Statins and small GTPases: Koch’s postulates and chronic kidney disease

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Introduction

Statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) are attracting more and more attention as cardiovascular and renal protective agents. Evidence from in vitro and rodent experiments indicates that this is not mediated via cholesterol-lowering effects, simply because culture dishes and rodents do not respond to statins with a decrease in plasma cholesterol. However, these so-called pleiotropic effects of statins are much harder to prove in humans, where in secondary prevention trials even...
normo-cholesterolemic subjects exhibit a decrease in LDL cholesterol [1]. Pleiotropic effects of statins appear to depend partly, but not wholly, on the activation of small guanosine triphosphate (GTP)-binding proteins such as Ras, Rho and Rac. The activation depends on a post-translational modification termed isoprenylation. Isoprenoid intermediates of the cholesterol biosynthetic pathway, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) must be attached to these small GTP-binding proteins to achieve activation (Figure 1) [2]. Small GTP-binding proteins display a host of deleterious effects that all impact on the cardiovascular system and the kidney, including downregulation of eNOS, upregulation of NADPH oxidase and activation of the AT1 receptor, to mention just a few.

Other pleiotropic actions of statins include enhancement of eNOS phosphorylation by activating phophatidylinositol 3 (PI3)-kinase/protein kinase Akt [3] and inhibition of caveolin-1 and thus enhancement of eNOS activity [4]. However, statin effects on renal caveolin-1 have not yet been described. Heme oxygenase-1 (HO-1) is also induced by statins [4]. Appropriately for a heat shock protein (HSP); HO-1 is HSP32; the mechanism is hotly debated. Most support exists for the PI3 kinase/Akt, ERK and p38 MAP kinase [5,6] pathways, but direct antioxidant effects of some statins [7] may also induce HO-1 [8]. In renal ischaemia/reperfusion (IR) antioxidant effects of statins may be linked to HO-1 formation [9]. It should be noted that protection has only been observed when statins were administered prior to IR.

**Koch’s postulates**

In 1993, Robert Koch’s postulates were reformulated, in order to establish criteria implicating a cytokine in renal pathology [10,11]. However, just like Koch’s original microbial postulates, the ‘cytokine postulates’ are monocausal. At the Winter School of the Dutch Kidney Foundation (Scheveningen, NL, 11 January 2007), Daha and I debated on whether application of the ‘cytokine postulates’ is useful in complex disease states. Their broad spectrum of effects makes statins hard to resist as add-on medication in chronic kidney disease (CKD) [12–14]. However, to establish causality, it is my thesis that reformulating the ‘cytokine postulates’ is a useful exercise in relation to the pleiotropic effects of statins. To satisfy Koch’s postulates, namely, to prove that activation of a small GTP-binding protein such as RhoA is deleterious in a certain condition, one must identify which substance or pathway is the crucial target in relation to the desired effect. This may well vary from disease to disease, and even between individual subjects. This opinion paper focuses on the application of Koch’s postulates in relation to the effects of statins on small GTP-binding proteins in the setting of CKD.

Koch’s original first postulate was: *The bacteria must be present in every case of the disease.* In the statin context, the first postulate can be rephrased as follows: *Expression or activity of small GTP-binding proteins is increased in renal diseases that respond to statins. This expression or activity correlates inversely with the response to a statin.*

Some *in vivo* studies on CKD exist that fulfil the first postulate. Rats with chronic NO synthase (NOS) inhibition, due to exposure to a high dose of the arginine analogue l-NAME, developed severe hypertension, loss of GFR and albuminuria. The animals also showed increased expression of VEGF and RhoA activity. Co-administration of a statin with l-NAME prevented albuminuria and loss of GFR, and normalized the VEGF expression and RhoA activity. Interestingly enough, these effects occurred without impact on the pressor effects [15]. AngII also induces RhoA, and blocking the AngII AT1 receptor provides a very effective antihypertensive and renoprotective effect in the model of chronic NOS inhibition [16]. Lecian *et al.* indirectly dissected the RhoA stimulating effect of AngII from its hypertensive effect [15]. However, other groups have described antihypertensive effects of statins during chronic NOS inhibition [17,18]; thus this issue remains unsettled. Statins also protected podocyte morphology in puromycin aminonucleoside nephrosis and reduced activated RhoA expression [19]. Note that, when measured, statins had no hypolipidemic effect [17,18], other than that associated with less proteinuria [19]. In streptozotocin-induced diabetic nephropathy, fluvastatin reduced renal injury and Rho activity in association with a pronounced hypocholesterolemic effect, to levels that were numerically but not significantly lower than cholesterol in non-diabetic controls [20]. Thus indeed, the first postulate has been verified in different models of CKD, although in diabetic nephropathy this was not strictly a pleiotropic effect.

Koch’s second postulate was: *The bacteria must be isolated from the host with the disease and grown in pure culture.* For statins this can be rephrased as follows: *Activation of small GTP-binding proteins should elicit an effect relevant to renal pathophysiology in target cells or in tissue (in vitro). This effect should be reversible by statins.*

This second postulate has been verified for RhoA activation in a number of *in vitro* studies that mimic different aspects of renal disease. These studies include glomerular endothelial cells exposed to the permeability factor VEGF [21], endothelial cells exposed to an HLA antibody as in chronic allograft nephropathy [22], mesangial cells exposed to high glucose as in diabetic nephropathy [23,24] or VEGF to stimulate matrix production [25], podocytes exposed to puromycin aminonucleoside as in experimental nephrotic syndrome [20], proximal tubular cells (PTC) exposed to albumin as in the nephrotic syndrome [26,27] and renal fibroblasts exposed to the profibrotic factor CTGF [28]. Even the effects of renal ageing can be blocked *in vitro* by statins. AngII downregulates the *in vitro* expression of klotho, an anti-ageing protein. Statins can block this downregulation in conjunction with RhoA deactivation in collecting duct cells [29].

Similar findings have been reported for statins and Rac1 activation in renal cells. Thus statins block Rac1 activation in PTC exposed to albumin [27], and Rac1 and RhoA activation and epithelial-to-mesenchymal transition (EMT) in activated PTC [30]. Studying circulating cells of subjects treated with a statin made an important link between *in vitro* and *in vivo* results. Using 2D electrophoresis, Cicha *et al.* [31] could show a shift towards the non-isoprenylated form for both RhoA and Rac1 in freshly isolated human
Fig. 1. Biological actions of isoprenoids. A diagram of the cholesterol biosynthesis pathway showing effects of inhibition of HMG-CoA reductase by statins. Decrease in isoprenylation of signalling molecules, such as Ras, Rho and Rac, leads to modulation of various signalling pathways [2]. BMP-2: bone morphogenetic protein-2; eNOS: endothelial nitric oxide synthase; t-PA: tissue-type plasminogen activator; ET-1: endothelin-1; PAI-1: plasminogen activator inhibitor-1.

peripheral mononuclear cells after treatment with a statin in vivo.

Koch’s third postulate was: The specific disease must be reproduced when a pure culture of the bacteria is inoculated into a healthy susceptible host; this can be rephrased as follows: Overexpression of small GTP-binding proteins (in vitro or in transgenic animals) should elicit an effect relevant to renal pathophysiology. This effect should be reversible by statins. There is a temporal relation between overexpression of the small GTP-binding proteins and pathophysiologic manifestations, and conversely reversal of the pathology by statins.

Statins increase eNOS expression and inhibit Rho membrane translocation and GTP-binding activity in endothelial cells. Conversely, activation of Rho by E. coli cytotoxic necrotizing factor-1 decreases eNOS expression. Finally, inhibition of Rho by the exotoxin, C. botulinum C3 transferase, or by overexpression of a dominant-negative RhoA mutant increased eNOS expression [32]. In vascular smooth muscle cells, statins increased apoptosis by inhibiting expression of the (constitutive) anti-apoptotic protein Bcl-2. This is associated with decreased isoprenylation of RhoA. Overexpression of RhoA partially prevented both downregulation of Bcl2 and apoptosis [33]. However, no studies have addressed the third postulate in renal cells, let alone in a model of CKD. It is not known whether overexpression of small GTP-binding proteins in transgenic animals indeed leads to (enhanced sensitivity for) renal injury. To my knowledge such models are not available.

Finally, Koch’s fourth postulate was: The bacteria must be recoverable from the experimentally infected host.

After the discovery of antibiotics, the fourth postulate was commonly appended with the statement: Disease symptoms should be reversed by treatment with a specific antibiotic. For statins this can be rephrased as follows: Inhibition of small GTP-binding proteins (in vitro or in transgenic animals) should reverse or prevent renal pathophysiology. This effect should be mimicked by statins and refractory to statins after specific inhibition of small GTP-binding proteins.
Statins reduced AngII AT1 receptor mRNA and protein expression in vascular smooth muscle cells and this can be mimicked by overexpression of the dominant-negative form of RhoA, or by inhibiting RhoA with a specific inhibitor C3 exotoxin [34]. Fasudil, a specific rho kinase inhibitor, ameliorated puromycin aminonucleoside-induced podocyte damage and proteinuria [20], prevented renal injury and proteinuria in AngII-induced hypertension without affecting blood pressure [35]. Interestingly, Rho kinase inhibition prevented the acute hypertensive effect of AngII [36], which appears to involve calcium-dependent constriction of the afferent but not efferent arterioles [37,38]. Fasudil can prevent the development of diabetic nephropathy either induced by streptozotocin [20] or occurring spontaneously in obese OLETF rats with type II diabetes, but it did not appear to induce regression of established renal injury [39]. Protection by Rho kinase inhibition was also observed in unilateral ureter obstruction, in hypertensive Dahl salt-sensitive rats, in CKD induced by renal ablation in spontaneously hypertensive rats (SHR) [40] and in salt-loaded stroke-prone SHR [41]. In all cases, protection was achieved without any change in blood pressure. Thus the first part of the fourth postulate has been extensively addressed. However, no studies have really challenged the fourth postulate by combining a Rho kinase inhibitor with a statin in an established model of CKD.

Meta-analysis provides support for the idea that statins can reduce albuminuria or proteinuria above 30 mg/day [42]. Rho-kinase inhibitors such as Fasudil are already in clinical trials for pulmonary hypertension. Thus, the fourth postulate can theoretically also be addressed in humans. If pleiotropic mechanisms are important for renal protection, proteinuric patients undergoing treatment with a Rho-kinase inhibitor, for instance for pulmonary hypertension, should be refractory to the antiproteinuric effects of statins.

Thus, based on the first and second postulates, statins appear to have pleiotropic effects, mediated by the small GTP-binding proteins RhoA and Rac1 in many relevant models of CKD. However, to clinch the argument, the third (temporal relation) and fourth (reversibility) postulates require more testing.

**Pleiotropic effects of statins in humans**

At least two other important questions need to be addressed. First, and most importantly, do beneficial pleiotropic effects of statins on renal function and injury occur in humans?

Dissecting pleiotropic and hypolipidemic effects of statins *in vivo* requires kidney tissue; thus the first and third postulates cannot be addressed in humans. However, as mentioned above Cicha et al. [31] elegantly tackled the second postulate, by studying peripheral mononuclear cells after treatment of healthy human subjects with a statin for 5 days and found decreased isoprenylation of small GTP-binding proteins. This cell population includes monocytes, the precursors of infiltrating macrophages that play an important role in renal inflammation [43]. My proposal is that finding decreased isoprenylation of small GTP-binding proteins in these cells after a short period of statin treatment may well predict whether pleiotropic effects could benefit patients with CKD during prolonged treatment with the drug. Promising data are appearing on the use of circulating karyocytes, particularly monocytes, in individual care in patients with cardiovascular disease [44].

**Differences between statins**

Second, do the pleiotropic effects vary between different statins? With respect to their hypolipidaemic effects in humans, all statins appear to be qualitatively equal, although differences in potency require dose adjustment [45]. However, to paraphrase George Orwell, regarding their pleiotropic effects, some statins are more equal than other statins. More than 10 years ago, we observed that lipophilic simvastatin and lovastatin induced severe muscle toxicity in young rats whereas hydrophilic pravastatin did not [46]. Different statins have different antioxidant capacities [7]. Fluvastatin, a statin with a known hydroxy radical scavenging effects [47], had renoprotective and anti-oxidative effects and increased renal glutathione peroxidase activity in streptozotocin-induced diabetic nephropathy [48] and in unilateral ureteral obstruction [49]. Differences in anti-oxidative capacity may explain some differences in renal protection, but other properties also play a role. In salt-loaded stroke-prone SHR, the hydrophilic rosvastatin was renoprotective without any change in blood pressure or plasma lipids, whereas the lipophilic simvastatin was not [50]. Interestingly, only rosvastatin decreased the number of macrophages in this model.

**Conclusion**

Statins appear hard to resist as add-on medication in CKD. However, in normolipidaemic CKD subjects, it would theoretically be more efficient, pharmacologically safer, and intellectually more satisfying, if, by sampling peripheral blood monocytes before and after a short ‘challenge’ with a statin, one could ascertain whether in a particular subject isoprenylation can be suppressed, before relying on the pleiotropic effects of statins.

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**References**


Haemodiafiltration: promise for the future?

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Introduction

During haemodiafiltration (HDF), diffusive and convective transport are combined for the removal of waste solutes. Fluid removal exceeds the desired weight loss, and fluid balance is maintained by infusion of a sterile pyrogen-free solution. This dialysis modality may offer advantages, as compared to haemodialysis (HD) or haemofiltration (HF) used separately. This brief editorial comment summarizes currently available knowledge on technical and (pre-)clinical aspects of HDF, as well as currently ongoing trials.

Theoretical background

In HDF, not only small molecules (<5 kDa) are removed more effectively as compared to low-flux HD, but in addition, a considerable clearance of so-called middle molecular weight (MMW) substances (5–50 kDa) is obtained [1]. Beta2-microglobulin (B2M, MW 11.8 kD) is a typical example of this category and is strongly associated with the presence of carpal tunnel syndrome and dialysis-related amyloidosis in chronic HD patients. In the HEMO study (see details below), predialysis B2M levels were associated with all-cause mortality, even when adjusted for residual renal clearance [2]. These data suggest that B2M can be used as a marker for MMW toxins which contribute to the extremely high mortality in chronic HD patients, although a direct relationship between B2M levels and mortality is lacking.

Other examples of MMW molecules include markers of inflammation such as IL-6, TNF-a and complement factor D and other molecules that might be relevant in the pathogenesis of cardiovascular morbidity and mortality, such as advanced glycation end products (AGEs) and mediators of oxidative stress [3].

HD using high-flux membranes can be considered as a form of HDF, because the pressure drop along the fibres induces filtration that can be considerable (8–10 L per treatment). The total amount of ultrafiltration exceeds the required weight loss and is compensated by backfiltration. However, the exact volume of filtration in high-flux HD is unpredictable, unmeasurable and fluctuates per treatment.

In HDF, the volume of ultrafiltration can be larger (10–30 L per treatment in the postdilution mode) and can be controlled. The substitution volume infused into the patient compensates for the total ultrafiltration volume (i.e. convective volume) minus the desired weight loss. It can be added downstream (postdilution) or upstream (predilution) from the dialyser. In the latter mode, less small molecular clearance is obtained for a given filtration volume, as diffusion is less effective when compared to the postdilution mode. In predilution HDF, the concentration-gradient driven