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Increased size and cellularity of advanced atherosclerotic lesions in mice with endothelial overexpression of the human TRPC3 channel

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In previous in vitro studies, we showed that Transient Receptor Potential Canonical 3 (TRPC3), a calcium-permeable, nonselective cation channel endowed with high constitutive function, is an obligatory component of the inflammatory signaling that controls expression of the vascular cell adhesion molecule-1 (VCAM-1) and monocyte adhesion to coronary artery endothelial cells. Also, TRPC3 expression in these cells was found to be up-regulated by proatherogenic factors, which enhanced inflammation and VCAM-1 expression. However, it remained to be determined whether these in vitro findings were of relevance to atherosclerotic lesion development in vivo. To answer this important question in the present work, we generated mice with endothelial-specific overexpression of human TRPC3 in an Apoe knockout background (TgEST3ApoeKO) and examined lesions in the aortic sinus following 10 and 16 wk on a high-fat diet. No significant differences were found in size or complexity of early stage lesions (10 wk). However, advanced plaques (16 wk) from TgEST3ApoeKO mice exhibited a significant increase in size and macrophage content compared with nontransgenic littermate controls. Remarkably, this change was correlated with increased VCAM-1 and phospho-IkBα immunoreactivity along the endothelial lining of lesions from transgenic animals compared with controls. These findings validate the in vivo relevance of previous in vitro findings and represent, to our knowledge, the first in vivo evidence for a proatherogenic role of endothelial TRPC3.

TRPC3 channels | atherosclerosis | endothelial inflammation

Members of the Transient Receptor Potential Canonical (TRPC) family of calcium-permeable, nonselective cation channels are involved in numerous molecular and cellular events associated to cardiovascular physiology and disease (1, 2). A great deal of experimental evidence documents the participation of TRPC channels in signaling mechanisms linked to the pathogenesis of essential hypertension, cardiac hypertrophy, intima hyperplasia and endothelial dysfunction, among others (1, 2). TRPCs belong to the TRP superfamily of channel forming proteins and are grouped as follows: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 (3, 4). Regardless of the expression system TRPCs form nonvoltage gated, calcium-permeable nonselective cation channels activated downstream of receptor-stimulated phospholipases (3, 4). However, among all TRPCs, TRPC3 also forms channels endowed with significant constitutive function that is manifest both in overexpression and native expression conditions (4–6). Despite their recognized roles in cardiovascular disease, the potential functions of TRPCs in atherosclerosis, the major cause of death due to coronary artery disease in western societies, has remained largely unexplored (discussed by us in refs. 2 and 7). Atherosclerosis is a chronic disease of the arterial wall with a clear maladaptive inflammatory component, and implies a complex interplay between mechanisms intrinsic to the endothelium and genetic and environmental factors (8). Endothelial inflammatory signaling, monocyte recruitment, and the balance between survival and apoptosis of lesional macrophages, are all fundamental in initiation and progression of atherosclerotic lesions as well as in determining the fate of the plaque at advanced stages (9, 10). A paramount piece of experimental evidence supporting a role of TRPC3 in atherogenesis was provided by our recent findings in a mouse model of atherosclerosis demonstrating that TRPC3 contributes to mechanisms underlying necrotic core growth in advanced atherosclerotic plaques of Apoe knockout (ApoeKO) mice by influencing the rate of apoptosis of lesional macrophages (11). However, the expression of TRPC3 is ubiquitous and macrophages are not the sole atherorelevant cells where this channel is present. In previous in vitro studies using human coronary artery endothelial cells, we showed that native TRPC3 forms calcium-permeable channels that mediate both constitutive and regulated calcium influx (12). In these cells, TRPC3 expression and its constitutive function are obligatory components of the proinflammatory, nuclear factor kappa B alpha (NFkBα)-dependent signaling that drives expression of the vascular cell adhesion molecule-1 (VCAM-1), one of the key adhesion proteins that in vivo mediate monocyte recruitment into the arterial wall (13). In addition, we also reported that lesions in the aortic root of atherosclerotic ApoeKO mice exhibited robust TRPC3 expression compared with aortic cross-sections from nonatherosclerotic animals (13). Nevertheless, because of the several factors that underlie lesion development in vivo a definitive relationship between increased expression of TRPC3 and atherogenesis could not be established. It was thus obvious that generation of mouse models of atherosclerosis in which TRPC3 expression could be manipulated was required (i.e., conditional transgenic or knockout animals for TRPC3) to properly appreciate the contribution of endothelial TRPC3 to lesion development in vivo.

To address this contribution, in the present work we generated mice with endothelial-specific overexpression of human TRPC3 in an ApoeKO background (TgEST3ApoeKO) and

Significance

Atherosclerosis is a chronic disease of the arterial wall with a dominant inflammatory component. Endothelial cell inflammation and recruitment of circulating monocytes are critical processes during atherosclerotic lesion progression. In this manuscript, we generated a mouse model of atherosclerosis with endothelial-specific overexpression of TRPC3, a calcium permeable channel, and provide evidence indicating that augmented expression/function of TRPC3 supports, in vivo, endothelial inflammation and increased macrophage infiltration, resulting in atherosclerotic lesions of bigger size and complexity. These findings support the notion that endothelial TRPC3 channels may represent attractive targets for development of novel therapeutic strategies in the treatment of atherosclerosis.

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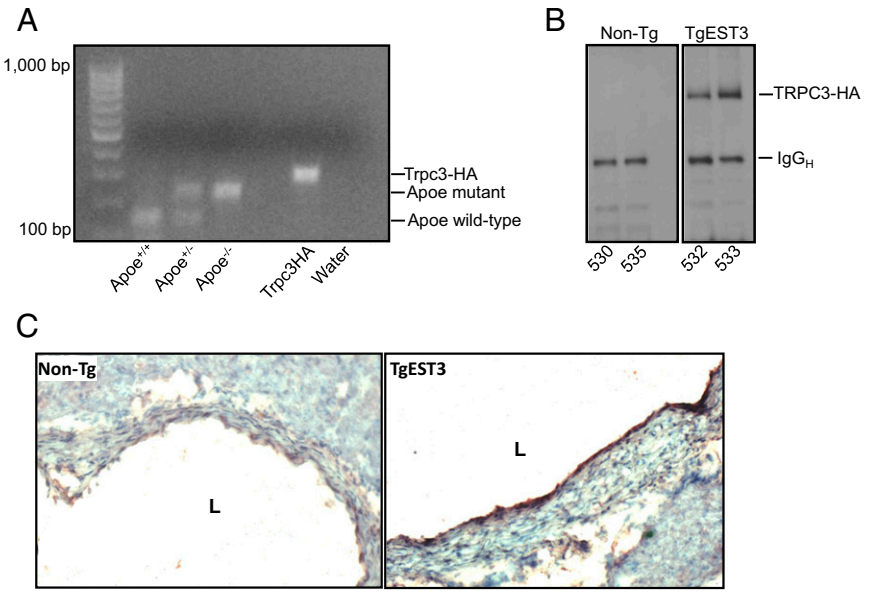

Fig. 1. Transgene expression. (*A*) PCR performed on gDNA derived from tail biopsies of TgEST3ApoeKO mice ("TgEST3") using the genotyping primers described in Methods which amplify the expected amplicon of 334 bp spanning the last 92 bp of Trpc3-HA plus 212 bp of the polyA tail. Primers and PCR conditions for Apoe were as recommended by Jackson Labs ($Apoe^{+/+}$ and $Apoe^{+/-}$ are included as controls). (*B*) Lung lysates from endothelial-specific TRPC3-HA transgenic mice ("TgEST3") or their nontransgenic littermates ("Non-Tg") were subjected to immunoprecipitation using anti-HA antibody (12CA5) and immunoprecipitated proteins were subjected to Western blot analysis using the same anti-HA antibody (numbers below the blots correspond to in-house numbering of the mice); IgG_H band corresponds to the heavy chain of the immunoprecipitating IgG. (*C*) Immunohistochemistry for TRPC3-HA in aortic root sections from endothelial-specific TRPC3-HA transgenic mice ("TgEST3") or their nontransgenic littermates ("non-transgenic"). The fuchsia-colored deposit on the endothelium in the TgEST3 section denotes positive immunoreactivity for the HA epitope. Background staining in sections from nontransgenic mice was not different from the negative control. L, arterial lumen.

examined lesions in the aortic sinus following 10 and 16 wk on a high-fat diet to accelerate development of atherosclerosis. Whereas no significant differences were found in the characteristics of early stage atherosclerotic lesions (10 wk), advanced plaques (16 wk) from TgEST3ApoeKO mice were larger and with higher macrophage content than in control animals. Remarkably, this correlated with increased density of VCAM-1 immunoreactive areas and NFkB activation along the endothelial lining of lesions from transgenic animals compared with those in nontransgenic littermates. These findings validate the in vivo relevance of our previous in vitro findings and represent, to our knowledge, the first in vivo evidence for a proatherogenic role of endothelial TRPC3.

Results

In this study, we examined the impact of endothelial-specific overexpression of human TRPC3 on the characteristics of early and advanced atherosclerotic lesions in Apoe knockout (ApoeKO) mice. As described in *Methods*, endothelial-specific overexpression of the human TRPC3 gene (hTRPC3) was achieved by having hTRPC3 cDNA under control of the human Flt-1 promoter that confers endothelial-specific gene expression (14). Expression of hTRPC3 at the protein level was confirmed by immunoblot of proteins from lung lysates from TgEST3ApoeKO mice with an anti-HA antibody (12CA5), which detects expression of the HA-tagged hTRPC3 in TgEST3ApoeKO but not in nontransgenic littermates (Fig. 1*B*). Lungs have a high content of endothelial cells, facilitating immunoblot detection of HA-tagged TRPC3 of endothelial origin. Contrarily to what is observed with other endothelial promoters (e.g., Tie2, Ve-cadherin), there was no expression of TRPC3-HA in bone marrow-derived macrophages from TgEST3ApoEKO mice, which is of importance as we recently showed that macrophage TRPC3 influences the characteristics of early and advanced atherosclerotic lesions of ApoeKO mice by virtue of its role in mechanisms associated to macrophage apoptosis (11, 15). Immunohistochemistry for HA epitope in aortic root sections from the transgenic mice confirmed endothelial localization of TRPC3-HA (Fig. 1*C*).

At 8 wk of age, female TgEST3ApoeKO mice or their nontransgenic littermates were placed on a Western-type high-fat diet (HFD; TD.88137, Harlan Laboratories) for 10 or 16 wk to allow for development, respectively, of early and advanced atherosclerotic lesions. At sacrifice, body weight, plasma cholesterol, and triglycerides were determined. As shown in Table 1, there were not significant differences in these parameters between transgenic and nontransgenic mice after either diet period.

To examine the impact of endothelial-specific overexpression of hTRPC3 on the characteristics of atherosclerotic lesions we performed histological analysis of heart sections at the level of the aortic sinus, as we described in detail in refs. 11 and 13. Based on morphometric evaluation of hematoxilin-eosin–stained sections, after 10 wk on HFD, lesions in transgenic mice were somewhat larger than in control mice, although not reaching statistical significance [358,983 ± 27,088 μm^2 ($n = 9$) vs. 279,054 ± 35,240 μm^2

Table 1. Body weight and lipid profile for ApoeKO and TgEST3ApoeKO mice

Parameter	ApoeKO	TgEST3ApoeKO
Total cholesterol (mg/dL)	1,138 ± 105	1,016 ± 55
	1,040 ± 55	1,103 ± 105
Triglycerides (mg/dL)	329 ± 64	322 ± 31
	229 ± 39	220 ± 40
Body weight after diet (g)	29.1 ± 2.16	26.6 ± 1.3
	28.6 ± 1.10	29.7 ± 1.3

Mice were fed a HFD for 10 (first row of values for each parameter) and 16 wk (second row of values for each parameter), and body weight and total plasma cholesterol and triglycerides were measured as described in *Methods*. Values are mean ± SEM, $n = 10$ for all parameters. All P values were >0.05 (not statistically significant) according to the Mann–Whitney u test.

(n = 10), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.094]. Neutral lipid content, as assessed by Oil Red O (ORO) staining, was not different [267,823 ± 38,379 μm^2 (n = 10) vs. 256,274 ± 39,266 μm^2 (n = 10), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.57]. At this stage, lesion macrophage content was similar between the two groups [MOMA2-positive area: 285,357 ± 66,471 μm^2 (n = 10) vs. 258,782 ± 41,833 μm^2 (n = 10), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.80]. At this time point, areas of necrosis were not evident in lesions examined throughout the entire aortic root.

We next examined the aortic root lesions in mice maintained on HFD for 16 wk. As expected, plaques at this stage were larger than those observed after 10 wk on HFD (P = 0.002), and areas of necrosis were clearly identifiable. As shown in Fig. 2, morphometric analysis of H&E stained plaques showed a marked increase in total lesion area in TgEST3ApoeKO mice compared with control animals [574,407 ± 55,416 μm^2 (n = 9) vs. 372,724 ± 62,787 μm^2 (n = 9), respectively; P = 0.019]. A similar result was obtained when analyzing ORO stained-sections, which were larger in the transgenic animals [355,614 ± 23,520 μm^2 (n = 10) vs. 224,993 ± 36,398 μm^2 (n = 10), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.026, Fig. 2]. Notably, the macrophage content of these plaques was markedly augmented compared with the nontransgenic littermates [MOMA2-positive area: 524,606 ± 29,766 μm^2 (n = 6) vs. 263,469 ± 76,483 μm^2 (n = 6), for TgEST3ApoeKO vs. nontransgenic littermates, respectively; P = 0.015, Fig. 3]. The significant overlap between ORO and MOMA2 positive areas suggests that lesional lipid is contained, to a great extent, in macrophages. In those lesions where necrosis was present, the areas of necrosis were, on average, significantly larger in the transgenic mice compared with those in the nontransgenic littermates [32,703 ± 3,956 μm^2 (n = 6) vs. 16,134 ± 3,362 μm^2 (n = 8), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.019]. In advanced atherosclerotic plaques, macrophage apoptosis plays a key role in formation and growth of areas of necrosis (11, 16). Using a modified in situ TUNEL technique we examined if endothelial overexpression of hTRPC3 affected accumulation of apoptotic cells in mice maintained on HFD for 16 wk. Whereas apoptotic (TUNEL$^+$) cells were clearly evident in lesions from both groups of animals, the total number of apoptotic cells was similar [24.1 ± 5.8 cells per mm^2 (n = 8) vs. 13.5 ± 2.0 cells per mm^2 (n = 8), for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.094]. To determine whether differences in macrophage content between lesions of TgEST3ApoeKO and nontransgenic littermates was related to differences in the extent of proliferation of lesional macrophages, aortic root sections from animals fed HFD for 16 wk were subjected to immunostaining for the nuclear antigen Ki67, a marker of cell proliferation. Evidence of actively proliferating cells was barely detectable and there were no differences between TgEST3ApoeKO and ApoeKO mice (1.1 ± 0.9 vs. 2.0 ± 0.8 Ki67$^+$ cells per mm^2, for TgEST3ApoeKO vs. ApoeKO, respectively; n = 8 for both groups, P = 0.42).

In previous in vitro studies using human coronary artery endothelial cells we found that increased expression of TRPC3 resulting from either exposing cells to proatherogenic stimuli or by overexpressing TRPC3-HA, resulted in higher expression levels of VCAM-1 in a NFkB dependent manner (12, 13). To determine whether, in vivo and in the setting of atherosclerosis, endothelial overexpression of hTRPC3 affected lesional expression of VCAM-1, we performed immunofluorescence on aortic root sections from TgEST3ApoeKO mice and their nontransgenic littermates to evaluate VCAM-1 immunoreactivity and its distribution along the endothelial lining. Representative stained sections are shown in Fig. 4. In vivo, endothelial VCAM-1 immunoreactivity can appear patchy and/or evenly distributed, and is not restricted to endothelium but it is also evident in other lesional cells (17, 18). Four independent operators blind to the study group scored the VCAM-1 staining that localized to endothelium as of low, intermediate, or high intensity, and as of having diffuse or even distribution along the endothelial lining. After analyzing the categorical scores in contingency tables, lesions from TgEST3ApoeKO mice maintained on HFD for 10 wk exhibited a more robust staining for VCAM-1, mostly at the expense of an increase in the number of sections showing staining with intermediate intensity of immunoreactivity (48% vs. 30%, for TgEST3ApoeKO vs. ApoeKO, respectively; P = 0.023,

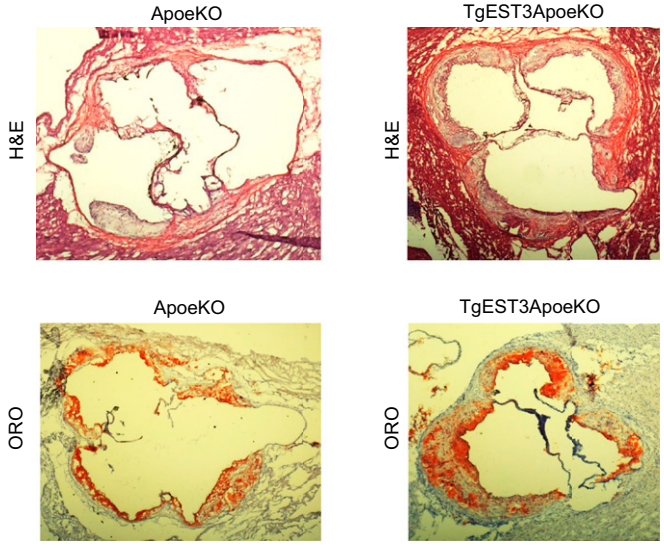

Fig. 2. Lesion area and lipid content. Aortic root sections from TgEST3ApoeKO mice or their transgenic littermates (ApoeKO) that were maintained on a 16 wk high fat diet, were stained with hematoxylin-eosin (H&E) or Oil-Red-O (ORO) to evaluate lesion area and neutral lipid content, respectively. Mean values and corresponding SEs, n numbers and P values are provided in the text.

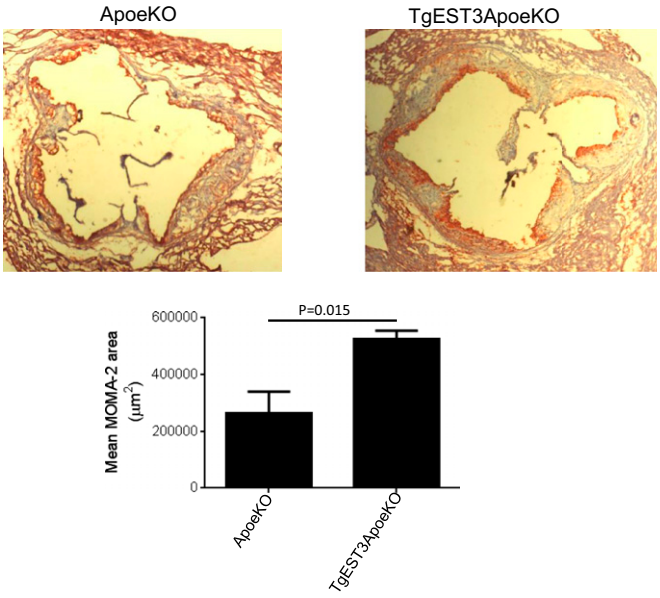

Fig. 3. Macrophage/monocyte content. Atherosclerotic lesions in aortic root sections from TgEST3ApoeKO mice or their transgenic littermates (ApoeKO) that were maintained on a 16 wk high fat diet, were stained with MOMA-2 antibody to evaluate macrophage content. Shown are representative sections and corresponding quantitations of the stained areas for each group of animals. P values were determined using the Mann–Whitney u test.

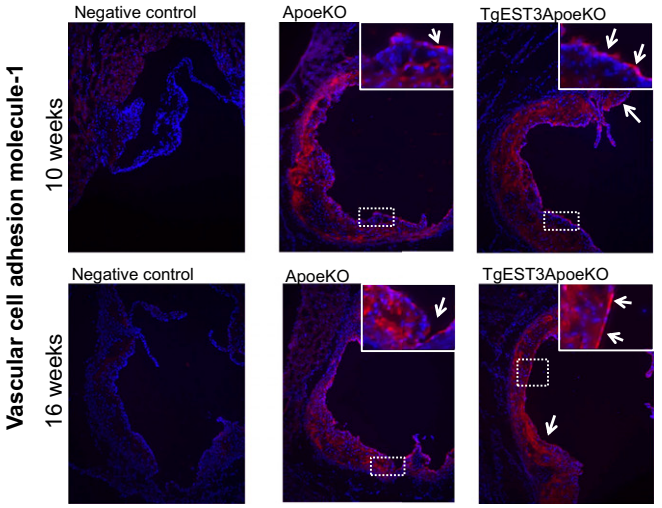

Fig. 4. VCAM-1 expression. Aortic root sections from TgEST3ApoeKO mice or their transgenic littermates (ApoeKO) that were maintained on a 10 or 16 wk high fat diet regimen were stained for VCAM-1 (red) and DAPI (nuclei). In the negative control, VCAM-1 antibody was replaced by an equivalent dilution of normal rat serum (see *Methods* for details). Typical immunoreactive areas (white dotted box in full image) are shown enlarged in the insets. The VCAM-1 staining that localized to endothelium was scored by four independent operators blind to the study groups, as of low, intermediate or high intensity, and as of having patchy or even distribution along the endothelial lining. The categorical scores were analyzed in contingency tables as detailed in the text.

χ^2 test). However, at this time point, there were no significant differences in the distribution of VCAM-1 along the endothelial lining. The above difference in VCAM-1 immunoreactivity was also noted in lesions from mice maintained for 16 wk on HFD, although in this case it did not reach statistical significance (57% vs. 42%, for TgEST3ApoeKO vs. ApoeKO, respectively; $P = 0.09$, χ^2 test). Notably, the distribution pattern of VCAM-1 staining in lesions from mice on 16-wk HFD was clearly shifted toward an increase in the presence of patches of VCAM-1 expression (82% vs. 58%, for TgEST3ApoeKO vs. ApoeKO, respectively; $P = 0.0003$, Fisher's exact test; Fig. 4). Interestingly, immunostaining for phospho-IkBα, an indirect indication of NFkB activation, was evidently more robust and noticeable along the endothelial lining of sections from TgEST3ApoeKO mice than those from controls after both 10 and 16 wk on HFD (Fig. 5).

Discussion

A number of previous studies from other groups, although not directly aimed at studying atherosclerosis, provided evidence somewhat supportive of the notion of a potential relationship between expression and/or function of members of the TRPC family of cation channels in the endothelium and events associated to the pathogenesis of atherosclerosis (reviewed by us in ref. 2). For instance, it is well established that TRPC3 participates in oxidative stress signaling, a recognized factor in vascular inflammation (19, 20). In bovine aortic endothelial cells migration is inhibited by lysophosphatidylcholine, a major component of oxidized low density lipoprotein, through a mechanism that requires sustained calcium influx mediated by the sequential activation of TRPC6 and TRPC5 (21). TRPC6 is also required for the angiogenic actions of vascular endothelial growth factor in endothelial cells (22, 23) suggesting a role of this TRPC in neoangiogenesis, a process often observed in advanced atherosclerotic plaques. Our previous in vitro studies showing that TRPC3 is an obligatory component of the mechanism driving expression of VCAM-1 and monocyte adhesion in human coronary artery endothelial cells, and that expression of native endothelial TRPC3 is up-regulated by proatherogenic factors, was to our knowledge the first direct evidence linking expression/function of endothelial TRPC3 to molecular and cellular events of relevance to atherosclerosis (12, 13). In addition, we reported that murine atherosclerotic lesions exhibit robust expression of TRPC3 compared with arterial cross-sections from nonatherosclerotic animals (13). Despite these findings, the multifactorial nature of lesion development in vivo impeded for any definitive association to be made between endothelial TRPC3 and atherogenesis. Hence, the necessity of generating a mouse model of atherosclerosis in which TRPC3 expression could be manipulated (i.e., conditional transgenic or knockout animals for TRPC3) was obvious. In the present studies, we introduced a unique mouse model of atherosclerosis with endothelial-specific overexpression of human TRPC3 to examine the impact of endothelial overexpression of this channel on the characteristics of atherosclerotic lesions.

Our findings show that whereas lesion size and macrophage content of early stage lesions in TgEST3ApoeKO were not different from those in nontransgenic littermates, advanced plaques were significantly larger and with markedly increased cellularity in the transgenic mice compared with control animals. In previous work we demonstrated that the immediate consequence of the augmented expression of TRPC3 that follows exposure of endothelial cells to proatherogenic factors, is a higher number of plasma membrane resident TRPC3 channels operating in constitutive mode, and this in turn enhanced NFkB-dependent expression of VCAM-1 and monocyte adhesion (12, 13). The present findings show that in advanced lesions from TgEST3ApoeKO mice the presence of patches of VCAM-1 expression along the endothelial lining was greater than in lesions from nontransgenic littermates, with the increased immunoreactivity for VCAM-1 being already evident after 10 wk on high fat diet. Interestingly, the appearance of endothelial patches of VCAM-1 expression in vivo has been correlated to clusters of newly expressed molecules (24–26). Remarkably, compared with control animals the extent of NFkB activation in endothelium from TgEST3ApoeKO, as evidenced by the extent of phospho-IkBα immunoreactivity, was also significantly increased. Recent

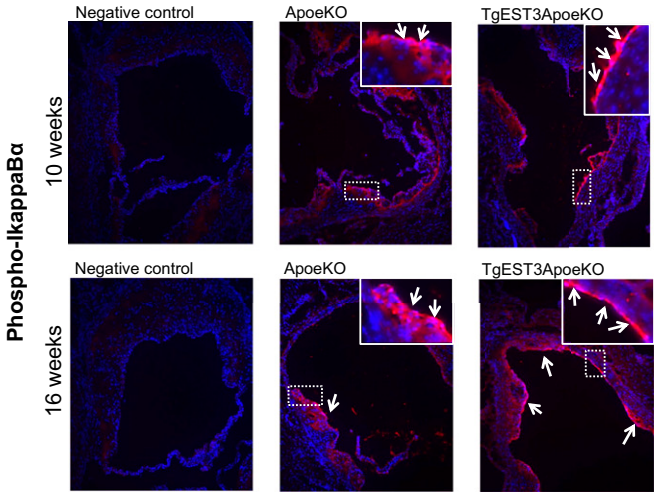

Fig. 5. NFkB activation. Aortic root sections from TgEST3ApoeKO mice or their transgenic littermates (ApoeKO) that were maintained on a 10 or 16 wk high fat diet regimen were stained for phospho-IkBα (red) and DAPI (nuclei). The negative control was processed as indicated in Methods, replacing the primary anti-phospho-IkBα antibody by an equivalent dilution of normal goat serum. Typical immunoreactive areas (white dotted box in full image) are shown enlarged in the insets.

observations in a mouse model of atherosclerosis revisited the mechanism of macrophage accumulation during lesion development, suggesting that as lesion progresses proliferation of lesional macrophages, rather than continuous monocyte influx, accounts for a significant proportion of the macrophage content in the lesion (27). When examining immunoreactivity for the nuclear antigen Ki67 in advanced aortic root lesions from both TgEST3ApoeKO and nontransgenic mice we did not find differences in the number of proliferating lesional cells, indicating that the marked increase in plaque cellularity exhibited by the TgEST3ApoeKO animals cannot be attributed to changes in macrophage proliferation. Altogether, our results suggest that the increased cellularity of lesions from TgEST3ApoeKO mice is likely due, at least in part, to augmented monocyte recruitment that follows increased endothelial inflammatory signaling and VCAM-1 expression. It is reasonable to speculate that, similar to what we observed in human coronary artery endothelial cells (12, 13), much of the impact of endothelial overexpression of TRPC3 on VCAM-1 expression and the inflammatory status of the endothelium of TgEST3ApoeKO mice is presumably subsequent to increased TRPC3-mediated constitutive calcium influx into the endothelial cells. This adds to previous examples of the biological consequences of a gain in TRPC3 constitutive function. Indeed, mice with global deficiency of TRPC6 exhibit compensatory up-regulated expression of TRPC3 which results in elevated vessel tone in both aorta and cerebral arteries mostly due to augmented constitutive calcium influx (28). In mouse skeletal myocytes increased TRPC3 expression and the gain in constitutive calcium entry correlates with enhanced basal NFAT activity (29). In this context, the present findings can be taken as, to our knowledge, the first in vivo evidence illustrating the impact of augmented expression of endothelial TRPC3 on atherosclerosis, and support our previous postulate that up-regulated expression of a calcium-permeable channel endowed with significant constitutive function may have pathological relevance (13). However, a contribution also from regulated TRPC3 activity cannot be ruled out here, because of the existence of a myriad of circulating factors that can, in vivo, further increase channel activity. Remarkably, and regardless of the extent of contribution of constitutive vs. regulated TRPC3 function in vivo, the positive correlation observed between larger plaque burden, cellularity, VCAM-1 expression and NFkB activation in the TgEST3ApoeKO mice are in line with our previous in vitro findings (13) and validate our prediction that in vivo and in the setting of atherosclerosis, endothelial TRPC3 supports inflammatory signaling and contributes to lesion cellularity (13).

Interestingly, the lipid content of advanced lesions was greater in TgEST3ApoeKO mice compared with their nontransgenic littermates. Although a definitive assignment of lipid location to a particular cell type cannot be made based solely on histological evaluation, the significant overlap between ORO and MOMA2 positive areas indicates that the majority of lesional lipid is contained in macrophages. Because plasma lipids are similar between TgEST3ApoeKO and nontransgenic mice, this observation suggests that augmented endothelial expression of TRPC3 somehow influences lipid accumulation in the plaque, possibly by promoting alterations in endothelial lipid transport and/or in the production of endothelial-derived cytokines that, directly or indirectly, may influence the lipid handling capability of lesional macrophages.

Accumulation of apoptotic macrophages in advanced atherosclerotic lesions is mostly due to impairment of the efferocytic functions of resident phagocytes as the lesion progresses and contributes to formation of necrotic cores (30). Interestingly, whereas the density of apoptotic macrophages was similar in advanced lesions from TgEST3ApoeKO mice compared with those from controls, the areas of necrosis were larger in the transgenic mice. Whether an association exists between expression/function of endothelial TRPC3 and the development of lesional necrosis, one that could be related to endothelial-derived factors directly or indirectly affecting the composition of the necrotic core, warrants further investigation.

Despite compelling evidence accumulated over the last two decades documenting the participation of TRPCs in mechanisms associated to the pathogenesis of cardiovascular diseases (reviewed by us in (1, 2)), the role of these channels forming proteins in atherosclerosis has remained largely unexplored. Our recent studies showing that bone marrow deficiency of TRPC3 reduces macrophage apoptosis and necrosis of advanced plaques in a mouse model of the disease (11) constitute, to our knowledge, the first available experimental in vivo evidence supporting a role of TRPC3 in murine atherosclerosis. In a more recent study, we found that the impact of TRPC3 deficiency on macrophage apoptosis is due to its supportive role in mechanisms that drive endoplasmic reticulum stress-induced apoptosis of M1 macrophages (15). We now show that, as predicted in previous in vitro studies in coronary artery endothelial cells (12, 13), augmented expression of endothelial TRPC3 also has profound effects in the characteristics of advanced atherosclerotic lesions. Taken together, our previous and present observations suggest that in the setting of atherosclerosis TRPC3 has distinctive proatherogenic effects by virtue of its unique functions in two atherorelevant cell types.

Interestingly, analysis of expression profile studies using human genomic microarrays shows significant up-regulation of TRPC3 mRNA levels in plaques derived from patients with atherosclerotic lesions in the left anterior descendent coronary artery (31). In circulating monocytes from three out of four patients homozygous for familial hypercholesterolemia, the TRPC3 mRNA was notoriously up-regulated compared with heterozygous or control patients (32). Despite these suggestive data showing a correlation between increased expression levels of TRPC3 and presence of atherosclerosis, whether TRPC3 has an active role in the pathogenesis of the human lesion, remains unknown. In that context, the present and our previous work provide a platform to start defining, by analogy to the roles of TRPC3 in murine lesions, cellular and molecular correlates in the human pathology that might be influenced by TRPC3 expression and/or function.

Methods

Aortic root sectioning, morphometric analysis of atherosclerotic lesions, and immunohistochemistry and immunofluorescence evaluation of lesion composition was performed essentially as we described in refs. 11 and 13. Additional details on protocols and source of antibodies are provided below.

Experimental Animals and Generation of Endothelial TRPC3 Transgenic Mice. All studies involving animals described in this work conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and have been approved by University of Toledo IACUC. C57BL/6 mice and the ApoeKO (B6.129P2-Apoe^{tm1Unc}/J) mice were obtained from Jackson Labs, and colonies were maintained in our animal facility. Euthanasia was performed by i.p. injection of sodium pentobarbital (150 mg/kg, plus 10 Units/mL of heparin). Mice with endothelial-specific overexpression of human TRPC3 (hTRPC3) were generated by using the human Flt-1 promoter (InVivoGen) to drive endothelial expression of wild-type human TRPC3. TRPC3 cDNA with C-terminal hemagglutinin (HA) epitope (33) was cloned into pDRIVE (InVivoGen) between the Flt-1 promoter and the SV40 polyA. Human and mouse TRPC3 are more than 93% identical at the gene and protein level and indistinguishable in terms of functional and pharmacological properties (5). TRPC3-HA has been shown to be functional in mice with cardiomyocyte overexpression of TRPC3HA (34) and in several cell types in vitro (5, 33, 35). The Flt-1/TRPC3-HA minigene (5,178 bp) was sequenced, excised, purified, and injected into C57BL/6 oocytes (Diabetes and Endocrinology Research Molecular Core, Yale School of Medicine, New Haven, CT). PCR of tail gDNA from founders (primers TgF: 5′-AAGACAAGAGCCAAGCAACTGA; TgR: 5′-CTCCCCCTGAACCTGAAACA) rendered the expected 334 bp amplicon corresponding to the last 92 bp of Trpc3+HA+212 bp of the polyA. Founders with the endothelium-specific TRPC3 transgene (TgEST3 - F0, 2 males, 3 females) represented fully inbred F0 lines on C57BL/6 background. Once transferred to our facility F0 mice were mated with C57BL/6 breeders; all were fertile and passed the transgene to progeny with no gross phenotypes being observed. At F4 high and low expressors were identified by Western blot analysis of TRPC3-HA in lung

lysates and these lines were maintained on C57BL/6 background; high expressor lines were intercrossed with ApoEKO mice (C57BL/6 background) to generate TgEST3ApoEKO mice (see Fig. 1*A*; genotyping for ApoE allele was performed according to Jackson Labs recommendations).

Determination of Plasma Cholesterol and Triglycerides. After a 12-h fasting period blood was collected by submandibular vein puncture. Total plasma cholesterol and triglyceride concentrations were determined using Cholesterol-E and L-Type Triglyceride-M (Wako Chemicals) following manufacturer's instructions.

Aortic Root Sectioning. Aortic root sections were prepared as we described in refs. 11 and 13. Briefly, euthanized mice were perfused through the left ventricle with 4% (wt/vol) paraformaldehyde followed by PBS. The heart was cut so that all three aortic valves were in the same geometric plane, and the upper portion was embedded in O.C.T., frozen in the Peltier stage of the cryostat (Thermo Scientific R. Allan HM550 Cryostat) and sectioned. Sections (10 μm) were collected onto Fisher Superfrost Plus-coated slides, starting from where aorta exits the ventricle and moving toward the aortic sinus (~650–700 μm). Lesion analysis, Oil Red O (ORO), hematoxylin and eosin (H&E) and trichrome staining, as well as evaluation of necrotic cores and in situ TUNEL (in situ cell death detection kit, Roche) were as described (11).

Immunohistochemistry. Immunohistochemistry was essentially as we described in detail in refs. 11 and 13. Briefly, sections were fixed in acetone and processed for immunostaining for MOMA-2 (Santa Cruz Biotechnology, catalog no. sc-59332) followed by incubation with biotinylated rabbit anti-rat antibody (Dako). After treatment with secondary antibodies sections were incubated with alkaline phosphatase-conjugated streptavidin (Dako). Counterstaining was with hematoxylin. Negative controls were performed by substituting the primary antibody with nonimmune IgG from the same species and at the same concentration. Under these conditions, nonspecific immunostaining was minimal or not detected. Stained areas were captured (Micropublisher 3.3 Megapixel Cooled CCD Color Digital Camera) and measured (NIS Elements D).

Immunofluorescence for VCAM-1 and Phospho-IkBα. Sections were fixed in ice-cold acetone (15 min) and treated with 0.1% hydrogen peroxide in TBS (15 min). After blocking nonspecific binding sites with 3% BSA (1 h) sections were incubated with anti-VCAM-1 antibody (1:100; Southern Biotech, catalog no. #1510-01) overnight at 4 °C. Following washes in PBS, sections were incubated with anti-rat Alexa-fluor 555 (Cell Signaling, catalog no. 4417; 1:2,000, 1 h), washed, and mounted with Prolong Gold Antifade Reagent containing DAPI (Cell Signaling, catalog no. 8961). For phospho-IkBα immunostaining, acetone-fixed sections were soaked in PBS containing 1% Triton X-100 for 30 min (room temperature) and blocked in 5% normal goat serum in PBS (30 min) before overnight incubation (4 °C) in unconjugated AffiniPure Fab Fragment Goat Anti-Mouse IgG (H +L) (1:10; Jackson ImmunoResearch Laboratories). Sections were washed in 0.1% PBS-Tween-20 (30 min) and incubated with anti-phospho-IkBα antibody (Cell Signaling, catalog no. 9246) for 1 h (room temperature), followed by an additional 1 h incubation with anti-mouse Alexa-fluor 555 (Cell Signaling, catalog no. 4409). After washing in 0.1% PBS-Tween-20 (30 min) slides were mounted with Prolong Gold Antifade Reagent containing DAPI (Cell Signaling, catalog no. 8961). A similar treatment was performed on sections processed for immunohistochemistry using an anti-HA mouse monoclonal antibody (12CA5, as in ref. 33) followed by incubation with biotinylated goat anti-mouse and alkaline phosphatase-conjugated streptavidin (Dako). Counterstaining was with hematoxylin.

Statistical Analysis. Values are shown as mean ± SEM. Comparisons between groups was performed by Mann–Whitney *u* test using GraphPad Prism version 6 for Windows 2007. *P* values below 0.05 were considered significant.

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