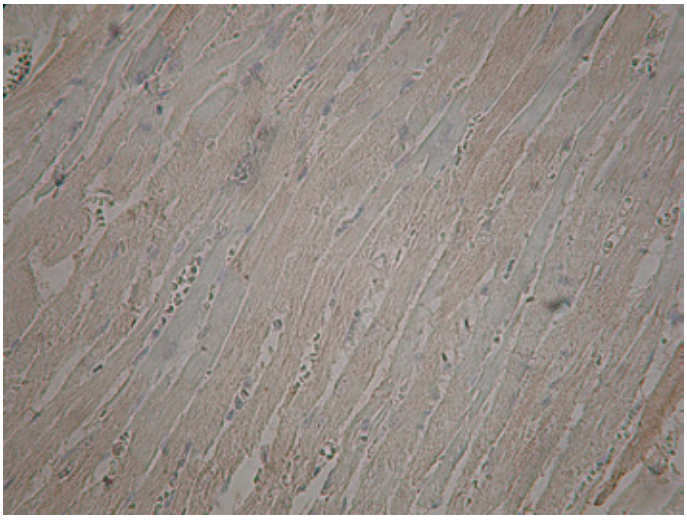

Fig. 2. Experimental group 1. Immunopositive reaction of Fas receptor in rat's cardiac muscular tissue.
Magn. ca. x400

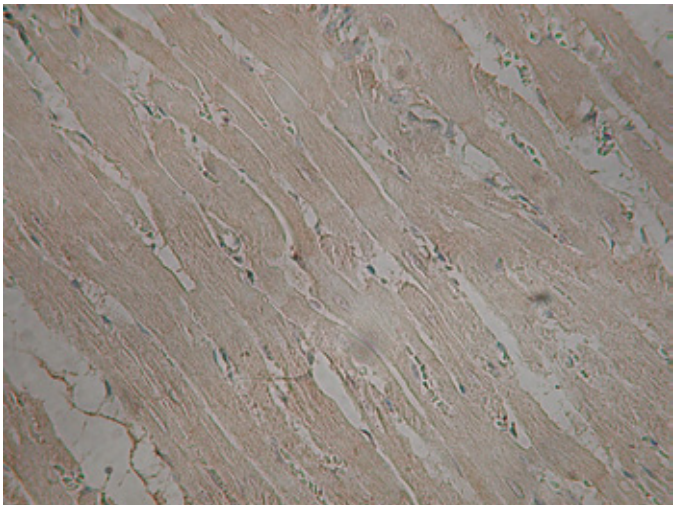

Fig. 3. Experimental group 3. Immunopositive reaction of Fas receptor in rat's cardiomyocytes.
Magn. ca. x400

DISCUSSION

The professional literature reported using statins to treat cardiovascular diseases, leading to decrease of mortality rate, and also to decrease the risk of strokes and atherosclerosis [10]. The statins therapy in women and men gives similar advantageous effects [16]. In study which was conducted by B. Uznańska and Plewka [17], it was proved that these drugs are safe and give good results in therapy of children and young people. The experiment was carried on patients who belong to high risk group e.g. with familial hypercholesterolemia. The obtained results were similar to adult patients. Statins have been proven to be generally well tolerated in elderly population. It was observed that advantages are slightly larger in comparison to younger people [1, 16]. However, these patients are very often treated with other drugs. So there is risk of drug-drug interactions, but it is mainly pharmacokinetic, than pharmacodynamic action of Atorvastatin [1]. The numerous investigations have proven that effectiveness of Atorvastatin is greater in comparison to other medicines from this group. During 5-years studies we observed, that the number of deaths due to heart diseases while using Atorvastatin decreased about 44%, in comparison to Pravastatin 36%, and Lovastatin just about 24%. Predominance of Atorvastatin was evident in reducing of total cholesterol about 25–30%, LDL fraction 25–61%, and triglycerides about 14–27%. It should be mentioned that greater decrease in cholesterol levels occurs during the first 2 weeks of therapy [16].

Statins have an influence on stabilization of atheromatous plaque, anti-inflammatory and antithrombotic effects, and also have pleiotropic effects. They are used during circulatory system diseases therapy, and concurrent diseases treatment. Multidirectional statins action creates the base to using this drug in other fields of medicine [6,7]. The anti-inflammatory effects are used during

autoimmune diseases therapy. Additionally they might have advantageous influence on the course of Alzheimer disease [6].

Extralipids action of statins and induction of apoptosis made the authors undertake estimation of expression of Fas receptor in rats' cardiomyocytes after Atorvastatin and ethanol administration. The strongest expression of the examined receptor was observed in experimental groups 1 and 3, who were given both of substances during experiment.

Apoptosis is the programmed cell death, which proceeds on 2 main pathways. One of them is the Extrinsic Pathway, which is initiated upon action of cell surface death receptors of superfamilies of tumor necrosis factor (TNF). The signal transduction is best characterized for Fas, which belongs to the Extrinsic Pathway apoptosis marker. Fas protein is stimulated upon action of toxic substances, infections, exposure of radiation, and oxygen deficiently in tissues. The second way of the Intrinsic Pathway is connected with mitochondrions [4,8]. This Pathway is initiated upon action of oncogenes, elevated concentration of Ca^{2+} ions, oxidation stress, and elevated levels of reactive forms of oxygen [4,14]. The oxidation stress and free radicals are formed during ethanol alcohol metabolism [3]. Activation of the Intrinsic Pathway makes the cells become more sensitive to stimuli of death ligands, so the Extrinsic Pathway of apoptosis can be initiated [4]. The action of both substances which were used during experiment (Atorvastatin and ethyl alcohol) can induce the superposition of 2 apoptosis pathways – it means the proapoptosis signal was strengthened. So we can make a statement that joined Atorvastatin and ethanol administration can cause the activation of the Extrinsic and the Intrinsic apoptosis Pathways which was observed in elevated expression of Fas receptor especially on the specimens from experimental group 1.

CONCLUSIONS

Conducted studies indicate that Atorvastatin administrated in white rats causes changes in heart tissue morphology. Administration of Atorvastatin and ethyl alcohol can activate the apoptosis, which can be observed in the strongest expression of examined receptor on the heart samples from experimental group 1 and 3. The increased activity of 2 simultaneously applied substances can be explained by its cumulative activity through different mechanisms.

Annotation

HMG-CoA, hydroxy-methylglutaryl-coenzyme A
TNF, Tumor Necrosis Factor
LDL-C, low-density lipoprotein cholesterol
HDL-C, high-density lipoprotein cholesterol
ACEI, angiotensin converting enzyme inhibitors
NO, nitrogen oxide
CK, creatine kinase

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SUMMARY

Atorvastatin belongs to statins which act by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Statins are the main substances which are used in the disturbed fat metabolism therapy, in the treatment and prevention the illness of cardiovascular system. Fas receptor belongs to superfamilies of TNF receptors and is one of the best known markers of the Extrinsic Pathway of apoptosis.

The aim of the work was estimate the expression of the Extrinsic Pathway apoptosis marker in cardiac muscle of rats after Atorvastatin and ethanol administration.

The experiment was carried on 30 female Wistar rats. They were divided into: 2 control groups (K and KA) and 4 experimental groups. During the study all animals were given standard granulated fodder and drinking water ad libitum. Moreover, the rats from all experimental groups were given Atorvastatin in 2 different doses independently from groups: 1.14 mg/kg of body mass and 11.4 mg/kg of body mass. The medicine was administered by successive 4 week periods in 1ml water suspension, once by day, by stomach tube. The rats from experimental groups 1 and 3 were given Atorvastatin and ethyl alcohol. The animals from all experimental groups were decapitated 24 hours following the last dose of the drug; next the heart samples for immunohistochemistry examination were taken. The Ethical Committee of the Medical University of Lublin gave consent to this experiment.

The strongest expression of Fas receptor was observed on the heart samples from experimental groups 1 and 3 which were given Atorvastatin and ethanol.

One of the advantageous pleiotropic statins actions is indication of apoptosis. The reader must be reminded that the strongest expression of investigated receptor was observed on the specimens from experimental groups 1 and 3 which were given Atorvastatin and ethanol. The obtained results may be connected with metabolism of ethyl alcohol. During this process free radicals may be formed. Additionally, the effect of consumption of great amount of ethanol may be oxidation stress. Reactive forms of oxygen and oxidation stress are the factors which can activate the Intrinsic Pathway of apoptosis. So the action of both substances which were used in experiment may stimulate the Extrinsic and the Intrinsic Pathway of apoptosis in the cardiac muscular tissue of rats.

Keywords: Atorvastatin, apoptosis, Fas receptor, cardiac muscle

STRESZCZENIE

Atorwastatyna należy do statyn, inhibitorów reduktazy 3-hydroksy-3-metyloglutarylo-koenzymu A (HMG-CoA). Są to podstawowe związki stosowane w leczeniu zaburzeń gospodarki lipidowej oraz w terapii i profilaktyce chorób układu sercowo-naczyniowego. Receptor FAS należy do nadrodziny receptorów TNF i jest jednym z najlepiej poznanych markerów zewnątrzpochodnego szlaku apoptozy.

Celem pracy była próba oceny ekspresji markera zewnątrzpochodnego szlaku apoptozy w tkance mięśnia sercowego szczurów, którym podawano łącznie Atorwastatynę i alkohol etylowy.

Doświadczenie przeprowadzono na 30 samicach szczurów białych rasy Wistar. Zwierzęta zostały przypisane do 2 grup kontrolnych (K i KA) i 4 grup doświadczalnych. Podczas trwania doświadczenia zwierzętom ze wszystkich grup podawano granulowaną paszę oraz wodę bez ograniczeń. Poza tym szczury z grup doświadczalnych otrzymywały Atorwastatynę w 2 różnych dawkach: 1,14 mg/kg m.c. oraz 11,4 mg/kg m.c. w zależności od przydziału do grupy. Lek podawany był przez 4 tygodnie w postaci zawiesiny wodnej w objętości 1ml, 1 raz dziennie dożołądkowo za pomocą sondy. W przypadku szczurów z grup doświadczalnych 1 i 3, otrzymywały one łącznie z Atorwastatyną alkohol etylowy. Szczury ze wszystkich grup dekapitowano 24h od podania ostatniej dawki leku, a następnie pobrano wycinki mięśnia sercowego do badań immunohistochemicznych. Na wykonanie doświadczenia uzyskano zgodę Lokalnej Komisji Bioetycznej.

Najsilniejszą ekspresję receptora FAS wykazywały komórki mięśnia sercowego pochodzące od zwierząt, które oprócz Atorwastatyny otrzymywały alkohol etylowy.

Reasumując można stwierdzić, że indukowanie procesu apoptozy jest jednym z korzystnych plejotropowych działań statyn. Na uwagę zasługuje jednak fakt najsilniejszej ekspresji badanego receptora uzyskanej na preparatach pochodzących od zwierząt z grup doświadczalnych 1 i 3, czyli otrzymujących lek i etanol. Otrzymane wyniki mogą być związane z metabolizmem alkoholu etylowego, podczas którego powstają wolne rodniki. W przypadku spożywania dużych ilości alkoholu może także dochodzić do powstawania stresu oksydacyjnego. Reaktywne formy tlenu oraz stres oksydacyjny należą do czynników aktywujących wewnętrzny szlak apoptozy. Zatem działanie obu zastosowanych w doświadczeniu substancji może pobudzać 2 niezależne szlaki apoptozy w tkance mięśnia sercowego szczurów.

Słowa kluczowe: Atorwastatyna, apoptoza, receptor Fas, tkanka mięśnia sercowego.