Hepatocyte-specific IKKβ expression aggravates atherosclerosis development in APOE*3–Leiden mice
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A B S T R A C T

Objective: The liver is the key organ involved in systemic inflammation, but the relation between hepatic inflammation and atherogenesis is poorly understood. Since nuclear factor-κB (NF-κB) is a central regulator of inflammatory processes, we hypothesized that chronically enhanced hepatic NF-κB activation, through hepatocyte-specific expression of IkB kinase-β (IKKβ) (E3L.IKKK), will aggravate atherosclerosis development in APOE*3-Leiden (E3L) mice.

Methods and results: E3L.IKKK and E3L control littermates were fed a Western-type diet for 24 weeks. E3L.IKKK mice showed a 2.3-fold increased atherosclerotic lesion area and more advanced atherosclerosis in the aortic root with less segments without atherosclerotic lesions (11% vs. 42%), and more segments with mild (63% vs. 44%) and severe (26% vs. 14%) lesions. Expression of IKKβ did not affect basal levels of inflammatory parameters, but plasma cytokine levels tended to be higher in E3L.IKKK mice after lipopolysaccharide (LPS) administration. E3L.IKKK mice showed transiently increased plasma cholesterol levels, confined to VLDL. This transient character resulted in a mild (+17%) increased cumulative plasma cholesterol exposure.

Conclusion: We conclude that selective activation of NF-κB in hepatocytes considerably promotes atherosclerosis development which is (at least partly) explained by an increased sensitivity to proinflammatory triggers and transiently increased plasma cholesterol levels.

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1. Introduction

Increased inflammation, in addition to disturbances in lipid metabolism, is the other main contributor to the development of atherosclerosis [1]. Nuclear factor-κB (NF-κB) has been identified as the most important transcription factor in the regulation of inflammatory processes during atherosclerosis development [2]. In unstimulated cells, NF-κB p65/p50 dimer is kept inactive by its inhibitory protein: inhibitor of κB (IκB). A wide range of extracellular stimuli, including cytokines, microbial components, and also free fatty acids, induce activation of the IκB kinase complex, which consists of two kinases (IKKα and -β) and a regulatory subunit, NEMO/IKKγ. This complex mediates the phosphorylation of IκB, resulting in its ubiquitination and degradation, leaving the NF-κB dimer free to translocate to the nucleus and activate its target genes [2].

While general inhibition of the NF-κB pathway by pharmacological agents reduces atherosclerosis development in mice [3,4], the relative contribution of NF-κB may differ at cellular- or tissue-specific level. Suppression of the NF-κB pathway in endothelial cells by ablation of NEMO/IKKγ has been shown to decrease atherosclerosis development [5]. In murine bone marrow transplantation models, inhibition of the NF-κB pathway at distinct levels in hematopoietic cells can have different outcomes, i.e. deficiency of the NF-κB p50 subunit resulted in smaller atherosclerotic lesions [6], whereas deletion of IKKβ increases atherosclerosis development [7]. Surprisingly, the role of the NF-κB pathway in hepatocytes on atherosclerosis development has not been investigated thus far.

The liver plays a central role in both lipid metabolism [8] and inflammation [9]. Disturbances in lipid metabolism and increased inflammation are the two main risk factors for atherogenesis [1]. Hepatocytes form the largest population of cells in the liver and execute most of its important functions. During inflammation, acute phase proteins are mainly synthesized by the hepatocytes [10]. Interestingly, hepatocyte-specific deficiency of gp130, a receptor component of IL-6 signaling which signals independent of the NF-κB pathway, decreases atherosclerosis in mouse models [11], suggesting that reduced hepatic inflammation is associated with less atherosclerosis development.

Despite ample evidence implicating the involvement of NF-κB in atherogenesis, the hepatocyte-specific role of NF-κB in atherosclerosis has not been investigated directly. Therefore, in this study we aimed to investigate whether chronic activation of hepatocyte-specific NF-κB aggravates atherosclerosis development. We used transgenic mice with hepatocyte-specific expression of human IKKβ (liver-specific IKKβ or LIKK mice), resulting in an increase of active NF-κB [12], crossed with atherosclerosis-prone apoE−/− mice. E3L mice exhibit a human-like lipoprotein distribution on a cholesterol-rich diet due to transgenic expression of a human mutant of the apoE3 gene, and are therefore susceptible to atherosclerosis development [13]. Collectively, our results show that hepatocyte-specific NF-κB activation markedly aggravates atherosclerosis development in E3L mice.

2. Methods

Brief descriptions of the most important procedures of this study are provided in this section. An expanded description is available in the supplemental data (available online at http://atherosclerosis-journal.com).

2.1. Animals

Transgenic LIKK mice expressing constitutively active human IKKβ in hepatocytes under the control of an albumin promoter [12] were crossed with E3L mice [13] to generate heterozygous E3LLIKK and control E3L littermates, as described before [14]. Ten-12 weeks old female mice were fed a Western-type diet for 24 weeks. Blood was drawn every 4 weeks after a 4-h fast.

2.2. Plasma analysis

Plasma levels of serum amyloid A (SAA), inflammatory cytokines, total cholesterol (TC), triglycerides (TG) and phospholipids (PL) levels were determined.

2.3. Lipopolysaccharide stimulation

Mice were injected i.v. with Salmonella minnesota Re595 lipopolysaccharide (LPS) (50 mg/kg body weight). Blood was collected 90 min after injection and plasma was assayed for cytokines.

2.4. Atherosclerosis quantification

The extent of atherosclerosis was assessed in the aortic root area. After staining with hematoxylin–phloxine–safron (HPS), atherosclerotic lesion severity and area were determined.

3. Results

3.1. LIKK causes low-grade inflammation

The overall appearance of E3L and E3LLIKK mice during the study was similar. To assess whether expression of LIKK affects body weight gain, we measured food intake and body weight weekly. Both were not different between E3LLIKK and E3L control mice (Supplemental Fig. 1A and B). The liver- and spleen weight and histological morphology of the liver were also comparable between E3LLIKK and E3L mice (data not shown). To gain more insight in the effects of LIKK on inflammation, we determined whether LIKK expression increased the inflammatory state of the liver and systemic inflammatory markers in E3LLIKK mice on a Western-type diet. We confirmed previous findings [14] showing that the enhanced expression of hepatocyte-specific human IKKβ (Supplemental Fig. 2A) resulted in a 1.4-fold increased hepatic NF-κB activation, as shown by an increase in the phosphorylated p65 subunit (pNF-κBSer536) (Supplemental Fig. 2B). IKKβ kinase phosphorylates subunit p65 of NF-κB at the position Ser536, which activates the transcriptional activity of NF-κB [15]. The transgenic expression of human IKKβ mRNA was present only in E3LLIKK mice and did not alter murine IKKβ mRNA expression (Supplemental Fig. 2C and D). The enhanced hepatic NF-κB activation in E3LLIKK mice did not result in increased IL-6 expression in whole liver, but did result in a tendency towards increased IL-1β expression (P = 0.085) and a significant increase in MCP-1 expression (Supplemental Table 2).

To evaluate whether the increased hepatocyte-specific NF-κB activation in E3LLIKK mice enhanced the systemic inflammatory state, we determined the plasma inflammation marker SAA and plasma cytokines under basal conditions. LIKK expression did not affect SAA before (3.1 ± 0.17 μg/mL vs. 3.4 ± 0.15 μg/mL) and after 8 weeks (4.4 ± 0.28 μg/mL vs. 4.2 ± 0.31 μg/mL) and 24 weeks (4.9 ± 0.51 μg/mL vs. 5.4 ± 0.78 μg/mL) of Western-type diet feeding (Fig. 1), and neither the determined plasma cytokine levels (Supplemental Fig. 3A–F). SAA levels increased with Western-type diet feeding in both E3L and E3LLIKK mice, but this difference only reached statistical significance in E3LLIKK mice (Fig. 1).

Since we did not observe a clear increased systemic proinflammatory state under basal conditions, we challenged the mice with LPS to boost the inflammatory response. Interestingly, after injection of LPS, proinflammatory cytokines (e.g. IL-1β, IFNγ) showed

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**Likk** does not increase plasma SAA levels. SAA levels were determined in plasma from E3L.LIKK (black bars) and E3L (white bars) mice fed a Western-type diet for 0, 8 and 24 weeks. Values are means ± SEM; n = 15/group; **P < 0.01.

To study whether this chronic low-grade inflammation in E3L.LIKK mice also resulted in increased inflammatory cell counts in liver and plasma, we determined the hepatic mRNA expression of various cell-type markers of inflammatory cells present in the liver, which are likely to influence atherogenesis [16], and the number of circulating monocytes. Hepatic mRNA expression of CD68 (Kupffer cells), CD3 (T cells), and Vα14 (NKT cells) were not different between the genotypes (Supplemental Table 2), neither were the total number of circulating monocytes, the proinflammatory Ly6C-hi monocyte subset, the intermediate Ly6C-med monocyte subset and the less inflammatory Ly6C-lo monocyte subset (Supplemental Fig. 4A–D). Together, the above findings indicate that the enhanced hepatocyte-specific NF-κB activation in E3L.LIKK mice results in a tendency towards increased plasma levels in E3L.LIKK mice as compared to E3L mice (Fig. 2A–F). The anti-inflammatory IL-10:IL-1β ratio was significantly lower in E3L.LIKK mice (Fig. 2G). Overall, these data indicate that E3L.LIKK mice are more sensitive to proinflammatory triggers compared to their E3L littermates.

**Lik** tends to enhance plasma cytokines after LPS stimulation. LPS was injected intravenously in E3L.LIKK (black bars) and E3L (white bars) mice. Cytokine levels were measured 90 min after LPS injection (A–F). The IL-10:IL-1β ratio was calculated (G). Values are means ± SEM; n = 7/group; *P < 0.05.
Fig. 3. LIKK transiently increases VLDL. Plasma cholesterol levels of E3LIKK (black symbols) and E3L (white symbols) mice fed a Western-type diet were assessed (A), and cumulative total cholesterol exposure was calculated (B). Lipoprotein profiles were determined at 8 (left) and 16 (right) weeks (C). Values are means ± SEM; n = 15/group; *P < 0.05, **P < 0.01, ***P < 0.001.

a tendency towards a mildly enhanced hepatic proinflammatory state and an elevated sensitivity to proinflammatory stimuli as compared to E3L littersmates.

3.2. LIKK transiently enhances VLDL cholesterol levels

To assess the effect of hepatocyte-specific NF-κB activation on plasma lipid levels, TC, TG and PL concentrations were determined every 4 weeks in E3LIKK and E3L mice. LIKK expression caused a transient increase of plasma TC levels only at 8 weeks (+50%; P < 0.0001) and 12 weeks (+28%; P < 0.05) of Western-type diet feeding (Fig. 3A). Accordingly, the cumulative total cholesterol exposure was higher in E3LIKK than in E3L mice (+17%; P < 0.05; Fig. 3B). A similar transient increase was found for plasma TG and PL levels (Supplemental Fig. 5A and B).

To determine which lipoproteins contribute to the transient elevated plasma TC levels, lipoproteins were size-fractionated by FPLC, and cholesterol was measured in the individual fractions. The transient increase in plasma TC levels at 8 weeks of Western-type diet feeding in E3LIKK mice was confined to (V)LDL, whereas at 16 weeks the lipoprotein distribution in the E3LIKK mice was similar to that of the E3L mice, in line with the plasma lipid levels (Fig. 3C). Consistent with our previous finding that expression of LIKK increased the VLDL production in male mice on chow diet [14], we found that expression of LIKK increased, albeit not significantly, the VLDL-TG production rate (+24%) (Supplemental Fig. 6A), and tended to increase the VLDL-apolipoprotein B (apoB) production rate (+33%) (Supplemental Fig. 6B). No differences were observed in the liver lipid content between E3LIKK and E3L mice (Supplemental Fig. 7A–E). Taken together, these findings indicate that hepatocyte-specific NF-κB activation results in a modest and transient increase in plasma lipid levels in E3L mice.

3.3. LIKK enhances atherosclerosis development

To investigate the effect of LIKK expression on atherosclerosis development, E3LIKK and E3L mice were sacrificed after 24 weeks of Western-type diet feeding, and lesion size and severity were measured in the aortic root. Representative pictures of both groups are shown in Fig. 4A. E3LIKK mice developed more than 2-fold
larger atherosclerotic lesions (+131%; \( P < 0.05 \); Fig. 4B) as compared to their E3L littermates. This increased lesion area coincided with more advanced lesion progression, since we found markedly fewer segments without atherosclerotic lesions (11% vs. 42%; \( P < 0.001 \)) and more segments with mild (63% vs. 44%; \( P < 0.001 \)) and severe lesions (26% vs. 14%; \( P = 0.001 \)) as compared to E3L mice (Fig. 4C). Examples of mild and severe lesions are shown in Supplementary Fig. 8. These data indicate that chronic hepatocyte-specific NF-κB activation severely augments atherosclerosis development in E3L mice.

3.4. LIKK aggravates atherosclerotic lesion composition

We next evaluated whether LIKK expression would affect monocye adherence and recruitment to the vascular wall, as well as the composition of the atherosclerotic lesions with respect to the macrophage, smooth muscle cell, and collagen content of the lesions. Adherence of monocytes to the vessel wall and the content of the chemokine monocyte chemoattractant protein–1 (MCP–1) of the atherosclerotic lesions was not significantly enhanced in E3LLIKK mice as compared to E3L mice (Fig. 5A and B). LIKK expression did not affect the relative macrophage and collagen content of the lesions (Fig. 5C and E), but did result in an increased smooth muscle cell content of the lesions (+79%, \( P < 0.05 \); Fig. 5D).

3.5. Aggravated atherosclerosis development in E3LLIKK mice does not solely depend on the transient increase in plasma cholesterol levels

In E3L mice on Western-type diet, the cumulative plasma cholesterol exposure is highly predictive for the atherosclerotic lesion area (unpublished data, J.F.P. Berbée, P.C.N. Rensen). To verify if the transient increase in plasma TC levels (Fig. 3) alone could account for the aggravation in atherosclerosis development observed in E3LLIKK mice, or whether additional mechanism(s) could contribute, including the low-grade systemic inflammation, we assessed the correlation between the cumulative plasma total cholesterol exposure and the atherosclerotic lesion area of the E3LLIKK and E3L mice. As expected, there was a significant positive logarithmic correlation between the atherosclerotic lesion area and the cumulative plasma cholesterol exposure in the control E3L mice (Supplemental Fig. 9A; \( r^2 = 0.757, P = 0.002 \)). However, we did not observe such a correlation in the E3LLIKK mice (Supplemental Fig. 9B; \( r^2 = -0.250, P = 0.369 \)), indicating that in addition to the transient increase in plasma TC levels in E3LLIKK mice, additional mechanism(s), most likely the increased sensitivity to proinflammatory stimuli, contributed to the aggravated atherosclerosis development in these mice.

4. Discussion

NF-κB is regarded as a potential therapeutic target in atherosclerosis [3,4] and studying tissue- and cell-specific effects of NF-κB in atherogenesis will expand our knowledge in the comprehensive actions of NF-κB on atherosclerosis development. The present study demonstrates for the first time that chronic, hepatocyte-specific expression of IKKβ (LIKK) and subsequent activation of NF-κB aggravates atherosclerosis development in E3L mice. In addition, the atherosclerotic lesion composition with respect to the macrophage and collagen content was not affected by LIKK, but in line with the presence of more advanced lesions, the smooth muscle cell content was increased. Expression of LIKK resulted in transiently increased plasma cholesterol levels and an enhanced sensitivity to proinflammatory triggers, which both are likely to have contributed to the increased atherosclerotic lesion size and severity. Since lesion size and severity are often correlated in atherosclerosis studies with different murine models [13,17], the increased lesion severity in E3LLIKK mice is likely to be mainly attributed to the larger size of the lesions.

Expression of LIKK in E3L mice increased the activation of the NF-κB pathway in the liver, in line with our previous report [14]. In addition, hepatic mRNA expression of inflammatory parameters was increased or tended to be increased in E3LLIKK mice, indicating that inflammatory mediators at local tissue level were enhanced in E3LLIKK mice. This enhanced activation of hepatocyte-specific NF-κB in E3LLIKK mice, however, did not result in a significant increased systemic proinflammatory state as compared to their E3L littermates. Importantly, Cai et al. [12] demonstrated that in LIKK mice on a wild-type background, systemic levels of IL–6 were only mildly elevated, while IL–1β and TNFα levels were similar as in wild-type mice. Our results show that LIKK expression on an E3L background resulted in a less pronounced hepatic inflammatory state as compared to LIKK expression on a wild-type background as described by Cai et al. [12], as reflected in a smaller increase in active NF-κB (1.4– vs. 2.2-fold) and mRNA levels of proinflammatory cytokines levels in the liver. Furthermore, under basal conditions E3L mice have lower levels of active NF-κB present in the liver as compared to wild-type mice (unpublished data, J.A. van Diepen, M.C. Wong, P.J. Voshol). This implies that E3L mice have a lower chronic inflammatory state than wild-type mice, which could interfere with the proinflammatory effects caused by expression of LIKK in the present study. Also, in comparison with other murine atherosclerosis models, e.g. the apoE–/– and ldlr–/– mice, E3L mice display a milder phenotype with respect to hyperlipidemia and increased inflammation [18,19]. In the current study, basal circulating levels of some cytokines were at borderline of the detection limit of current assays (Supplemental Fig. 3) and, as expected, the cytokine levels increased approximately 5– to 3700-fold after LPS injection (Fig. 2). Furthermore, after stimulation with LPS, E3LLIKK mice showed a tendency towards a higher systemic inflammatory state than E3L mice.

There is a strong interaction between inflammation and lipid metabolism [20]. For example, lowering inflammation using salicylate did not only reduced NF-κB activation, but concomitantly also reduced circulating cholesterol levels in E3L mice [21]. In line with this observation, in the present study we found higher plasma lipid levels at 8 weeks of Western-type diet feeding in female E3LLIKK compared to E3L mice, which were confined to (V)LDL. We hypothesize that the increased lipid levels at this time point are accompanied by increased systemic inflammation, which is in line with previous findings showing that lipid metabolism and inflammation strongly influence each other [20]. A possible cause for the increased plasma lipid levels at 8 weeks of diet is therefore a more enhanced inflammation in the liver, possibly due to an increased activation of the NF-κB pathway in the liver.

We recently reported that male E3LLIKK mice on chow diet also showed enhanced (V)LDL levels as a result of an increased hepatic VLDL-TG production rate [14], and found in the current study a trend towards an enhanced VLDL-apoB production in female E3LLIKK mice on Western-type diet, with a similar effect-size. Possible reasons for the less apparent increase of VLDL-TG production in females compared to males are differences in gender and/or diet. Although the increase in VLDL-TG production is more apparent in male E3LLIKK mice, we used female mice in the present study. The main reason for this is that female E3L mice are more susceptible to develop atherosclerosis. In order for male E3L mice to become similarly atherosclerosis-prone they need to be fed Western-type diets not only with higher percentages of cholesterol, but also containing cholate. In addition, fructose was added to the drinking water to further raise their (V)LDL-cholesterol levels [22]. The increase in (V)LDL levels in females in the current study was only transient at 8 weeks of Western-type diet feeding and disappeared at 16
weeks. Since no differences in plasma lipid levels and hepatic mRNA expression of genes involved in lipid metabolism were detected between both groups at 24 weeks of diet, the increased VLDL-TG production at 8 weeks of diet is likely to be transient. At present, we cannot explain the transient nature of this increase in VLDL levels, but it may be the result of a progressive negative feedback mechanism to reduce the hepatic VLDL production which takes place during long-term Western-type diet feeding.

Dyslipidemia is regarded as the classical risk factor for atherosclerosis development. The transiently enhanced total cholesterol levels, resulting in a modest increase (+17%) in cumulative total cholesterol exposure upon LIKK expression, thus likely contributed to the enhanced atherosclerosis development. Previous diet-induced atherosclerosis studies in E3L mice have consistently demonstrated that there is a positive logarithmic relation between the cumulative cholesterol exposure during the study and the atherosclerotic lesion area (J.F.P. Berbée, P.C.N. Rensen, unpublished data). In agreement with these previous observations, we did observe such a significant logarithmic relation in E3L mice but not in E3LLIKK mice. This suggests that the increase in atherosclerotic lesion area in E3LLIKK mice can only partly be attributed to the transiently enhanced plasma cholesterol levels and that additional mechanisms are involved.

Inflammation is the second main risk factor for atherosclerosis. Enhanced extravascular or systemic inflammation, by the periodontal pathogen Porphyromonas gingivalis [23] or by repeated administration of LPS [24], respectively, promotes atherosclerosis development. In addition, in humans, low-grade systemic inflammation is associated with enhanced risk of coronary artery disease [25,26]. It is thus likely that, as discussed above, the increased sensitivity for proinflammatory triggers such as LPS in E3LLIKK mice also directly contributed to the enhanced atherosclerotic lesion formation.

We excluded higher circulating levels of proinflammatory Ly6C-hi monocytes as being another possible contributor to the aggravated atherosclerosis development in E3LLIKK mice. Adhesion of monocytes to endothelial cells and subsequent migration into the vessel wall is one of the crucial steps in atherosclerotic lesion formation. Ly6C-hi monocytes are more prone to adhere to activated endothelium than Ly6C-lo monocytes and are, therefore, associated with enhanced atherosclerosis development [27]. We found that E3LLIKK mice had similar levels of circulating subsets of monocytes as compared to their E3L controls, which is consistent with the observed similar number of adhering monocytes to the vascular wall.

In line with the enhanced atherosclerosis development that we observed in E3LLIKK mice, Luchtefeld et al. [11] have reported that gp130-deficient mice with defective IL-6 signaling specifically in hepatocytes, develop less atherosclerosis, indicating that modulation of hepatic inflammation can have profound effects on atherogenesis. These studies also underscore that enhanced inflammation in the liver, e.g. due to viral hepatitis or steatohepatitis, may augment atherosclerosis development. Indeed, in several clinical studies, such hepatic pathological conditions are associated with an elevated occurrence of CVD [28–30]. Even after adjustment for classical risk factors for CVD, such as LDL cholesterol levels,
chronic hepatitis C infection was still significantly associated with increased atherosclerosis in a cross-sectional study [30]. Together, these findings suggest that there is a direct effect of hepatic inflammation on atherosclerosis development, independent of systemic lipid levels. Moreover, they suggest that in addition to the currently used lipid-targeted drugs such as statins, reducing NF-κB activity in the liver may be a promising additive therapeutic strategy against atherosclerosis development.

In conclusion, we have shown that hepatocyte-specific activation of NF-κB leads to larger and more advanced atherosclerotic lesions. Our studies furthermore suggest that both the transient elevated (V)LDL cholesterol levels as well as the increased sensitivity to proinflammatory stimuli are most likely responsible for this aggravating effect on atherosclerosis. These findings contribute to the present understanding of the role of the liver, and more specifically the role of hepatic NF-κB, in atherosclerosis development and may help to develop new innovative anti-atherosclerotic strategies.

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Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.06.055.

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