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Loss of housekeeping selenoprotein expression in mouse liver modulates lipoprotein metabolism

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Abstract

Selenium is incorporated into proteins as selenocysteine (Sec), which is dependent on its specific tRNA, designated tRNA[Ser]Sec. Targeted removal of the tRNA[Ser]Sec gene (*Trsp*) in mouse hepatocytes previously demonstrated the importance of selenoproteins in liver function. Herein, analysis of plasma proteins in this *Trsp* knockout mouse revealed increases in apolipoprotein E (ApoE) that was accompanied by elevated plasma cholesterol levels. The expression of genes involved in cholesterol biosynthesis, metabolism and transport were also altered in knockout mice. Additionally, in two transgenic *Trsp* mutant mouse lines (wherein only housekeeping selenoprotein synthesis was restored), the expression of *ApoE*, as well as genes involved in cholesterol biosynthesis, metabolism and transport were similar to those observed in wild type mice. These data correlate with reports that selenium deficiency results in increased levels of ApoE, indicating for the first time that housekeeping selenoproteins have a role in regulating lipoprotein biosynthesis and metabolism.
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The importance of trace elements in human health can be assessed by the fact that their reduction in the diet may lead to various disorders. One important dietary trace element is selenium, which has potent cancer chemo-preventive properties [1]. It also has protective roles against viral infection [2], cardiovascular and muscular disorders along with roles in mammalian development, male reproduction and immune function [3]. Selenium is incorporated into a select group of proteins, selenoproteins, in the form of the amino acid, selenocysteine (Sec). The biological function of selenium is thought to be exerted primarily by these proteins [4]. Of the 24 and 25 selenoproteins iden-

tified in mice and humans, respectively [5], several are known to have roles in cellular redox regulation (e.g., methionine sulfoxide reductase B1 (Msrb1), glutathione peroxidases (Gpxs), and thioredoxin reductases (TRs)) [6]. Selenoprotein synthesis is dependent on the unique tRNA, designated tRNA[Ser]Sec [4] which is modified post-transcriptionally for proper functioning [7]. Two bases and one nucleoside modification occur within the anticodon loop of tRNA[Ser]Sec: A37 is modified to N^6-isopentenyladenosine (i^{6}A) and U34 is modified to methylcarboxyl-5′-methyluridine (mcm^{5}U); mcm^{5}U is further modified on its ribosyl moiety to Um34 [8]. Interestingly, the synthesis of Um34 on tRNA[Ser]Sec is responsive to selenium status [9].

Removing the gene for tRNA[Ser]Sec (designated *Trsp*) causes complete loss of selenoprotein expression and Δ*Trsp* is embryonic lethal [10,11]. In addition, we generated

transgenic mice in which $\Delta Trsp$ mice were rescued with a mutant *Trsp* transgene lacking i[6]A at position 37 (generated by an A37 → G37 mutation and the resulting transgenic mouse was designed as *G37*) [12] or mcm[5]U at position 34 (generated by an T34 → A34 mutation and the resulting transgenic mouse was designed as *A34*) in the Sec tRNA[Ser]Sec transgene product [13]. Interestingly, housekeeping, but not stress-related selenoproteins are synthesized by A34 and G37 Sec tRNAs[Ser]Sec that are transcribed and modified from the corresponding *A34* and *G37* transgenes [12].

We have also selectively removed *Trsp* in hepatocytes using *loxP-Cre* technology which demonstrated that proper liver function is dependent on selenoprotein expression [14]. The mean life span of the hepatocyte $\Delta Trsp$ knockout mice was significantly reduced compared with their wild type counterparts [14]. These mice died suddenly as a result of hepatocellular degeneration/necrosis. Most hepatocytes had vacuolated cytoplasm and mineralization with a majority of them being apoptotic. In contrast, the liver of mice carrying *A34* or *G37* transgenes appeared to be normal, with their lifespan being similar to that of the corresponding phenotypically normal litter mates (designated herein as wild type).

In the present study, we examined the plasma protein profile of hepatocyte $\Delta Trsp$ and the corresponding wild type mice and observed an elevation in the level of a protein, later identified as apolipoprotein E (ApoE). The elevated level of ApoE was accompanied by an increase in plasma cholesterol levels in hepatocyte $\Delta Trsp$ mice. A comparative gene expression analysis of hepatocyte $\Delta Trsp$ and wild type mice revealed an enhanced expression of genes involved in cholesterol biosynthesis and a decreased expression of genes involved in cholesterol metabolism or transport. Thus, the increase in plasma cholesterol levels accompanied by alteration of genes involved in cholesterol biosynthesis in the hepatocyte $\Delta Trsp$ mice reflects a link between selenoproteins and cholesterol biosynthesis. Interestingly, the levels of ApoE and cholesterol in the *A34* and *G37* transgenic and wild type mice were similar, as demonstrated by immunodetection and plasma lipid analysis, respectively. Since *A34* and *G37* transgenes restore housekeeping, but not stress-related selenoprotein synthesis, the data suggest a relationship between selenoproteins and lipoproteins, wherein housekeeping selenoproteins affect lipoprotein biosynthesis and metabolism.

Materials and methods

Materials. NuPage polyacrylamide gels, polyvinylidene difluoride membranes, Trizol reagent, and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA). SuperSignal West Dura substrate was obtained from Pierce (Rockford, IL, USA); goat polyclonal antibodies against ApoE and bovine anti-goat horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fairplay® II Microarray Labeling kit was obtained from Stratagene (La Jolla, CA, USA), RNA Storage Solution from Ambion Inc. (Austin, TX, USA), MinElute column

from Qiagen (Valencia, CA, USA) and SYBR green supermix from Bio Rad Laboratories (Hercules, CA, USA). All other reagents were commercially available products of the highest grade.

Mice and their genotyping. Control mice (genotype $Trsp^{+/+}$-$AlbCre^{+/+}$, designated wild type), liver $\Delta Trsp$ knockout mice (genotype $Trsp^{fl/fl}$-Alb-$Cre^{+/+}$, designated $\Delta Trsp$) and the *A34* and *G37* mutant *Trsp* transgenic mice (designated *A34* and *G37* mice and described above) were obtained as given [14]. Mice were either siblings or of similar ages, ranging between 8 and 9 weeks. The care of mice was in accordance with the National Institutes of Health institutional guidelines under the expert direction of Dr. Kyle Stump (NCI, National Institutes of Health, Bethesda, MD, USA). DNA was extracted from mouse tail clippings and genotyped by PCR with appropriate primers described earlier [14].

Microsequencing of protein. Plasma proteins were subjected to 10% SDS/PAGE and visualized by Coomassie blue staining. The elevated protein band observed on gels in plasma from $\Delta Trsp$ mice was excised, digested with trypsin, and subjected to ESI-MS/MS analysis at the mass spectrometry core facility, Redox Biology Center, University of Nebraska. The resulting spectra were analyzed against mouse protein database and expressed sequence tag databases.

Analysis of plasma lipids and western blotting. The analysis of plasma lipids was carried out at the Diagnostic and Research Services Branch and Department of Laboratory Medicine, National Institutes of Health, Bethesda, MD, USA. Plasma samples from wild type, $\Delta Trsp$, *A34*, and *G37* mice were electrophoresed on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes and immunoblotted with antibodies against ApoE (1:1000 dilution). Following washes with TBST (Tween supplemented TBS), the membrane was incubated with bovine anti-goat horseradish peroxidase-conjugated secondary antibodies (1:20,000) and then washed with 0.1% TBST, incubated in SuperSignal West Dura substrate and exposed to X-ray film.

Sample preparation for microarray analysis. Total RNA from the liver of wild type, $\Delta Trsp$, *A34*, and *G37* mice was isolated using Trizol reagent according to manufacturer's protocol and dissolved in the RNA Storage Solution. To generate cDNA, 10 μg RNA was incubated with 1 μl oligo dT at 70 °C for 5 min and cooled on ice for 1 min. In a separate tube, 2 μl of 10× StrataScript reaction buffer, 1 μl of 0.1 M dithiothreitol, 1.0 μl of 20× aminoallyl dioxy-ribonucleotide triphosphate, 0.5 μl of 40 U/μl RNAase inhibitor and 2 μl of 400 U/μl StrataScriptII reverse transcriptase were mixed. This mixture was added to the RNA and oligo dT mix and reverse transcription was carried out at 48 °C for 2 h. The cDNA was purified on MinElute columns and eluted from the column with 10 μl elution buffer and dried for 15 min using Speed-Vac. Finally, 5 μl of 2X coupling buffer and 5 μl Cy3 and Cy5 dye were mixed into control and experimental cDNAs, respectively, and incubated in dark at room temperature for 1 h. Following incubation, the labeled cDNA was purified on a MinElute column and eluted with 10 μl of elution buffer.

Gene expression analysis. Mouse oligonucleotide glass arrays were procured from the NCI microarray facility, Frederick, MD, USA. These high-quality oligonucleotide arrays were designated Mm-MEEBO-v1.3px, with each slide having 48 blocks containing 28 rows and 28 columns each. Each slide had 36,960 oligonucleotide spots with a spacing of 155 μm. Arrays were prehybridized with 40 μl prehybridization buffer (5X SSC, 1% BSA and 0.1% SDS) under a coverslip for 1 h at 42 °C. The slides were then washed with deionized water and isopropanol (each wash 2 min), spin-dried and kept at room temperature.

For hybridization, the Cy3 and Cy5 labeled cDNA were combined together and mixed with 1 μL [10 μg] COT-1 DNA, preheated at 100 °C for 1 min to denature the target and cooled on ice for 2 min. This mixture was added to 20 μl of 2X F-hybridization buffer (50% formamide, 10X SSC, 0.2% SDS) and warmed to 42 °C. The total cDNA probe (40 μl) was added to the prehybridized array and covered with a coverslip. The slides were placed in hybridization chambers and incubated at 42 °C overnight (12–16 h). Following hybridization, the slides were washed for 2 min each in 2X SSC and 0.1% SDS, 1X SSC and 0.2X SSC and spin-dried.

Microarray slides were scanned in both Cy3 (532 nm) and Cy5 (635 nm) channels using an Axon GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA) with a 10 μM resolution. Scanned

microarray images were exported as TIFF files to GenePix Pro 3.0 software for image analysis. For data analysis, data files (in gpr format) and images (in jpeg format) were imported into the microarray database (mAdb), and analyzed by software tools provided by NCBI.

Quantitative real-time PCR analysis. Gene expression was verified by real-time PCR, using the DNA Engine Opticon® 2 Real-Time PCR Detection System (MJ-Research/Bio Rad Laboratories, Hercules, CA, USA) in combination with primer sequences outlined in Table 1. Two micrograms of total RNA from each sample was used to synthesize first strand cDNA in a 20 μl reaction mixture by using SuperScript II reverse transcriptase enzyme and random primers. Twenty nanogram of cDNA was utilized for the PCR reaction, using iQ™ SYBR green supermix and 500 nM of each primer, under the following conditions: initial denaturation for 5 min at 95 °C, followed by 40 cycles consisting of 20 s at 94 °C, 20 s at 55 °C and 30 s at 72 °C. Amplifying known amounts of a PCR-product generated a standard curve at the same time as the samples. The expression of various mRNAs in each sample was normalized to the expression of 18 S rRNA.

Results

Analysis of plasma proteins

Many proteins are synthesized in liver and transported to the plasma, and as we had shown previously the importance of selenoproteins in liver function [14], the plasma protein profiles of Δ*Trsp* and wild type mice were examined. Equal amounts of plasma proteins from both mouse

Table 1
Primers used for real-time PCR

Gene	Forward sequence	Reverse sequence
Apoe	5′-GAGGAACAGACCCAGCAAAT-3′	5′-GCCACAGAGGCCTGTATCTT-3′
Cyb5r3	5′-CCCGACATCAAGTACCCTCT-3′	5′-GCCATCGATCCTAGTCGAG-3′
Dhcr24	5′-TGCGAGTCGGAAAGTACAAG-3′	5′-TGGAGTTCAGCAAAGCTGTC-3′
Ebp	5′-TTGGCCTCTTCTCCATCTCT-3′	5′-CCTCGATCACAAGGTGAATG-3′
Ldlr	5′-TGGCCATCTATGAGGACAAA-3′	5′-GTGTGACCTTGTGGAACAGG-3′
Pctp	5′-TGGCATACTGGGAAGTGAAG-3′	5′-GACTTCTCGGGAAACTGAGG-3′
Pmvk	5′-AGGCTGAAGAGCAGACTTGG-3′	5′-CATGTCCCTCCGATAGGTCT-3′
Star	5′-CATTGGCCAAGAGCTCAAC-3′	5′-TGCTGGATGTAGGACAGCTC-3′
Stard3	5′-CCCAGGAAGAGAACTGGAAG-3′	5′-CAGGATCACCTCCTGGTACA-3′
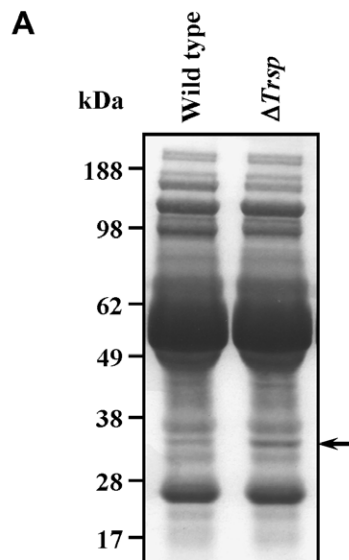
Fig. 1. Analysis of plasma proteins in wild type and Δ*Trsp* mice. (A) Plasma proteins from wild type and Δ*Trsp* mice were electrophoresed and stained with Coomassie blue. The elevated protein (∼35 kDa) in Δ*Trsp* mice is indicated by an arrow. (B) Mouse ApoE sequence. Peptides detected by MS/MS analysis are underlined and shown in bold.

lines were electrophoresed on polyacrylamide gels and stained with Coomassie blue. Staining revealed elevated levels of a protein at ∼35 kDa in the plasma of Δ*Trsp* mice (Fig. 1A). The corresponding band was excised from the gel and protein identity determined by tandem mass spectrometry sequencing. This procedure revealed that the 35 kDa protein is ApoE (Fig. 1B).

ApoE protein and mRNA levels in knockout mice

To verify that ApoE levels were increased in plasma of Δ*Trsp* mice, equal amounts of plasma protein from both male and female wild type and Δ*Trsp* mice were electrophoresed and stained with Coomassie brilliant blue (Fig. 2A). Separately, the samples were examined in immunoblot assays using polyclonal anti-ApoE antibodies (Fig. 2A, lower panel). Increased ApoE was detected in both male and female Δ*Trsp* mice compared to their corresponding wild type counterparts. In addition, real-time PCR revealed a 1.4-fold increase in the levels of *Apoe* mRNA in the liver of Δ*Trsp* mice (Fig. 2B). Interestingly, *Apoe* mRNA levels were also significantly higher (∼3.75-fold) in kidneys of Δ*Trsp* mice as compared to the wild type mice.

Analysis of cholesterol levels and genes involved in cholesterol biosynthesis

Cholesterol levels in plasma were examined in male and female wild type and Δ*Trsp* mice (Fig. 3A). The elevated level of ApoE was also reflected in plasma lipids with an increase in plasma cholesterol levels of Δ*Trsp* mice. Total plasma cholesterol was increased by 38.9% in Δ*Trsp* males and 35.5% in Δ*Trsp* females. We further focused on male mice as they had a lower mean lifespan than their female counterparts [14].

The elevation of cholesterol in the plasma of Δ*Trsp* mice prompted us to examine the expression profile of genes associated with cholesterol biosynthesis. Comparative analyses of gene expression using microarrays revealed altered expression of genes involved in cholesterol biosynthesis, metabolism and transport (Fig. 3B). Genes displaying a change of twofold or more were placed in a tabular form. The loss of *Trsp* in liver was associated with an increased expression of several genes involved in cholesterol biosynthesis (e.g., *Cyb5r3*, *Dhcr24*, *Ebp*, and *Pmvk*) and a decreased expression of genes involved in cholesterol metabolism or transport (e.g., *Ldlr*, *Pctp*, *Star*, and *Stard3*). A quantitative analysis of these genes confirmed
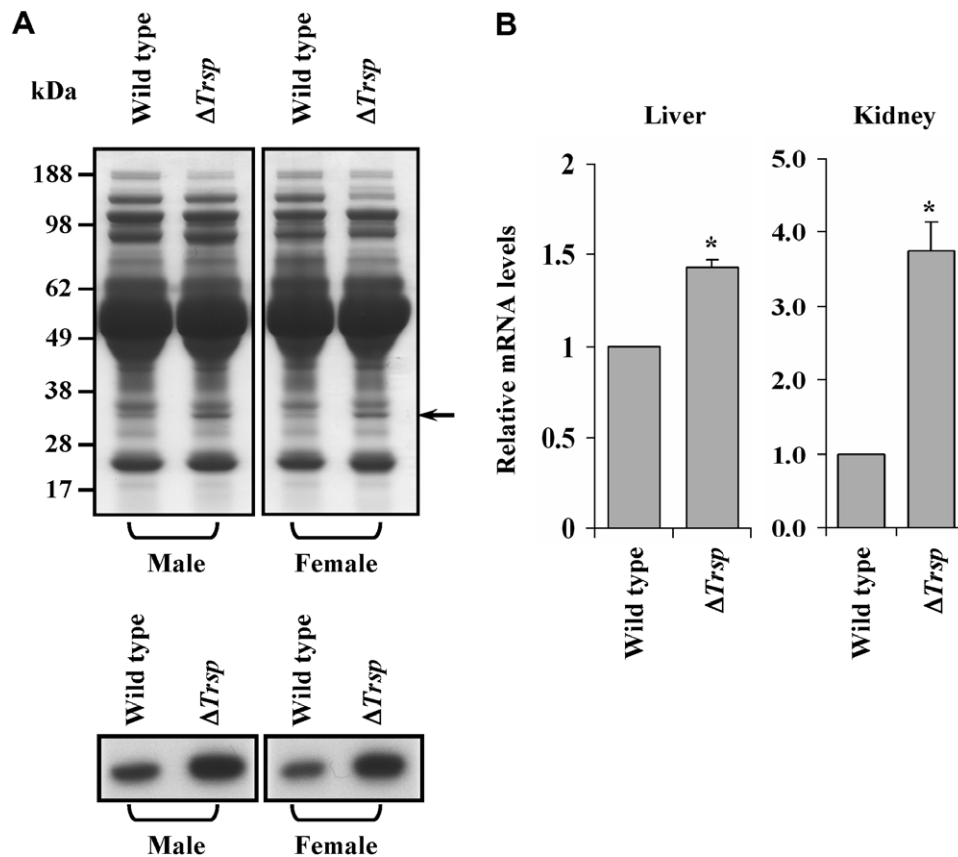

Fig. 2. Analysis of ApoE and *Apoe* mRNA in wild type and Δ*Trsp* mice. (A) Coomassie blue staining of plasma proteins from male and female wild type and Δ*Trsp* mice (upper panel) and immunodetection of ApoE using polyclonal anti-ApoE antibodies in plasma from the same mice (lower panel). (B) The relative *Apoe* mRNA levels in liver and kidney samples of wild type and Δ*Trsp* mice, determined by real-time PCR. The level of *Apoe* mRNA in each sample was normalized to that of 18 S rRNA and the normalized value for *Apoe* mRNA in Δ*Trsp* mice was then plotted relative to that of wild type mice along with the error bars. The results are representations of 4 independent experiments, each carried out in triplicate (*$p < 0.0005$ versus wild type mice).
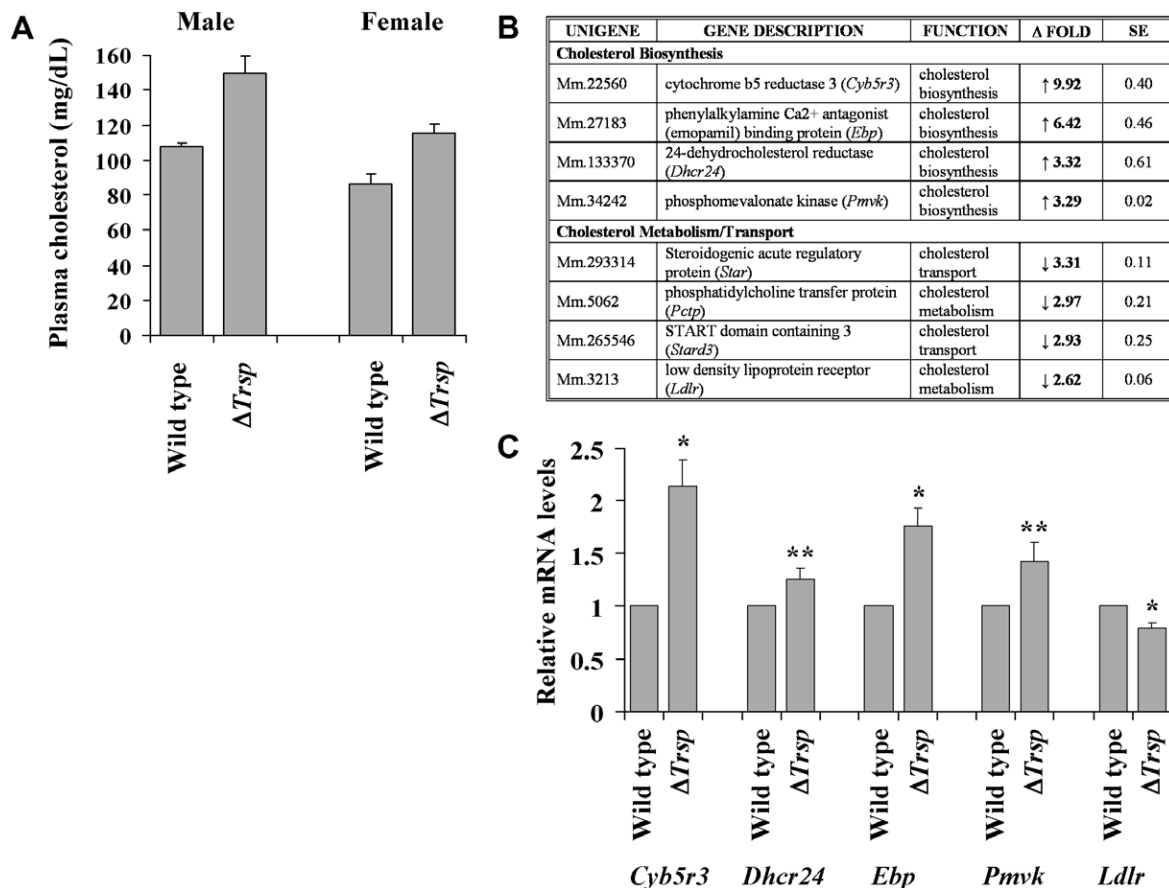
Fig. 3. Cholesterol levels and analysis of genes involved in cholesterol metabolism in wild type and ΔTrsp mice. (A) Examination of plasma cholesterol levels in wild type and ΔTrsp male and female mice. The values represent means ± SE, $n = 5$ mice/sex genotype^{-1} ($p < 0.01$ for both genders). (B) Microarray analysis, using total RNA from liver of wild type and ΔTrsp mice showing genes with altered expression involved in cholesterol biosynthesis, metabolism or transport. Results from four independent hybridizations are shown in the column designated ΔFOLD wherein an arrow pointing up indicates a relative increase, and an arrow pointing down, a relative decrease in mRNA expression; and the standard error is shown in the column designated SE. (C) Real-time PCR analysis of mRNA levels of some of the genes tabulated in (B). The results represent 3–4 independent experiments, each carried out in triplicate and shown along with the error bars ($^{*}p < 0.005$; $^{**}p < 0.1$).

the microarray data for *Cyb5r3*, *Dhcr24*, *Ebp*, *Pmvk*, and *Ldlr* (Fig. 3C). The transcript levels of *Pctp*, *Star*, and *Stard3* were too low to be detected under these experimental conditions and were not further examined.

Analysis of ApoE levels and cholesterol levels in selenoprotein replacement mice

To assess whether the elevation in ApoE and cholesterol levels was due to altered expression of housekeeping (e.g., TR1 and GPX4) or stress-related (e.g., GPX1 and SELT) selenoproteins, the plasma protein profiles of wild type and ΔTrsp mice were compared to the corresponding levels in *A34* and *G37* transgenic mice, which are known to express the former class of selenoproteins, but express the latter class poorly [14]. Equal amounts of plasma proteins from each mouse line were electrophoresed and stained with Coomassie brilliant blue (Fig. 4A). Western blot analysis detected ApoE levels in these mice and we found that *A34* and *G37* mice had normal levels of ApoE (Fig. 4A, lower panel).

Plasma lipid analysis showed that although the levels of cholesterol were elevated in ΔTrsp mice with respect to wild type mice, they were virtually identical in wild type and the *A34* and *G37* mice (Fig. 4B). Furthermore, expression of genes associated with cholesterol biosynthesis was restored in selenoprotein replacement mice and was more in line with that in wild type mice (data not shown).

Discussion

Selenoproteins are critical for proper liver function as their loss in this organ leads to severe necrosis and hepatocellular degeneration [14]. This observation suggested that the absence of selenoproteins in liver may influence the function of other proteins. To examine whether the levels of secreted proteins are altered in the mouse model of hepatic selenoprotein deficiency, we compared the levels of major plasma proteins from ΔTrsp and wild type mice and observed an increase in a ~35 kDa protein in liver knockout mice. Microsequencing and western blotting identified this protein as ApoE. The increase in ApoE
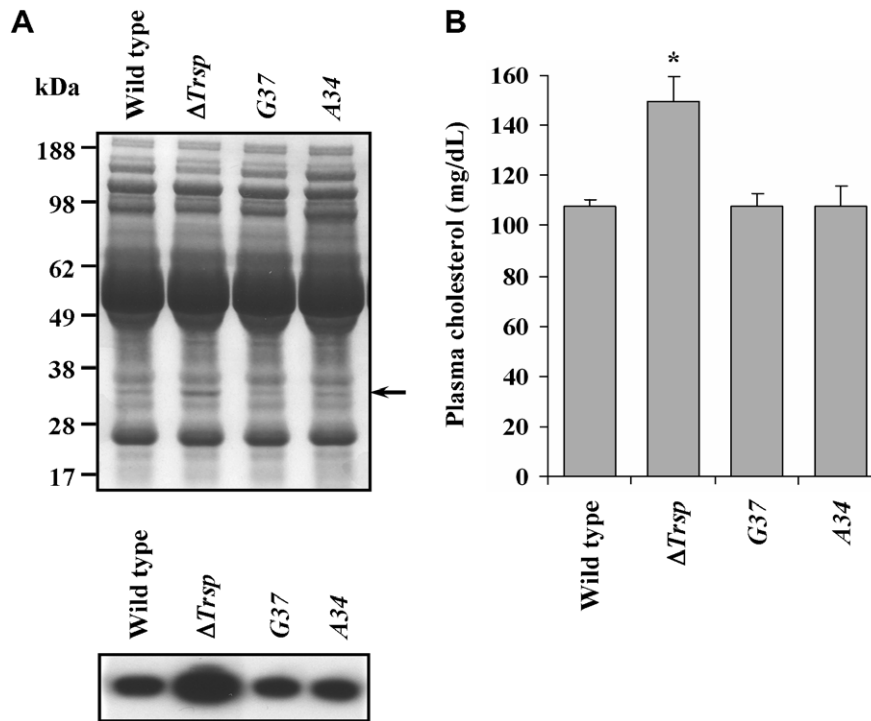
Fig. 4. ApoE and cholesterol levels in *A34* and *G37* mice. ApoE was assessed by Western blotting and cholesterol levels were measured in plasma lipids of wild type, ΔTrsp, *A34*, and *G37* mice. (A) Coomassie blue staining of plasma proteins from wild type, ΔTrsp, *A34*, and *G37* male mice (upper panel) and western blotting carried out using polyclonal anti-ApoE antibodies (lower panel). (B) Plasma cholesterol levels. Values represent means ± SE, $n = 5$ mice/genotype for wild type and ΔTrsp mice and $n = 4$ mice/genotype for *A34* and *G37* mice (*$p < 0.01$).

was accompanied by an increase in cholesterol levels in the plasma of ΔTrsp mice, which did not appear to be gender specific. A comparative gene expression analysis in livers of ΔTrsp and wild type mice revealed an enhanced expression of genes involved in cholesterol biosynthesis and a decreased expression of genes involved in cholesterol metabolism and transport in ΔTrsp mice. The *Apoe* mRNA levels were also significantly higher in the kidney of ΔTrsp mice as compared with the kidney from wild type mice. Earlier reports suggested that *Apoe* is synthesized in both liver and kidney [15], and the increased mRNA levels observed in kidney could also account for elevated levels of the protein in plasma.

It was previously reported that selenium deficiency results in an increased plasma cholesterol concentration [16,17] along with an increase in ApoE levels [18]. It was speculated that this increase was related to an increase in the HDL1 fraction [18] which is rich in ApoE. The present study analyzed the effects of the targeted removal of *Trsp* in liver of mice fed a selenium sufficient diet. Interestingly, the findings of increased cholesterol and elevated levels of ApoE in the ΔTrsp mice are similar to those reported earlier in rats maintained on selenium deficient diets [19]. Thus, these observations could be attributed to the absence of selenoproteins in liver, even though our mice were fed selenium sufficient diets. Since stress-related selenoproteins are more susceptible to selenium status than housekeeping selenoproteins, one might expect that the former subclass

of selenoproteins is responsible for the observed effect. To test this possibility we examined levels of ApoE and cholesterol in the *A34* and *G37* transgenic mice. The housekeeping, but not stress-related selenoprotein population was replaced in these transgenic mice, yet the levels of ApoE and plasma cholesterol were restored to those observed in the corresponding wild type mice. Thus, the observed changes in ApoE and cholesterol levels and their restoration in mice in which housekeeping selenoproteins were expressed, suggests that stress-related selenoproteins could not account for this effect.

Several selenoprotein mRNAs were restored in *A34* and *G37* transgenic mice, such as *Dio1*, *Selk*, *Sepp1*, and *Sep15* [13], while others like *TR1* and *TR3* were restored partially. Although mRNA levels might not necessarily match protein expression levels, our data suggest that some of these or other restored selenoproteins are responsible for the role of selenium in ApoE and cholesterol metabolism.

Earlier studies have suggested a role of selenium [20] and/or selenoproteins in cardiovascular disorders [21] and reports indicate that cholesterol levels increase in selenium deficient animals compared to adequate selenium fed animals [22]. Our studies suggest that these effects are most likely executed through a select group of selenoproteins as our mouse models have shown that the loss of Sec tRNA[Ser]Sec elicits similar conditions, even under selenium sufficient diets. It is of interest to note that the selenium content of liver from ΔTrsp and the mutant *Trsp* transgenic

mice are similar and only about 30% of wild type. This observation provides further evidence that the selenium effect is mediated through (a) housekeeping selenoprotein(s) providing evidence for a novel role of this subclass in human health through modulation of lipoprotein and cholesterol metabolism.

Acknowledgments

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