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The anti-carcinogenic effect of statins in a rat model correlates with levels and synthesis of ubiquinone

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ABSTRACT

Ubiquinone (Q) is a product in the cholesterol synthesis pathway and is an essential component of the respiratory chain in the mitochondrial membrane. In addition, extra-mitochondrial Q has anti-oxidative properties and this fraction is increased during carcinogenesis. The aim of the present study was to investigate if extra-mitochondrial level of Q is affected by statin treatment in a rat model for liver cancer, and if this change correlates with inhibited carcinogenesis. To do this we isolated sub-cellular fractions of rat livers from a previous experiment where we have shown anti-carcinogenic effects of statins. The levels of Q_8, Q_9 and Q_{10} were analysed with liquid chromatography–mass spectrometry. The Q_9-levels, constituting the major part of Q in rats, were not significantly affected in any of the sub-cellular compartments. The levels of Q_{10}, constituting a minor part of Q in rats but the major part of Q in humans, were significantly decreased by about 60% in the statin treated rats. The decrease was present in all sub-cellular compartments, but was most pronounced in the cytosol. There was a significant correlation between extra-mitochondrial Q_{10} levels and inhibited carcinogenesis. No such correlation was observed with extra-mitochondrial Q_9. The reduced Q_{10}-levels might be explained by the reduced availability of isoprene units during statin treatment, shifting the synthesis towards isoforms with shorter side-chains. In line with this hypothesis there were increased levels of Q_8-levels during statin treatment.

The results support our previous suggestion that at least part of the anti-carcinogenic effect of statins in our rat model is mediated by effects on synthesis of Q. We also demonstrate a shift in the Q-synthesis pathway towards isoforms with shorter side-chains during statin treatment. The ratio between the different Q-isoforms might be used as a more sensitive marker of statin-induced inhibition of Q than measuring total Q levels.

1. Introduction

Statins are competitive inhibitors of hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase, thus inhibiting the endogenous synthesis of cholesterol in the body. In humans statins efficiently reduce serum cholesterol levels and are protective against cardiovascular disease [1]. Statins are today some of the most prescribed drugs. During recent years it has been proposed that statins have beneficial anti-carcinogenic effects [2–4]. Recently we showed that lovastatin could efficiently inhibit carcinogenesis in a rat model of liver cancer [5].

Statins do not only inhibit the synthesis of cholesterol but all components in the mevalonate pathway are affected (Fig. 1). Ubiquinone (Q) is one of these products. Q is a lipid that is crucial in the respiratory chain and thus a large part of Q is found in the mitochondria [6]. The extra-mitochondrial Q has anti-oxidative properties [7] and the extra-mitochondrial, but not the mitochondrial, ubiquinone levels are increased in preneoplastic liver tissue [8].

The number of isoprenoid units of the side-chain is different in different species. In rat the most prevalent Q is Q_9 and Q_{10} represents only a small fraction of the total Q. In humans Q_{10} is the most prevalent Q and Q_9 constitutes only a small fraction of the total Q [9]. However, Q_9 and Q_{10} are considered to have the same function in the cell and are interchangeable. The mechanism behind the regulation of synthesis of the different Q-isoforms is unknown [9]. However, it is reasonable to think that if the synthesis of Q in the rat is reduced

Abbreviations: Q, ubiquinone; HMG-CoA, hydroxymethyl-glutaryl coenzyme A; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy.

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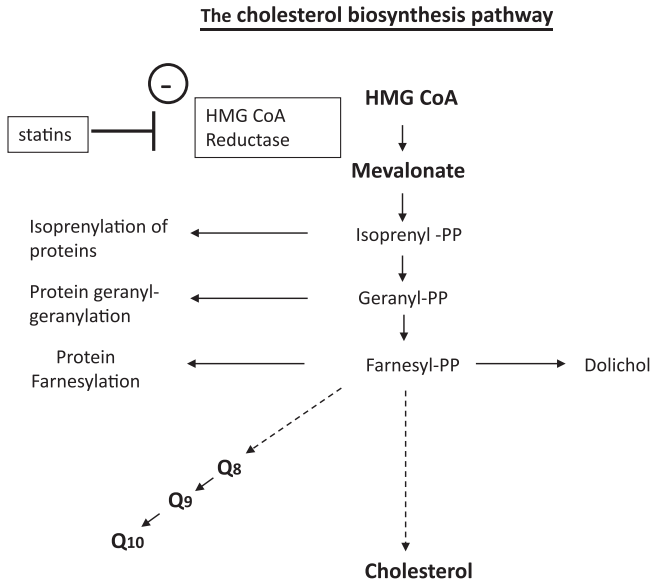

Fig. 1. A scheme of the cholesterol biosynthesis pathway. Q, ubiquinone.

due to a lack of isoprene units, the levels of Q_{10} would be expected to be reduced more than Q_9. If Q_8, the precursor of Q_9 is present, a lack of isoprene units would result in increased Q_8-levels.

In our previous work showing anti-carcinogenic effects of lovastatin [5] we hypothesised that the anti-carcinogenic effect might be mediated by the statin-induced inhibition of Q. In rats fed with lovastatin the preneoplastic liver tissue was reduced by 50% and the cell proliferation within the tissue was reduced to one third compared to non-statin treated animals. In rats fed with both lovastatin and Q_{10} the cell proliferation rate was totally reversed to normal levels. Thus, inhibition of Q-synthesis might explain a part of the anti-carcinogenic effects of statins. However, in that study the total levels of Q, measured with a high-performance liquid chromatography (HPLC) method, were not significantly affected by statin treatment. The aim of this study was to determine the levels of Q_9 and Q_{10} in sub-cellular fractions of liver tissue to investigate if the extra-mitochondrial fraction of Q was affected by statin treatment. Here, we used a liquid chromatography–mass spectrometry (LC–MS) method with better sensitivity and specificity than the previously used HPLC method. We also wanted to correlate a possible change in extra-mitochodrial levels of Q with inhibition of carcinogensis mediated by statins.

2. Material and methods

2.1. Animal experiments

In this study we used liver tissue from rats from the study described previously [5]. Male Fischer-344 rats, weighing 160 g, were purchased from Charles River, Germany. Animals were maintained in a 12 h light and 12 h dark cycle and fed standard chow diet R36 (Lactamin AB, Linköping, Sweden) until the start of the experiment (week 1). The chow diet given to the "statin-treated" animals during the experiment contained lovastatin (150 mg/kg R36) produced by Lactamin AB, Linköping, Sweden. The animals were allowed to acclimatise in the animal room for four days before the start of the experiment. Temperature, humidity and ventilation in the animal room were controlled in accordance with international standards. In this study we used liver tissue from five rats treated with lovastatin per os for 5 weeks (dose approximate 13 mg/kg bodyweight/day) and five rats treated with standard diet for 5 weeks.

All rats were treated according to the modified Solt and Farber-hepocarcinogenesis protocol described previously [10,11]. Briefly, initiation was performed using diethylnitrosamine injection and promotion was achieved by administration of 2-acetylaminofluorene (2-AAF) administered as intra-gastric injections. During the fourth week, 2/3 partial hepatectomy (PH) was performed to trigger cell proliferation. This was followed by another two intra-gastric injections of 2-AAF on day 2 and 4 after PH.

2.2. Preparation of sub-cellular fraction

Frozen liver tissue stored at −80 °C was cut into pieces with a pair of scissors in cold 0.25 M sucrose and homogenised in a Potter–Elvehjelm glass-Teflon homogenizer with 4 up-and-down strokes at a speed of 440 rev./min. The homogenate was centrifuged at 350*g* for 10 min to remove cell-debris. The supernatant was subsequently centrifuged at 2800*g* for 20 min. The pellet was collected as "mitochondria" and was washed and resuspended in sucrose-solution. The supernatant was centrifuged at 30,000*g* in 20 min to remove disruptet mitochondria (in the pellet). The supernatant was thereafter centrifuged at 105,000*g* in 90 min. The supernatant was collected as the "cytosol" and the pellet as "microsomes" that was washed and resuspended in the sucrose-solution. Protein-measurements in the different fractions and liver homogenate were performed using the method described by Lowry [12].

2.3. Ubiquinone analysis by LC–MS

2.3.1. Chemicals

All chemicals and reagents were of the highest purity available. Methanol (J.T. Baker, Denventer, The Netherlands), 2-propanol and 1-propanol (Merck KGaA, Darmstadt, Germany) were of HPLC grade. Coenzyme standards Q_{10} and Q_9 with Q_4 as internal standard were obtained in oxidised form from Sigma Aldrich (St. Louis, MO, USA). Sodium borohydride (Merck Schuchardy OHG, Huhenbrunn, Germany) was used for reduction of Q_{10} and Q_9. Preparation of Q_{10}, Q_9 and Q_4 standards was achieved by dissolving the powder in toluene (Merck KGaA, Darmstadt, Germany) and further diluted with 2-propanol.

2.3.2. Reduction procedure

Q_{10} and Q_9 were reduced with sodium borohydride. Appropriate amounts of Q_{10} and Q_9 standards dissolved in 2-propanol were evaporated in glass vials under stream of Ar and 2 ml of hexane was added. After addition of 50 μl of methanol and 20 mg of sodium borohydride, the mixture was stirred and mixed for 20 min in the dark. After addition of 1 ml water containing 100 μM EDTA, the tube was shaken and centrifuged for 5 min. The hexane phase was evaporated under the stream of Ar and dissolved in 50 μl of water and 100 μl of 1-propanol containing 100 ng/μl of Q_4 internal standard.

2.3.3. Extraction of Qs

Hundred microlitre of 1-propanol, containing 100 ng/μl of Q_4 internal standard, was added to 50 μl of sample, then wortexed for 1 min and the solution centrifuged at 5000*g* for 5 min to separate proteins from solution. Liquid phase was directly injected into LC–MS.

2.3.4. LC–MS

The samples were run on Waters 2695 separation module coupled with Micromass Quattro micro mass spectrometer. Hundred microlitre of each sample (temperature was 6 °C) was injected into the liquid chromatograph inlet via autosampler. Separation was performed on a Waters SunFire column (C18, 3.5 μm,

4.6 × 100 mm) at 40 °C, using solvents A (95% methanol, 5% 2-propanol) and B (100% 2-propanol). The gradient at a flow of 1.2 ml/min was as follows: isocratic A solvent for 3 min, gradient from 100% A to 70% A and 30% B for 1 min then isocratic 50% A and 50% B for 4 min, gradient from 50% A and 50% B to 100% A for 1 min and isocratic 100% A for 1 min, giving a total runtime of 10 min.

The mass spectrometer was optimised for different Qs using positive atmospheric pressure chemical ionisation mode (APCI+). Probe temperature was 525 °C for all the Qs analysed. The total ion current was collected from 454.0 to 458.5 mass-to-charge ratio (m/Q) with cone voltage 24 (V) for Q_4. For Q_{10} oxidised and Q_{10} reduced total ion current from 862.0 to 869.0 with cone voltage 40 (V) was collected. The total ion current was from 726.0 to 732.0 with cone voltage 40 (V) for Q_8 oxidised and Q_8 reduced. For Q_9 oxidised and Q_9 reduced total ion current from 794.0 to 800.5 with cone voltage 36 (V) was collected.

Retention times were 2.40, 5.73, 6.61, 6.30, 7.37, 6.92 and 8.27 min for Q_4, Q_8 reduced, Q_8, Q_9 reduced, Q_9, Q_{10} reduced and Q_{10}, respectively.

The concentrations of each Q form were estimated on the basis of adequate standard curve using its respective standard with MassLynx V4.1 software (Waters). Due to the lack of the standard Q_8 and Q_8 reduced were quantified as total ion current (arbitrary units) of each individual form. Thus, the Q_8/Q_9 and Q_{10}/Q_9 ratios were calculated from the total ion currents.

2.4. Statistical analysis

The R statistical programming language (version 2.15.1) was used for statistical analysis, and the R package ggplot2 for the graphical presentation [13]. Statistical significance was tested by using Mann–Whitney–Wilcoxon test for the comparison of Q_9 and Q_{10} levels in the different sub-cellular fractions and homogenates (Fig. 2 and SM1 (Supplementary Material)) as well as for comparison of Q_8/Q_9 and Q_{10}/Q_9 ratios (Fig. 4). Spearman's rank-correlation coefficient was used in the evaluation of correlation between the nodule density and Q_9 and Q_{10} levels (Fig. 3).

2.5. Ethical approval

Animal experiments received full approval from The Animal Stockholm Experimentation Ethics Committee.

3. Results and discussion

In the rat model used here we have previously demonstrated an anti-carcinogenic effect of statins [5]. We speculated that part of the effect was mediated by decreased synthesis of Q since addition of Q_{10} to the rats reversed some of the anti-carcinogenic effects. However, in the previous study we could not show any significant decrease of the Q-levels in the statin treated rats, measured with a HPLC method. This method has not optimal sensitivity and specificity. In the present study we used a LC–MS method in a scan mode with a registration of specific range for each Q analysed. To increase accuracy and sensitivity we examined the isotopomer distribution (M; M + 1; M + 2) in each individual sample. In addition, we analysed different sub-cellular compartments.

Approximately 10% of the total Q in all homogenates and fractions were in oxidised form and the rest in reduced form. The percentage relation between reduced and oxidised Q_9 and Q_{10} was similar in all samples analysed and no statistically significant differences could be detected (data not shown). Thus, the total Q_9 and Q_{10}, respectively (oxidised + reduced form) is presented in all of the figures.

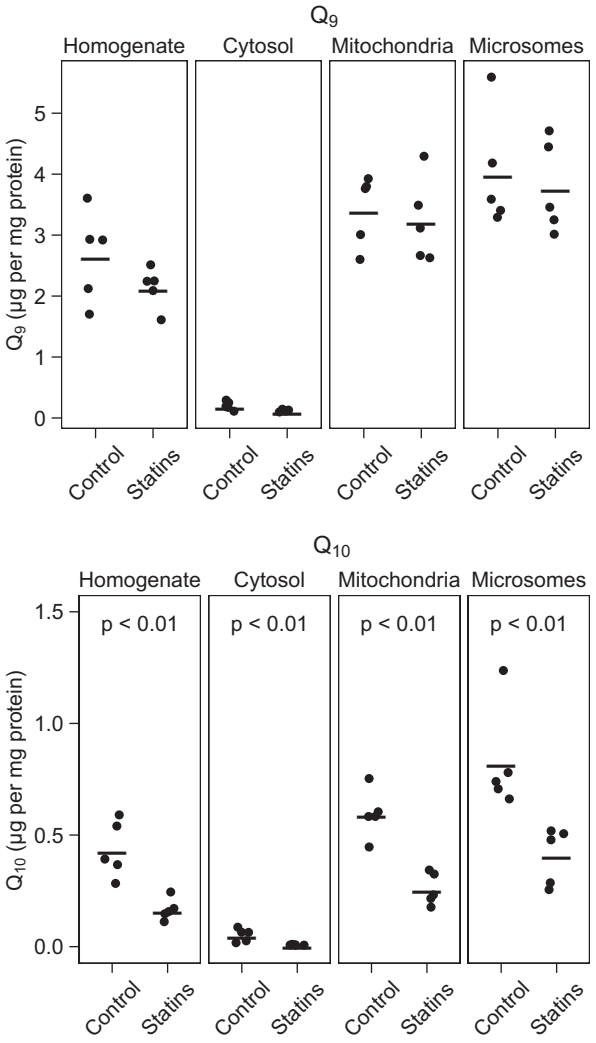

Fig. 2. Levels of Q_9 and Q_{10} in rat liver homogenate and sub-cellular fractions from rats in a hepatocarcinogenic model. Raw data (filled circles) and mean values (lines) as µg per mg protein of Q_9 and Q_{10} for each group in liver homogenate and sub-cellular fractions.

The levels of extra-mitochondrial but not mitochondrial Q are increased in preneoplastic liver tissue (liver nodules) compared to normal liver tissue [6]. This is regarded to be an important factor in a resistant cell phenotype and may be important during carcinogenesis [6].

The primary aim of this study was to investigate if the extra-mitochondrial levels of Q were reduced during stain treatment and if this correlated with inhibited carcinogenesis.

There was no significant difference of Q_9 levels between statin treated and non-treated rats in rat liver tissue homogenate (Fig. 2). However, the Q_{10} levels were reduced by approximately 60% after statin treatment in the homogenate ($p < 0.01$) (Fig. 2). Since the Q_{10} levels represent only a small fraction of total Q in rats this reduction could not be detected when analysing "total- ubiquinone" ($Q_9 + Q_{10}$).

In all sub-cellular fractions there was a significant reduction of Q_{10} but not of Q_9 levels (Fig. 2). The most pronounced decrease of Q_{10} levels was found in the cyotsol, with a reduction by 85% ($p < 0.01$). The cytosolic Q_9 levels were not significantly reduced (Fig. 2 and Fig. SM1 in Supplementary Material). However, there was a tendency towards decreased levels of total cytosolic Q ($Q_9 + Q_{10}$) during statin treatment ($p = 0.056$) while the mitochondrial levels were unaffected. This implicates that statin treatment

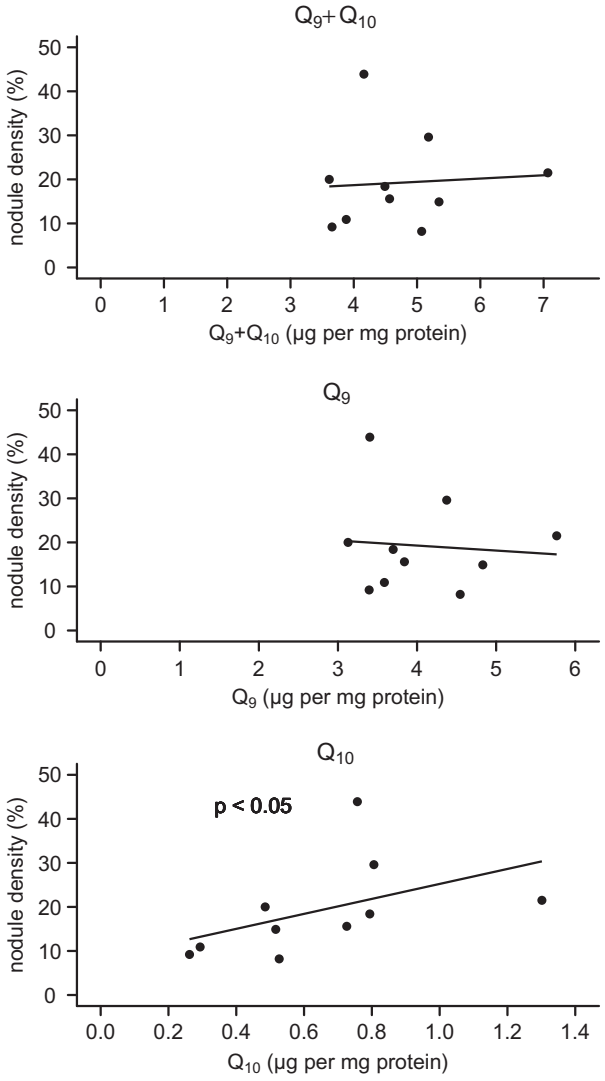

Fig. 3. Nodule density versus extra-mitochondrial $Q_9 + Q_{10}$, Q_9 and Q_{10} levels. Scatter plot of nodule density (%) versus µg per mg protein of $Q_9 + Q_{10}$, Q_9 and Q_{10} in the liver extra-mitochondrial fractions of rats with the corresponding correlation line of best fit.

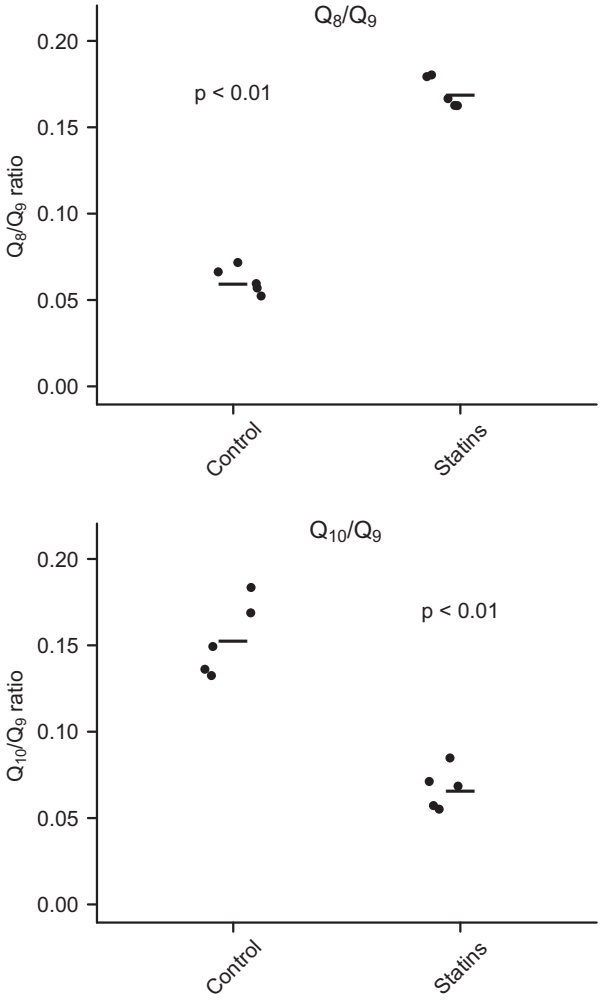

Fig. 4. Ratios of Q_8/Q_9 and Q_{10}/Q_9 levels in rat liver homogenate from rats in a hepatocarcinogenic model. Raw data (filled circles) and mean values (lines) as ratios of Q_8/Q_9 and Q_{10}/Q_9 for each group in liver homogenate.

leads to decreased anti-oxidative capacity in the cytosol while the cell favours to with-hold normal levels of Q in the mitochondria. Decreased levels of cytosolic Q during statin treatment might contribute to the inhibition of carcinogenesis achieved by statins in this model.

To investigate the association between carcinogenesis and the levels of extra-mitochondrial Q (cytosolic and microsomal) a correlation analysis was performed. A well-established marker for the measurement of carcinogenesis in this model is the nodule density (preneoplastic tissue) within the liver [14]. The total levels of extra-mitochondrial Q ($Q_9 + Q_{10}$) were not significantly reduced and there was no correlation with liver nodules (Fig. 3). However, there was a significant correlation between the extra-mitochondrial Q_{10} levels and liver nodules ($p < 0.05$). Also after compensating for the reduced volume of liver nodule tissue in the statin treated rats, which could explain approximate 12% decreased levels of extramitochondrial Q, this reduction was significant. High extra-mitochondrial Q_{10} levels were associated with increased burden of preneoplastic tissue and increased carcinogenesis. Consequently, reduced Q_{10} levels, achieved by statins, correlated with reduced burden of liver nodules and inhibited carcinogenesis (Fig. 3). There was no significant correlation between the liver nodule density and extra-mitochondrial Q_9 levels (Fig. 3).

The most likely explanation for the effect of statins on Q_{10} is that formation of this compound corresponds to the last step in the synthesis of the side-chain of Q (Fig. 1). Reduced availability of the isoprene units required for the synthesis would be expected to reduce the level of Q_{10} and increase the level of Q_8. Previous studies have also shown that the activity of the prenyltransferase enzyme responsible for the formation of the side-chain in Q is dependent on intracellular concentrations of isoprene units [15]. To evaluate *de novo* synthesis of Q we measured the levels of Q_8 in liver homogenate and calculated the ratios of Q_8/Q_9 and Q_{10}/Q_9. In accordance with our hypothesis the Q_8/Q_9 ratio was significantly increased ($p < 0.01$) in the statin-treated group and the Q_{10}/Q_9 ratio was significantly decreased ($p < 0.01$) (Fig. 4). Thus, it is possible that Q_{10} is a more sensitive marker for changes in the over-all rate of synthesis than Q_9.

The reduction of Q_{10} levels during statin treatment have also been shown in humans [16–18]. Recently we confirmed this in a Swedish cohort of statin-treated patients where statin-treatment for 6 weeks reduced the Q_{10}-levels in serum by approximately 25% in average (unpublished data).

This study supports our previous findings that at least part of the anti-carcinogenic effect of statins in our rat model is mediated by the reduced synthesis of Q. We also show a shift in the

Q-synthesis pathway towards isoforms with shorther side-chains during statin treatment. The ratios between the different isoforms of Q could be used as a more sensitive marker for statin-mediated inhibition of Q than measurement of total Q.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.094.

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