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Tissue specific up regulation of ACE2 in rabbit model of atherosclerosis by atorvastatin: Role of epigenetic histone modifications

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ABSTRACT

Growing body of evidence points out the crucial role of ACE2 in preventing atherosclerosis. However, data on how atherosclerosis affects ACE2 expression in heart and kidney remains unknown. Atherosclerosis was induced by feeding New Zealand White rabbits with high cholesterol diet (HCD – 2%) for 12 weeks and atorvastatin was administered (5 mg/kg/day p.o) in last 3 weeks. ACE2 mRNA and protein expression was assessed by Western blotting and real time PCR. HCD fed rabbits developed atherosclerosis as confirmed by increase in plasma total cholesterol, LDL and triglycerides as well as formation atherosclerotic plaques in arch of aorta. The ACE2 protein but not mRNA expression was reduced in heart and kidney of HCD rabbits. Interestingly, atorvastatin increased the ACE2 protein expression in heart and kidney of HCD rabbits. However, atorvastatin increased ACE2 mRNA in heart but not in kidney of HCD rabbits. Atorvastatin increased the occupancy of histone H3 acetylation (H3-Ac) mark on ACE2 promoter region in heart of HCD rabbits indicating direct or indirect epigenetic up-regulation of ACE2 by atorvastatin. Further, atorvastatin suppressed Ang II-induced contractile responses and enhanced AT_2 receptor mediated relaxant responses in atherosclerotic aorta. We propose that atherosclerosis is associated with reduced ACE2 expression in heart and kidney. We also show an unexplored potential of atorvastatin to up-regulate ACE2 via epigenetic histone modifications. Our data suggest a novel way of replenishing ACE2 expression for preventing not only atherosclerosis but also other cardiovascular disorders.

1. Introduction

Renin Angiotensin system (RAS) plays a crucial role in formation of atherosclerotic lesions as it drives endothelial dysfunction, vascular smooth muscle cell (VSMC) proliferation and migration [1]. The main peptide of RAS, Ang II, promotes atherosclerosis and therefore therapies degrading or antagonizing Ang II would be effective. Moreover, pharmacological inhibition of RAS reduces both experimental and human atheroscleorosis [2,3] which supports the role of RAS.

RAS can be divided into ACE/AT_1/Ang II and ACE2/Mas/Ang-(1-7) axes having opposite functions. Two carboxypeptidases, ACE1 and ACE2, are responsible for endogenous regulation of Ang II levels and activity. ACE2 opposes the actions of ACE1 by converting Ang I to Ang-(1-9) and Ang II to Ang-(1-7), having vasodilator and anti-proliferative properties [4]. The ability of ACE2 to degrade local Ang II and produce Ang-(1-7) limits the patho-physiological activation of RAS and identifies it as potential therapeutic target for treatment of cardiovascular disorders.

Till date, several studies have demonstrated the role of ACE2 in reducing atherosclerosis. ACE2 is localised in smooth muscles, endothelium and macrophages in aortic and coronary atherosclerotic lesions of hypercholesterolemic rabbits [5,6]. Adenoviral overexpression of ACE2 in apo$E^{-/-}$ mice or hypercholesterolemic rabbits decreased atherosclerosis [7,8]. On the other hand, deficiency of ACE2 exaggerated atheroma formation indicating vital role of ACE2 in preventing atherosclerosis [9]. Thus therapies up-regulating ACE2 can be used as a potential approach in treating human atherosclerosis.

ACE2 is subjected to both transcriptional and translational regulation. Ang II and endothelin-1 have been shown to down

Abbreviations: ACE2, angiotensin converting enzyme-2; Ang II, Angiotensin II; HCD, high cholesterol diet; H3-Ac, histone H3 acetylation (Lysine 9/14); p-H3, histone H3 phosphorylation (serine 10).

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regulate ACE2 mRNA expression by elevating ERK/p38 MAP kinases [10]. Further, hypoxia increases the levels of Ang II, which in turn down regulates ACE2 expression [11]. Our group previously observed that high fat consumption decreases the ACE2 expression and heat shock prevents it [12]. Still there is need to investigate the detailed transcriptional machinery regulating ACE2. Apart from transcriptional regulation, ACE2 is subjected to posttranslational regulation. ADAM 17 cleaves ACE2 at the cell membrane resulting in shedding of ACE2. Myocardial dysfunction has been correlated with increased levels of shed ACE2 [13]. Understanding of ACE2 regulation would open new ways to manipulate this angel enzyme.

Several studies have shown that ACE2 overexpression reduces atherosclerosis but very little is known about its regulation. Some epidemiological studies show that epigenetic mechanisms such as histone modifications, promoter DNA methylation and miRNAs reprogram later development of disease *in utero* [14]. Interestingly, ACE1 is regulated by histone acetylation and DNA methylation *in vitro* and *in vivo* [15]. We hypothesized that ACE2 may also be regulated by histone modifications in heart and kidney of atherosclerotic rabbits where it is abundantly expressed.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were approved by Institutional Animal Ethics Committee (IAEC no: 12/51) and were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Ministry of Environment and Forests, Government of India.

2.2. Animals and treatment

Male New Zealand White rabbits weighing 1.5–2.0 kg were procured from disease free small animal house, College of Veterinary Sciences Hisar, India. They were housed individually in stainless steel mesh bottom cages and maintained under standard environmental conditions – room temperature 22 ± 2 °C, relative humidity 50 ± 10% and 12 h light and dark cycles with free access to food and water *ad libitum*. All the animals were quarantined for 2 weeks prior to the study and fed normal pellet diet during this time. Fourteen male New Zealand White rabbits were divided into three groups, i.e., a control group ($n = 4$) receiving commercially available standard pellet diet (Pranav Agro Industries Ltd., Pune, India), a hypercholesterolemic group or high cholesterol diet group (HCD, $n = 5$) receiving atherogenic diet (2% cholesterol and 10% vegetable oil) for 12 weeks, and a treatment group (HCD + A, $n = 5$) receiving atherogenic diet along with 5 mg/kg/day atorvastatin (Dr. Reddy's Labs, India) orally added from 9th week to end of 12th week. The rabbits were also provided with cabbage and sprouted gram daily. The study was pursued in two phases; (1) Aortae obtained from first study were used exclusively for oil O red staining and the other tissues such as heart and kidney were frozen at −80 °C for Western and real-time PCR. (2) In second study, aortae were used for vascular reactivity and Western blotting studies. The other tissues such as heart and kidney were frozen at −80 °C for Western and real-time PCR. Biochemical measurements were performed in all the animals of both the studies in order to validate the model.

2.3. Oil O red staining

Oil O red (Sigma, USA) staining was performed as described earlier [7]. In brief, fixed aortas were cut longitudinally and put into distilled water for 2 min. After that aortas were kept in 60% isopropanol (Sigma, USA) for 5 min, followed by staining with prewarmed oil O red working solution (Sigma, USA) at 60 °C for 5 min. Extra staining was removed by washing with 60% isopropanol for 15 min. Aortas were stored in normal saline and photographs were taken by digital camera.

2.4. Biochemical estimations

Fasting plasma cholesterol, triglyceride, HDL and LDL were estimated strictly as per manufacturer's instructions (Accurex, India).

2.5. Vascular reactivity

Aortic ring preparation and isometric tension measurement studies were preformed with Panlab (Harvard apparatus, USA) and Powerlab data acquisition system (AD instruments, Australia) as described previously [12,16]. Briefly, after two KCl (Sigma, USA) challenges, cumulative concentration response curves (CRCs) to Ang II (Sigma, USA) (1 nM–1 μM) were recorded. The Ang II (AT_2 receptor) mediated relaxation was studied in the presence of AT_1 receptor antagonist Losartan (Gift from Novartis, India) (100 μM, contact time: 15 min) and AT_2 receptor antagonist PD123319 (Sigma, USA) (10 μM, contact time 30 min). The tissues were precontracted with either 100 nM phenylephrine (PE) (Sigma, USA) following which CRC to Ang II (1 nM–1 μM) was recorded.

2.6. Western blotting

Protein isolation and Western blotting were performed as previously described [12]. The whole heart and kidney homogenates consist of different cell types. To maintain the homogeneity we have used left ventricular portion of heart and cortex of kidney for Western, PCR and ChIP experiments and aortic tissues were pooled for protein extraction (as less quantities of protein we get from aorta). The experiments were performed at least three times. Briefly, frozen tissues were homogenized in liquid nitrogen and transferred to lysis buffer. Protein samples were resolved using 7.5, 10 and 14% sodium dodecyl sulphate polyacrylamide gels (SDS–PAGE) (Sigma, USA) depending on the molecular weight of desired proteins. These were then transferred to PVDF membranes (Pall, USA) and were probed with primary antibodies. Antibodies used were: ACE2 (Cat no:-sc-20998), phospho-H3 (Serine 10) (Cat no:-sc-8656-R) (SantaCruz, USA) and acetylated-H3 (lysine) (Cat no:−39139) (Active Motif, USA). Actin (Cat no:-sc-1616) and H3 (Cat no:-sc-8654) (SantaCruz, USA) were used as loading controls. The antigen-primary antibody complexes were incubated with horseradish-peroxidase conjugated secondary antibodies (Santa Cruz, USA) (Cat no:−2030) and visualised using enhanced chemiluminescence system and ECL hyperfilm (Amersham, England). Blots were scanned and analysed using ImageJ software (NIH, USA).

2.7. Real time PCR

RNA was isolated from heart and kidney using RNA extraction kit (Ambion, USA). After reverse transcription with Superscript II (Invitrogen, USA), real-time PCR was performed with LightCycler 2.0 (Roche Diagnostics, Switzerland) using SYBR master mix (Invitrogen, USA) and the specific forward and reverse primers (Eurofins Mwg operon, USA) (ACE2*:- Forward-CATATGCCACG-CAGCCTTTC*; *Reverse-CAAGTGCTCAGGGGTAGCTG*). After amplification, a melting curve analysis was performed to verify the specificity of the reaction. 18s gene (*Forward*: GTAACCCGTT-GAACCCCATT; *Reverse*: CCATCCAATCGGTAGTAGCG) was used as internal control and results determined by $2^{-\Delta\Delta Ct}$ method and expressed as fold change over control rats.