ORIGINAL RESEARCH

Atorvastatin reduces the expression of aldo-keto reductases in HUVEC and PTEC. A new approach to influence the polyol pathway

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Tobias F Ruf MD¹

Susanne Quintes²

Paula Sternik³

Uwe Gottmann MD³

¹ Department of Cardiology, Herzzentrum Dresden GmbH, University Hospital Dresden, Fetscherstraße 76, 01307 Dresden, Germany.

² Department of Neurogenetics, Max-Planck-Institute of Experimental Medicine, Hermann-Rein-Strasse 3, D-37075 Goettingen, Germany

³ Vth Department of Medicine (Nephrology /Endocrinology/Rheumatology), University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68135 Mannheim, Germany

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Abstract

Purpose: Increased flux of glucose via the polyol pathway, oxidative stress and ischaemia lead to the upregulation of the aldose reductase (AR), the key enzyme of the polyol pathway. This adversely affects the organism and can in part be reduced by inhibition of the enzyme.

Methods: In this study, we examined the effect of the HMG-CoA-reductase inhibitor atorvastatin on the expression of aldose reductase (AR, AKR1B1), aldehyde reductase (AldR, AKR1A1) and small intestine reductase (SIR, AKR1B10) in human umbilical vein endothelial cells (HUVEC) and human proximal tubular epithelial cells (PTEC) by RT-PCR.

Results: In HUVEC, atorvastatin reduces the expression of aldehyde reductase and aldose reductase compared with control medium (-20% and -12% respectively, $P < 0.05$), while small intestine reductase is not expressed. In PTEC no regulation of aldehyde reductase and aldose reductase by atorvastatin could be measured, while the expression of small intestine reductase was reduced by 37% compared

with control medium ($P < 0.05$). The reduction observed was not abolished by the addition of mevalonic acid.

Conclusion: The reduction of members of the aldo-keto-reductase family by atorvastatin is a novel way to influence the polyol pathway and a new pleiotropic effect of atorvastatin.

The activity of the *aldose reductase* (AR, AKR1B1) has extensive effects on different tissues in the organism. It is the key enzyme of the polyol pathway and transforms D-glucose into D-sorbitol. It is a member of the aldo-keto-reductase-family which also includes *aldehyde reductase* (AldR, AKR1A1) and *small intestine reductase* (SIR, AKR1B10). They are expressed in various tissues of the organism and seem to have similar functions.¹⁻³

It has been shown that high concentrations of glucose, as well as oxidative stress and ischaemia lead to an upregulation of AR.⁴⁻⁶ During ischaemia, oxygen supply is too low for cells to produce ATP via the normal aerobic pathways. Therefore, anaerobic pathways have to be utilised to produce the energy needed. The result is the so-called ischaemic cascade, leading to the production of excessive concentrations of lactate. Aside from direct damage to cells, the high concentration of lactate leads to a number of detrimental effects, such as a general inflammation reaction in the tissue, inter alia by activating the protein kinase C (PKC). Moreover, failure of the cell's ion pumps results in an overly high concentration of intracellular Ca^{2+} which causes the release and production of reactive oxygen species (ROS), ATPases, endonucleases and phospholipases.⁷⁻⁹ The ultimate consequence is the destruction of the cell's membranes causing further release of agents which damage the surrounding tissue, inducing apoptosis.¹⁰ Chronic hyperglycaemia causes an increased flux of glucose via the polyol pathway yielding high concentrations of sorbitol.¹¹ Firstly, this gives rise to an oedematous swelling of the cells and an imbalance of ions due to the osmotic properties of sorbitol³ and secondly, a vast change in metabolic function is induced with accumulation of 3-Deoxyglucosone (DOGzn) and Glycerolaldehyde-3-phosphate (GA3P)¹², central precursors of Advanced Glycation End products (AGEs).¹³ Finally, the production of excessive reductive and more oxidative agents in the cell, such as NADH and O_2^- result in further reductive and oxidative stress in the cell fuelling a vicious cycle, entailing cell death. These derogatory mechanisms are associated with a diverse line of diseases such as chronic renal failure^{14, 15}, ischaemic injury¹⁶ or Alzheimer's disease and amyotrophic lateral sclerosis¹⁷⁻¹⁹, some of which are associated with the increased expression of AR.^{14, 16, 20-23}

In several studies the inhibition of AR diminished some of the damage described, making it an excellent target for novel drug modulation. In diabetic rats, inhibition of the AR had anti-oxidative effects²⁴ and

damage to the nerve tissue induced by diabetes was partly reversed²⁵. Also, inhibition of the AR in rabbit hearts undergoing ischaemia resulted in a decreased level of serum creatine kinase and a smaller infarction area^{4, 22, 26, 27}. Also, the ion balance and distribution of ATP was more favourable^{27, 28}.

In a study of ischaemia/reperfusion injury with rats receiving isogenic or allogenic renal transplantation, our group has recently shown that pre-treatment of organ donors with the aldose reductase inhibitor (ARI) epalrestat improved kidney function and reduced renal inflammation after prolonged cold storage and transplantation. The same effect was created through therapy with the Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor atorvastatin. Moreover, a genome wide gene expressing profile of donor kidneys from atorvastatin or vehicle treated rats, revealed a fivefold down regulation of AR in renal tissue of atorvastatin treated rats.²⁹ Renal ischemia is characterized predominately by tubular injury and PTECs are the major site of injury during renal ischemia, so that we were interested whether aldose reductase is reduced in PTECs, as the major site of tissue injury during renal ischemia. Also, endothelial cells are injured during ischemia/reperfusion, so we investigated human umbilical vein endothelial cells for expression of aldose reductase.

In order to translate the latest findings to the human organism, this present study aims to show the effects of atorvastatin on the expression of AR, AldR and SIR in tissues of human umbilical vein endothelial cells (HUVEC) and human proximal tubular epithelial cells (PTEC).

Materials and Methods

Reagents

Reagents were purchased from PromoCell GmbH (Heidelberg), Perkin Elmer (Jügesheim), BD Bioscience (Heidelberg), Fluka/Sigma-Aldrich (Steinheim), Invitrogen Life Technologies (Karlsruhe), Sigma

(Taufkirchen), Serva (Heidelberg), Roche (Mannheim), Pfizer AG (Karlsruhe), all in Germany and Stratagene (La Jolla, CA, USA).

Cell culture

The HUVEC cells were harvested from umbilical cords by a modified method of Jaffé's³⁰, as previously described.³¹ For each series, the cells of one approximately 10 cm long cord were obtained and cultured in 2 ml of Endothelial Cell basal Medium, spiked with the Supplement pack C-39210 and 10 % Vol. FCS (all PromoCell GmbH, Heidelberg, Germany). After reaching confluence, secondary cultures were produced using a one-to-three-ratio. In fourth generation, the cells of one series were treated for 24 hours either with plain medium solution, 10 µM of atorvastatin or 10 µM of atorvastatin and 200 µM of mevalonate.

PTEC cells were obtained from kidneys from patients undergoing nephrectomy due to tumor formation. After transversal slicing of the organ, the cortex was inspected for an area without macroscopically pathological findings. From here, a block measuring about three cubic centimetres was resected and then minced into smaller bits which then were seeded into the flasks for culturing until the cells were confluent. The 2 ml of medium used for each flask consisted of renal epithelial cell growth medium spiked with the supplement pack C-39605 and 10 % Vol. FCS (all PromoCell GmbH, Heidelberg). The secondary cultures were spawned in a one-to-two-ratio. Treatment was carried out in third generation: as in HUVEC, the PTEC were treated for 24 hr either with plain medium solution, 10 µM of atorvastatin or 10 µM of atorvastatin and 200 µM of mevalonate. All experiments proceeded in an environment of 37 °C with an ambient CO₂-concentration of 5 % Vol.. A total of n=4 series was each created for the experiments with HUVEC and PTEC.

RT-PCR

After pre-treatment the cell culture for 24hr with atorvastatin, atorvastatin and mevalonate or control medium we measured the expression of AldR, AR and SIR by RT-PCR. Total RNA was isolated using a modified method from Chomczynski and Sacchi³²: First, the cells were dissolved in 1 ml of the TRIzol agent (Invitrogen Life Technologies, Karlsruhe). Then, 200 µl chloroform were added and after centrifuging at a speed of 12000 rpm at 4 °C the aqueous phase of about 500 µl was transferred to a fresh tube followed by the addition of 500 µl isopropylol and glycogen. After incubation for two minutes at room temperature and another centrifugation for ten minutes, the pellet formed was subsequently washed three times with 75 % Vol. ethanol. Then, the pellets were dissolved in RNase-free DEPC-water. The yield of RNA was checked in each sample spectrophotometrically at an absorbance of A=260 nm. cDNA was transcribed using one microgram of the RNA attained as a template as described in the instructions from the DNaseI-, RNaseOUT-, SuperScript II RT-reaction kits (all Invitrogen Life Technologies, Karlsruhe, Germany).

The oligonucleotides used for PCR were 5' – AGC CTC GCC TTT GCC GA – 3' (β-Aktin fwd), 5' – CTG GTG CCT GGG GCG – 3' (β-Aktin rev), 5' – GGC CTG TCC AAC TTC AAC AGT C – 3' (AKR1 A1 fwd), 5' – CGG CCA TAC TTT TCA GCC AAT – 3' (AKR1 A1 rev), 5' – CCC ATG TGT ACC AGA ATG AGA ATG – 3' (AKR1 B1 fwd), 5' – CAT TGC CCG ACT CAT CCA AT – 3' (AKR1 B1 rev) 5' – GAT GCA GGA TAT CGG CAC ATT G – 3' (AKR1 B10 fwd) 5' – TCC CCA GAC TTG AAT CCC TGT – 3' (AKR1 B10 rev). The primers labeled "β-Aktin" transcribed for Actin, which was used as a housekeeping gene in all experiments.

Amplification of 0.5 µL of the cDNA solution was performed in a total volume of 25 µL containing 19.6 pmol of each primer, 5 mM of dNTPs, 2.5 U Taq polymerase, 10 mM Tris HCl, 7.5 mM KCl, 1.5 mM

MgCl₂. PCR reactions were initiated at 94°C for 3 min, followed by 30 cycles of amplification, each consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C (AKR1B1, AKR1A1 and AKR1B10) or 60°C (β -actin) and primer extension for 2 min at 72°C. At the end of the amplification cycles, the products were incubated for 10 min at 72°C. Control samples were constructed either by omitting cDNA synthesis or without addition of cDNA. PCR products were separated on a 1% agarose gel.

Statistical analysis

The digital images from the gel electrophoreses were loaded into ImageJ 1.37v (National Institute of Health, USA, <http://rsb.info.nih.gov/ij/>), analysing the intensities of the different bands. The results were then standardised.

The program used for statistical analysis was SAS® Version 8.02 (SAS Institute GmbH, Heidelberg, Germany).

The data is shown as means $\pm$ SEM. Differences between groups were assessed by Kruskal-Wallis test. Statistical significance was defined as $P < 0.05$.

Results

Atorvastatin treatment reduces expression of AldR and AR in HUVECs

Actin was used as a control for all experiments (figure 1). Atorvastatin treatment led to a reduction in expression of AldR (table 1) and AR (table 2) by 21 % and 12 % respectively when compared with the control group treated with medium only ($P < 0.05$ for each group vs. control/medium group). To test if this effect could be abolished by adding a downstream metabolite, we treated HUVECs with a concentration of 200 μ M mevalonic acid in addition to atorvastatin. This did not reverse the attained reduction in AldR and AR expression. There was no expression of SIR in any of the cell cultures (data not shown).

FIGURE 1. Gel electrophoresis showing the expression of the housekeeping gene β -Actin in HUVEC. The concentrations used were 10 μ M of atorvastatin, and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. The samples were diluted before transcription, using the dilution factor shown. In the columns, all bars are nearly equal, showing that the amount of cDNA used was nearly equal in each one of the groups.

Atorvastatin has no effect on the expression of AldR and AR in PTECs

We treated PTECs with 10 μ M atorvastatin only or 10 μ M atorvastatin and 200 μ M mevalonic acid using the same paradigm as for the HUVECs. Interestingly, there was no effect of atorvastatin on the expression of AldR (table 3) and AR (table 4) in PTECs. The addition of mevalonic acid had no effect in this respect as well. Here also, the quantity of the expressed housekeeping gene actin was equal in each group (figure 2).

Unlike HUVECs, PTECs express the SIR and, being treated with 10 μ M of atorvastatin, the cells' production of the SIR's mRNA is down regulated by 37

FIGURE 2. Gel electrophoresis showing the expression of the housekeeping gene β -Actin in PTEC. The concentrations used were 10 μ M atorvastatin, and 10 μ M atorvastatin + 200 μ M mevalonic acid. All bars' intensities are nearly equal, showing that the amount of cDNA used was equal in each one of the groups.

HUVEC - AldR (AKR1 A1)

TABLE 1. Relative change in the expression of the mRNA of AldR (aldehyde reductase, AKR1A1) in HUVEC treated with medium only, 10 μ M of atorvastatin and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. Treatment lasted 24 hours in each group. Treatment with atorvastatin or atorvastatin + mevalonic acid reduced the expression of the enzyme by 21 % and 35 % respectively (* $P < 0.05$ for each group vs. the control group/Medium). The data is shown as means $\pm$ SEM.

HUVEC - AR (AKR1 B1)

TABLE 2. Relative change in the expression of the mRNA of AR (aldose reductase, AKR1B1) in HUVEC treated with medium only, 10 μ M of atorvastatin and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. Treatment lasted 24 hours in each group. Treatment with atorvastatin or atorvastatin + mevalonic acid reduced the expression of the enzyme by 12 % and 22 % respectively (* $P < 0.05$ for each group vs. the control group / Medium). The data is shown as means $\pm$ SEM.

PTEC - AldR (AKR1 A1)

TABLE 3. Relative change in the expression of the mRNA of AldR (aldehyde reductase, AKR1A1) in PTEC treated with medium only, 10 μ M of atorvastatin and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. Treatment lasted 24 hours in each group. There is no significant change in between the groups. The data is shown as means $\pm$ SEM.

PTEC - AR (AKR1 B1)

TABLE 4. Relative change in the expression of the mRNA of AR (aldose reductase, AKR1B1) in PTEC treated with medium only, 10 μ M of atorvastatin and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. Treatment lasted 24 hours in each group. There is no significant change in between the groups. The data is shown as means $\pm$ SEM.

TABLE 5. Relative change in the expression of the mRNA of SIR (small intestine reductase, AKR1B10) in PTEC treated with medium only, 10 μ M of atorvastatin and 10 μ M of atorvastatin + 200 μ M of mevalonic acid. Treatment lasted 24 hours in each group. Treatment with atorvastatin or atorvastatin + mevalonic acid reduced the expression of the enzyme by 37 % and 45 % respectively (* $P < 0.05$ for each group vs. the control group / Medium). The data is shown as means $\pm$ SEM.

% when compared to PTEC treated with medium only (table 5; $P < 0.05$ vs. control/medium group). Here also, treatment with 10 μ M atorvastatin in combination with 200 μ M mevalonic acid did not abolish the reduced expression of SIR.

Discussion

The present study shows that the expression of some of the Aldo-Keto-Reductase family members – AKR1A1 (AldR), AKR1B1 (AR) and AKR1B10 (SIR) in particular – is reduced by treatment with atorvastatin, translating our previous findings, showing that atorvastatin pre-treatment reduces the expression of AR in rat kidneys²⁹, to human tissue.

Of particular interest is the fact that the downregulation was not reversed by the addition of mevalonic acid, suggesting an underlying mechanism independent of the established inhibition of the HMG-Co-A-Reductase. In first line, the pleiotropic effects include

stabilisation of arterial plaques and consequently the reduction of cardiovascular and cerebrovascular events.^{33, 34} Worth mentioning is the fact, that these effects do not correlate directly to the mean serum cholesterol level or its reduction³⁵⁻³⁸ nor to the lowering of LDL-cholesterol alone, as the timeframe of the measured positive effects on atherosclerosis, ischaemia/reperfusion damage and tissue function was too short.^{34, 39} Also, there are positive effects of statins on diseases that are not associated with elevated serum cholesterol levels at all, such as Alzheimer's disease and dementia⁴⁰⁻⁴² and even osteoporosis.⁴³ Most of these so called pleiotropic effects of statins are contingent on the inhibition of the enzyme HMG-Co-A-Reductase, leading to the regulation of small GTPases that seem to mediate the effects.⁴⁴⁻⁴⁶ Therefore, the described influence of statins on the tissues can be abrogated by the addition of mevalonic acid. However, there are some implications that are conveyed in a different manner. For instance, statins

block the leukocyte function antigen 1 (LFA-1) by binding at the allosteric centre of the molecule.⁴⁷ Also, statin-mediated gene regulation leading to the activation of pro-inflammatory transcriptional factors is borne autonomously⁴⁸, as well as the direct inhibition of the cytokine-induced activation of NF- κ B.⁴⁹ It remains to be unravelled in what way the down regulation of the AKRs studied here is achieved explicitly.

The next ambiguity is the observation, that the AldR (AKR1A1) and AR (AKR1B1) are being regulated by statins in HUVEC, while they are not affected in PTEC. In our opinion, the explanation of this contrariety lies in the different tissue functions, in which PTEC have a unique role. In the kidney, they are subjected to extreme osmotic stress, especially in anti-diuresis, with intracellular sorbitol ensuring protection.^{23, 50} Thus, it is plausible, that in PTEC additional mechanisms must exist, guaranteeing the enzymes' function in order to keep a basic level of sorbitol concentration. Renal ischemia is characterized predominately by tubular injury and PTECs are the major site of injury during renal ischemia. We have previously shown that treatment with atorvastatin led to a down regulation of aldose-reductase in rat renal tissue. Furthermore, treatment with atorvastatin or an aldose-reductase inhibitor improved functional and morphological signs of renal ischemia in rats.²⁹ Maybe AR or AldR are down regulated in other renal celltypes like glomerular epithelial cells, mesangial cells or fibroblast, which we have not investigated so far. The PTEC observed express the SIR, AKR1B10, while HUVEC do not. It is possible, that this enzyme as well is employed for special cell functions that are innate to PTEC. However, as the SIR in PTEC is downregulated by atorvastatin analogously to the AldR and AR in HUVEC while in PTEC they are not, this possibility seems rather unlikely. Another explanation is that the SIR is expressed in PTEC due to micrometastases since the cells were obtained from kidneys that had to be removed due to tumor formation. Expression of SIR has long been associated with malignancy such as hepatocellular and renal cell

carcinomata.^{51, 52} The inhibition of SIR showed an enhancement of the cytotoxic effects of the anticancer agents doxorubicin and cisplatin in HeLa cervical carcinoma cells.⁵³ Therefore the modulation of SIR by atorvastatin gives reason to more profound research in the field of anticancer therapy.

This study has limitations. We have not performed studies on the cells' protein levels leaving it unclear if there are more effects downstream. Also, we do not know if higher statin concentrations could lower the level of AldR and AR expression in PTEC after all, or if it really is different pathways that regulate their expression.

In summary, we showed a novel approach to modulate the expression of some of the human Aldo-Keto-Reductase family members, including aldose reductase AKR1B1. Its down regulation has been shown to have beneficial effects in the treatment of several diseases. Coevally we disclosed an additional attribute of the pleiotropic effect of statins independent of the drugs' ability to inhibit HMG-Co-A-Reductase, highlighting potentially new areas for medical treatment.

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Correspondence to:

Uwe Göttmann, M.D.
 Fifth Department of Medicine (Nephrology/Endocrinology/
 Rheumatology)
 University Hospital Mannheim, University of Heidelberg
 D-68135 Mannheim, Germany
 E-Mail: Uwe.goettmann@umm.de