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IMMUNOHISTOCHEMISTRY OF EXTRACELLULAR SOD AND GPX1 ENZYMES IN RABBIT ATHEROSCLEROTIC LESIONS

Oxidative stress plays an important role in arising, development and progression of atherosclerosis as the main cause of ischemic heart disease (IHD) and myocardial infarction (MI) manifestations. Experimental studies on model objects continue to be a significant tool for revealing molecular and genetic aspects of atherogenesis. However, many questions remain unanswered and results are controversial. In this study, we characterized expression of two major vascular wall proteins – extracellular superoxide dismutase (ex-SOD) and glutathione peroxide dismutase 1 (GPX1) in rabbit atherosclerotic lesions compared to normal rabbit aorta. Using immunohistochemistry, we identified the presence of EC-SOD and GPX1 proteins in rabbit normal aorta and atheroma lesions and established their expression in specific atherosclerotic areas distinctive for both enzymes. Also, we confirmed the higher expression level as for EC-SOD, as for GPX1 proteins in aorta tissues affected by atherosclerosis.
Keywords: *oxidative stress, antioxidant system, rabbit, ES-SOD, GPX1*

Redox signaling pathways influence many physiological processes providing a strict crosstalk within and between cells in a vessel wall. Oxidative stress induced by inflammation in the coronary artery wall affects excessive oxidizing of lipoproteins, nitric oxide (NO) molecules, DNA and other cell's proteins leading to normal homeostatic state disruption and atherosclerosis development.The main free radical of inflammation – superoxide anion (O_2^-), was found as a prominentfeatureof the atherosclerotic plaques, produced by activated SMC, macrophages, endothelial cells and some otherscells in abundant amounts. Polyunsaturated fatty acids of lipids in cells membranes, lipoprotein particles (LP) became oxidized in a chain-reaction manner.Highly regulated endothelium derived NO, involved in vascular dilatation, reacts rapidly with O_2^- forming toxic peroxynitrite ($ONOO^-$).High dose and/or inadequate generation of superoxide anion lead to activation of enzymes involved in O_2^- removal –superoxide dismutases (SODs), catalase, peroxiredoxins (Prxs), or glutathione peroxidases (GPx). SODs are the first line of antioxidant defense through dismutation of O_2^- to H_2O_2. Isoenzyme EC-SOD is of great interest for vascular homeostasis. Its unique role in vasculature is determined by theextracellular localization, anchoringirreversibly to type I collagen and heparan sulfate and thus on guard of vascular tissue integrity. EC-SOD was found to be highly expressed in human[1], mouse and rabbit atherosclerotic lesions [] with time-dependent downregulationtendency.

Formed through dismutation reaction H_2O_2 is a second signaling molecule in tissues, but generated in high amounts under oxidative stress, became a dangerous agent. Enzymatic inactivation of derived H_2O_2 is achievedprincipally by glutathione peroxidases superfamily. Clinical and experimental studies suggest a crucial role for GPX1 isoenzyme in atherogenesisand cardiovascular disease promotion[2], [3], [4]. But, available data on expression and regulation of GPX1 protein under atherosclerosis especially in rabbit experimental model are rare and incomplete.

Based on the importance of these proteins for the development of atherosclerosis pathology, we sought to determine and compare the exact locations of GPX1 and EC-SOD proteins and itsexpression level under atherosclerotic lesions. In our study we used a suitable atherosclerotic model object – rabbit, normal and after six-months fed high cholesterol diet.

Materials and methods.

Rabbit experimental atherosclerosis model

Total 10 male Chinchilla rabbits of 6 months old, initial weight 3500g and without any physical pathology was investigated. Atherosclerosis was induced in 5 male Chinchilla rabbits by high 2% cholesterol diet "Purina rabbit chow" (Dytes Inc., USA) lasting for 6 months. Five rabbits were on a regular diet (food "Purina rabbit chow", Dytes Inc., USA) and served as a control. After 6 months, cervical dislocation under anesthesia were performed and thoracic aorta tissue (0.5×0.7 mm)were prefixed in 10% buffered formalin solution and embedded in paraffin blocks by classical method.

Animal experimental procedures were approved by the Local Bioethics Committee of the Institute of Cardiology and Internal Diseases (Protocol № 10 of March 12, 2009).

Immunohistochemistry.

Immunohistochemistry was done according to avidin-biotin peroxidase complex methodby using rabbit monoclonal antibodies to Glutathione Peroxidase 1(Abcam, USA) and extracellular Superoxide Dismutase 3 (Abcam, USA). Before immunostaining, blocking of endogenous peroxidase activity was performed by incubation with 3% H_2O_2 for 15 min at room temperature with subsequent antigen retrieval by heating slides in 10mM citrate buffer (pH-6).

Protein extraction from paraffin embedded rabbit aorta tissues

Total protein extracts from paraffin embedded rabbit aorta tissues were isolated according to Kimimasa Ikeda et al.[5].

Protein assay.

Concentration of total protein lysates was measured by M. Bradford method [6]. BSA solution in different dilutions was used as a reference standard.

SDS-PAGE and Western blot (WB) analysis 20 ng of total protein extracts were separated in 12% SDS-PAGE by electrophoresis at a constant 160 volts. Proteins were transferred into PVDF membrane overnight at constant 30mA, overnight, +4C°. Nonspecific binding was inhibited by blocking buffer (0.1% casein in 0.2× PBS, 0.1% Tween-20) during overnight at +4C°. The membranes were incubatedwith the primary antibody at +4C° for 2 hr at the 1:3000 dilutions. After being washed with TBST (50 mMTris-HCl, pH 7.5, 150 mMNaCl, 0.1% Tween-20), the membranes were incubated for 1 h with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP).Membranes were developed with the Blue Basic Autorad Film 8×10 (ISC Bio Express) in 1.25mM luminol, 0.68mM coumaric acid and 0.01% H2O2 buffer.

Statistical analysis.

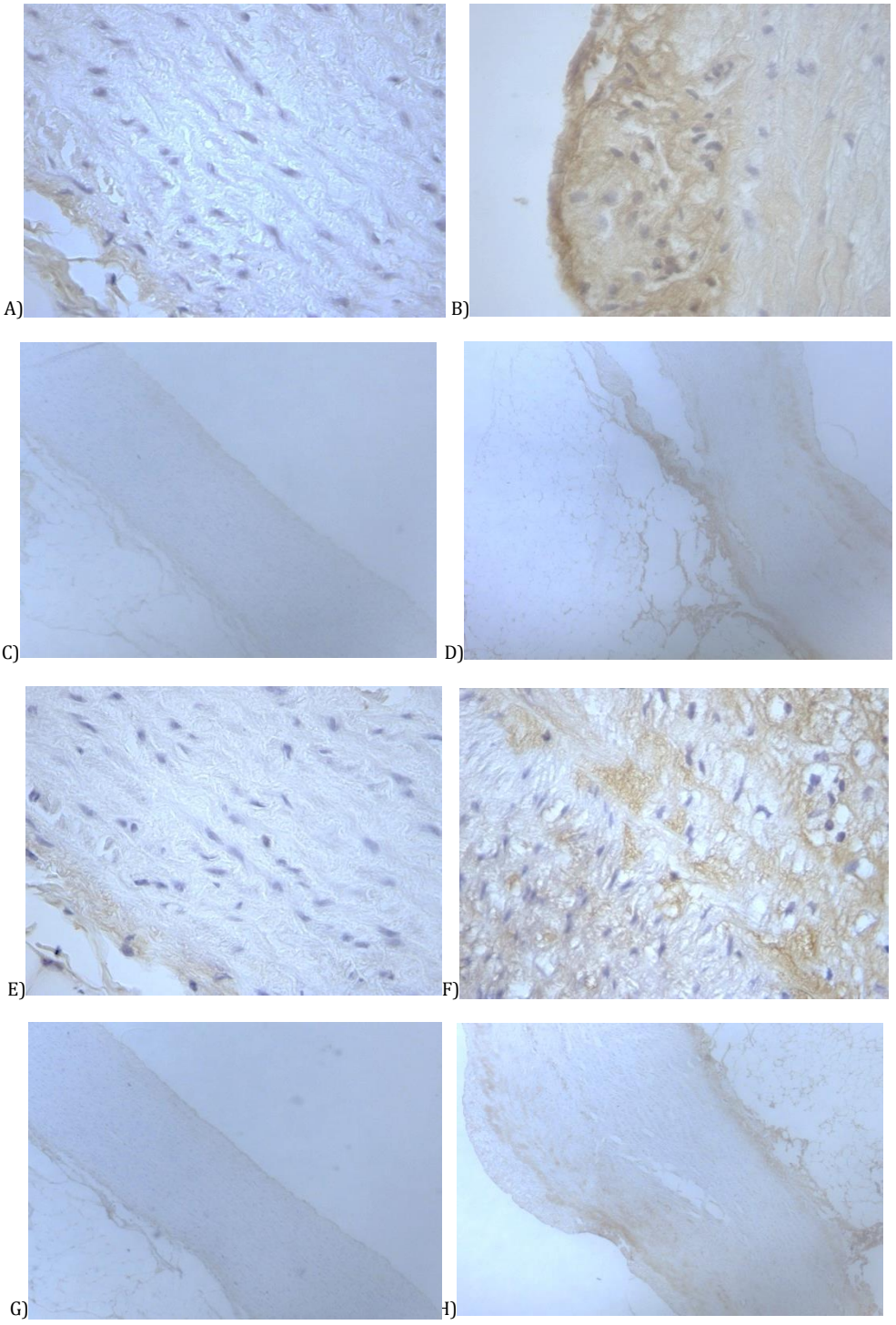
All data results are expressed as mean ±SD.A p-value of <0.05 was considered statistically significant. Distribution of variables between two-rabbit groups was compared by using a Student's t-test.

Results.

Immunohistochemical identification of EC-SOD and GPX1 proteins were studied in 10 different sets of rabbit thoracic aorta tissue samples.Positive EC-SOD and GPX1 proteins immunostaining were found in normal and atherosclerotic aortas. Pic 1A through 1D shows representative microphotographs of GPX1 immunostaining in serial sections of normal (Picture A, C) and atherosclerotic aortas (B, D). A strong immunostaining of GPX1 was seen in intimalayer under advanced atheroma lesions (Picture 1D). Also, positive, but less intensity GPX1 immunoreactivity was detected in early atherosclerotic lesions in the intima layer (Picture 1B). In control sections there were no intensive immunoreactivity to GPX1 protein.

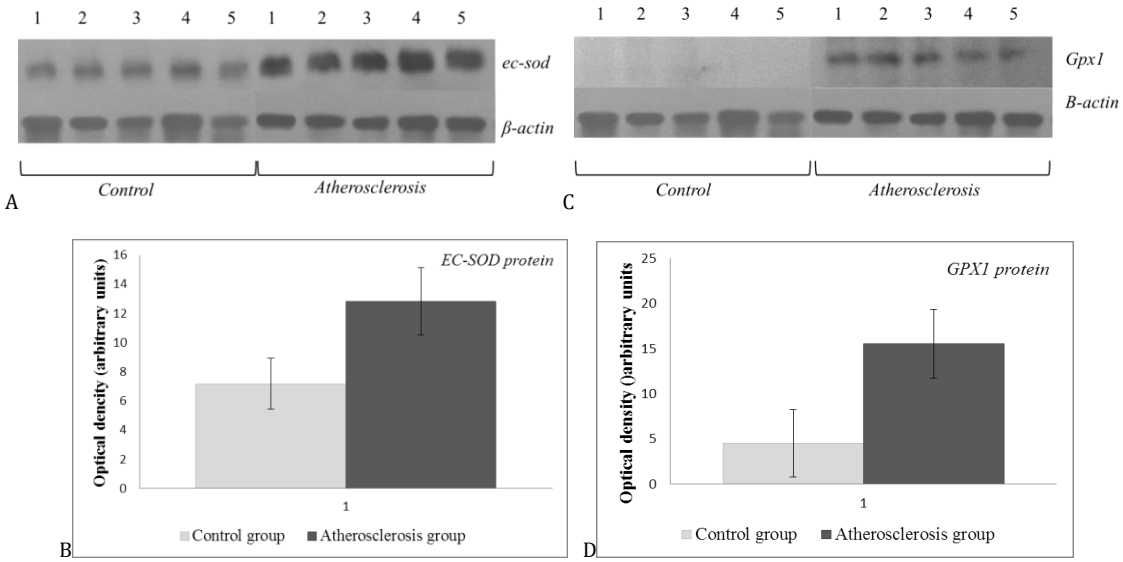
Staining with EC-SOD antibody was also positive in normal rabbit aorta tissues sections, but low intensity (Picture 1 E). EC-SOD signal was observed only in the intima layer mostly directly under epithelium. There were no visible EC-SOD signals in media and adventitia layers (Picture 1 G). In contrast, atherosclerotic aorta tissues sections showed positive and intense staining signal across the intima, especially

under advanced atheromas (Picture 1 F). Also, the intensive EC-SOD antibody signal was noted in the media layer near the adventitia (Picture 1H).



Picture 1 – GPX1 and EC-SOD immunohistochemical localization in aorta tissue. A – GPX1 in normal aorta ×86; B – GPX1 in atherosclerotic aorta ×86; C – GPX1 in normal aorta ×20; D – GPX1 in atherosclerotic aorta ×20; E – EC-SOD in normal aorta ×86; F – EC-SOD in atherosclerotic aorta ×86; G – EC-SOD in normal aorta ×20; H – EC-SOD in atherosclerotic aorta ×20

Western blots revealed bands of EC-SOD and GPX1 proteins at the expected molecular size 22kDa and 26kDa respectively (Picture 2 A and B). Densitometryanalysis of expression also showed increased levelsfor both enzymes in atherosclerosis compared with the control group (Picture 2 A and B).



Picture 2 – Western blot (WB) analysis of EC-SOD and GPX1 in normal and atherosclerotic aorta tissues. A –expression of EC-SOD protein in aorta tissue from control and atherosclerosis groups (SDS-PAGE and immunoblotting with rabbit monoclonal antibody to SOD3). B – densitometric analysis of WB for EC-SOD protein, p=0.000. C – expression of GPX1 protein in aorta tissue from control and atherosclerosis groups (SDS PAGE and immunoblotting with rabbit monoclonal antibody to GPX1). D – dencitometric analysis of WB for GPX1 protein, p=0.002.

Discussion.

Chinchilla rabbit atherosclerosis status. In our study, we used experimental model of atherosclerosis in rabbits (Chinchilla breed). Historically, rabbits were the first and successful model for atherosclerosis basic research [7]. Despite the existing differences between rabbits and human in terms of lipid metabolism [8], [9] and in arterial susceptibility to atherosclerosis[10], studies in translational medicine show that this model of atherosclerosis is the most suitable than mice and rats[11].Hypercholesterolemia and atherosclerosis promotion in rabbits is directly proportional to the dietary cholesterol and/or fats uptake [12], [13], [14]. TakingintoaccountthesefeaturesandtheChinchillabreedhypercholesterolemiainduction[15],we used a standard "Purina rabbit chow" with the addition of 2% cholesterol for 6 months. This time was enough for advanced atherosclerotic plaques formation, without systemic lipid degeneration in other organs. Also, due to differences in plasma cholesterol metabolism between rabbit males and females [16], we used only males in the study.

EC-SOD and GPX1 proteins expression profile. According to numerous studies, EC-SOD and GPX1 proteins areundoubtedly cardiovascular diseases associated proteins. Manifestation of arterial hypertension[17][18][19], atherosclerosis[20][21][22], ischemia[23], myocardial infarction and other cardiovascular disorders provoking states may be aggravate by an expression and regulation of EC-SOD and GPX1[24].The precise regulatory mechanisms for the EC-SOD and GPX1 genes expression under normal and atherosclerosis states are unknown. Several mechanisms for the EC-SOD have been proposed[24][25][26], [27]. Mikko O. Laukkanen et al., assumedthe existence of the additional possible mechanism throughde/methylation pattern ofthe EC-SOD gene in atherosclerosis[28].

The data obtained by us also support the above-listed studies and views on the important role of antioxidant defense enzymes in the development of atherosclerosis. The increased expression of EC-SOD and GPX1 proteins in rabbit aortic tissues indicates a shift in the oxidation-reduction potential in the tissue and intensification of antioxidant processes. Deficiency and/or overexpression of these proteins may speed up[22][29]or slow down[30]the atherosclerosis pathological states.Identification of specific biochemical pathways requires further studies on suitable experimental models and human.

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ҚОЯНДАРДЫҢ АТЕРОСКЛЕРОЗБЕН ЗАҚЫМДАНҒАН ҚАН ТАМЫРЛАРЫНДАҒЫ КЛЕТКАДАН ТЫС GPX1 ГЛУТАТИОН ПЕРОКСИДАЗА ЖӘНЕ SODСУПЕРОКСИДДИСМУТАЗА АҚУЫЗДАРЫН ИММУНОГИСТОХИМИЯЛЫҚ ЗЕРТТЕУ

Түйін: Тотығу стрессы жүректің ишемиялық аурулары мен жүрек инфарктісінің неізгі себебі болып табылатын атеросклероздың пайда болуында, дамуында және күшейе түсуінде маңызды рөл атқарады. Атерогенездің генетикалық және молекулярлы көріністерін анықтау үшін эксперименталъді зерттеуге модельді объектілерді пайдалану маңызды инструмент болыпта былады. Осыған қарамастан көптеген мәселелер әлі күнге дейін өз жауабын тапқан жоқ, кейбір алынған нәтижелер бір-біріне қарама-қайшы келіп жатады. Көрсетілген зерттеу жұмысында біз экспериментальді модель ретінде қоянның атероскелерозы зерттелді. Мұнда атероскелерозбен зақымданған және зақымданбаған аорта қантамыры қабырғасындағы қантамырлардың негізгі екі ақуызы болып табылатын клеткадан тыс GPX1 глутатионпероксидаза 1 (GPX1) және супероксиддисмутаза (ex-SOD) ақуыздарының экспрессиясы сипатталды. Иммуногистохимиялық зерттеу нәтижесінде спецификалық атеросклерозды аймақта екі ферменттің де экспрессиясы жүретіні анықталды. Сонымен қатар біз атероскелерозбен зақымданған аорта қантамыры ұлпасында GPX1 және EC-SOD ақуыздарының жоғарғы деңгейде экспрессияланауын дәлелдедік.
Түйінді сөздер: тотығу стрессі, антиоксиданттық жүйе, қояндар, ES-SOD, GPX1.

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ИММУНОГИСТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ БЕЛКОВ ВНЕКЛЕТОЧНОЙ СУПЕРОКСИДДИСМУТАЗЫ SOD И ГЛУТАТИОН ПЕРОКСИДАЗЫ GPX1 В АТЕРОСКЛЕРОТИЧЕСКИХ ПОРАЖЕНИЯХ СОСУДОВ КРОЛИКА

Резюме: Окислительный стресс играет важную роль в возникновении, развитии и прогрессировании атеросклероза как основной причины проявлений ИБС (ИБС) и инфаркта миокарда (ИМ). Экспериментальные исследования на модельных объектах, по-прежнему являются важным инструментом для познания молекулярных и генетических аспектов атерогенеза. Однако, многие вопросы, до сих пор остаются без ответа, а получаемые результаты, зачастую противоречивы. В данном исследовании, на экспериментальной модели атеросклероза у кроликов, мы охарактеризовали экспрессию двух основных белков сосудистой стенки - внеклеточной супероксиддисмутазы (ex-SOD) и глутатионпероксидазы 1 (GPX1) в нормальной стенке аорты и пораженных атеросклерозом. Иммуногистохимически, было установлена их экспрессия в специфических атеросклеротических областях, характерных для обоих ферментов. Кроме того, мы подтвердили высокий уровень экспрессии как для EC-SOD, так и для белков GPX1 в тканях аорты, пораженных атеросклерозом.
Ключевые слова: окислительный стресс, антиоксидантная система, кролики, ES-SOD, GPX1