Treatment with atorvastatin partially protects the rat heart from harmful catecholamine effects

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1. Introduction

Heart failure is accompanied by chronic activation of the sympathetic nervous system, the magnitude of which is inversely correlated with survival.1 Noradrenaline acutely augments cardiac contractile function, but chronically induces adverse effects such as apoptosis, hypertrophy, and energy depletion. It also leads to downregulation of β1-adrenergic receptors (β1-AR) and causes desensitization to further stimulation, which is generally viewed as a beneficial, protective response.2–4

Statins inhibit HMG CoA (3-hydroxy-3-methylglutaryl-coenzym-A) reductase, the key enzyme in the cholesterol synthesis pathway, which catalyzes the conversion of HMG CoA to mevalonate. In consequence, statins not only inhibit the synthesis of cholesterol, but also the formation of isoprenoids. The latter serve as lipid attachments of key signalling molecules including monomeric GTPases of the Rho and Ras families. Reduced isoprenylation is assumed to account for most of the cholesterol-independent effects of statins.5

We have recently shown that atorvastatin reduces isoprenylation of heterotrimeric G protein γ-subunits in neonatal rat cardiac myocytes. This effect was accompanied by a reduction in total Gγs, cAMP generation, and contractile response to isoprenaline as tested in engineered heart tissue and was seen without a decrease in β-AR density.6

The effect of atorvastatin was cholesterol-independent and reversed by geranylgeranylpyrophosphate. Given the eminent role of the β1-AR/cAMP/PKA pathway in regulating heart function, this effect of statins could be clinically meaningful. The present study examined two questions. (i) Does pre-treatment of normal rats with atorvastatin affect the in vivo heart rate under normal or stressed conditions or the inotropic response of isolated heart preparations to isoprenaline? (ii) Does pre-treatment with atorvastatin affect the detrimental effects of chronic catecholaminergic stimulation on the heart?

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2. Methods

2.1 Animals and experimental design

A total of 90 male Wistar rats (Charles River Laboratories) were maintained with water and food ad libitum at constant humidity and temperature with a light/dark cycle of 12 h. They were held in separate cages. The animals (150–200 g) were randomized into six groups (n = 15 each) to receive 1 or 10 mg/kg × day atorvastatin (SORTIS®, Pfizer) or water per gavage. For the last 4 days, rats were additionally treated with isoprenaline (1 mg/kg × day) or vehicle (2 mM HCl in isotonic NaCl) via osmotic minipumps (model 2002, Alzet, Direct) implanted subcutaneously in the neck under isoflurane (0.5–2%) anaesthesia. After 14 days, animals were sacrificed by carbon dioxide inhalation and cervical dislocation. The time of treatment was chosen with regard to the half-life of atorvastatin (18 h in humans) and the aim to get stable conditions before start of the isoprenaline/NaCl infusion (assumed steady state after 5 × half-life, security factor of 3). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Ambulatory ECG recordings

ECG transmitters (PhysioTel CA-F40, DataSciences) were implanted under general anaesthesia (isoflurane). The negative lead was placed subcutaneously in the area of the right shoulder and the positive lead left of the xiphoid space caudal the rib cage approximating an Einthoven Lead II configuration. All rats were allowed to recover for 7 days before the drug regimen was started. Data were acquired using Dataquest A.R.T. (v 3.01, DataSciences). For evaluation of the circadian rhythm, the average heart rate of 1 min was recorded every 5 min resulting in 12 data points per hour. After implantation of ECG transmitters, rats were individually housed in cages placed on a telemetric receiver (RPC1, DataSciences) in a temperature-controlled room (22 ± 1°C).

2.3 Restraint stress test

Rats were subjected to a restraint stress test for 60 min followed by 10 days of treatment with atorvastatin or water. Animals were placed in a plexiglass tube (6 cm diameter) with holes for fresh air supply. The length of the tubes was adjusted by plastic plugs such that the animals were unable to move/turn around. Heart rate was recorded continuously and averaged for each minute. Baseline heart rate was recorded for a period of 10 min immediately before the confinement. Following the restraint test, animals were transferred to their cage for recovery.

2.4 Quantitative RT–PCR (qRT–PCR)

Total RNA was prepared from left ventricles and quantitative RT–PCR (qRT–PCR) was done essentially as described previously. Probes were designed to cross exon/intron boundaries of the atrial natriuretic peptide (ANP) gene (GAPDH forward AACTCCCTCAA-GATTGTGACAA, reverse CAGCTTCTGAGCGGATGATG, probe AT GGACTGTGGTCATGAGCCCTTCCA; ANP forward CTGGGACCCCTC CTGATAG, reverse TGCTTACC3GGAACCTGTTG, probe TAGTCGGCTC TGGGCTCAATCCT). GAPDH transcript levels were identical in the study groups and were used to normalize for differences in RNA quantity and RT-efficiency. Standard curves were performed in triplicate with serially diluted cDNA (100 pg-100 ng) to determine PCR efficiency. Quantification was performed by the standard curve and 2–ΔΔCt method.

2.5 Force measurement in isolated left atria

Experiments were performed on isolated, electrically stimulated left atria, as previously described, in parallel (one animal per group per day) and in varying order. Left atria were chosen as an easily accessible, stable cardiac muscle preparation. Briefly, the heart was quickly removed under carbon dioxide narcosis after cervical dislocation, rinsed in Tyrode’s solution, carefully stripped from buffer between paper towels, and weighed to determine heart-to-body weight ratio. During preparation of left atria, the heart was kept in continuously oxygenated (95% O₂, 5% CO₂, 37°C, pH 7.4) modified Tyrode’s solution containing 119.8 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.05 mM MgCl₂, 0.42 mM NaH₂PO₄, 22.6 mM NaHCO₃, 0.05 mM Na₂EDTA, 0.5 mM ascorbic acid, and 5 mM glucose. The atria were individually mounted vertically in a glass tissue bath (25 mL bath volume) and attached to an isometric force transducer (Ingenieurbu√®cke Jäckel). Muscles were electrically stimulated (rectangular, 1 Hz, 5 ms, 80–100 mA) directly after mounting, equilibrated for about 10–45 min and, after exchange of Tyrode’s solution, stretched stepwise to Lmax. Force was measured under cumulative increases in extracellular calcium (1.8–7.2 mM) and isoprenaline (0.001–3 μM) in the presence of 1.8 mM calcium.

2.6 Radioligand binding experiments

Total β-AR density was determined in crude membrane fraction as previously described. One hundred microgram protein per assay was incubated with 3 nM of the non-specific β₁/β₂ antagonist [³H]-CGP 12177 ([3-tertiarybutylamino-2-hydroxypropoxy]-benzimidazole-2-on) for 90 min at room temperature. Non-specific binding was determined with 10 μM of the non-selective β-AR antagonist nadolol.

2.7 Subcellular fractionation and western blot

Frozen left ventricle (100–150 mg) was powdered and homogenized in 1.5 mL homogenization buffer (20 mM Tris–HCl pH 7.4, 1 mM EDTA, 1× complete) using a Qiagen TissueLyser. The supernatant of the low spin centrifugation step (15 min, 4°C, 2000 rpm) was subjected to ultracentrifugation (1 h, 4°C, 34000 rpm). The resulting pellet (particulate fraction) was resuspended in 60 μL homogenization buffer with 1% Triton-X 100, the supernatant was termed cytosolic fraction. Protein concentrations were determined by Bradford assay. For SDS–PAGE analysis, 100 μg protein was loaded onto the gel to determine Gβγ, Gγ, and Go, and 40 μg for the detection of Gβ. Equal amounts of denatured protein were subjected to SDS–PAGE on a 10% polyacrylamide gel (Gαs and Gββ, αγ, or to gels with polyacrylamid concentrations of 10% in the top half and 17% in the bottom half (Gγ) and Gγ). The following antibodies were used: anti-Gαs, polyclonal antibody (1:400, Santa Cruz), anti-Gβ (1:500, Santa Cruz), anti-Gγ, anti-γ (1:300, Santa Cruz), and horseradish peroxidase-conjugated secondary antibodies (1:10,000, Sigma). All antibodies were diluted in 5% fat-free milk powder in TBST (140 mM NaCl, 10 mM Tris, 1% Tween 20). Bands were visualized by enhanced chemiluminescence (for Gαs and Gββ, αγ, SuperSignal West Pico Substrate and for Gγ, SuperSignal West Dura, Pierce).

2.8 Statistical analysis

Data were calculated as mean ± SEM. Unpaired Student’s t-tests (two-sided) were used to compare means between two independent groups or samples. Paired t-test (two-sided) was used to compare values in rats before and after treatment. Concentration response curves were analysed by F-test. Differences in heart rate were analysed using one-way ANOVA followed by Bonferroni’s post hoc test. A P-value < 0.05 was considered significant.

3. Results

3.1 Atorvastatin had no effect on heart rate

Heart rate ranged between 380 and 450 bpm under unrestrained conditions and showed a clear day–night rhythm. No differences were observed between the six groups (measured at day 9, Figure 1A). Under stress induced by...
restraint, heart rate quickly rose from ~380 to ~500 bpm in all groups without apparent differences (Figure 1B). Figure 1C and D displays the heart rate of animals receiving NaCl (Figure 1C) or ISO (Figure 1D) via minipumps. Since heart rate at days 12, 13 and 14 was similar for each animal, it was averaged over these 3 days. Compared with NaCl, infusion of ISO caused a marked increase in heart rate at day time (resting period: ISO ~500 bpm, NaCl ~375 bpm) and a smaller increase at night (activity period: ISO ~500 bpm, NaCl ~440 bpm). Pre-treatment with atorvastatin did not affect the heart rate response to ISO (Figure 1D). Animals pre-treated with atorvastatin and infused with NaCl showed a slightly lower maximum heart rate during the activity period than water-pre-treated ones (Figure 1C). Such difference would be compatible with the expected anti-adrenergic effect of atorvastatin. However, a further series of experiments (atorvastatin high dose vs. water, n = 6 each) did not reveal any consistent effect on heart rate of unrestrained animals of atorvastatin (data not shown), suggesting that the small difference occurred by chance or was related to pump implantation.

3.2 Atorvastatin attenuated hypertrophic effects of ISO infusions

Chronic infusion of ISO in water-pre-treated rats caused an increase in heart-to-body weight ratio by 19% compared with vehicle (Figure 2A). Atorvastatin pre-treatment reduced this hypertrophic effect to 14% (low dose, ~26%, n.s.) and 11% (high dose, ~42%, P < 0.05 vs. water), respectively. Atorvastatin alone did not alter heart-to-body weight ratio. Chronic ISO infusion increased the transcript levels of ANP in the left ventricle 4.5-fold. The low and the high dose of atorvastatin reduced the increase to 2.3- (~63%) and 2.8-fold (~49%), respectively (Figure 2B).

3.3 Atorvastatin had minor effects on contractile force in left atria

Force of contraction under basal and cumulatively increasing concentrations of extracellular Ca$^{2+}$ did not differ between the six groups (Figure 3A and B) and was therefore used to normalize the force of contraction under increasing isoprenaline concentrations (Figure 3C and D). According to our previous data in neonatal rat heart cells, we had expected that atorvastatin treatment would reduce the inotropic effect of β-AR stimulation. Indeed, the positive inotropic effect of isoprenaline was lower in atria from rats pre-treated with atorvastatin at high dose (Figure 3C). However, the difference was rather minor (~10%) and did not apply to the low dose group. EC$_{50}$ values were identical in the three groups (~20 nM; data not shown).

3.4 Atorvastatin attenuated the desensitizing effect of ISO infusions

Chronic infusion of ISO induces subsensitivity of the heart to further β-AR stimulation, but generally does not affect the response to Ca$^{2+}$. Indeed, the 4 day infusion of ISO resulted in almost 50% reduction of the positive inotropic response in the group pre-treated with water (compare Figure 3C and D) without significant differences in the Ca$^{2+}$ response (compare Figure 3A and B). The EC$_{50}$ values were four-fold higher in the ISO treated groups (~80 vs. 20 nM). Thus, ISO infusion caused the expected...
of the membrane content of $G_{\alpha 3}$ (long and short form) by 4% (n.s.) and 37% at low and high doses, respectively (Figure 4B). Accordingly, $G_{\alpha 3}$ in the cytosolic fraction was increased (Figure 4A). Neither high nor low dose atorvastatin administration affected $G_{\beta}$ distribution (Figure 4C and D). $G_{\gamma 3}$ was detectable in the membrane fraction with an apparent weight of 10 kDa, but was undetectable in the cytosolic fraction. Atorvastatin treatment dose-dependently induced a reduction in $G_{\gamma 3}$ membrane content by 24% and 50% in the low and high dose group, respectively (Figure 4E). Similar changes were seen in animals treated by atorvastatin and ISO infusion (Figure 4F). $G_{\alpha 12}$ protein levels were not affected by atorvastatin (data not shown).

4. Discussion

The present study aimed at evaluating potential antiadrenergic effects of atorvastatin in rats. Oral treatment with atorvastatin for 14 days induced partial drop-out of $G_{\gamma 3}$ and $G_{\alpha 3}$ from cardiac membranes. This was associated with a 10% reduction of the maximal inotropic effect of $\beta$-AR stimulation in isolated left atria and partial prevention of the desensitizing, hypertrophic, and ANP-increasing effects of chronic ISO infusion. The overall effect size was small. This may be related to dosing of atorvastatin. Compared to other studies with atorvastatin (using doses between 2 and 50 mg/kg × day in rats), the two doses of 1 and 10 mg/kg × day can be considered as low and moderate. Atorvastatin or water was given by daily gavage, excluding problems of drug intake via the food. Analysis of the lipid profile by HPLC revealed no differences between the groups (data not shown). This is in accordance with previous studies showing that atorvastatin delivered to normal Chow-fed rats did not affect plasma cholesterol and lowered triglycerides only at 25 mg/day.12 Similarly, rosuvastatin at 20 mg/kg/day did not affect the lipid profile in rats.13 The maximal licensed dose of atorvastatin in patients (80 mg/day ~1 mg/kg × day) achieves maximal plasma levels of 0.3–0.5 μM in humans,14,15 but only 0.03 μM in rats.15 Thus, the high dose atorvastatin used in our study (10 mg/kg × day) can be expected to achieve 0.3 μM, corresponding to the normal high dose regimen in humans. Our previous study in cultured cardiac myocytes showed that significant desensitization started between 0.1 and 1 μM.6 Thus, the moderate effect size seen in this study appears realistic.

Treatment with atorvastatin was not associated with a significant reduction in heart rate. This may seem at variance to previous studies showing that statins lowered sympathetic activity in patients with stable ischaemic heart disease,16 stroke-prone spontaneously hypertensive rats,17 and rabbits with heart failure.18 However, these data were obtained in models with increased sympathetic drive, whereas we tested atorvastatin in normal Wistar rats. Statins may also increase cardiac parasympathetic responsiveness.19 This mechanism is lipid-dependent and involves increased expression of acetylcholine-activated potassium channels and $G_{\alpha 12}$. The fact that $G_{\alpha 12}$ levels were unchanged in the heart of atorvastatin-treated rats argues against the involvement of this mechanism under our conditions. The lack of heart rate effects suggests that the reduction in functional $G_{\gamma 3}$ in cardiac membranes is either not relevant for the regulation of beating rate or that $G_{\gamma 3}$ levels in the

3.5 Atorvastatin did not affect total $\beta$-AR density, but reduced membrane localization of $G$ proteins

Infusion of ISO resulted in a reduction of total $\beta$-AR density by 37% (36 ± 8, $n = 13$, vs. 56 ± 8 fmol/mg protein in NaCl/water, $n = 13$). Atorvastatin pre-treatment alone did not change $\beta$-AR density (50 ± 8 fmol/mg protein in NaCl/Ator low, $n = 13$; 60 ± 10 fmol/mg protein in NaCl/Ator high, $n = 13$) nor its downregulation by ISO (36 ± 6 fmol/mg protein in ISO/Ator low, $n = 13$; 40 ± 7 fmol/mg protein in ISO/Ator high, $n = 13$). However, atorvastatin pre-treatment was associated with a decrease
sinoatrial node cells (in contrast to left ventricle) were not affected by atorvastatin.

The effects of atorvastatin on isoprenaline-induced cardiac hypertrophy and ANP were robust, amounting to a relative reduction by 26–63%. Similarly, the reduction in the membrane content of $G_\gamma_3$ and $G_\alpha_s$ amounted to 40–50% in the high dose group, providing direct biochemical evidence for an action of the drug on the heart. Fourteen different $G_\gamma$ isoforms exist\(^20\) of which $G_\gamma_3$, $G_\gamma_5$, $G_\gamma_7$, and $G_\gamma_{12}$ have been detected in the heart.\(^21\)–\(^23\) We concentrated on $G_\gamma_3$ which is known to be cardiac myocyte-specific.\(^21\) $G_\gamma_7$ was detectable as well and was also reduced in atorvastatin-treated rats (data not shown). The $\gamma$-subunit requires geranylgeranylation for membrane anchorage and normal function.\(^24\) On the basis of our previous study in neonatal rat cardiac myocytes,\(^6\) the present data suggest that treatment with atorvastatin partially depletes the pool of isoprenoid moieties needed for geranylgeranylation in the cell and consequently results in a decrease in $G_\gamma_3$ (and $G_\gamma_7$) membrane content. Since newly synthesized palmitoylated $G_\alpha_s$ requires fully processed $G_\beta_\gamma$ to be inserted into the plasma membrane correctly,\(^24\) the drop-out of $G_\alpha_s$ from the membrane and the accumulation in the cytosol are likely consequences of the reduced membrane anchorage of $G_\gamma_3$ and $G_\gamma_7$. Unexpectedly, we did not observe a change in $G_\beta$ content, neither in the cytosolic nor in the particulate fraction. Possibly, the amount of $G_\beta$ in the membrane and in the cytosol is too high to detect such slight effects generated by atorvastatin. Alternatively, $G_\beta$-subunits are able to attach to the cell membrane independently of correctly modified $\gamma$-subunits.\(^25\)

It is interesting to note that atorvastatin treatment partially protected the heart from the consequences of a prolonged infusion of a moderate dose of isoprenaline (less hypertrophy and ANP, slightly larger inotropic response to isoprenaline) despite the lack of heart rate effects and only very minor effects on $\beta$-AR responsiveness when given alone. This could indicate that protection from the 4-day ISO infusion represents an integrated effect over the entire period which is easier to determine than acute inotropic responses. Alternatively, the effects of atorvastatin on ISO-induced hypertrophy and ANP are unrelated to the effect on G proteins and due to other mechanisms, e.g. effects on small G proteins.\(^26\) The effect of atorvastatin on G proteins was also more pronounced than the effect on $\beta$-AR contractile responses. This could indicate a signalling reserve, i.e. the phenomenon that a full inotropic $\beta$-AR response in the rat heart requires only a fraction of receptors to be occupied.\(^27\) The present data would suggest that such reserve also exists in terms of G proteins.

Do our findings have clinical implications? The effects of atorvastatin alone on cardiac contractile responses to isoprenaline were small and likely not meaningful. On the other hand, the antihypertrophic effects seen under continued high $\beta$-AR stimulation could add to the beneficial cardiovascular profile of statins, particularly under excessive catecholamine stimulation as in heart failure.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Isometric force of contraction in isolated, electrically driven left atria. The respective 4 day infusion via minipump is indicated above [NaCl for (A) and (C) and isoprenaline (ISO) for (B) and (D)]. Response to cumulative increases in extracellular [Ca\(^{2+}\)] in atria from NaCl- (A) and ISO- (B) treated animals. Effect of atorvastatin pre-treatment on the positive inotropic effect of increasing concentrations of isoprenaline normalized to individual maximal Ca\(^{2+}\) responses (C and D).}
\end{figure}
Statins increased parameters of pump function in non-ischaemic heart failure, but did not reduce mortality in older patients with systolic heart failure in the CORONA study. This argues against the idea that statins generally exert beneficial effects in patients with heart failure. Of note, 75% of patients in this study were on β-blockers which likely obscures any anti-adrenergic effect of statins. In conclusion, atorvastatin exerts a mild anti-adrenergic effect in rats at a dose equivalent to the maximal licensed dose in humans. This effect is most likely mediated by a partial drop-out of Gγ and Gαs from the membrane and can be viewed as a potentially beneficial one. The relevance of this finding remains open, particular in view of the widespread use of β-blockers.

Figure 4  Cellular localization of G proteins. The long and short form of Gαs (A and B) and Gβ (C and D) were determined by western blot in cytosolic (A and C) and particulate fractions (B and D) of left ventricles of rats treated for 14 days with water or atorvastatin and for 4 days with infusion of NaCl. β-actin was used as loading control. (E and F) Gγ3 in particulate fractions of rats treated with NaCl (E) or ISO (F). Number in columns is equal to number of animals studied.
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