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Atorvastatin Increases miR-124a Expression: A Mechanism of Gamt Modulation in Liver Cells

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ABSTRACT

Atorvastatin is used to control cholesterol and lipid levels in hyperlipidaemic and hypercholesterolaemic patients. Myopathy and hepatotoxicity, however, have been reported as side effects in a small percentage of statin users. This study aimed to investigate the cytotoxicity and the effect of atorvastatin on microRNA expression in HepG2 cells. The methylthiazol tetrazolium assay was used to assess hepatocyte viability and at 20 μM atorvastatin (24 h) treatment were 82 ± 1.5% viable ($P = 0.0002$). Levels of intracellular ATP in cells treated with 20 μM atorvastatin were reduced by 1.25-fold, $P = 0.002$. Cytotoxicity, measured by the release of intracellular lactate dehydrogenase, was increased from 0.95 ± 0.29 units in control cells to 1.12 ± 0.02 units ($P = 0.002$) in atorvastatin treated cells. A panel of 84-miRNA species was used to evaluate the effect of atorvastatin on miRNA expression. MiR-124a was significantly up-regulated by atorvastatin (12.94-fold). A significant decrease in GAMT expression (3.54-fold) was observed in atorvastatin treated cells following quantitative PCR analysis. In addition, western blotting data showed GAMT protein levels were significantly lower than the controls (3.02-fold) and analysis of creatine levels in treated cells showed a significant decrease in the atorvastatin treated culture supernatant compared to control culture supernatant (32.33 ± 3.51 μM/l vs. 59.67 ± 1.52μM/l, $P = 0.0056$). This is the first study to show that atorvastatin up-regulates miR-124a levels and consequently modulates GAMT expression in hepatocytes. J. Cell. Biochem. 116: 2620–2627, 2015.

KEY WORDS: ATORVASTATIN; HEPATOTOXICITY; GUANIDINOACETATE-N-METHYLTRANSFERASE; CREATINE

Statins, inhibitors of cholesterol synthesis, are the most prescribed medications for the treatment of dyslipidaemia. Large clinical trials have shown a significant reduction in the proportion of cardiovascular deaths in patients on statin therapy [Wright et al., 1994; Shepherd et al., 1995, Schwartz et al.,2001]. It is well known that statins reduce the risk of myocardial infarction, stroke, and death, primarily competitively inhibiting hydroxymethylglutaryl-CoA Reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis [Strandberg et al., 2004]. It is well established that the liver is the central organ for the metabolism of macromolecules, including cholesterol, and detoxification of xenobiotics such as statins. The first pass metabolism of statins occurs via the cytochrome P450 system hence the bioavailability of statins to hepatocytes is greatest following oral administration.

In 2005, Chalasani, assessed the hepatotoxicity of statins and concluded that "statins are remarkably safe from a hepatic standpoint, but there are several issues related to their usage in humans that require further research and scrutiny" [Chalasani, 2005]. Further, an assessment by the Statin Liver Safety Task Force recommends clinicians to evaluate levels of liver enzymes (alanine aminotransferase (AST) and aspartic aminotransferase (ALT)) prior to, and during statin therapy [Bays et al.,2014]. A recent review on the epigenetic effects of common pharmaceuticals assessed possible mechanisms of drug-induced epigenetic changes [Csoka and Szyf, 2009]. This review focussed on drug-induced epigenetic changes either directly, by DNA methylation and histone modification or indirectly via transcription factor activation or receptor expression. They concluded that statins interact directly with the epigenome due

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to the increased production of low density lipoprotein (LDL) receptors, myaglias, muscle cramps and liver enzyme derangements associated with statin use [Csoka and Szyf, 2009]. The epigenetic mechanisms postulated to be responsible for these changes included DNA methylation and histone modification. To date, there is limited evidence available on the effects of statins on a third, recently described arm of epigenetics: microRNA (miRNA) expression [Wang et al., 2011].

It has been established that miRNAs coordinate metabolism by directly affecting the transcriptome, modulate multiple target transcripts and thus heavily influence gene expression patterns [Asirvatham et al., 2008]. A recent study found that miR-33, may be key to controlling HDL levels via repression of sterol transporters in the liver [Marquart et al., 2010]. In a separate study miR-33 was found to influence cholesterol homeostasis by down-regulation of cholesterol efflux transporters, ABCA1 and ABCG1 in HepG2 and Hepa liver cells [Rayner et al., 2010]. Other inflammation-, metabolism- and endothelial-associated miRNAs, affected by statins includes miR-34a, miR-92a, miR-124a, miR-146a/b, and miR-221/222 [Raitoharju, et al., 2011; Kin et al., 2012; Takwi et al.,2012; Wang et al.,2012]. In the study by Kin et al. [2012] patients with atherosclerotic abdominal aneurysms had lower levels of miR-124a, but when statistically adjusted for statin usage the miR-124a levels were found to be elevated [Kin et al., 2012]. The mechanism by which miR-124a has been implicated in inflammation is via the regulation of kruppel-like factor 2–shown to mediate rapamycin-induced arterial thrombosis in vivo [Manoharan et al., 2014]. The activation of kruppel-like factor two represses pro-inflammatory activation in monocytes [Das et al., 2006], and as a consequence is said to prevent monocyte entry into the vessel wall reducing atherogenesis in mouse models of atherosclerosis [Mestas and Ley, 2008]. MiR-124a also targets gaunidinoaceto-N-methyltranserase, a critical enzyme involved in the creatine synthesis pathway. The synthesis, release and availability of creatine from the liver are essential for optimum energy release and muscle function. The effect of statins on miR-124a expression and creatine synthesis in the liver has not been investigated to date. Limited data is available on the effects of statins on miRNAs involved in the regulation of specific biological pathways. The liver plays an integral role in the metabolism of statins and the effects thereof. In this study the effect of atorvastatin on the metabolism (ATP, LDH) and miRNA profile in $HepG_2$ cells, was evaluated.

MATERIALS AND METHODS

TREATMENT

Approximately 1.5×10^6 HepG2 cells were plated in sterile 25 cm^3 flasks in complete culture media [Eagle's minimum essential medium, 10% foetal calf serum, 1% L-Glutamine and 1% penstrep-fungizone] and incubated overnight (37°C, 5% CO_2). A stock solution of atorvastatin (Sigma Aldrich, SA) was prepared in 20% dimethylsulphoxide (DMSO) and a working solution of 1 mM atorvastatin was prepared in 0.1 M phosphate buffered saline (PBS). The effect of atorvastatin in $HepG_2$ cells was measured using a methyl tetrazolium dye reduction assay, the [3, (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay. HepG2 cells (15,000/well) were incubated for 24 h with atorvastatin at 1 μM, 10 μM, 20 μM and 40 μM in triplicate in a microtitre plate together with vehicle control (0 μM). The concentration and incubation time for this experiment was based on the half–life of atorvastatin (20–30 h) and the recommended physiological dose between 10 mg and 40 mg/day (bioavailability is 14%). These concentrations are commonly used in in vitro models assessing the effects of statins [Wilcox et al., 1999; Funatsu et al., 2001; Maejima et al., 2004, Mestas and Ley, 2008; Stormo et al., 2014]. Each experiment was conducted twice and with three replicates. The cells were then incubated (37°C, 5%CO_2) with the MTT substrate (5 mg/ml in PBS) for 4 h. Thereafter all supernatants were aspirated, and 100% DMSO (100 μl/well) was added to the wells. Finally the optical density was measured at 570 nm with a reference wavelength of 690 nm using an ELISA plate reader (Bio–Tek μQuant).

HepG2 cells were seeded (1×10^6cells/flask) in 25 cm^3 flasks and grown to approximately 90% confluency. For subsequent assays, three 25 cm^3 flasks of untreated cells and three 25 cm^3 flasks of 20 μM atorvastatin treated cells were incubated for 24 h (37°C, 5%, CO_2) and harvested for subsequent assays. All treatments were prepared in triplicate.

THE ATP QUANTIFICATION ASSAY

The levels of intracellular ATP were measured using a CellTiter-Glo® kit (Promega, United States, Madison, WI). Treated cells were seeded into an opaque microtitre plate (20,000 cells/ well) in triplicate followed by the addition of 100 μl/well of the reconstituted Cell Titre Glo reagent. The plate was agitated and incubated in the dark (30 min; room temperature (RT)) to allow for cell lysis and the luciferace-based reaction to occur. Thereafter, the luminescent signal was measured on a microplate luminometer (Turner Biosystems) and the results were expressed as relative light units (RLU).

THE LACTATE-DEHYDROGENASE (LDH) ASSAY

The LDH cytotoxicity detection kit (Roche) was used to measure cell death/damage of atorvastatin treated HepG2 cells. To measure LDH activity, supernatant (100 μl) was transferred into a 96-well microtitre plate in triplicate. Thereafter, substrate mixture (100 μl) containing catalyst (diaphorase/NAD^+) and dye solution (INT/sodium lactate) was added to the supernatant and allowed to react at ambient temperature for 25 min. Optical density was measured at 500 nm (ELISA plate reader -Bio-Tek uQuant). Results are represented as mean optical density.

QUANTITATIVE PCR ARRAY BASED PROFILING OF microRNA

Following atorvastatin treatment, HepG2 cells were rinsed (0.1 M PBS) and 500 μl 0.1M PBS was added to each flask. An aliquot of 500 μl Tri Reagent® Solution (Ambion) was added to each flask for isolation of RNA. Total RNA was isolated using the RNeasy Isolation Kit (Qiagen) as per standard methods and quantified using a Nanodrop 2000. Copy DNA (cDNA) was synthesized using the miScript II RT kit (Qiagen) and the miScript miRNA PCR Array

Human miFinder (MIHS-001Z, Qiagen) which contains 84 mature miRNA-specific primers.

STATISTICAL ANALYSIS AND SELECTION OF SIGNIFICANT miRNA AND COMPUTATIONAL ANALYSIS OF miRNA TARGETS

Data were normalized using U6 small RNA expression. Difference in miRNA expression was determined by fold change analysis using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001]. To stringently select miRNAs significantly up- or down-regulated in atorvastatin treated cells, a significance of analysis of microarray (SAM) strategy was used. Significant ($P < 0.05$) deviation of residuals from normal distribution was used to identify miRNAs of interest. Data were analysed with Microsoft excel (2011) Statplus plug-in and R.

Pathway analysis was conducted by first identifying miRNA targets using the Targetscan and PicTar algorithms and thereafter, scanning for ontological enrichment in the KEGG database.

TRANSFECTION OF HepG2 CELLS WITH miR-124a MIMIC AND miR-124a INHIBITOR

In order to directly assess the effect of miR-124a on GAMT mRNA levels, cells were transfected with mimics and inhibitors (Syn-hsa-miR-124a-3: MYS0000422, Anti-hsa-miR-124a-3p, MYS0000422, Qiagen) to miR-124a. Briefly, HepG2 cells were seeded at a density of 400,000 cells/well in a 6-well plate and allowed to adhere for 24 h (37°C, 5% CO_2) until 80% confluent. Lyophilized miRNA mimics and inhibitors (5 nmol) were reconstituted to 20 μM in nuclease free water. For the transfection, 10 μl miRNA mimic or inhibitor was added to medium without serum, proteins, or antibiotics to a total volume of 60 μl to which 2 μl of HiPerFect Reagent (Qiagen) was added and mixed by reverse-pipetting. Samples were then incubated for 15 min (RT) to allow complex formation. During this time CCM (complete culture medium) was gently aspirated from the cells and 1,940 μl fresh CCM was added such that the final concentration of mimic/inhibitor per well was 50 nM. The transfection complex was added in a drop-wise fashion into the appropriate well with gentle swirling of the plate to ensure uniform distribution. An untreated control and a 20 μM atorvastatin treatment were included on the plate. All treatments were then incubated for 24 h (37°C, 5%, CO_2) and utilized for RNA isolation.

QUANTIFICATION OF GAMT mRNA LEVELS

To quantify GAMT qPCR was performed. Total RNA was first isolated from control and treated cells by adding 500 μl Tri reagent (Am9738) as per manufacturer's guidelines. Thereafter, RNA was quantified (Nanodrop 2000) and standardised to 100 ng/μl. RNA was reverse transcribed by reverse transcriptase into cDNA using the RT2 First Strand Kit (Qiagen, C-03) as per manufacturer's instructions. Briefly, a 20 μl reaction was prepared by adding 10 μl genomic DNA (gDNA) elimination mixture (Total RNA, 5 × gDNA elimination buffer, H_2O) to 10 μl of Reverse Transcriptase cocktail (5 × RT buffer three, primer and external control mix, RT enzyme mix, H_2O). The reaction was then subjected to 42°C (15 min) and 95°C (5 min) (GeneAmp® PCR System 9,700, Applied Biosystems) to obtain cDNA. Quantitative PCR was used to determine mRNA expression using RT2 SYBR® Green qPCR Master Mix (Qiagen). A 25 μl reaction consisting of 12.5 μl iQ™ SYBR® green supermix (cat. no. 170–8880), 8.5 μl nuclease-free water, 2 μl cDNA, and 1 μl sense and anti-sense primers as follows: GAMT: sense 5′-TGGCACACTCACCAGTTCA-3′, antisense5′-AAGGCATAGTAGCGGCAGTC-3′; 10 mM, Inqaba Biotechnical Industries™, South Africa) were used. The mRNA expression was compared and normalized to a housekeeping gene, GAPDH (sense: 3′-CAACAGCCTCAAGATCATCAGC-5′; anti-sense: 3′-TGAGTCCTTCCACGATACCAAAG-5′).

The reaction was subjected to an initial denaturation (95°C, 10 min). It was followed by 40 cycles of denaturation (95°C, 15 s), annealing at 57°C, 40 s; and extension (72°C, 30 s) (CFX Real-Time PCR detector, Bio–Rad). The data was analyzed using CFX analysis software V3.0, Bio–Rad. The mRNA expression was determined as described by Livak and Schmittgen, [2001] and is represented as fold changes relative to the control.

QUANTIFICATION OF GAMT

Samples (HepG2 crude protein extract standardized to 1 mg/ml) were denatured by boiling for 10 min with a 1:1 dilution with 1 × Laemmli sample buffer (0.375 M Tris–HCl pH 6.8; 10%, w/v SDS; 3%, v/v glycerol; 0.2%, w/v bromophenol blue; 12% β-mercaptoethanol in dH_2O). For each sample, 100 μg of total protein was loaded on a 10% polyacrylamide gel, run at 150V. Transfer onto PVDF membrane was conducted at 350 mA for 1 h. Membranes were blocked for 1 h with blocking buffer containing 5% non-fat dry milk TTBS (25 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween 20). Membranes were probed overnight at 4°C with anti-mouse GAMT (ab119269, Abcam) and anti-β actin antibody (A3854, Sigma) diluted to 1:500 in 1% BSA in TTBS. A horseradish peroxidase-conjugated secondary antibody diluted to 1:10,000 in 1% BSA in TTBS was used to allow detection of appropriate bands using LumiGLO® Chemiluminescent Substrate Kit (KPL). Images were captured on the Alliance 2.4 gel documentation system (UViTech). All experiments were conducted at least thrice and blots were analysed using UViBand analysis software (UViTech).

QUANTIFICATION OF CREATININE

The level of creatinine in cell culture supernatant was measured using the clinical chemistry analyzer Piccolo Xpress Chemistry Analyser (Abaxis, California). Briefly, 2 ml aliquot of cell culture supernatant from control and atorvastatin treated cells was freeze-dried and resuspended in 500 μl of 0.1 M PBS before 100 ul was transferred to a Piccolo Xpress Comprehensive Metabolic panel for analysis. Three samples from two independent experiments were evaluated.

RESULTS

CELL PROLIFERATION, METABOLIC ACTIVITY AND CYTOTOXICITY OF ATORVASTATIN TREATED HepG2 CELLS

The MTT assay was used as a measure of cell proliferation/metabolic activity of atorvastatin in HepG2 liver cells. Following a 24 h incubation a significant decrease in cellular metabolism from 99 ± 1.18% in control cells to 82 ± 1.49% in 20 μM atorvastatin treated cells was observed ($P = 0.0002$, Fig. 1A). Next we investigated the effect of atorvastatin on liver cellular ATP levels.

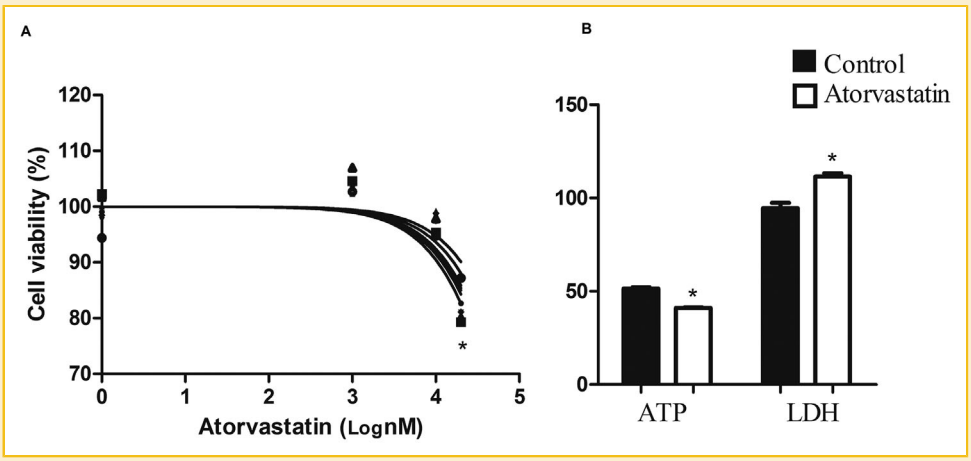

Fig. 1. Cell viability, ATP and LDH levels of $HepG_2$ cells treated with atorvastatin for 24 h. ATP: Adenosine triphosphate; LDH: Lactate dehydrogenase; RLU: relative light units, OD: optical density. *$P < 0.05$, Mann Whitney test.

Atorvastatin significantly decreased ATP levels compared to the controls ($5.148 \times 10^6 \pm 0.064 \times 10^6$ RLU to $4.11 \times 10^6 \pm 0.04 \times 10^6$ RLU, $P = 0.0022$, fig. 1B). In addition, atorvastatin was cytotoxic to HepG2 cells as LDH levels were significantly elevated compared to untreated cells (1.12 ± 0.02 units to 0.95 ± 0.03 units, $P = 0.0022$, fig. 1B).

microRNA EXPRESSION PROFILE IN STATIN TREATED HepG2 CELLS

We assessed the effect of atorvastatin on miRNA profiles in metabolically active liver (HepG2) cells. The miRNA pathway finder was used to evaluate atorvastatin induced differential regulation of miRNAs in HepG2 cells (Fig. 2).

MiRNA-124a was significantly up-regulated by 12.94-fold in the expression profile of miRNA's in the pathway finder array. Further analysis of miR-124a-3p by quantitative PCR using a miR-124a specific primer assay found a significant up-regulation by atorvastatin in HepG2 cells (5.8-fold, $P = 0.005$). The standard miRNA target prediction tools, targetscan and pictar, were used to search for potential candidate genes that may be transcriptionally regulated by miR-124a. Targetscan returned 1654 transcripts with conserved sites and PicTar, 787 mRNA targets. A list of targets related to metabolism was evaluated. GAMT was identified as an enzyme of interest in the context of this study as literature shows an elevation in creatine kinase levels in patients on statin therapy.

QUANTITATIVE ANALYSIS OF GAMT mRNA LEVELS AND WESTERN BLOT ANALYSIS FOR GAMT PROTEIN LEVELS

MiRNA-124a binds to the 3′ UTR of GAMT, influencing its stability and translation, which may compromise creatine synthesis. Thus the mRNA expression level (using qPCR) of the GAMT enzyme was analysed.

A significant decrease in GAMT mRNA levels was observed in HepG2 cells treated with atorvastatin (3.54 fold, $P < 0.05$, Fig. 4A). In HepG2 cells transfected with mimics of miR-124a-3p the changes in mRNA levels, was enhanced compared to the statin treatment (4.56-fold decrease). Interestingly, in cells transfected with the miR-124a inhibitor a slightly higher level of GAMT mRNA was found compared to the control (1.12-fold increase, Fig. 3).

The binding of miRNAs to the target mRNA at the 3′ UTR does not necessarily result in complete translational repression. Therefore, the protein levels of GAMT in atorvastatin and miR-124a-3p mimic transfected liver cells were assessed by western blotting.

The GAMT protein levels in atorvastatin treated cells were significantly lower than the untreated controls (3.02-fold, $P = 0.0005$). HepG2 cells transfected with 50 nM of miR-124a-3p mimic showed a (5.02-fold, $P = 0.0005$) decrease and cells transfected with miR-124a inhibitor, a 2.29-fold increase in GAMT protein expression (Fig. 4B).

Analysis of creatine levels in treated cells showed a significant decrease in the atorvastatin treated cell culture supernatant compared to untreated control cell culture supernatant (32.33 ± 3.5 μM/l vs. 59.67 ± 1.52 μM/l, $P = 0.0056$).

DISCUSSION

Statins are unequivocally the most widely prescribed drug for the primary and secondary prevention of coronary artery disease worldwide [Eidelman et al., 2002]. Atorvastatin is the most commonly prescribed statin drug due to its lower therapeutic dose compared to simvastatin, lovastatin, and cerivastatin. The well-tolerated and high benefit-risk ratio of atorvastatin has been demonstrated in clinical trials [Strandberg et al., 2004]. The liver, being the central hub of metabolism, plays a crucial role in glucose homeostasis, cholesterol and lipoprotein synthesis, and xenobiotic metabolism. Atorvastatin is also metabolized in liver and there is limited data on the mechanism of statin-induced hepatoxicity.

Statin therapy has major positive health benefits in coronary artery disease patients, but also has adverse effects [Chalasani, 2005]. It was reported that liver function enzymes rose asymptomatically after therapy. The guidelines for patients on atorvastatin

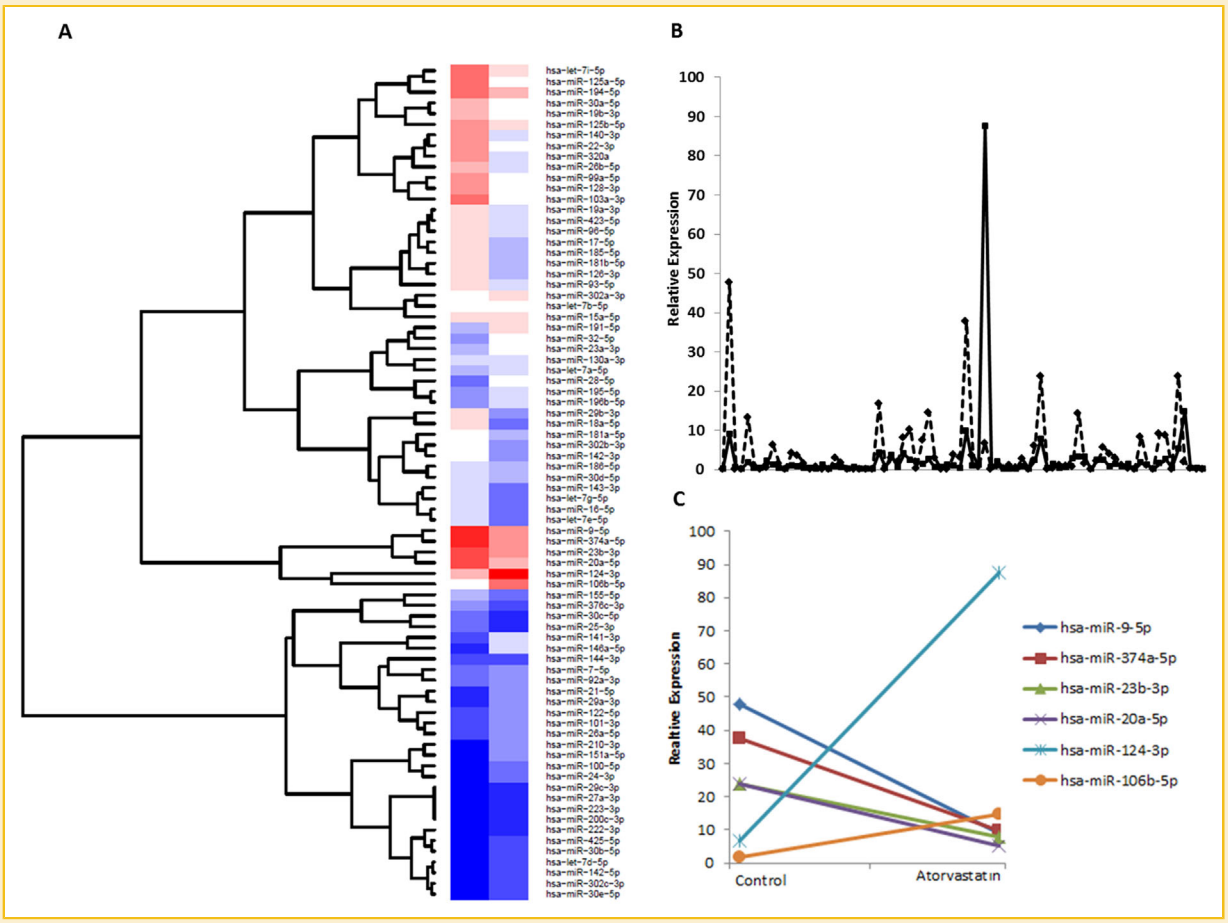

Fig. 2. Relative expression of miRNAs in atorvastatin treated HepG2 cells. (A) Hierarchical clustering of miRNA expression. Heatmap shows a descending ordered list of significant miRNA clusters in control cells. (B) Relative expression of miRNA's in HepG2 cells treated with atorvastatin. (C) Relative expression profile of six clustered miRNAs. Plot shows a significant increase in miR-124a in HepG2 cells by atorvastatin.

therapy recommended a liver function test be performed before administration, followed by biannual monitoring of liver function [Chalasani, 2005].

In our study, 20 μM atorvastatin increased LDH activity in HepG2 cells after 24 h (Fig. 1B), strongly suggesting that atorvastatin induced membrane damage/ leakage due to its lipophilic nature. In addition, atorvastatin compromised metabolic activity in HepG2 cells as evidenced by the MTT assay (Fig. 1A) and decreased levels of ATP (Fig. 1B). The inhibition of HMGCR and subsequent accumulation of acetyl-coA in the mitochondria can be transported back into the cytosol following conversion to citrate. Statins decrease production of superoxide by inhibiting geranylgeranyl pyrophosphate (GPP) activation of ras, preventing the stimulation of NADPH oxidase [Wagner, Martijnez-Rubio et al., 2002; Wassmann et al., 2002; Wang et al., 2008]. Also, cytosolic citrate undergoes a two-step reaction catalysed by ATP-citrate lyase and malate dehydrogenase to form pyruvate. The first reaction utilizes NADH and the second produces NADPH. The availability of reducing equivalents, NAD(P)H, is directly proportional to cellular metabolic activity and results in increased conversion of the tetrazolium salts in cell viability assays. It is possible that the decrease in cell metabolism and ATP levels may be due to the effect of atorvastatin on the GPP pathway. The positive effects of statins (LDL-C reduction, the protection against oxidative stress, vaso-relaxant properties of increased NO synthesis and anti-inflammatory potential), however, arguably out-weigh the minor adverse effects (elevated liver enzymes, creatine kinase, muscle fatigue, cramps, and rarely, rhabdomyolysis), for which there is limited mechanistic evidence [Phillips et al., 2002; Rao and Milbrandt, 2010].

The duration of myopathy from statin therapy ranges from a few weeks to two years [Phillips et al., 2002]. To date, little is known about the mechanism by which statin therapy leads to muscle weakness or fatigue. A study on statin-associated myopathy found pathology even in the presence of normal creatine kinase (CK) levels [Phillips et al., 2002]. Creatine kinase is abundant in energy-demanding tissues such as skeletal muscle. The reaction catalysed by CK is the reversible conversion of creatine to phosphocreatine which serves as an energy reservoir for the instant regeneration of ATP in situ and serves as a source of high energy phosphate, released during anaerobic metabolism. Creatine, however, is produced primarily in

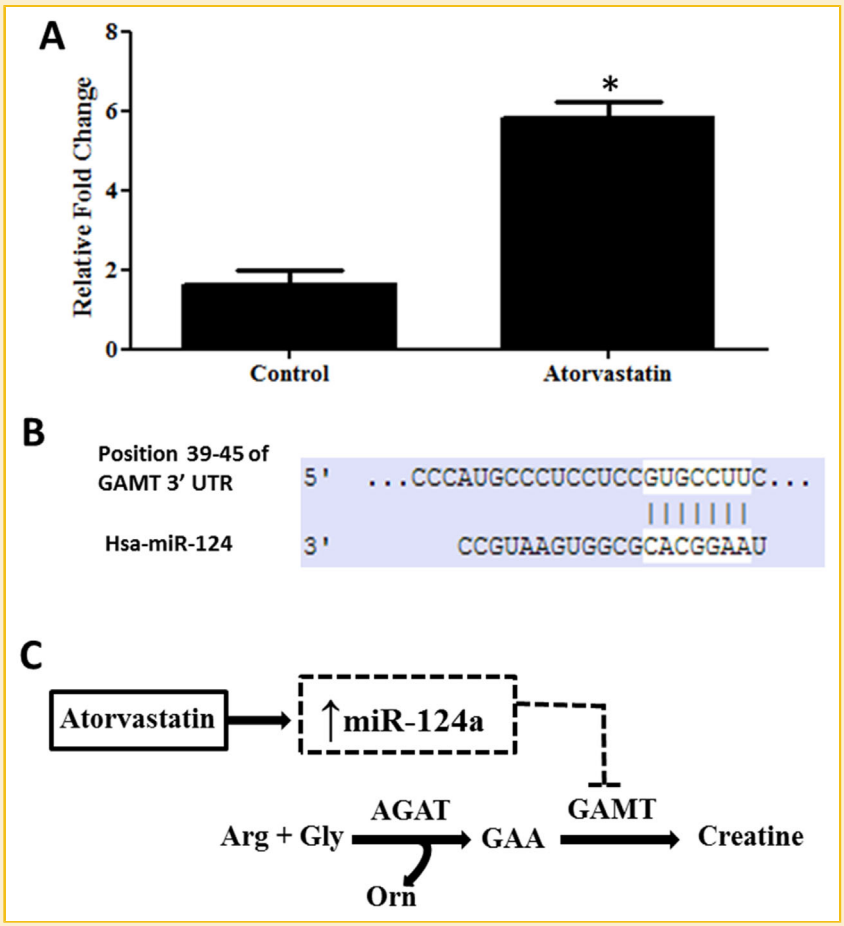

Fig. 3. Atorvastatin increases miR-124a and targets the 3′ UTR of GAMT. (A) Quantification of increased expression of miR-124a in atorvastatin treated HepG2 cells ($P = 0.005$). (B) TargetScan analysis of miR-124a target site of the 3′ UTR of GAMT (C) Proposed model of the effect of atorvastatin on miR-124a levels which targets GAMT, affecting the creatine synthesis pathway. GAA: guanidinioaminoacetate, AGAT: arginine-glycine amidinotransferase.

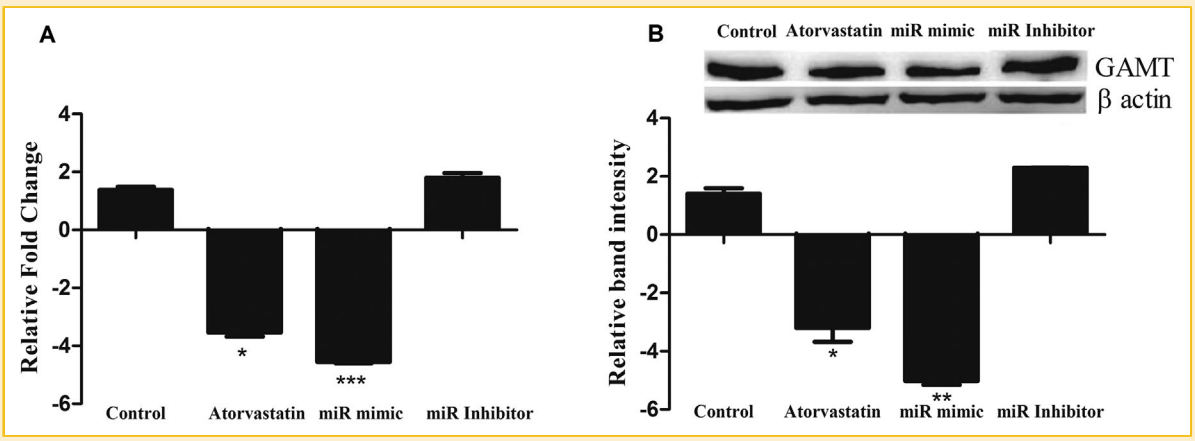

Fig. 4. Relative fold changes in (A) mRNA expression and (B) relative fold changes in protein expression of GAMT (normalized against actin) following HepG2 treatment with atorvastatin, and miR-124a-3p mimic and miR-124a-3p inhibitor.

the liver and kidney and is transported in circulation to peripheral tissues including muscle.

Our study assessed the effect of atorvastatin on 84 well characterized miRNAs in liver (HepG2) cells and showed a significant increase (12.94 fold) in miR-124a (Figs. 2 and 3). Following an extensive analysis of predicted targets of up-regulated miRNAs, several molecules involved in energy metabolism were identified. The elevated miR-124a, targets a key enzyme in creatine synthesis (GAMT).

A plausible mechanism of atorvastatin induced energy depletion and inhibition of creatine synthesis in liver cells is presented (Fig. 3C). In order to validate the proposed mechanism, the mRNA levels of GAMT—an enzyme involved in creatine synthesis—was assessed and found to be decreased by 2.54-fold (Fig. 4A). The lower expression of GAMT mRNA by atorvastatin was confirmed by western blotting (Fig. 4B). This decrease in GAMT protein levels reduces the cellular capacity for creatine synthesis. The relative increase in GAMT mRNA and protein levels in cells transfected with the inhibitor of miR-124a indicates that regulation of GAMT levels may be dependent on miR-124a.

This is the first study to demonstrate a mechanism by which atorvastatin increases miR-124a levels which targets subunits of the electron transport chain, and GAMT—both essential for ATP and creatine synthesis, respectively. The post-transcriptional regulation by miR-124a compromises translation and availability of GAMT and creatine. The up-regulation of creatine kinase levels in patients on statins may thus be a compensatory mechanism to preserve creatine stores.

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