EARLY STEATOHEPATITIS IN HYPERLIPIDEMIC MICE WITH ENDOTHELIAL-SPECIFIC GAIN OF TRPC3 FUNCTION PRECEDES CHANGES IN AORTIC ATHEROSCLEROSIS

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Running head: steatohepatitis and atherosclerosis

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) and its more advanced form non-alcoholic steatohepatitis (NASH), are the most common chronic liver diseases in developed countries. Moreover, NAFLD and NASH are considerable risk factors for atherosclerosis, the most frequent vascular pathology in these and other metabolic diseases. Despite this strong connection, current knowledge of the relationship between NAFLD/NASH and atherosclerosis is scarce. Recently, we studied hyperlipidemic Apoe knockout mice with Endothelial-Specific gain of Transient Receptor Potential Canonical 3 channel function (TgESTRPC3/ApoeKO) and found that these animals had increased burden of advanced aortic atherosclerosis (16 weeks on high fat diet) compared to non-transgenic ApoeKO littermate controls (non-Tg/ApoeKO), whereas early lesions (10 weeks on high fat diet) were not different. Here, we report that at the early stage when differences in aortic atherosclerosis are not yet manifest, the livers of TgESTRPC3/ApoeKO mice show steatosis, fibrosis and altered hepatic enzymes compared to non-Tg/ApoeKO animals. Because differences in liver pathology were noticeable long before differences in atherosclerosis were evident, our studies suggest that TRPC3-related endothelial mechanisms that promote steatohepatitis may also contribute to atherosclerosis progression. In vitro, downregulation of TRPC3 in liver sinusoid endothelial cells reduces their susceptibility to ER stress-induced apoptosis, suggesting that a pro-apoptotic effect of TRPC3 may add to other fibrogenic factors in vivo. These novel findings show a positive association between augmented expression of an endothelial TRPC channel, development of early steatohepatitis and
atherosclerotic burden in a hyperlipidemic mouse model of NAFLD fed conventional western-type diet.

**KEY WORDS:** TRPC channels; NAFLD; NASH; atherosclerosis; endothelium
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has an extensive histological spectrum ranging from simple fat accumulation in liver (steatosis) to steatohepatitis (NASH), the latter being characterized by liver inflammation, presence of apoptotic cells and fibrosis (1). NAFLD is the most common chronic liver disease in adults and children of developed countries (2). About thirty percent of individuals in western societies have NAFLD, and it is estimated that twenty percent of them will progress to NASH (12). NAFLD and NASH also contribute to development and/or progression of atherosclerosis, the main cause of coronary artery disease and most frequent vascular complication in these and other metabolic disorders. Likewise, atherosclerosis is a major determinant of mortality in NAFLD/NASH patients (12). In individuals with NAFLD, progression of liver inflammation and fibrosis correlates positively with plaque burden, independently of cardiometabolic risk factors (6). Despite the strong association between NAFLD/NASH and atherosclerosis, how these two diseases interplay remains poorly understood, to a great extent due to lack of animal models that simultaneously develop the liver and the vascular pathologies.

Transient Receptor Potential Canonical 3 (TRPC3) is a non-selective Ca²⁺-permeable channel that belongs to the TRPC family (TRPC1-7) of cation channels (15). TRPC3 has important functions in endothelium and participates in numerous events associated to cardiovascular physiology and disease (15). TRPC3 is activated downstream of receptor-stimulated phospholipases, and also exhibits significant constitutive function (15). Understanding the roles of TRPC3 in metabolic and cardiovascular diseases requires the identification of cellular and/or molecular events that are affected by
TRPC3 function. Existing evidence on potential roles of TRPC3 in regulating metabolic processes is only of an associative nature and direct causative roles have not been demonstrated (22). There is however compelling *in vitro* and *in vivo* work demonstrating functions of TRPC3 in cardiovascular pathologies, many of which are often complications of metabolic diseases (15). In a recent study using hyperlipidemic Apoe knockout (ApoeKO) mice with Endothelial-Specific gain of TRPC3 function (TgESTRPC3/ApoeKO) we found that advanced aortic atherosclerotic plaques (after 16 weeks on high fat diet, "HFD") in these mice were of bigger size and cellularity compared to non-transgenic ApoeKO littermates (non-Tg/ApoeKO; (17)). Early atherosclerotic lesions (10 weeks on HFD) were not different between the two groups of animals, but notably, at this early stage the endothelium of TgESTRPC3/ApoeKO mice already showed augmented inflammation and signs of increased endoplasmic reticulum (ER) stress compared to non-Tg/ApoeKO animals (17). In the present work we report that at the early stage of atherosclerosis, when differences in aortic plaques are not yet noticeable, the livers of TgESTRPC3/ApoeKO mice show, compared to non-Tg/ApoeKO, significant steatosis, fibrosis, augmented cell apoptosis and altered liver enzymes, *i.e.*, long before differences in aortic atherosclerosis become evident. This suggests that endothelial TRPC3-dependent effects exist that, directly or indirectly, promote steatohepatitis and may also contribute to atherosclerosis progression. This is also the first evidence of a positive association between augmented expression of an endothelial TRPC channel and development of steatohepatitis in a hyperlipidemic mouse model of NAFLD fed conventional western-type diet.
MATERIALS AND METHODS

**Animals:** all animal studies described in this work conform to the NIH Guide for Care and Use of Laboratory Animals and were approved by University of Toledo IACUC.

Generation and characterization of ApoeKO mice with endothelial-specific overexpression of human TRPC3 (TgESTRPC3/ApoeKO) is described in (17). Aortic root sectioning and histomorphometric analysis of atherosclerotic lesions was described in detail in (17).

**Measurement of metabolic parameters:** blood was collected by submandibular vein puncture after 12 hour fasting period. Total plasma cholesterol and triglycerides were determined as in (17) using commercial kits (Wako Chemicals USA, Inc.) following manufacturer’s instructions. Non-esterified fatty acids were determined as described in (11).

**Liver histology and function:** hepatic triglycerides and cholesterol were determined as in (11). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated with commercial kits (Abcam, MA) following manufacturer’s instructions. Wet liver weight was determined immediately after sacrifice. Livers were embedded in O.C.T., frozen in the Peltier stage of the cryostat (Thermo-Scientific R.Allan-HM550) and sections (10 μm) collected onto Fisher Superfrost-Plus-coated slides. Histological features were examined by hematoxilin-eosin (H&E) staining as in (17). Steatosis and inflammation were graded by two operators blinded to the study, using the 0–3 scale (3=highest; NASH scoring system, NIDDK-NASH Clinical-Research-Network (5)). Alternatively, images of Oil-Red O (ORO)-stained sections were captured with a digital camera (Micropublisher 3.3-Megapixel Cooled-CCD Color-Digital-Camera coupled to
Zeiss-Axiovert40CL inverted microscope) and ORO-stained areas were measured with image analysis software (NIS-Elements-D). The inflammatory genes TNFα, IL-1β and IL-6 were evaluated by qRT-PCR using protocols and primers described in (18).

Presence of apoptotic cells was determined by in situ TUNEL as in (17).

Liver fibrosis: liver sections were stained for collagen deposition (Masson's trichrome) and the extent of fibrosis estimated from relative area of collagen-staining respect to liver area, as we described in (17). Hydroxyproline was determined by photometry in liver hydrolysates as in (20). mRNA levels of collagen type I (Col1A) and transforming growth factor β (TGFβ) were determined by qRT-PCR using primers described in (10).

Isolation and transfection of liver sinusoid endothelial cells (LSECs): LSECs were isolated by collagenase perfusion, iodixanol-density-gradient centrifugation and centrifugal elutriation as in (21). Viability was >95%, purity >98% based on uptake of formaldehyde-treated albumin, a function specific to LSECs (4). siRNA oligonucleotides for TRPC3, 6 or 7 (siGENOME-SMART-pool, Dharmacon) were delivered to LSECs with Lipofectamine2000 (Invitrogen, CA), 100 nM final concentration (as in (16)) and cells were used 48 hours later. Downregulation of TRPC3, 6 and 7 was confirmed by qRT-PCR and western-blot as in (16).

Statistical analysis: values were compared using a two-tailed t test for two means (Graph Pad Software, San Diego CA). P values below 0.05 were considered significant.
RESULTS
In a recent study using ApoeKO mice with Endothelial-Specific gain of TRPC3 function (TgESTRPC3/ApoeKO) we observed that whereas early atherosclerotic lesions (10 weeks on HFD) were not different between transgenic and non-transgenic mice (17), at this early stage the endothelium of TgESTRPC3/ApoeKO mice showed more inflammation and increased ER stress compared to non-Tg/ApoeKO animals (17). Importantly, this changes occurred long before differences in aortic atherosclerotic burden were evident between TgESTRPC3/ApoeKO and non-Tg/ApoeKO mice, which occurred after 16 weeks on HFD. Based on this and considering that when maintained on HFD ApoeKO mice develop NAFLD (14), we wished to examine whether endothelial gain of TRPC3 had an impact on the liver of TgESTRPC3/ApoeKO animals, and if so, how this related to progression of atherosclerosis burden. Six week-old female TgESTRPC3/ApoeKO mice or their non-Tg/ApoeKO gender-matched littermates were placed on a high fat diet (HFD: 21% fat, 0.2% cholesterol; TD.88137-Harlan Laboratories, IN) for 10 or 16 weeks to allow for development, respectively, of early and advanced atherosclerotic lesions. Figure 1 (upper panels) shows that after 10 weeks on HFD the livers of TgESTRPC3/ApoeKO mice had significant steatosis, as assessed by a NASH scoring system (5) (score: 1.71 ± 0.3 vs. 0.35 ± 0.15, for TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO, respectively; p<0.01, n=8) or by quantification of ORO-stained areas (20 ± 3 vs. 8 ± 2 % ORO-stained area, for TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO, respectively; p<0.01, n=8). As reported by others (13, 14) ApoeKO fed regular chow diet did not develop steatosis, regardless of the TRPC3 expression status (not shown). In agreement with our previous findings (17), after 10 weeks on HFD there
were no differences in the already hyperlipidemic profile of the two groups of animals (Table I) or in plaque burden (Fig. 2). In line with the histological observations, hepatic triglyceride content after 10 weeks on HFD was higher in transgenic vs. non-Tg/ApoeKO mice (Fig. 1). Hepatic cholesterol (Fig. 1), gain in body weight and circulating non-esterified fatty acids (NEFAs) were similar between the two groups (Table I). After 16 weeks on HFD liver steatosis was slightly augmented (Fig. 1, lower panels), which coincided with significant progression of atherosclerosis. Similar to our previous observations (17), advanced aortic plaques in TgESTRPC3/ApoeKO mice were larger compared to non-Tg/ApoeKO animals (Fig. 2). After 16 weeks on HFD hepatic triglycerides remained higher in transgenic vs. non-transgenic mice (Fig. 1); hepatic cholesterol, although slightly increased compared to levels at 10 weeks, was not significantly different between groups (Fig. 1). Gain in body weight and lipid profile were also similar between groups (Table I). H&E staining revealed scattered inflammatory infiltration (score: 1.38 ± 0.32 vs. 0.51 ± 0.21, for TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO, respectively; p<0.01, n=8). Levels of the inflammatory genes TNFα, IL-1β and IL-6 were augmented by 2.3-, 3.1- and 2.8-fold respectively, in livers from TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO mice (n=5, P<0.01).

Next we examined whether the livers of TgESTRPC3/ApoeKO mice showed evidence of fibrosis. Figure 3 shows that already after 10 weeks on HFD there is collagen deposition in livers from TgESTRPC3/ApoeKO mice, but not in non-Tg/ApoeKO mice (Fig. 3, upper panels). The fibrosis augmented and was more disseminated by 16 weeks on HFD (Fig. 3, lower panels), i.e., as aortic atherosclerosis progressed. Hepatic hydroxyproline content was higher in TgESTRPC3/ApoeKO mice vs. non-Tg/ApoeKO
respectively, 0.91 ± 0.02 vs. 0.58±0.03 mg/100 mg liver; n=6, p<0.01). These histological and biochemical findings were positively correlated with increased levels of fibrogenic genes Col1A and TGFβ in livers from transgenic mice (5.22 ± 1.8 and 3.24 ± 0.9 fold increase in normalized mRNA levels in livers from TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO mice, for Col1A and TGFβ, respectively; n=5, p<0.01). These results indicate that, compared to non-Tg/ApoeKO, TgESTRPC3/ApoeKO mice develop early steatohepatitis with fibrosis. Wet liver weights were similar between TgESTRPC3/ApoeKO and non-Tg/ApoeKO (not shown). Plasma AST and ALT levels were higher in TgESTRPC3/ApoeKO mice compared to non-Tg/ApoeKO (AST: 162 ± 12 vs. 55 ± 9 U/L; ALT: 115 ± 9 vs. 45 ± 7 U/L, for TgESTRPC3/ApoeKO vs. non-Tg/ApoeKO, respectively; p<0.01, n=7), again long before differences in atherosclerosis burden were evident. Counting of TUNEL+ cells in liver sections showed higher number of apoptotic cells in livers of TgESTRPC3/ApoeKO mice compared to non-Tg/ApoeKO (Fig. 4A). The identity of these cells –i.e., endothelial, parenchymal- remains to be determined. Recently, we showed that TRPC3 contributes to mechanisms of ER stress-induced apoptosis in coronary endothelial cells (3). To examine if a similar effect exists in liver sinusoid endothelial cells (LSECs), we isolated LSECs from livers of ApoeKO mice and transfected them with siRNA specific for TRPC3, its close relatives TRPC6 and 7, or scrambled oligonucleotides (controls) under conditions previously shown by us to efficiently downregulate these proteins (16). After transfection LSECs were exposed (24 hours) to oxidized-LDL or tunicamycin to trigger ER stress-induced apoptosis. Figure 4B shows that downregulation of TRPC3, but not TRPC6 or 7, markedly reduced the susceptibility of LSECs to apoptosis.
DISCUSSION

The present studies show that ApoeKO mice with endothelial-specific gain of TRPC3 (TgESTRPC3/ApoeKO) progress from NAFLD to steatohepatitis with fibrosis after only 10 weeks on a conventional high fat diet. The pathological liver phenotype of the TgESTRPC3/ApoeKO mice was manifest long before differences in aortic atherosclerotic burden were noticeable. This suggests that in a hyperlipidemic setting, TRPC3-dependent effects occur in the hepatic endothelium contributing to mechanisms mediating the progression of NAFLD to NASH. The early aggravation of the liver pathology might then add to the existing atherogenic factors to accelerate atherosclerosis progression. Similar to our previous findings (17), the early atherosclerotic lesions -10 weeks on HFD- in aortic roots of TgESTRPC3/ApoeKO mice were not different from non-Tg/ApoeKO animals. However, at this time point, the steatohepatitis, fibrosis and altered liver function were already present in TgESTRPC3/ApoeKO mice when compared to non-Tg/ApoeKO animals. Importantly, this occurred without significant differences between the two groups of mice in regards to body weight or lipid profiles. Although parameters of energy intake/expenditure were not evaluated, these results suggest that the overall energy metabolism is not different between TgESTRPC3/ApoeKO and non-Tg/ApoeKO mice. In ApoeKO mice, often used as a model of NAFLD, transition from simple steatosis to steatohepatitis, and in particular, the appearance of fibrosis, requires either extended periods on HFD or drastic dietary manipulations (9, 13, 14). In this context, the outcome of our studies is also especially interesting, as we observe progression from NAFLD to NASH with fibrosis after feeding the mice with a conventional HFD for only 10 weeks.
The livers in TgESTRPC3/ApoeKO mice were clearly more steatotic than in non-
Tg/ApoeKO mice. Because steatosis can promote liver fibrosis (19), it is possible that
the increased steatosis in TgESTRPC3/ApoeKO mice serves as a pro-fibrogenic factor
in these animals. Increased circulating NEFAs are a consistent steatogenic factor in
NAFLD, however, plasma NEFAs and lipid profiles were similar between transgenic and
non-transgenic mice. Of note, TNFα, which is pro-steatogenic, was elevated in livers
from TgESTRPC3/ApoeKO mice. Additional studies are needed to determine what
causes increased steatosis in the TgESTRPC3/ApoeKO mice. Apoptosis of LSECs can
also lead to fibrosis (7). In NAFLD/NASH the hepatic endothelium is chronically
exposed to ER stressors and endothelial cell apoptosis occurs throughout most stages
of the disease (8). Our *in vitro* studies in isolated LSECs, although limited in that they do
not recapitulate the environment to which these cells are exposed *in vivo*, suggest that
TRPC3 has a permissive role in ER stress-induced apoptosis. We speculate that by
virtue of this role, augmented TRPC3 expression in LSECs of TgESTRPC3/ApoeKO
mice may add to the plethora of mechanisms mediating fibrosis *in vivo*.

The pathophysiological/translational relevance of these findings in the context of
excessive caloric intake, NAFLD/NASH and atherosclerosis, can be better appreciated
in the light of our previous studies showing that the pro-inflammatory conditions found in
hyperlipidemic ApoeKO mice result in upregulation of endothelial TRPC3 (16). Thus, the
findings here add to those in our previous report (17) supporting the notion that
augmented TRPC3 expression may have pathological relevance.

In sum, the present studies in a hyperlipidemic mouse model of NAFLD show that
endothelial-specific gain of TRPC3 function is positively associated with early transition
of the liver pathology to steatohepatitis with fibrosis. This suggests that endothelial
TRPC3 participates in mechanisms mediating progression of NAFLD to NASH. Since
this transition precedes the aggravation of atherosclerosis, our data support the notion
that NAFLD/NASH are important atherogenic factors. Despite the strong connection
between NAFLD/NASH and atherosclerosis, current understanding of the relationships
between these diseases is poor. Our results indicate that the TgESTRPC3/ApoeKO
mouse is an attractive model to further investigate these interactions and the functions
of TRPC3, in a hyperlipidemic setting and without drastic dietary manipulations.

ACKNOWLEDGMENTS

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DISCLOSURES

None.

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Table I. Mice were fed a high fat diet for 10 (first row of values) and 16 weeks (second row of values) and the indicated parameters were measured as described in Methods. Values are mean ± SEM, n=10 mice.

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<thead>
<tr>
<th></th>
<th>non-Tg/ApoeKO</th>
<th>TgESTRPC3/ApoeKO</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>1,121 ± 93</td>
<td>1,105 ± 90</td>
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<tr>
<td></td>
<td>1,096 ± 84</td>
<td>1,117 ± 89</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>335 ± 37</td>
<td>319 ± 42</td>
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<td></td>
<td>341 ± 40</td>
<td>309 ± 36</td>
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<td>Non-esterified fatty acids (mmol/l)</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.7</td>
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<td>1.1 ± 0.8</td>
<td>1.6 ± 0.5</td>
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<tr>
<td>Body weight before diet (g)</td>
<td>19.5 ± 1.0</td>
<td>18.2 ± 1.1</td>
</tr>
<tr>
<td>10 weeks on high fat diet</td>
<td>28.7 ± 2.2</td>
<td>27.7 ± 1.8</td>
</tr>
<tr>
<td>16 weeks on high fat diet</td>
<td>29.4 ± 1.2</td>
<td>29.6 ± 2.0</td>
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**FIGURE LEGENDS**

**Figure 1.** ApoeKO mice with endothelial-specific gain of TRPC3 function (TgESTRPC3/ApoeKO) or their non-transgenic littermates (non-Tg/ApoeKO) were maintained on a high fat diet for 10 (upper panels) or 16 (lower panels) weeks. At the end of the diet periods mice were sacrificed and liver sections (10 µm) prepared and stained with Oil-Red O (H&E counterstaining) to evaluate lipid content. The bar graphs show values for hepatic triglycerides and cholesterol content (means ± SEM, n=8) for these groups of mice after the indicated diet periods. *P<0.001 vs. controls.

**Figure 2.** Aortic root sections from TgESTRPC3/ApoeKO mice (n=10) or their transgenic littermates (non-Tg/ApoeKO, n=10) that were maintained on a high fat diet for 10 or 16 weeks, were subjected to histomorphometric analysis to determine total plaque area as in (17). Shown are mean values and corresponding standard errors (SEM).

**Figure 3.** TgESTRPC3/ApoeKO or their non-transgenic littermates (non-Tg/ApoeKO) were maintained on a high fat diet for 10 (upper panels) or 16 (lower panels) weeks. At the end of the diet periods mice were sacrificed and liver sections (10 µm) prepared and stained with Mason’s trichrome to evaluate collagen content. Fibrotic areas (blue staining) are indicated by arrows. The bar graph shows relative fibrosis area (% of total liver area) as means ± SEM, n=8. *P<0.001 vs. controls.
**Figure 4.** In “A” liver sections (10 µm) from TgESTRPC3/ApoeKO mice or non-transgenic littermates (non-Tg/ApoeKO) that were maintained on a high fat diet for 10 or 16 weeks were stained for in situ TUNEL and the number of apoptotic (TUNEL+) cells were counted and normalized per mm² of section area. Shown are means ± SEM (n=5), *P<0.05 vs. corresponding control. In “B”, liver sinusoid endothelial cells isolated from ApoeKO mice (mLSECs) were incubated in EBM-2 (Control) or EBM-2 containing oxidized-LDL (“oxLDL”, 50 µg/ml) or tunicamycin (“Tun”, 10µg/m) for 24 hours and processed for TUNEL assay (black bars). Alternatively, mLSECs were transfected with scrambled oligonucleotides (gray bars) or with siRNA specific for TRPC3, 6 or 7 (siT3, siT6, or siT7, empty bars), and 48 hours post-transfection subjected to the above described treatments and processed for TUNEL assay. Shown are means ± SEM (n=3), *P<0.001 vs. corresponding control. Differences for “siT3+oxLDL” and “siT3+Tun” vs. corresponding scrambled-transfected controls have P<0.0001. In the presence of siT6 or siT7, the effect of oxLDL and Tun was not different from corresponding scrambled-transfected controls.
Figure 3

non-Tg/ApoeKO  TgESTRPC3/ApoeKO

10 weeks

16 weeks

Relative fibrosis area (%)

- non-Tg/ApoeKO
- TgESTRPC3/ApoeKO

16 wk HFD

10 wk HFD

*