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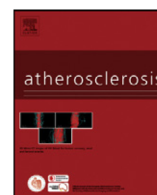

Effects of atorvastatin on serum soluble receptors for advanced glycation end-products in type 2 diabetes

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ABSTRACT

Objective: The receptor for advanced glycation end-products (RAGE) plays an important role in the pathogenesis of diabetic complications and atherosclerosis. Interfering with the activation of RAGE by using a soluble form of the receptor (sRAGE) ameliorates the vascular complications of diabetes in animal models. We have investigated whether statin can influence the expression of sRAGE and esRAGE (a splice variant of sRAGE) in vitro and in vivo.

Methods: THP-1 cells were incubated with atorvastatin in vitro and sRAGE and esRAGE in the medium was measured by Western immunoblot. Serum levels of sRAGE and esRAGE were measured by ELISA in archived serum samples from a previous randomized double-blind placebo-controlled clinical trial that explored the cardiovascular effects of atorvastatin in hypercholesterolemic Chinese type 2 diabetic patients.

Results: sRAGE and esRAGE were induced by atorvastatin in a time- and dose-dependent manner in THP-1 cells. In the diabetic patients, there was a significant increase in serum sRAGE ($p < 0.05$) and esRAGE ($p < 0.01$) in the atorvastatin group at 6-month, but no change in placebo group. Serum esRAGE was higher in atorvastatin group than placebo group [median 240.5 pg/ml (interquartile range 186.5–377.3) vs 194.8 pg/ml (124.1–347.9) respectively, $p < 0.01$] at 6-month, whereas the differences in sRAGE did not reach statistical significance ($p = 0.051$). There was a correlation between the increase of serum esRAGE and reduction of serum LDL ($r = -0.36$, $p = 0.001$).

Conclusions: Statins are known to have pleiotropic effects and we have shown that atorvastatin can increase circulating esRAGE levels in type 2 diabetic patients.

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1. Introduction

The receptor for advanced glycation end-products (RAGE) is a multi-ligand member of immunoglobulin superfamily of transmembrane cell surface molecule and binds a number of ligands including advanced glycation end-products (AGEs), amyloidogenic peptides/polypeptides, amphoterins and S100/calgranulins [1]. The activation of RAGE by AGEs has been shown to play an important role in the development of diabetic vascular complications [2,3]. Blockade of RAGE using a soluble form of the receptor (sRAGE) suppressed vascular hyperpermeability, atherosclerotic lesion development and enhance wound healing in diabetic rodents [4–7]. sRAGE contains only the extracellular domain of the receptor and lacks the transmembrane and intracytoplasmic domains. Hence, sRAGE has ligand-binding properties in the absence of a signalling cascade. Based on these experimental results, it has been suggested that sRAGE may have a protective function against the development of diabetic vascular complications by acting as a decoy for RAGE ligands [8–10].

The potential usefulness of genetically engineered sRAGE in the prevention and treatment of diabetic complications has been demonstrated in animal studies, but whether endogenous sRAGE species can be effectively increased to protect tissues against the harmful effects of AGEs in humans remains to be determined. Our current understanding of the pathophysiological mechanism regulating sRAGE production in humans is very limited. It has been suggested that sRAGE measured in peripheral blood may be released by several cell types such as endothelial cells and circulating leukocytes, and may result from the cleavage of the full-length native membranous receptor by proteinases like matrix metalloproteinases and/or from the expression of a RAGE splice gene variant that encodes the soluble form of the receptor [8–14]. Several alternatively spliced isoforms of RAGE which results in a secretory protein have been reported in the literature and the best characterized is endogenous secretory receptor for advanced glycation end-products (esRAGE) [15]. There is evidence to suggest that

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sRAGE level may be modulated by some currently available pharmacological agents [16]. Forbes et al. have shown that inhibition of the renin-angiotensin system by perindopril in patients with type 1 diabetes increased plasma sRAGE level [17]. We have reported that treating type 2 diabetic patients with the thiazolidinedione rosiglitazone can increase circulating esRAGE levels [18]. Santilli et al. have recently shown that atorvastatin increased serum sRAGE level in a small group of hypercholesterolemic non-diabetic subjects without vascular disease when compared with subjects being given pravastatin but esRAGE was not measured [19]. In the present study, we have (i) investigated the direct effect of atorvastatin on the expression of sRAGE and esRAGE in macrophages in vitro and the mechanisms(s) involved; (ii) evaluated the impact of atorvastatin on circulating total sRAGE and esRAGE in vivo in blood samples from Chinese type 2 diabetic patients who participated in a randomized double-blind placebo-controlled clinical trial that explored the cardiovascular effects of atorvastatin [20].

2. Methods

2.1. Antibodies

The mouse monoclonal antibody against human RAGE MAb55413 (R&D, Minneapolis, MN) was raised against N-terminal Gln 24 through Ala 344 of the extracellular domain of RAGE and therefore recognizes all isoforms of sRAGE including esRAGE in conditioned medium. To detect esRAGE specifically, anti-esRAGE antibody (Sigma, M.A) was raised according to Yonekura et al. [11]. Briefly, the anti-human esRAGE antibody was raised in rabbit against the unique C-terminal 16-amino-acid peptide (amino acids 332 to 347, EGFDKVREAEDSPQHM) of esRAGE and then purified by the immune-affinity column which contained the antigen peptide.

2.2. In vitro study

To investigate the effect of atorvastatin on esRAGE and sRAGE in macrophages, human monocytic leukemia cell line THP-1 (ATCC, Manassa., VA) was used. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and cells at 5–20 passages were used for the experiments. THP-1 macrophages were differentiated using phorbol 12, 13-dibutyrate (PMA) at 100 nM for a total of 3 days and then followed by 1-day fasting before being incubated with increasing doses of atorvastatin (0–30 μM) in serum-free and PMA-free medium. After 24 h of incubation, cells and medium were then harvested and sRAGE and esRAGE was measured by Western blot analysis. For the time-dependent experiments, THP-1 macrophages were tested with increasing incubation time (0–24 h) at a fixed concentration of atorvastatin in (30 μM) in serum-free and PMA-free medium. To determine whether the effect of atorvastatin on the generation of soluble forms of RAGE was mediated by proteolytic cleavage by metalloproteinases rather than by alternative splicing, GM6001 (Calbiochem), a broad-spectrum hydroxamate metalloproteinase inhibitor which can block the shedding of RAGE by metalloproteinases [21], was used to pretreat THP-1 macrophages 1 h before the addition of atorvastatin (30 μM).

Western blot analysis was performed to measure RAGE and esRAGE in cell lysates, and sRAGE and esRAGE in medium. Conditioned media was first concentrated by precipitating with 100% cold acetone and separated by 7.5% SDS-polyacrylamide electrophoresis gel (SDS-PAGE). The gel was subsequently electrotransferred onto the polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked in Tris-buffered saline Tween 20 (TBST) with 10% powdered skimmed milk. The blocked membrane was probed with primary antibody diluted in TBST with 1% of powdered skimmed milk and then with horseradish peroxidase conjugated secondary antibodies. Bandings were visualized by an enhanced chemiluminence advanced detection system (GE healthcare) and then exposed to X-ray film (Kodak, Rochester, NY).

2.3. Human serum samples

The effect of atorvastatin on circulating sRAGE and esRAGE in type 2 diabetic patients was examined in stored frozen serum samples from Chinese type 2 diabetic patients who participated in a randomized double-blind placebo-controlled trial that explored the cardiovascular effect of atorvastatin [20]. In brief, the inclusion criteria were HbA1c <10%, fasting LDL >3.4 mmo/l, triglyceride (TG) < 4.0 mmol/l, total cholesterol to HDL ratio > 4.0, and blood pressure ≤ 160/90 mmHg. Exclusion criteria were the current use of lipid lowering therapy, secondary hyperlipidemia, deranged liver or renal function, and a major cardiovascular event within the last 6 months. Patients were randomised in a double-blind manner to atorvastatin 10 mg daily, increasing to 20 mg daily after the first 3 months or to matching placebo. Serum sRAGE, esRAGE and AGEs were determined at baseline and 6-month from serum samples stored at −70 °C which have never been thawed.

Laboratory personnel were blinded to the treatment status of the subjects and samples were assayed in duplicate and in random order. Serum sRAGE levels were determined using enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. This assay measures serum total sRAGE level. Briefly, a monoclonal antibody raised against the N-terminal extracellular domain of human RAGE consisting of amino acids Gln 24 through Ala 344 was used to capture sRAGE from serum. Captured sRAGE was detected with a polycloncal antihuman sRAGE antibody raised against the same immunogen. After washing, plates were incubated with streptavidin-HRP, developed with appropriate substrate and OD450 was determined using an ELISA plate reader. The intra- and inter-assay coefficients of variation were 1.9% and 5.5%, respectively. Serum esRAGE levels were determined using a different ELISA (B-Bridge International Inc., CA, USA). The capture antibody in the ELISA assay for esRAGE uses the monoclonal antibody 278-13G4 and the detection antibody is a rabbit esRAGE-specific polyclonal antibody raised against the unique C-terminal 16-amino-acid peptide (amino acids 332 to 347) of esRAGE. The intra- and inter-assay coefficients of variation were 3.7% and 5.4%, respectively. We have compared measurements of sRAGE and esRAGE in serum and EDTA plasma samples in a random subgroup of 20 subjects. There was a strong correlation between serum sRAGE and plasma sRAGE levels ($r = 0.99$, $p < 0.01$) and the mean difference ± standard deviation of the differences between serum and plasma samples was 14.7 ± 31.2 pg/ml. The correlation coefficient between serum esRAGE and plasma esRAGE was 0.99, $p < 0.01$ and mean difference was 8.8 ± 18.7 pg/ml. Serum and EDTA plasma levels of soluble RAGE were comparable and serum samples were used in the measurement of sRAGE and esRAGE in our study.

Serum AGEs were measured by a competitive ELISA developed in-house using a well-characterised polyclonal rabbit antisera raised against AGE-RNase as previously described [18]. The polyclonal anti-AGE antibody recognizes N^{ε}-(carboxymethyl)lysine as well as the major non-fluorescent, AGE-crosslink arginine-lysine imidazole and therefore detects pathologically relevant AGEs. The intra- and inter-assay coefficients of variation were 2.5% and 7.4%, respectively.

2.4. Data analysis

Data analysis was performed using Statistical Package for Social Sciences (SPSS for Windows, version 16.0). Results are expressed

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