The Antioxidant Activity of Recombinant Rat Hepatic Fatty Acid Binding Protein T94A Variant

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ABSTRACT - PURPOSE: Liver fatty acid binding protein (FABP1) is a cytoplasmic polypeptide that transports substrates throughout the cytosol and functions as an antioxidant. A common polymorphic variant, FABP1 T94A has a minor allele frequency of 26-38%, 8.3±1.9% homozygous in the human population. The purpose of this study was to mutate and isolate recombinant rat FABP1 to the T94A variant to evaluate the mutant's antioxidant activity using *in vitro* studies. **METHODS:** Site-directed mutagenesis was used to generate a mutation in rat cDNA within a pGEX-6p-2 vector. This plasmid was transformed into competent cells and cultured for expression of FABP1 T94A mutant. The mutated protein was purified using GSTrap Fastflow columns within an ÄKTA FPLC system. A 2,7-dichlorofluorescein (DCF) assay was used to screen the T94A variant antioxidant activity. Additionally, Thiobarbituric Acid Reactive Substances (TBARS) assay was used in determining T94A mutant antioxidant activity in hydrophilic and lipophilic environments through the use of the azo compounds AAPH and MeO-AMVN, respectively and in the presence and absence of the long-chain fatty acid palmitate and α-bromo palmitate. **RESULTS:** Although the FABP1 T94A (20 μM) mutant significantly reduced DCF fluorescence compared to control (no protein; P< 0.001), there were no significant difference when compared to the wild-type (WT) FABP1. T94A was able to diminish the formation of malondialdehyde (MDA) in both lipophilic and hydrophilic systems. There were significant differences between T94A mutant and WT FABP1 at concentrations 1 and 10 μM (P< 0.05) in the hydrophilic milieu, however, this was not seen at 20 μM and also not seen in the lipophilic milieu at all concentrations. When T94A was pre-incubated with the long-chain fatty acids palmitate or α -bromo palmitate, MDA formation was decreased in both lipid peroxidation systems. There were no statistical differences between the WT FABP1 and T94A bound with fatty acids in both lipid peroxidation systems, however, there was a slight statistical difference when the T94A and WT FABP1 bound α-Br-PA in the AAPH lipid peroxidation system only. **CONCLUSIONS:** The T94A has antioxidant activity in both hydrophilic and lipophilic environments. The T94A variant of FABP1 does not have a loss of function in regard to acting as an antioxidant but the extent of function may be influenced by ligand binding. We conclude that populations having the minor T94A allele frequency would have similar ROS scavenging potential as those with nascent FABP1.

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INTRODUTION

FABP1 is the intracellular equivalent to plasma albumin, binding lipophilic endogenous and exogenous compounds and transporting them throughout the cytosol. The protein is found in various tissues such as liver, intestine, pancreas, kidneys, lung, and stomach (0). FABP1 has been shown to be an effective intracellular antioxidant. A study showed the importance of FABP1 as an antioxidant utilized Chang cells, which are normally devoid of FABP1. Stably transfected cells with the FABP1 cDNA, showed lower concentrations of reactive oxygen species (ROS) than those without FABP1, suggesting that the protein can scavenge ROS (1).

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It was further shown that FABP1 levels can be increased using clofibrate or decreased with dexamethasone, thus affecting ROS levels in models of H_2O_2 incubation or hypoxia-reoxygenation (2). FABP1 was shown to act as a potent hepatic antioxidant in animal models of cholestasis (3), alcohol liver disease (4), acetaminophen toxicity (5), in *in vitro* models of NAFLD (6). FABP1 may thus play a critical role in oxidative homeostasis within the liver. The molecular mechanism for FABP1's antioxidant function was reported to centre on the solvent accessible methionines (five out of seven) and single cysteine being responsible for scavenging ROS (7). Others have subsequently reported the antioxidant role of FABP in other species (8, 9). Reports showed that when liver damage was induced by environmental stress on orange-spotted groupers, FABP10 (a paralog of FABP1) acts as an antioxidant (8). Decreased expression of FABP1 in zebrafish larvae also was associated with lower superoxide dismutase activity and higher levels of malondialdehyde (MDA) (9).

FABP1 T94A is a missense mutation in the human *Fabp1* gene where the 94th amino acid, a polar moderate sized threonine, is substituted for a non-polar, small sized alanine residue (11). The single nucleotide polymorphism (SNP) is considered common within humans having a minor allele frequency of 26-38%; 8.3% homozygous MAF for 1000 genomes in NCBI dbSNP database; ALFRED database (11). Carriers are associated with higher triacylgycerol, elevated free fatty acid in plasma, and lower body mass index (11). Moreover, the mutant variant is linked with atherothrombotic cerebral infarction (12) and non-alcoholic liver disease (13). Initially, the T94A substitution was thought to completely abolish the binding and function properties of the protein. Recent studies revealed the substitution did not alter ligand binding affinity, however, it significantly altered conformational and functional response to fibrates as well as stability (14). The altered function was also demonstrated with fatty acids and triglyceride intermediates (15). The FABP1 T94A variant also has an increased affinity for cholesterol that may be due to a change in the binding site's polarity (16).

Since it has been established that FABP1 acts as a critical antioxidant in the liver and that the T94A variant is associated with altered functional response to drug binding, determining whether the T94A variant would be associated with impaired antioxidant function is important and has yet to be elucidated. As FABP1 accounts for about 2% of total cytosolic protein (17), the loss of the protein's antioxidant ability would compromise carriers during states of oxidative stress. Therefore, the current study aims in determining the antioxidant ability of FABP1 T94A using *in vitro* assays.

METHODS

Materials
Triton X-100, phenylmethylsulfonyl (PMSF), aprotinin, leupeptin, reduced glutathione, dithiothreitol (DTT), 2, 7-dichlorofluorescein-diacetate (DCFH-DA), hydrogen peroxide (H_2O_2), low density lipoproteins (LDL), palmitate (PA), alpha-bromo palmitate (α-Br-PA), and DH5α-T1^R competent cells were purchased from Sigma (St. Louis, MO, USA). Site-Directed Mutagenesis system kit, LB broth, and isopropyl-β-D-thio-galactoside (IPTG), were purchased from Fisher Scientific (Ottawa, ON, CA). Mutagenic primers, pGEX 3' Sequencing Primer, 5', GSTrap Fastflow columns, and PreScission Protease were obtained GE Life Sciences, Chicago, USA). Thiobarbituric acid (TBA) was purchased from EMD Millipore (Billerica, MA, USA). 2, 2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) were purchased from Wako (Osaka, Japan).

Site-Directed Mutagenesis, Expression and Purification of Recombinant FABP1 and T94A
The introduction of the T94A mutation to the pGEX-6P-2 vector containing rat FABP1 cDNA was accomplished using the GENEART Site-Directed Mutagenesis System (Invitrogen, ThermoFisher Scientific, Mississauga, Canada) (18, 19). The pGEX-6P-2 vector containing rat FABP1 cDNA was previously constructed (7), sequences for mutagenic primers were $^{5'}$AAT AAA ATG GTG ACA GCT TTC AAA GGC ATA A$^{3'}$ and $^{5'}$T TAT GCC TTT GA A AGC TGT CAC CAT TTT ATT$^{3'}$.

pGEX-6P-2 with the FABP1 T94A mutant or wild-type (WT) FABP1 were transformed in *E. coli* DH5α cells for large scale production of the FABP1 T94A variant and WT FABP1 control. For each construct, a single colony from a freshly streaked plate was used to inoculated 3 mL LB with 100 µg/mL ampicillin and 20 mM dextrose as a starter culture and grown overnight by shaking (200 rpm) at 37°C. Each was then diluted into 500 mL LB broth

in 2.8 L Fernbach flask and incubated for 5-6 hours at 37°C with 200 rpm until an OD600 ≈ 1.0 was reached. FABP1 T94A expression was induced with the addition of 1 mM of isopropyl-β-D-thio-galactoside (IPTG) for six hours at 37°C. Cells were collected by centrifugation at 3500 g for 20 minutes, and resuspended in 20 mL of ice-cold 1 X PBS containing 1% Triton X-100 and protease inhibitors (PMSF, aprotinin, and leupeptin at concentrations of 25 μg/ml, 2 μg/ml, 1 μg/ml, respectively). Cells were then sonicated on ice for five cycles of 30 seconds with 30 seconds rest using a Branson Sonifier 450 (Danburry, Connecticut, USA) and centrifuged at 14,000 g for 15 minutes at 4°C. WT FABP1 and the T94A variant were purified from the cell lysate using the ÄKTA Fast protein liquid chromatography (FPLC) and GSTrap FF columns using the elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, 1 mM DTT, pH 8.0). Eluted protein sample was cleaved with the addition of 500 μL PreScission Protease (40 μL PreScission Protease and 460 μl of cleavage buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.5]). The pGEX-6P-2 has a cleavage site for PreScisson Protease to cleave the protein of interest from the GST tag leaving an N-terminal infusion linker, sequence of GPLGSIAT. The eluted fusion protein was incubated with protease at 4°C overnight with end over end rotation. After digestion, the FABP1 or T94A variant samples were re-added to an equilibrated GSTrap FF column, collected and concentrated using Amicon Ultra-15 Centrifugal Filter Devices with centrifugation at 5,000 g for 60 minutes. To validate DNA sequencing (FABP1 and T94A), the DNA plasmid was sent to the Manitoba Institute of Cell Biology for DNA sequencing using pGEX 3' Sequencing Primer, 5'.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed to identity the purified recombinant protein. A 15% separating and 5% stacking gel were used for the SDS-PAGE and Western blot. Composition of the separating gel was 2.3 mL double distilled water, 2.5 mL Tris-HCl (1.5M), 100 μL SDS (10%), 2.5 mL acrylamide mix (30%), 100 μL ammonium persulfate (APS) (10%), and 4 μL tetramethylethylenediamine (TEMED). Compostition of stacking gel was 3.4 mL double distilled water, 0.63 mL Tris-HCl (1M), 50 μL SDS (10%), 0.83 mL acrylamide mix (30%), 50 μL APS (10%), and 5 μL TEMED . Running buffer consisted of 25 mM Tris, 190 mM glycine, 0.1% SDS (250 mL). A total of 30 μg protein (15 μL) was added to (5 μL) 4x sample loading buffer (250 mM Tris-HCl pH6.8, 20% glycerol 8%, sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 5% β-mercaptoethanol). Samples were boiled for 5 minutes at 100°C and briefly centrifuged using a Mini Centrifuge (Mandel Scientific, Ontario, Canada). Samples (20 μL) were loaded into the wells. Electrophoresis was performed at a constant 100 Volts for 90 minutes using the Miniprotean II cell powered by Powerpac 1000 (Bio-Rad, Hercules, USA) afterwhich gels were rinsed with water (3X) before being stained with Coomassie (0.1% Coomassie R-250, 10% acetic acid, 40% methanol). Bands were viewed following destaining.

Western blot

Identity of proteins was confirmed by Western blot analysis. Proteins separated by SDS-polyacrylamide gel were transferred to 0.45 μm nitrocellulose membranes and blot run for 1 hr at 100V. Membranes were rinsed in water (3x) before being blocked with 5% (w/v) non-fat skim milk in Tris buffered saline with Tween 20 (TBST) solution (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 hour at RT with gentle agitation. Membranes were then washed (3x) with 30 mL 1X TBST for 5 minutes and then incubated with 10 mL rabbit polyclonal L-FABP antibody (Santa Cruz Biotechnology, Dallas, USA) (1:500 in 5% powdered non-fat skimmed milk in TBST) overnight with gentle agitation. After the application of the primary antibody, membranes were washed (5x) with 30 mL of 1X TBST for 5 minutes each and incubated with goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Dallas, USA) (1:500 in TBST) for 1 hour at RT with gentle agitation. After incubation membranes were washed (5x) with 30 mL TBST for 5 minutes each. Samples in the membrane were detected by enhanced chemiluminescence using the ECL Plus system from GE Lifesciences. Protein samples were also sent to the Pharmaceutical Analysis Laboratory at the University of Manitoba to confirm protein size by LC-MS.

Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLC-MS) Assay

Mass spectrometry was performed using a Shimadzu LCMS-8040 triple-quadrupole mass spectrometer; LC-MS/MS (Shimadzu, Kyoto, Japan)

coupled to a Nexera ultra high performance liquid chromatograph (Shimadzu, Kyoto, Japan) and analyzed using Shimadzu LabSolutions software. The MS was operated in DUIS mode (ESI/APCI) using a positive scan between 1000 and 2000 m/z. MS conditions consisted of a desolvation line temperature of 250 °C and heating block temperature of 400 °C. Nebulizing gas flow was 2 L/min and drying gas was 15 L/min. FABP1 and the T94A variant were analzed on a Waters Aquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) at 40°C using a binary gradient of mobile phase A (aqueous 0.1% trifluoro acetic acid, TFA) and mobile phase B (methanol with 0.1% TFA) with a total flow rate of 0.3 mL/min. 10% mobile phase B was held for 1 min followed by a 2 min gradient to 80% B, which was held for 12 min. The column was washed with 90% B for 5 min and stepped back to 10% B for 5 min to recondition the column. The retention time of WT FABP1 and T94A was 5.8 minutes. The mass spectra extracted from each chromatogram was deconvoluted using the Multi-Charded Ion Analysis to report mass.

DCF (2,7-dichlorofluorescein) Fluorescence Assay

A DCF assay was used in determining whether the FABP1 T94A variant possessed antioxidant capacity similar to that of the WT FABP1. Briefly, 2,7-dichlorofluorescein-diacetate (DCFH-DA) was de-esterified by reacting with ethanol and sodium hydroxide yielding 2,7-dichlorodihydrofluorescein (DCFH). This non-fluorescent derivative was the dye stock solution prepared daily. DCFH when oxidized by ROS produces the fluorescent compound DCF. Oxidation reactions were performed in 96 well CoStar plates where 50 μL of different concentrations of protein sample or control were added to 40 μL DCFH [60 μM], 5 μL iron (II) sulfate ($FeSO_4$) [1 mM], and 5 μL hydrogen peroxide (H_2O_2) [1 mM]. Sample fluorescence was read using a Synergy HT BioTeK microplate reader (Winooski, Vermont, USA) at 485 nm excitation and 590 nm emission.

TBARS Assay

Lipid peroxidation was assessed by investigating the presence of lipid peroxides that decompose into reactive malondialdehyde (MDA) compounds. LDL provides a platform for the free radicals to attack, resulting in lipid peroxides such as MDA. Lipid peroxidation products are highly reactive forming adducts with biologically important macromolecules. Hence, the TBARS assay illustrates the capacity of a protein to act as an antioxidant. Lipid peroxides are generated by incubating LDL with the azo compounds 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) or 2, 2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). These azo compounds thermally decompose into free radicals in hydrophilic and lipophilic environments, respectively. Malondialdehyde bis (dimethyl acetal 1, 1, 3, 3-tetramethoxypropane) was prepared fresh and used as a standard. LDL [0.5 mg/mL] was incubated with either AAPH [10 mM] or MeO-AMVN [1 mM] and varying concentrations of protein in a 37°C water bath for 90 minutes. The addition of a TBA solution (0.67% TBA, 15% trichloroacetic acid, 0.25 N HCl) halts the oxidation reaction. Samples were then heated at 100°C for 15 minutes to develop the colorimetric pink chromogen. Reactions were then cooled on ice for 5 minutes and centrifuged briefly to pellet the flocculates with the supernatant absorbance being analyzed at 535 nm. The TBARS assay was also used to evaluate whether proteins were affected by binding with the long-chain fatty acids palmitate and α-bromo palmitate, in which case 10 μM of the proteins were pre-incubated with the fatty acids (30 μM) for 1 hour before being subjected to oxidation reactions.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. The N value represents the number of replicates for each study. Statistical analysis used t-test (unpaired) where two groups were compared while a two-way ANOVA was employed for multiple comparisons followed by a Bonferroni post-test using GraphPad Prism 6.0. Results were considered significant when the p-value was less than 0.05.

RESULTS

Identification of the Plasmid Vector pGEX-6P-2 with FABP1 cDNA by Restriction Enzyme Mapping

FABP1 cDNA was initially ligated into the pGEX-6P-2 vector with BamHI and XhoI restriction enzyme recognition sequences (8). By using these endonucleases the isolated plasmid was cleaved into the vector and the cDNA permitting the separation of the DNA into bands of appropriate sizes with agarose

gel electrophoresis. Separation between the plasmid vector at an estimated 4900 bp and the FABP1 cDNA at 418 bp is shown in Figure 1. Figure 1 also shows that there was no difference between the original and mutated pGEX-6P-2 and FABP1/T94A bands, suggesting no unnecessary deletions or insertions occurred during site directed mutagenesis.
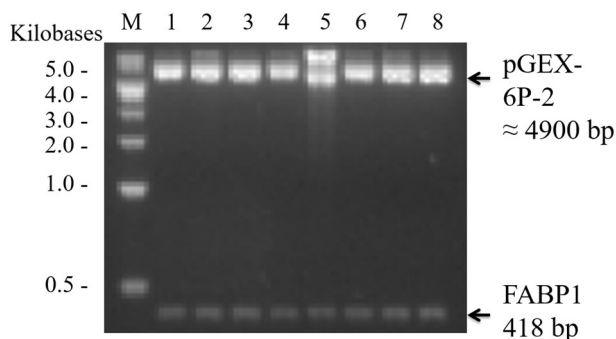

Figure 1. Analysis of recombinant pGEX-6P-2/FABP1 using restriction enzyme mapping after site-directed mutagenesis using GENEART Site Directed Mutagenesis System. Plasmids were purified using the plasmid midi kit from QIAGEN and digested with endonucleases at 37°C for 4 hours. DNA samples were loaded into 1.0% agarose gel and ran for 90 minutes at 100 V. The gel was stained with SYBR Safe and visualized with a UV transilluminator. Lanes 1 and 2 show the original plasmid while the mutated plasmid digested with BamH1 and Xho1 are in lanes 3-8 pGEX-6P-2/FABP1.

Expressing Recombinant proteins FABP1 and the FABP1 T94A Mutant Variant

Plasmids used in this study contained FABP1 and the mutated variant T94A cDNA fragments. Fragments were located between restriction enzymes site BamHI and XhoI, which is downstream of the GST tag sequence. The promoter for the GST tag was induced by introducing 1 mM IPTG to the bacterial culture and incubated for an additional 6 hours before collecting cells. Figure 2A shows results from the 15% SDS-PAGE gel comparing non-mutated and mutated bacterial sonicates with and without bacterial induction with IPTG. The dark band indicated by the arrow at 40 kDa shows the fusion protein FABP1 or T94A with the GST tag. Lanes 2 and 4 show incubation with IPTG resulting in darker bands than those in lanes 1 and 3. Figure 2B illustrates a histogram of band density of the gel seen in Figure 2A using ImageJ for analysis. From Figure 2B, samples that were induced with IPTG have clearly stronger band densities compared to samples that were not induced by IPTG.
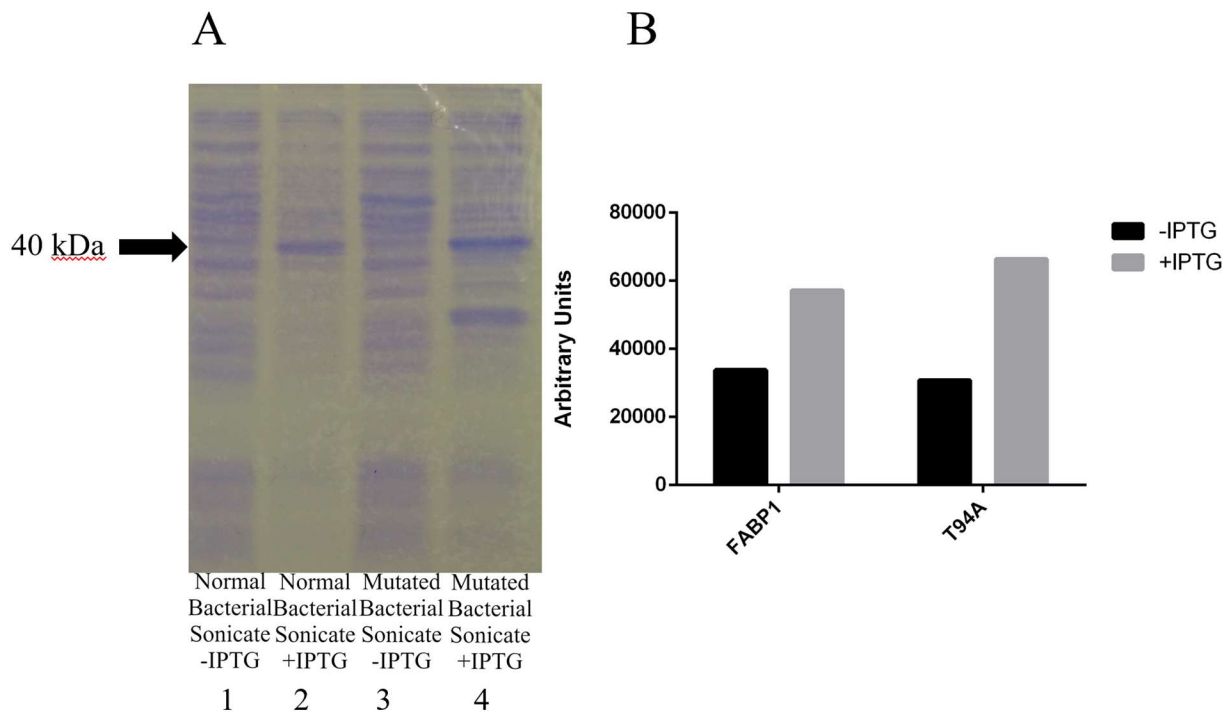

Figure 2. Protein induction with IPTG. The single experiment illustrates the use of IPTG to induce protein expression. (A) 15% Tri-Glycine SDS-PAGE gel. From left to right: Normal bacterial sonicate (1), induced normal bacterial sonicate (2), mutated bacterial sonicate (3), induced bacterial sonicate (4). (B) Histogram illustrating the band density from the 15% Tri-Glycine SDS-PAGE gel of Figure 2A between IPTG induced and uninduced bacterial cultures.

The Purification of Recombinant FABP1 and FABP1 T94A

The fusion protein was purified and the GST tag separated from FABP1 or T94A variant with PreScission protease. Figure 3 shows a 15% polyacrylamide gel loaded with Amercham™ ECL™ Rainbow™ Marker- Full Range, normal bacterial sonicate, induced normal bacterial sonicate, mutated bacterial sonicate, induced mutated bacterial sonicate, normal fusion protein, mutated fusion protein, WT FABP1, and the T94A variant protein. Figure 3A, lanes 6 and 7 show both the WT and mutated fusion proteins having a molecular mass of 40 kDa, which is consistent with FABP1 or its T94A mutant (14 kDa) and the GST tag (26 kDa). The next two lanes show purified recombinant proteins at 14 kDa after cleavage and additional purification to remove the tag and protease. The last four lanes show no contaminants present despite loading up to 30 μg of protein in each well. To further confirm identities of the purified proteins a western blot was conducted. Figure 3B shows the western blot of the purified recombinant proteins. The result shows that the recombinant protein samples are immunoreactive with the rabbit polyclonal antibody against rat FABP1. A second method of identifing purified proteins used LC-MS in determining the proteins' mass. Figure 4 is the total ion count chromatogram and the deconvoluted mass of FABP1 (A) and T94A (B). The deconvoluted mass of FABP1 was 1468 Da and the T94A was 1438 Da showing a difference of 30 Da, corresponding to the difference in mass between threonine and alanine.
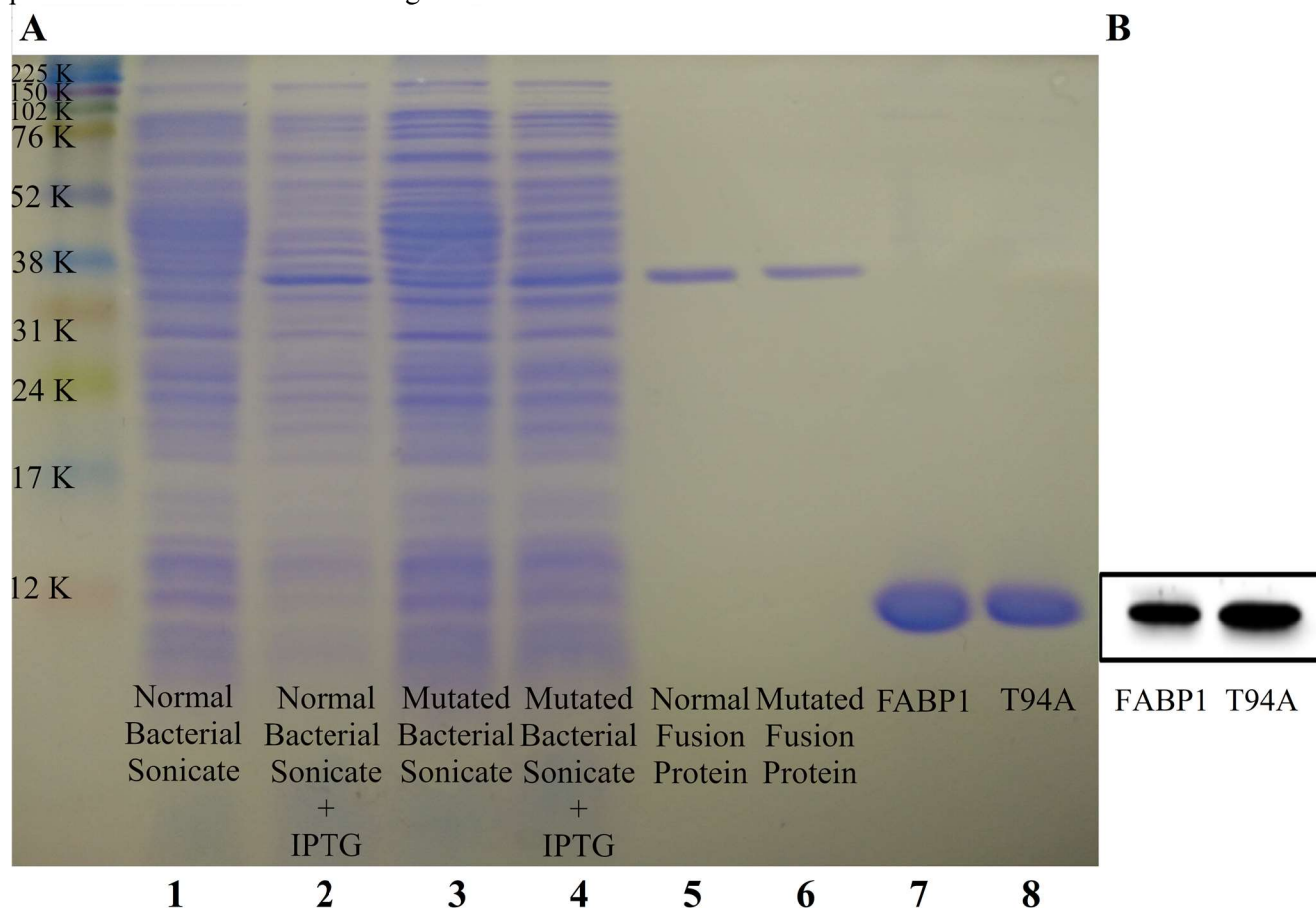

Figure 3. Identification of Proteins using SDS-PAGE (A) and Western Blot (B) using 15% Tri-Glycine SDS-PAGE gel. From left to right: Amercham™ ECL™ Rainbow™ Marker- Full Range, normal bacterial sonicate (lane 1), induced normal bacterial sonicate (lane 2), mutated bacterial sonicate (lane 3), induced mutated bacterial sonicate (lane 4), normal fusion protein (lane 5), mutated fusion protein (lane 6), FABP1 (lane 7), and the T94A variant protein (lane 8). B) Western Blot of the WT FABP1 and T94A variant proteins. Lanes 5 and 6 indicate the non-mutated WT and mutated fusion proteins have a molecular mass of 40 kDa, from FABP1 or its mutant (14 kDa) and the GST tag (26 kDa). Lanes 7 and 8 are purified recombinant proteins at 14 kDa after cleavage and additional purification to remove the GST tag and protease.

Antioxidant Capacity of Recombinant FABP1 T94A

One of the main objectives of this study was determining whether recombinant rat FABP1 T94A variant possesses the ability to act as an antioxidant similar to its non-mutated WT form. To evaluate the protein's antioxidant capacity, DCF fluorescence assay was employed using ascorbic acid and TROLOX™ as controls. Figure 5 shows that at the lowest concentration of T94A (1 μM), the protein was able to reduce DCF fluorescence to 75.8 ± 2.9%, compared to control (0 μM) while at its highest tested concentration of 20 μM a reduction to 36.3 ± 2.2% of control was seen (P< 0.001). There was a dose response relationship between increasing concentration of T94A mutant and decreasing DCF fluorescence, suggesting that the protein functions as an antioxidant. When comparing T94A mutant to WT FABP1, there were no significant differences in their ability to reduce DCF fluorescence at all concentrations, suggesting that the T94A mutation does not significantly affect the ROS scavenging properties of FABP1.

Effect of Recombinant FABP1 T94A on *in vitro* Lipid Peroxidation with AAPH and MeO-AMVN

To further evaluate the antioxidant activity of FABP1 T94A, AAPH and MeO-AMVN were used to release free radicals in the presence of LDL in hydrophilic or lipophilic environments, respectively. Since these compounds thermally decompose at different rates it was necessary to standardize their concentrations such that free radical release would be similar. Using a TBARS assay, LDL was incubated with 10 mM AAPH and found to produce 670.1±13.4 ng MDA/ mg LDL protein, which was not statitiscally different than MeO-AMVN at 1 mM generating 713.0±11.2 ng MDA/ mg LDL protein. Since these concentrations of azo compounds produced similar amounts of MDA they were used in determining T94A's antioxidant activity in hydrophilic and lipophilic environments. FABP1 was reported to have more activity in the hydrophilic environment (8), it is of interest and importance in determining whether the T94A mutation has similar activity. Figure 6A shows that at a concentration of 20 μM, T94A produced 72.1±4.4% of control MDA formation in the hydrophilic system. In the lipophilic system, the T94A protein was able to inhibit 23.4±5.5% of MDA from forming at 20 μM (Figure 6B). The variant was able to reduce MDA in a dose-dependent manner in both environments (Figure 6A

and B), suggesting the mutant can act as an antioxidant in both systems. Positive controls used for the assay included ascorbic acid and TROLOX™ in the AAPH system while in the MeO-AMVN system ascorbic acid acted as a negative control. When comparing FABP1 WT to T94A with AAPH being used as a source of ROS, there was a significant difference between proteins at concentrations at 1 μM (79.8%±1.5 and 105.0%±9.4 of the WT control) and 10 μM (65.8%±1.8 and 88.1%±5.7 of WT control) only (P< 0.05). When antioxidant activities of FABP1 and T94A were used in a lipophilic environment with MeO-AMVN, there was no statistical difference in MDA production (Figure 6B). Figure 7 shows a summary of the WT FABP1 and T94A data using AAPH and MeO-AMVN systems.

Influence of Long-Chain Fatty Acid Binding on FABP1 T94A's Antioxidant Function

Some of the FABP1 antioxidant residues are contained within its binding pocket. Consequently, binding with ligands may affect the protein's ability to act as an antioxidant (8). Previous work shows that FABP1 has very good antioxidant activity when the protein is in the unbound state but in the presence of fatty acids its activity is reduced (8). In this study we investigated whether FABP1 T94A's function as an antioxidant also could be influenced by binding fatty acids. In determining the effects of fatty acid binding, we pre-incubated purified recombinant proteins (10 μM) with either 30 μM of palmitic acid (PA) or α-bomo palmitate (α-Br-PA). Palmitic acid binds reversibly to FABP1 while α-bomo palmitate effectively binds irreversibly to FABP1. Figure 8A shows MDA production with unbound recombinant proteins while Figures 8B and C show proteins bound with palmitic acid and α-bomo palmitate, respectively.

In the AAPH environment, unbound WT FABP1 and T94A had statistically lower amounts of MDA produced compared to control (Figure 8A). Unbound WT FABP1 and T94A were associated with MDA production of 61.1±1.8% of control (p<0.001) and 83.2±6.1% of control (p<0.05), resepctively, which were also statistically different from each other (p<0.01). In the MeO-AMVN environment, unbound WT FABP1 and T94A had statistically lower amounts of MDA produced compared to control (Figure 8A). Unbound WT FABP1 and T94A were associated with MDA production of 75.4±6.3% of control (p<0.01) and 83.4±5.9% of

control (p<0.05), which were not statistically different from each other.

When WT FABP1 and T94 were bound to PA both proteins showed statistical differences from control (Figure 8B). In the AAPH environment, WT FABP1 was associated with MDA production of 79.8±3.7 (p<0.001) while T94A had 87.6±2.5% of control (p<0.01). There was no statistical difference between the recombinant proteins. In the MeO-AMVN environment both recombinant proteins showed statistically lower MDA production. MDA production with the WT FABP1 was 79.9±2.7% (p<0.001) while the T94A variant was 87.4±1.7% (p<0.01). As in the AAPH environment, there was no statistical difference between recombinant proteins in the AMVN environment.

Figure 8C shows results from binding of α-Br-PA to WT FABP1 or T94A in the AAPH environment. The WT FABP1 showed a decrease in MDA production to 86.9±1.9% (p<0.01) of control while the T94A variant was 97.3±1.4%. The T94A was not statistically different from control (p>0.05) but significantly different from the WT FABP1 (p<0.05). In the MeO-AMVN (lipophilic) environment, both recombinant proteins showed statistically lower MDA production. MDA production with the WT FABP1 was 85.2±3.8 (p<0.001) while the T94A variant was 87.1±5.5% (p<0.05). As in the AAPH environment, there was no statistical difference between recombinant proteins in the MeO-AMVN environment.

DISCUSSION

Oxidative stress has been implicated in the pathogenesis of several liver diseases such as NAFLD (20, 21), ALD (22, 23), Hepatitis C (24), and NASH (25, 26). The excessive amount of pro-oxidants such ROS and RNS being produced by cells at times overwhelms the body's antioxidant defense systems. FABP1, a small (14 kDa) ubiquitous cytosolic protein could reach concentrations of up to 400 µM in murine and 1000 µM in human liver (27, 27), has been shown to act as an antioxidant (1). FABP1 contains several methionine groups and a single cysteine that are responsible for the molecular mechanism of the protein's antioxidant capacity (7). Cysteine and methionine both contain sulfur atoms where its electronegativity makes them highly susceptible to oxidation. Not only are these residues easily oxidized they take part in redox cycling reactions that regenerate the residues. Solvent accessible methionine and cysteine residues can be oxidized by ROS into methionine sulfoxide and sulfenic acid/sulfinic acid/sulfonic acid, respectively. These residues are reduced back by methionine sulfoxide reductase (29) and thioredoxin (30). Thus, we investigated the antioxidant potential of FABP1 compared to its T94A mutant. FABP1 also binds LCFA and its peroxides products as well as many other ligands including peroxisome proliferators (31), bile acids (32), heme (33), and endocannabinoids (34). Being capable of functioning as an antioxidant and having the capability to partition fatty acids (free vs protein-bound), FABP1 is considered to be a cytoprotectant (35). Since FABP1 is important in the redox homeostasis of the liver and binding many ligands, the loss of one of these functions through SNP can be detrimental.

The recently discovered human T94A variant is a SNP that occurs in the coding region of the FABP1 gene (11). The FABP1 T94A variant's reported phenotypes can be inconsistent. An example is the BMIs and waist circumferences where it was reported the BMI was lower in carriers of the T94A variant (11) while others found it to be higher (36). Differences in such characteristics may be attributed to the diversity amongst the human population being studied. The T94A mutation has been associated with increased LDL cholesterol (37), hepatic TAG accumulation (38), higher basal plasma TAG levels (11) and NAFLD (36). An explanation for these characteristics was that the T94A variant resulted in a total loss of function (39). Others observed no change in ligand binding specificity or changes in the affinity for binding fibrates (14), steric acid or oleic acid (15) between the wild type and the T94A variant protein. Slight changes were seen in the secondary structure binding affinities between the T94A protein and palmitic or linoleic acid (15) and differences in cholesterol affinity and uptake (16). While research uncovered various differences between FABP1 and its T94A variant, none have explored the antioxidant function of the T94A variant. It is important in determining if the T94A mutation is associated with a loss of function with respect to its ability to act as an antioxidant since the pathogenesis of various diseases are linked to oxidative stress. The impairment of an essential cytoprotectant such as FABP1 can be deleterious to carriers. Therefore, gaining insight on this aspect of the T94A variant is important in developing new approaches for treating diseases.

The GST affinity tag system previously described (7) was used to purify FABP1 and the T94A variant. An N-terminal infusion linker, sequence of GPLGSIAT, remained when PreScission Protease was used to remove the GST tag. This made the weight of the purified rat FABP1 and T94A variant to be 14968.0 and 14938.0 Da, respectively (Figure 4). Weight of purified rat FABP1 in the current study was smaller when compared to results previously described (7) where the total weight was 15275.9 Da. The extra mass was attributed to having the cysteine glutathionylated, suggesting the current study's purification protocol did not have a gluthionylated cysteine residue and

was available for scavenging ROS. A potential limitation of our study was that MALDI-TOF was not conducted for protein confirmation, however, recombinant proteins were identified by SDS-PAGE, western blot, and LC-MS/MS.

The present study showed that the recombinant FABP1 T94A variant scavenged ROS to a similar degree as the WT FABP1, as shown by the decrease in DCF fluorescence intensity (Figure 5). The ability of WT FABP1 to reduce DCF fluorescence is consistent with a previous report (7) despite the lower range of concentrations used in the present study.
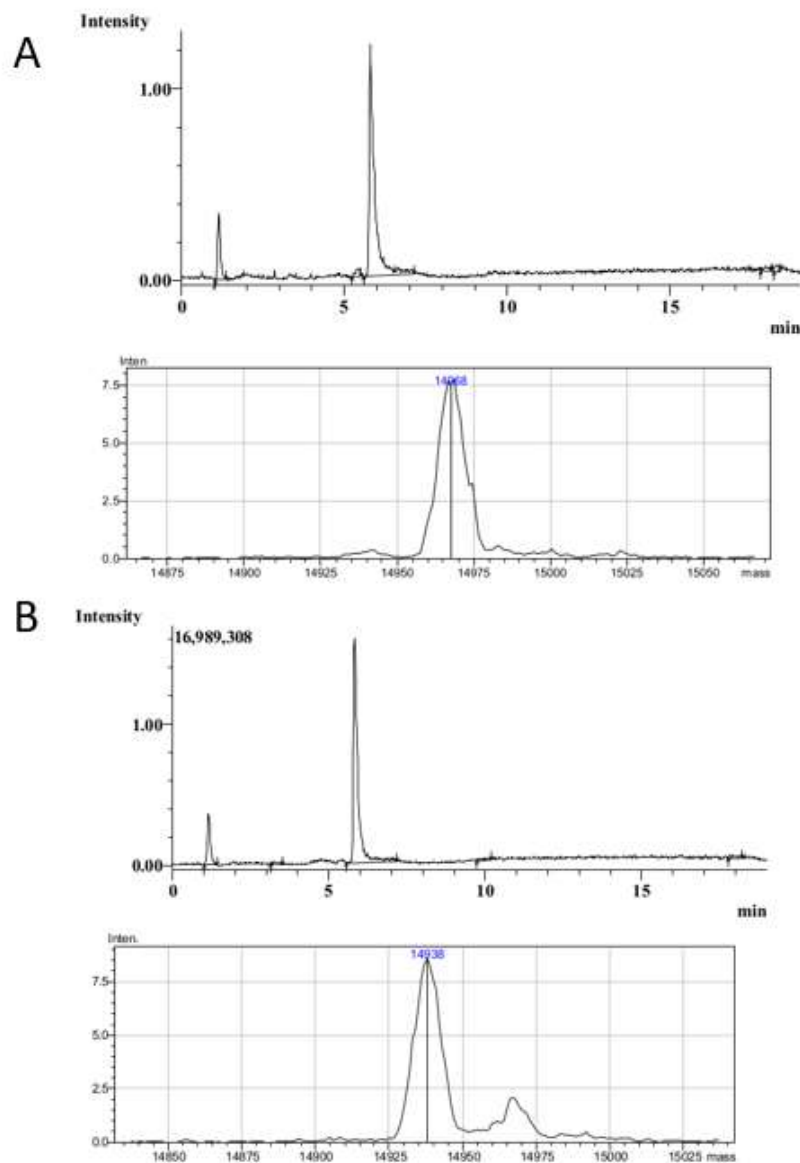

Figure 4. LC-MS Results. Total ion count chromatograms (top) and the deconvoluted mass (bottom) of WT FABP1 (A) and FABP1 T94A mutant (B). Samples were sent to the Pharmaceutical Analysis Laboratory at the College of Pharmacy, University of Manitoba for LC-MS analysis.
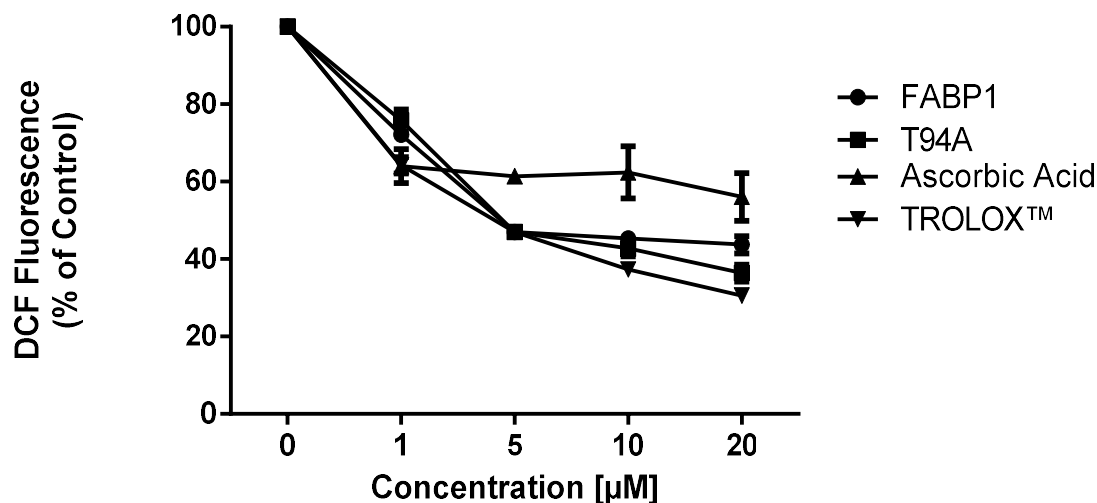
Figure 5. DCF fluorescence intensity versus antioxidants recombinant FABP1, FABP1 T94A, ascorbic acid, and TROLOX™ concentration. The parent compound DCFH-DA was de-esterified to the dye stock DCFH that was used to detect ROS in the reactions. Reactions were conducted in 96 well CoStar plates with 24 μM DCFH, 50 μM $FeSO_4$, 50 μM H_2O_2, and protein sample. Data are presented as mean ± SEM (n = 4). There was a statistical decrease with an increase in protein concentration; 1 μM vs 20 μM (p< 0.001). Overall, there were no statistical differences between WT FABP1 and T94A in their ability to reduce DCF fluorescence at all concentrations.

To assess the capabilities of the T94A mutant as an antioxidant, the protein was subjected to simulated lipid peroxidation reactions by compounds, AAPH and MeO-AMVN, that thermally decompose into free radicals in hydrophilic and lipophilic environments, respectively. Addition of the T94A protein into these oxidation reactions produced lower amounts of MDA as the concentration of the protein increased (Figure 6). This indicated that the T94A variant can act as both a hydrophilic and lipophilic antioxidant preventing the formation of MDA. Comparing the performance of the mutant variant to the wild type, significant differences were found at 1 μM and 10 μM but no differences were seen with 20 μM in the AAPH system (Figure 6A). Since FABP1's concentration in human hepatocytes may be as high as 1000 μM (27, 28) and 200–400 μM in rodents (34), the difference between the mutated and non-mutated forms may be considered negligible assuming that at concentrations higher than 20 μM, the protein behaves similarly in a hydrophilic environment.

When examining proteins in the lipophilic MeO-AMVN system, there were no significant differences observed (Figure 6B), suggesting that the T94A mutation did not alter protein structure in a way that inhibits the antioxidant moieties from scavenging ROS in a lipophilic environment. Interestingly, the WT FABP1 protein in the present study did not have a glutathionylated cysteine residue while that in Yan's study it was glutathionylated and hence not available for free radical scavenging. Antioxidant activity, however, was observed to be similar, suggesting that the cysteine residue may not be solvent accessible to scavenge ROS. A study done by Smathers *et al.,* (40) investigating 4-hydroxynonenal adduction on FABP1 used *in silico* simulations and PROPKA (http://propka.ki.ku.dk/) to calculate solvent accessibility. The study illustrated that the apo state of rat FABP1 had its cysteine 11% buried from the solvent while its holo state was at 37%, suggesting that the cysteine is partially solvent accessible and may not be readily available to scavenge ROS.

WT FABP1's antioxidant function was attenuated when bound with either PA or α-Br-PA in both AAPH and MeO-AMVN lipid peroxidation reactions but was still able to significantly decrease MDA production (Figure 8). The T94A variant did not differ significantly compared to control when bound with α-Br-PA (Figure 8C) in the hydrophilic milieu. Comparing WT FABP1 to T94A, MDA production showed significant differences in the unbound and bound only with α-Br-PA in the AAPH derived lipid peroxiditon system.
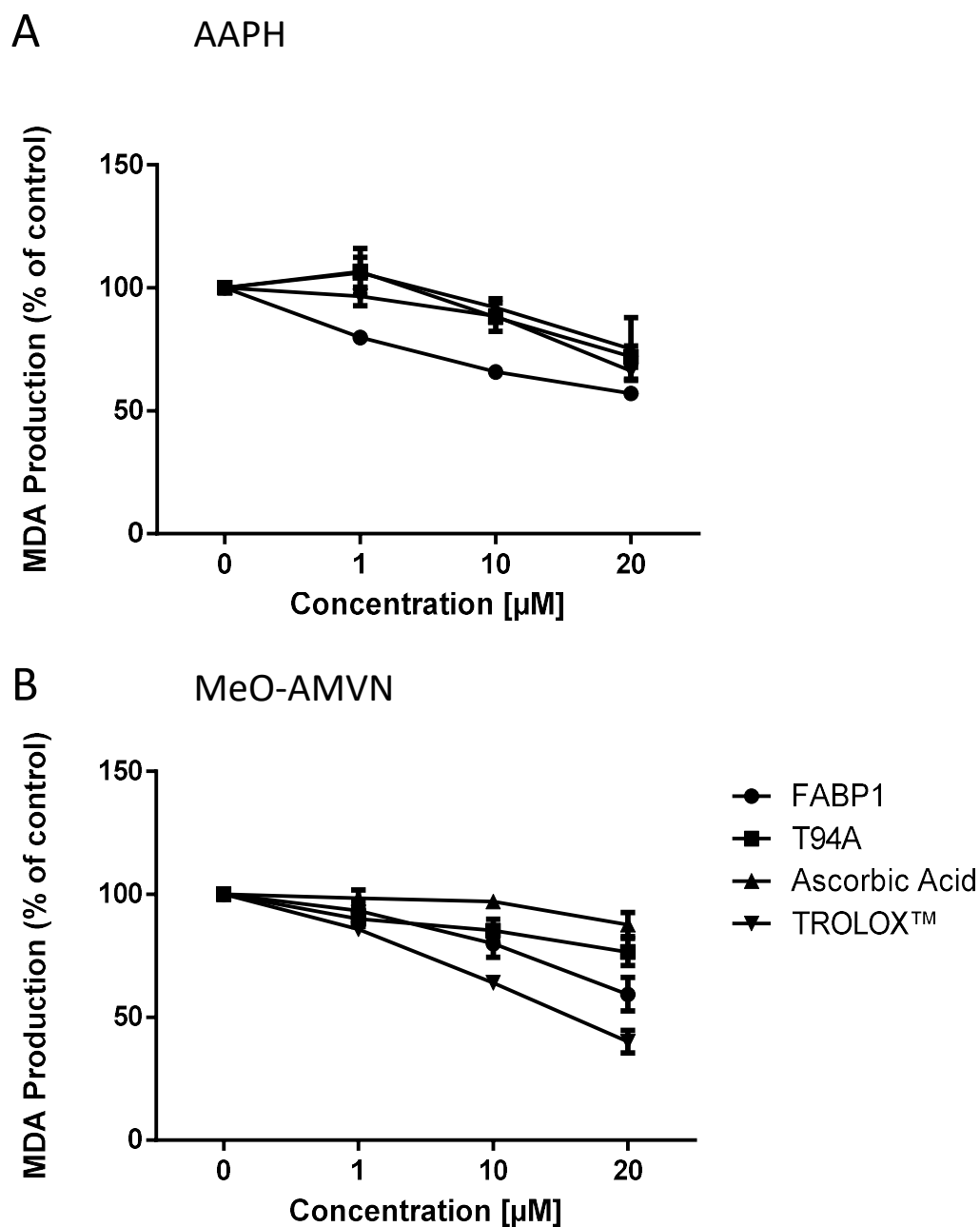
Figure 6. Antioxidants in lipid peroxidation reactions. The effect of WT FABP1, FABP1 T94A mutant, ascorbic acid, and TROLOX™ on AAPH (hydrophilic) (A) and MeO-AMVN (lipophilic) (B) induced MDA production. LDL was incubated with azo compounds and varying concentrations of WT FABP1, T94A mutant, ascorbic acid, or TROLOX™ for 90 minutes at 37°C. MDA was measured using the TBARS assay. Formation of MDA decreased with an increase in protein concentration. There was a statistical difference ($p<0.05$) between WT FABP1 and T94A using AAPH induced MDA production at 1 μM (79.8%±1.5 and 105.0%±9.4 of the control) and 10 μM (65.8%±1.8 and 88.1%±5.7 of control) ($p< 0.05$), but not at 20 μM. WT FABP1 and T94A were not different from each other in the MeO-AMVN system. Values represent mean ± SEM, n=8.
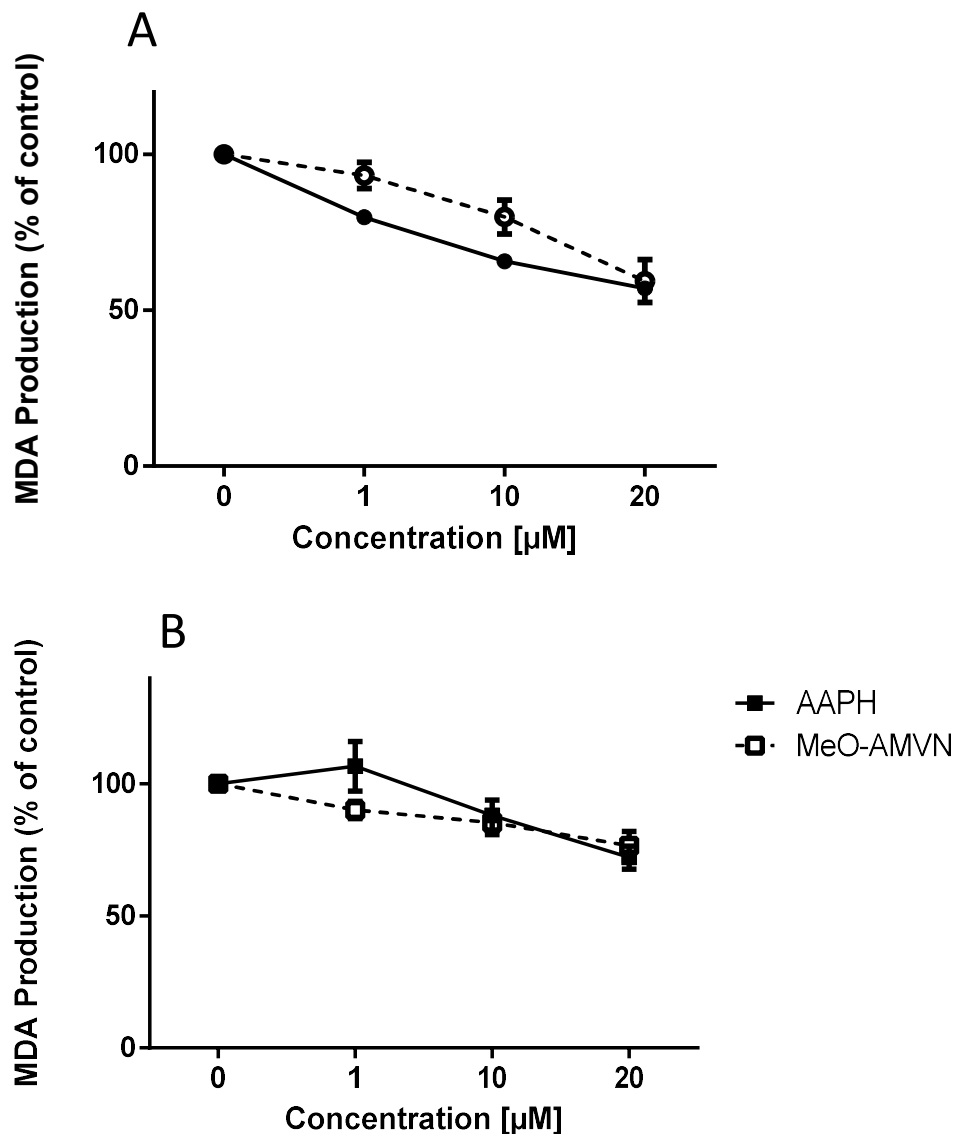
Figure 7. Antioxidant function of WT FABP1 (A) and T94A mutant (B) in hydrophilic and lipophilic environments. FABP1 T94A concentration (µM) is shown as a function of MDA production (% of control) in an AAPH and MeO-AMVN environment. Proteins were able to reduce MDA production similarly despite being induced by different azo compounds. Values represent mean ± SEM, n = 8.

Results suggest that when the T94A variant binds ligands, the protein's conformation may be unfavourable for scavenging ROS in certain conditions. Some of the antioxidant amino acids of the FABP1 T94A may have become unavailable when ligands are bound. Studies have shown no change in ligand specificity and affinity when the T94A variant binds ligands (14, 15), which implies that the mutation does not cause a loss of function.

However, the secondary structural response of the mutant when bound with ligands has been shown to influence the protein's downstream interaction with PPARα (14). Consistent with these findings, the present study found that although there was no loss in overall antioxidant activity (Figure 5), results seen in Figure 8 imply that ligand binding can influence the T94A variant from WT FABP1 in different environments.
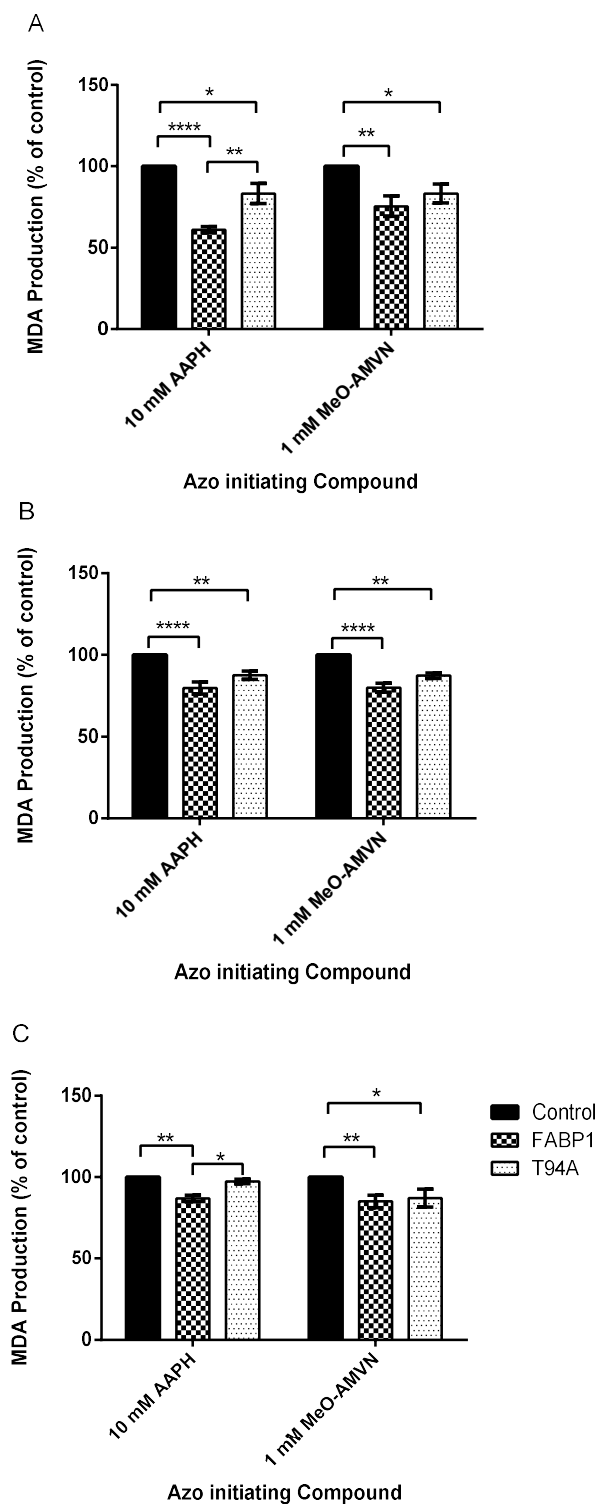
Figure 8. Comparison of the effect of unbound recombinant proteins (A), after binding with PA (B), and after binding with palmitate α-Br-PA (C), on azo compounds AAPH and MeO-AMVN free radical release. Recombinant proteins (10 μM) were pre-incubated with either no fatty acid (A), PA (B), or α-Br-PA (C) for 1 hour at 37°C before being used in the lipid peroxidation reactions. MDA levels were measured using the TBARS assay. Control reflect MDA levels produced in lipid peroxidation reactions without WT FABP1 or T94A mutant. Values represent mean ± SEM, n = 6, * p<0.05, ** p<0.01, ****p<0.001).

Previously reported, T94A variant has a lower binding affinity with palmitic and linoleic acid (15), which may illustrate that the T94A variant could be more readily available to act as an antioxidant. This was not observed as the antioxidant ability was similar to the WT when it was unbound and bound with α-Br-PA in the AAPH lipid peroxidation system. The secondary structural changes in response to LCFAs binding with the T94A variant is a possible reason for the weakened antioxidant response. While FABP1 is found to a lesser extent in other tissues (41) and presumed to have similar roles, example kidney (42), it is acknowledged that these studies were conducted with low protein concentrations. It may be that our results could be extended to higher protein concentrations but this needs verification. We also recognize that the use of recombinant rat instead of human FABP1 may be considered a limitation to our study, as the latter would have shown clinically relevance.

CONCLUSION

In the current study, recombinant rat FABP1 T94A variant was purified using the GST tag system and *in vitro* studies were used in determining the mutant protein's antioxidant activity. From these results, recombinant rat FABP1 T94A variant was shown to also act as an antioxidant similar to FABP1 and can do so in hydrophilic and lipophilic environments. There were no significant differences between the performance of the wild type and mutant, suggesting that the T94A substitution does not impact the protein's antioxidant function. However, it was found that when T94A was bound to α-Br-PA, the T94A protein was unable to significantly reduce the generation of MDA in a hydrophilic millieu, which may suggest there is an altered antioxidant function once ligands bind to the protein. Further studies using higher protein concentrations and larger n values will help verify these results.

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