Anti-Inflammatory and Antiatherogenic Effects of the NLRP3 Inflammasome Inhibitor Arglabin in ApoE₂.Ki Mice Fed a High-Fat Diet

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**Background**—This study was designed to evaluate the effect of arglabin on the NLRP3 inflammasome inhibition and atherosclerotic lesion in ApoE₂.Ki mice fed a high-fat Western-type diet.

**Methods and Results**—Arglabin was purified, and its chemical identity was confirmed by mass spectrometry. It inhibited, in a concentration-dependent manner, interleukin (IL)-1β and IL-18, but not IL-6 and IL-12, production in lipopolysaccharide and cholesterol crystal–activated cultured mouse peritoneal macrophages, with a maximum effect at ≈50 nmol/L and EC₅₀ values for both cytokines of ≈ 10 nmol/L. Lipopolysaccharide and cholesterol crystals did not induce IL-1β and IL-18 production in Nlrp3⁻/⁻ macrophages. In addition, arglabin activated autophagy as evidenced by the increase in LC3-II protein. Intraperitoneal injection of arglabin (2.5 ng/g body weight twice daily for 13 weeks) into female ApoE₂.Ki mice fed a high-fat diet resulted in a decreased IL-1β plasma level compared with vehicle-treated mice (5.2±1.0 versus 11.7±1.1 pg/mL). Surprisingly, arglabin also reduced plasma levels of total cholesterol and triglycerides to 41% and 42%, respectively. Moreover, arglabin oriented the proinflammatory M1 macrophages into the anti-inflammatory M2 phenotype in spleen and arterial lesions. Finally, arglabin treatment markedly reduced the median lesion areas in the sinus and whole aorta to 54% (P=0.02) and 41% (P=0.02), respectively.

**Conclusions**—Arglabin reduces inflammation and plasma lipids, increases autophagy, and orients tissue macrophages into an anti-inflammatory phenotype in ApoE₂.Ki mice fed a high-fat diet. Consequently, a marked reduction in atherosclerotic lesions was observed. Thus, arglabin may represent a promising new drug to treat inflammation and atherosclerosis. *(Circulation. 2015;131:1061-1070. DOI: 10.1161/CIRCULATIONAHA.114.013730.)*

**Key Words:** arglabin-dimethylaminohydrochloride ■ atherosclerosis ■ cytokines ■ inflammasomes ■ inflammation

Inflammation is generally a key factor in the development of a variety of diseases, including atherosclerosis. Although markers of chronic inflammation such as C-reactive protein are clearly predictive of clinical atherosclerosis, the cause or origin of inflammatory responses and the mechanisms by which inflammation leads to vascular disease are not fully understood. Cells of the innate immune system, namely macrophages and dendritic cells, express sensors for “danger” signals. These include the family of transmembrane Toll-like receptors, RIG-1-like helicases, and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs). They are involved in innate immune recognition of pathogen-associated molecular patterns and intracellular and extracellular damage-associated molecular patterns. Several members...
of the NLR family such as NLRP1, NLRP3, and NLRC4 have been shown to assemble into large multiprotein complexes called inflammasomes to control caspase-1 activity. The inflammasome is a multiprotein platform that activates procaspase-1 to caspase-1, and caspase-1, in turn, can convert pro–interleukin (IL)-1β and pro–IL-18 into their bioactive secreted forms. Both IL-1β and IL-18 induce severe atherosclerotic lesions in apolipoprotein E–deficient (ApoE−/−) mice. These findings suggest the involvement of the inflammasome in the development of atherosclerosis. This hypothesis gains further evidence from recent findings showing that caspase-1 deficiency decreases atherosclerosis in ApoE−/− mice.

NLRP3 is the most extensively studied inflammasome. It is activated in response to a variety of signals that are indicative of damage to the host, including tissue damage, metabolic stress, and infection.

It is well known that sterile inflammation is a crucial event in the pathological process that underlies atherosclerosis, whereas the role of inflammasomes in this process is still poorly explored. Cholesterol crystals (CCs) are important inflammatory stimuli in atherosclerosis development, acting as an endogenous danger signal via the NLRP3 inflammasome. Lyposomal injury after phagocytosis of CCs appears to be responsible for the activation of NLRP3. Besides CCs, a role for minimally modified low-density lipoprotein (LDL) in NLRP3 activation was suggested. Moreover, it has recently been demonstrated that amino-functionalized polystyrene nanoparticles with 100 nm in diameter trigger NLRP3 inflammasome activation.

Plants harbor a plethora of so-called secondary metabolites, which have been used traditionally to treat numerous ailments. Even today, plant-derived natural products and their derivatives and synthetic mimics make up a considerable portion of currently available drugs. Neutraceutical aspects are based on their favorable properties as small molecules and the fact that they very often possess rather unusual structural features, which cannot be easily mimicked, not even by combinatorial chemistry. On the basis of these properties, such compounds may serve as lead compounds for the development of new drugs.

Terpenoids are the largest known class of secondary metabolites in plants and possess a broad range of biological activities and potential therapeutic effects on atherosclerosis, psoriasis, and liver cirrhosis associated with schistosomiasis. On the basis of their structural diversity and biological activity, from a phytopharmaceutical point of view, specifically sesquiterpenes have been suggested to be of special interest among the terpenoids.

Sesquiterpene lactones can be categorized into 4 major classes: the germacranoledes, eudesmanolides, guaianolides, and pseudoguianolides. One of the guianolide sesquiterpene lactones that has been minimally investigated because of its limited availability (it is mainly synthesized by Artemisia glabrilla, which grows exclusively in Kazakhstan) is arglabin. The arglabin molecule consists of a cyclopentane ring with 5 contiguous stereocenters to which 5 membered rings are transannulated. The resulting strain can be released by ring opening of the γ-butyrolactone at C-2, which makes arglabin and its derivatives, similar to helenalin, prone to attack by nucleophiles, a mode of action that seems to play an integral role in its biological activity.

Arglabin shows anti-inflammatory and antitumor activity, yet only the antitumor activity is being explored in Kazakhstan in the form of a rather unstable dimethylamino hydrochloride derivative for the treatment of breast, colon, ovarian, and lung cancer. Preliminary modeling and pilot studies with primed macrophages exposed to CCs provided evidence for inhibition of the NLRP3 inflammasome by arglabin (T. Simmet, B. Büchele, T. Syrovets, Y. Yin, unpublished results, 2011). Therefore, we tested whether arglabin might prevent the development of atherosclerosis in a murine model through inhibition of the NLRP3 inflammasome activity.

As an in vivo atherosclerosis model, we have chosen ApoE2.Ki mice expressing human ApoE2 (2/2), which exhibits virtually all the characteristics of type III hyperlipoproteinemia in humans. Accordingly, their plasma cholesterol and triglyceride levels are 2 to 3 times those of normalolipidemic mice. These animals are markedly defective in clearing β-migrating very-low-density lipoprotein particles and spontaneously develop atherosclerotic plaques, even while on a regular diet. In ApoE2.Ki mice on an atherogenic diet or exposed to lipopolysaccharide, an exacerbation of atherosclerosis is observed.

In the present study, we tested whether the natural NLRP3 inflammasome inhibitor arglabin, administered intraperitoneally, would affect the development of atherosclerosis in ApoE2.Ki mice fed a high-fat diet (HFD).

**Methods**

Arglabin, a natural product, was purified, and its chemical identity has been confirmed by mass spectrometry. For in vitro studies, murine peritoneal macrophages were isolated from C57Bl/6 or Nlrp3−/− (C57BL/6 genetic background), cultured at 0.5×106 cells/mL, primed with lipopolysaccharide (10 ng/mL), treated or not with arglabin, and activated with CCs at 1 mg/mL. Cytokines in the supernatants were quantified with an ELISA or Multiplex approach. LC3 type II protein was analyzed by Western immunoblotting. Activation of autophagy by arglabin was evidenced by the formation of cellular autophagosome punctate containing LC3 type II with the use of confocal microscopy. Procaspase-1 and pro–IL-1β/pro–IL-18 processing was analyzed by Western immunoblotting. In vivo studies were performed on ApoE2.Ki mice and ApoE2.Ki/Nlrp3−/− mice. Female mice at 6 weeks of age were treated twice daily with arglabin or with vehicle for 13 weeks. Plasma cytokines, lipids, lipid subfractions, and autoantibodies against oxidized LDL (oxLDL) were determined. Heart and descending aortas were excised and fixed for immunohistochemical studies. Serial cryosections of the proximal aorta were stained, and the mean lesion area per animal was quantified. Staining of whole aorta (en face) and lesion quantifications were also determined. Statistical significance was calculated with either the Wilcoxon rank-sum test or, in the case of >2 comparisons, the Kruskal-Wallis test. To compare the specific pairs of outcome, the Wilcoxon rank-sum test combined with the Dunn-Sidak correction was used. Values of P<0.05 were considered significant.

All procedures involving animal handling and care were in accordance with the University of Pierre and Marie Curie Guidelines for Husbandry of Laboratory Mice.

**Results**

**Arglabin Inhibits the Production of IL-1β and IL-18 in Mouse Peritoneal Macrophages**

As shown in Figure 1B, arglabin inhibits IL-1β and IL-18 secretion in cultured mouse peritoneal macrophages in a...
concentration-dependent manner, with a maximum effect at \( \approx 50 \) nmol/L and EC\(_{50}\) values for both cytokines of \( \approx 10 \) nmol/L. The viability of cells was >95% during all the treatments (not shown). To rule out a general inhibition of MyD88/nuclear factor-kB activation by arglabin, levels of IL-6 and IL-12 were measured as a control in lipopolysaccharide/arglabin–treated macrophages. The result showed that arglabin does not significantly affect the expression levels of both cytokines (Figure 1B), indicating no general inhibition of nuclear factor-kB–dependent cytokines but rather of those depending on NLRP3 activation. To investigate the specificity of CCs as an activator of the NLRP3 inflammasome, we compared the levels of IL-1\( \beta \) and IL-18 in the supernatants of cultured peritoneal macrophages isolated from Nlrp3\(^{−/−}\), Nlrp3\(^{+/−}\), or Nlrp3\(^{−/+}\) mice, which were stimulated with lipopolysaccharide and CCs. As shown in Figure 1C, levels of both cytokines were decreased in Nlrp3\(^{−/−}\) by \( \approx 50\% \) versus control and almost completely abolished in Nlrp3\(^{−/+}\) cells. In addition, we have verified whether arglabin is a specific inhibitor of NLRP3 inflammasome or whether it could also inhibit other inflammasomes. Thus, we have stimulated cultured macrophages with anthrax, double-stranded DNA, or flagellin to specifically activate NLRP1, AIM2, and NLRC4, as reported earlier. The results did not show a significant effect of arglabin at 50 nmol/L on the activity of the 3 inflammasomes studied (not shown). Moreover, because CCs were reported to trigger IL-1\( \alpha \) production independently of NLRP3 and caspase-1,\(^{36}\) which has also been shown to promote atherosclerosis, we have measured the release of IL-1\( \alpha \) from mouse peritoneal macrophages primed with lipopolysaccharide and activated with CCs. The results indeed showed an increased IL-1\( \alpha \) secretion. In the presence of different doses of arglabin (10, 25, and 50 nmol/L), the levels of IL-1\( \alpha \) secreted in the supernatants were reduced (not shown). Finally, we stimulated the cells with ATP (5 nmol/L), a well- known potent and specific activator of NLRP3 inflammasome, and the results showed that macrophages increased IL-1\( \beta \) production and arglabin inhibited such an increase in a dose-dependent manner (Figure 1D). Nevertheless, in this study, we used CCs to activate NLRP3 inflammasome instead of ATP to mimic the activation of macrophages when they enter into arterial wall during atherosclerotic process.

Next, we investigated the reversibility of the effect of arglabin on IL-1\( \beta \) and IL-18 production. Peritoneal macrophages from mice of C57Bl/6 genetic background were first treated with lipopolysaccharide (10 ng/mL), and then arglabin (50 nmol/L) was added 1 hour before activation of the cells with CCs (1 mg/mL). The levels of IL-1\( \beta \) and IL-18 were evaluated by ELISA at different time periods. As shown in Figure 1 in the online-only Data Supplement, the maximum inhibition was observed 72 hours after exposure, with a gradual loss of the inhibitory effect thereafter.

**Arglabin Reduces IL-1\( \beta \) and Caspase-1 Production in Mouse Macrophages**

Cells were pretreated for 2 hours with lipopolysaccharide (10 ng/mL) and then treated with arglabin (50 nmol/L) or left untreated. One hour later, 1 mg/mL CCs were added to all samples, and cells were incubated for an additional 6 hours. Whole-cell lysates were analyzed for NLRP3, pro–IL-1\( \beta \), procaspase-1, and caspase-1 expression (Figure 2). The results showed complete abolition of mature caspase-1 in cells treated with arglabin. Concomitantly, arglabin reduced the expression of NLRP3 and pro–IL-1\( \beta \), which might indicate activation of autophagy, which has been shown to reduce the expression levels of both NLRP3 and pro–IL-1\( \beta \) by degradation.\(^{37}\) Supernatants of cells treated as above for 24 hours were analyzed by Western immunoblotting with the use of an antibody that recognizes the cleaved, active, 17-kDa form of IL-1\( \beta \). In agreement with ELISA data, the result indicated a reduction in the secretion of active IL-1\( \beta \) (Figure 2).

**Arglabin Induces Autophagy in Murine Macrophages**

Because it has recently been shown that atherosclerosis is characterized by impaired autophagy particularly in macrophages\(^{38}\) and because autophagy deficiency in macrophages promotes atherosclerosis in part by activation of the inflammasome,\(^{38}\) we have analyzed whether arglabin might induce autophagy in mouse macrophages. The results showed that, similar to the autophagy inducer amino acid–deficient...
media (Earl balanced salt solution sample) and bafilomycin A1, which induces autophagosome formation, arglabin strongly induced accumulation of LC3 type II protein in the absence of inflammasome activators (Figure 3A) but also in cells treated with lipopolysaccharide and CCs (Figure 3B). Additionally, arglabin induced clustering of LC3-II at autophagosomal membranes, which is characteristic for the autophagy, as evidenced by its punctuated pattern shown in confocal microscopic images of arglabin-treated macrophages (Figure 3C).

**Arglabin Reduces the Level of IL-1β and Increases the Level of IL-10 in the Plasma of ApoE<sub>2</sub>Ki Mice Fed an HFD**

Because arglabin inhibits the NLRP3 activation in vitro, we have further analyzed its activity in vivo in ApoE<sub>2</sub>Ki mice fed an HFD. The level of IL-1β was significantly reduced in the plasma of ApoE<sub>2</sub>Ki mice fed an HFD when treated twice daily with arglabin compared with untreated mice (11.7±1.1 versus 5.2±1.0 pg/mL; Figure 4). Unfortunately, measurement of plasma levels of IL-18 in these ApoE<sub>2</sub>Ki mice fed an HFD was not possible because of interference of the high levels of plasma lipids as claimed by the supplier. In contrast, the plasma levels of IL-10 increased significantly in arglabin-treated mice (12.1±0.3 versus 29.7±1.1 pg/mL), whereas the level of tumor necrosis factor-α remained basically unaffected (Figure 4).

**Arglabin Reduces the Plasma Levels of Total Cholesterol and Triglycerides in ApoE<sub>2</sub>Ki Mice Fed an HFD**

Female mice fed an HFD had plasma total cholesterol concentrations that were ≈3 times higher than those of mice fed a regular chow diet (Figure 5A). Intraperitoneal treatment of ApoE<sub>2</sub>Ki mice fed an HFD with arglabin significantly reduced the plasma level of total cholesterol by 59% (Figure 5A). Similarly, plasma triglyceride levels increased when ApoE<sub>2</sub>Ki mice were fed an HFD compared with mice fed a regular chow diet (194 versus 69 mg/dL, respectively; Figure 5B). Analogous to total cholesterol, the plasma level of triglycerides was significantly reduced in mice treated with arglabin (194 versus 82 mg/dL, respectively).

To identify the different lipoprotein fractions that were affected by the arglabin treatment, we used the LDL and high-density lipoprotein Quantimetrix Lipoprint Systems. The results show that all the different fractions of lipoprotein particles were affected by the treatment (Table I in the online-only Data Supplement). The decrease in plasma cholesterol and triglycerides cannot be explained by an increase in the LDL receptor expression because no difference was observed in control hepatic cells compared with hepatic cells from arglabin-treated mice (not shown). Likewise, we could not detect any difference in the hepatic steatosis of both groups of mice (not shown).
shown). In addition, by incubating the cells with $^{14}$C-acetate, we were not able to show a significant effect of arglabin on intracellular cholesterol biosynthesis (not shown). Finally, the relative distributions of cholesterol in the different lipoprotein fractions did not show any difference between treated and untreated mice (Table I in the online-only Data Supplement).

Because a direct link between circulating Ly-6C$^+$ monocytes and lesional macrophages was established earlier and because it has been reported that the Ly-6Chi monocyte subset increased dramatically in hypercholesterolemic apoE-deficient mice consuming an HFD, with the number of Ly-6Chi cells doubling in the blood every month,39 we explored by flow cytometry the Ly-6Chi and Ly-6Clo monocytes/macrophages in the spleens of ApoE$_2$.Ki mice fed an HFD either treated or untreated with arglabin. Our results clearly indicate a decreased number of Ly-6C$^+$ monocytes/macrophages and an increased number of Ly-6C$^+$ in arglabin-treated ApoE$_2$.Ki mice (Figure II in the online-only Data Supplement). However, a similar experiment conducted on ApoE$_2$.Ki/Nlrp3$^{-/-}$ mice fed an HFD and treated daily with vehicle or arglabin as described in the Methods section did not show any effect of arglabin on the number of splenic Ly6C$^+$ monocytes (not shown), confirming a direct link between Ly6C$^+$ phenotype and hypercholesterolemia. Indeed, because plasma total cholesterol did not decrease in ApoE$_2$.Ki/Nlrp3$^{-/-}$ mice fed an HFD and treated or not with arglabin, the splenic monocytes retained a proinflammatory phenotype characterized by Ly6C expression.39

Arglabin Reduces the Plasma Levels of Autoantibodies Directed Against OxLDL in ApoE$_2$.Ki Mice Fed an HFD

The titer of antibodies directed against oxLDL was found to be significantly increased in the plasma of mice fed an HFD compared with mice fed a chow diet (Figure 6). Treatment of ApoE$_2$.Ki mice fed an HFD with arglabin significantly reduced the plasma concentration of anti-oxLDL antibodies by $\approx 44\%$ compared with mice treated with vehicle (Figure 6). In contrast, levels of anti-oxLDL remained low in ApoE$_2$.Ki/Nlrp3$^{-/-}$ mice, even when fed an HFD (Figure 6). Not unexpectedly, arglabin did not affect those low levels of anti-oxLDL (Figure 6).

Arglabin Directs Lesional Macrophages of the Proinflammatory M1 Phenotype Into the Anti-Inflammatory M2 Phenotype in ApoE$_2$.Ki Mice Fed an HFD

Macrophages of control and arglabin-treated mice were analyzed with immunohistochemical analysis. The results show that arglabin strongly directed the proinflammatory M1
macrophage phenotype into the anti-inflammatory M2 phenotype (Figure 7).

Arglabin Reduces the Atherosclerotic Lesion Size in ApoE2.Ki Mice Fed an HFD

Consistent with the findings of Sullivan et al,34 we observed a significantly increased lesion size in HFD-challenged ApoE2.Ki mice compared with ApoE2.Ki mice fed a regular chow diet (not shown). Arglabin treatment of ApoE2.Ki mice fed an HFD significantly reduced lesion size in the aortic sinus (100% versus 54%; Figure 8B) and in whole aorta en face (100% versus 41%; Figure 8D). This inhibitory effect reduced the lesion size to the extent observed in ApoE2.Ki/Nlrp3−/− fed an HFD (Figure 8B and 8D). Treatment of ApoE2.Ki/Nlrp3−/− fed an HFD with arglabin did not further reduce the lesion area (not shown).

Discussion

Many clinical and experimental studies have reported a link between biologically active IL-1β and several sterile inflammatory diseases, including atherosclerosis.10,40 In addition, IL-18 has been shown to promote atherosclerosis in the ApoE−/− mouse model.3,41 The NLRP3 inflammasome complex regulates activation of caspase-1, which catalyzes the cleavage of the precursor cytokines pro–IL-1β and pro–IL-18 into their mature, active forms.41 In addition, several types of bone marrow–transplanted Ldlr−/− mice, which had Nlrp3−/−, Asc−/−, and Ila/b−/− bone marrow cells, showed a reduction in the atherosclerotic plaque size in mice deficient in inflammasome-related molecules, specifically in bone marrow cells.19 Moreover, it has recently been shown that the plaque size and macrophage infiltration were clearly inhibited in ApoE−/−Casp-1−/− double-deficient mice compared with ApoE−/− mice.12,13 In contrast, studies conducted by Menu et al42 showed that in ApoE−/−Nlrp3−/−, ApoE−/−Asc−/−, and ApoE−/−Casp−/− double-deficient mice, there are no significant differences in plaque size or macrophage infiltration compared with ApoE−/− mice. Despite this discrepancy, which could be attributed to the difference in the diet composition (0.15% cholesterol in the Usui et al13 study and 1.25% in the Menu et al42 study), the critical role of the NLRP3 inflammasome in atherosclerosis and in other inflammatory diseases such as type 2 diabetes mellitus and Alzheimer disease indicates the importance of this pathway for therapeutic targeting.

The major findings of this study are the following: (1) Nlrp3 deficiency significantly decreased the atherosclerotic lesions of the whole aortas and aortic sinuses in ApoE2.Ki mice fed an HFD; (2) the plant-derived compound arglabin inhibited the NLRP3 inflammasome activity and significantly reduced the production of IL-1β, IL-18, and IL-1α (not shown for IL-1α) in vitro and of IL-1β in vivo; (3) arglabin induces LC3-II formation but does not inhibit the final step of autophagy, the fusion of autophagosomes with lysosomes, and the generation of autolysosomes; (4) arglabin reduced total plasma cholesterol and triglyceride levels, as well as all lipoprotein subfractions, without affecting cellular cholesterol biosynthesis and hepatic LDL receptors expression; and (5) arglabin also reduced the plasma levels of antibodies against oxLDL and the number of M1 proinflammatory macrophages in spleen.
and arterial lesions in ApoE-Ki mice fed an HFD. In addition, arglabin increased the polarization of macrophages into the M2 anti-inflammatory phenotype in this mouse model.

Taken together, our results indicate that, by superimposing Nlrp3 deficiency onto ApoE-Ki mice, which are already susceptible to atherosclerosis, the loss of Nlrp3 reduced atherosclerotic lesion formation in mice fed an HFD. In addition, inhibition of the NLRP3 inflammasome activity by arglabin similarly reduced atherosclerotic lesions through a reduction in proinflammatory cytokines. Of note, IL-1α production, which was reported to be induced by CCs independently of NLRP3 and caspase-1, is also reduced by arglabin in a dose-dependent manner (not shown). This is not surprising because IL-1β can drive the release of IL-1α. Therefore, activation of autophagy by arglabin can inhibit IL-1α by at least these 2 mechanisms. Moreover, besides CCs, other more potent NLRP3 inflammasome activators such as ATP stimulated the production of IL-1β in cultured macrophages, and such stimulation was reduced by arglabin in a dose-dependent manner. We show that arglabin activates autophagy, which leads to increased degradation of NLRP3 and pro–IL-1β. Therefore, activation of NLRP3 by any activator is expected also to be inhibited by arglabin. Finally, inhibition of the NLRP3 inflammasome activity by arglabin reduced atherosclerotic lesions not only through a reduction in proinflammatory cytokines but unexpectedly also through a reduction in plasma total cholesterol and triglycerides. Arglabin has been reported to inhibit

Figure 8. Arglabin reduces atherosclerotic lesions in the aortic sinus and in the whole aortas (en face). At 6 weeks of age, female ApoE-Ki and ApoE-Ki/Nlrp3−/− mice were fed a high-fat diet. After 13 weeks, mice were euthanized, and hearts and proximal aortas were removed and fixed. Hearts were cut directly under and parallel to the leaflet, and the upper portions were embedded in optimal cutting temperature medium. A, One hundred sections 10 μm thick were prepared from the top of the left ventricle. The sections were stained for lipids with Oil Red O and counterstained with Harris hematoxylin. Ten sections of the 100, each separated by 90 μm, were used for specific morphometric evaluation of intimal lesions. B, The mean lesion size in these 10 sections was used to evaluate the lesion size in the aortic sinus of each animal. Data are shown as dot plots, with the line indicating the median; 100% represents the mean lesion area in control mice. The Kruskal-Wallis test (P=0.006), followed by the Wilcoxon rank-sum test with Dunn-Sidak correction for multigroup comparisons, was used for statistical analysis. C, Descending thoracic and abdominal aorta were cleaned, processed, and stained with Oil Red O to assess the extent of atherosclerosis with computer-assisted histomorphometry. D, The mean lesion area in whole aorta en face per animal was quantified; 100% represents the mean lesion area in control mice, which corresponds to ≈6% of the total surface aorta. The data are presented as dot plots, with the line indicating the median. Statistical analysis was performed with the Kruskal-Wallis test (P=0.001), followed by the Wilcoxon rank-sum test with Dunn-Sidak correction for multigroup comparisons, as indicated by the brackets.
farnesyltransferase, which could explain the finding of reduced cholesterol levels in arglabin-treated compared with untreated ApoE mice. Indeed, the inhibition of cholesterol synthesis by blocking enzymes downstream of 3β-hydroxy-3β-methylglutaryl coenzyme A reductase (HMGR) such as farnesyl diphosphate farnesyltransferase, which catalyzes the first committed step in the de novo cholesterol biosynthesis, has been suggested as a potentially good candidate to treat hypercholesterolemic patients. Such inhibitors decrease circulating LDL levels and consequently increase the expression of LDL receptors as reported for statins. Nevertheless, arglabin did not show any inhibitory effect on either intracellular cholesterol biosynthesis or LDL receptor expression in hepatocytes. This unexpected effect could be attributed to the fact that farnesyl diphosphate farnesyltransferase, unlike HMGR, is not the major regulatory enzyme of cholesterol synthesis and is less prone to feedback regulation. In addition, all the subfractions of lipoprotein particles (LDL, intermediate-density lipoprotein, and very-low-density lipoprotein) were reduced in the plasma of arglabin-treated mice. Therefore, these results are in favor of the hypothesis that arglabin might reduce plasma cholesterol and triglyceride levels through a blockage of its intestine absorption. Of note, in a recent publication, caspase-1 deficiency in mice was linked to intestinal lipid malabsorption. In fact, oral gavage of [1H] triglyceride-containing olive oil revealed that caspase-1 deficiency reduced triglyceride absorption and subsequent uptake of triglyceride-derived fatty acid in liver, muscle, and adipose tissue. Similarly, despite an elevated hepatic triglyceride content, caspase-1 deficiency reduced hepatic very-low-density lipoprotein and triglyceride production. Intestinal and hepatic gene expression analysis showed that caspase-1 deficiency did not affect fatty acid oxidation or fatty acid uptake but rather reduced intracellular fatty acid transport, thereby limiting lipid availability for the assembly and secretion of triglyceride-rich lipoproteins. These results reveal a novel function for caspase-1 or caspase-1-cleaved substrates in controlling intestinal triglyceride absorption and hepatic triglyceride secretion. Likewise, another recent study found that caspase-1-deficient mice have dramatically accelerated triglyceride clearance without an alteration in lipid production or absorption and a resultant decrease in steady-state circulating triglyceride and fatty acid levels. The authors also found that this effect is independent of IL-1 family signaling, supporting the concept that caspase-1 influences lipid metabolism through multiple mechanisms, not limited to cytokines. In this context, arglabin, through the inhibition of caspase-1, could affect either lipid absorption or lipid metabolism, which explains the result concerning the decrease in plasma total cholesterol and triglyceride in ApoE2.Ki fed an HFD.

On the other hand, caspase-1 deficiency is also known to lower the hepatic mRNA expression for genes associated with lipogenesis, even protecting mice from high fat-induced hepatic steatosis. In turn, IL-1 affects the expression of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase and cholesterol 7a-hydroxylase, rate-limiting enzymes for cholesterol biosynthesis and catabolism, respectively. As a result, IL-1 deficiency protected mice from hypercholesterolemia. Thus, arglabin might reduce serum cholesterol levels through the inhibition of caspase-1 activation and IL-1β maturation.

It is interesting to note that monocytes, as precursors of macrophages, display heterogeneity in mice and humans with 2 distinct subsets in which Ly-6C hi and Ly-6C lo mouse monocytes correspond to human CD14+ CD16− and CD14+CD16+ monocytes, respectively. Ly-6C lo cells selectively populate sites of experimentally induced inflammation. In contrast, Ly-6C hi can enter lymphoid and nonlymphoid tissues under homeostatic conditions. It has been reported that the Ly-6C lo monocyte subset increased dramatically in hypercholesterolemic apoE-deficient mice fed an HFD, with the number of Ly-6C lo cells doubling in the blood every month. Ly-6C hi monocytes adhered to activated endothelium, infiltrated lesions, and became lesional macrophages. On statin-induced cholesterol reduction, the number of Ly-6C hi decreased. In our study, we observed that in ApoE2.Ki mice fed an HFD and treated with arglabin, the number of spleen monocytes/macrophages of the Ly-6C lo phenotype dramatically decreased, whereas the number of Ly-6C hi increased, indicating that arglabin oriented monocytes/macrophages into an anti-inflammatory phenotype through the reduction in IL-1β levels or plasma lipid levels. However, experiments in ApoE2.Ki/Nlrp3−/− mice fed an HFD and daily treated with arglabin did not show any effect on the number of splenic Ly-6C hi monocytes, confirming a direct link between Ly-6C hi phenotype and hypercholesterolemia. Indeed, because plasma total cholesterol did not decrease in ApoE2.Ki/Nlrp3−/− mice fed an HFD and treated or not with arglabin, the splenic monocytes maintained a proinflammatory phenotype.

Conclusions
Our findings indicate that the NLRP3 inflammasome is an interesting target to treat atherosclerosis and that arglabin is a promising natural compound that not only reduced inflammation through NLRP3 inflammasome inhibition but also almost normalized plasma cholesterol and triglycerides in an ApoE2.Ki mice model.

Acknowledgments
The authors thank Felicitas Genze, Vimala Diderot, and Dr Nadir Benslimane, head of the transgenic animal facility at the IBPS, and his colleagues for their technical assistance and their professionalism.

Source of Funding
This work was supported in part by grant from the Nouvelle Société Francophone d’Athérosclérose (NSFA) to A. Abderrazak.

Disclosures
None.

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Inflammation is generally a key factor in the development of a variety of diseases, including atherosclerosis. The inflammasome is a multiprotein platform that activates procaspase-1 to caspase-1; in turn, caspase-1 can convert pro–interleukin (IL)-1β and pro–IL-18 into their bioactive secreted forms. Both IL-1β and IL-18 are major mediators of inflammation, and a number of laboratories have shown that both cytokines induce severe atherosclerotic lesions in mouse models. These findings suggest the involvement of the inflammasome in the development of atherosclerosis. NLRP3 is the most extensively studied inflammasome. It is activated in response to a variety of signals that are indicative of damage to the host, including tissue damage, metabolic stress, and infection. Cholesterol crystals are important inflammatory stimuli in atherosclerosis development acting as an endogenous danger signal via the NLRP3 inflammasome. In our study, we show that the natural NLRP3 inflammasome inhibitor arglabin reduces inflammation and plasma lipids, increases autophagy, and orients tissue macrophages into an anti-inflammatory phenotype in ApoE2.Ki mice fed a high-fat diet. Consequently, a marked reduction of atherosclerotic lesions was observed. Thus, arglabin may represent a promising new drug to treat inflammation and atherosclerosis.
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Circulation. 2015;131:1061-1070; originally published online January 22, 2015;
doi: 10.1161/CIRCULATIONAHA.114.013730

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/suppl/2015/01/22/CIRCULATIONAHA.114.013730.DC1

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SUPPLEMENTAL MATERIAL
Anti-inflammatory and anti-atherogenic effects of the inflammasome NLRP3 inhibitor, arglabin, in ApoE2.Ki mice fed a high fat diet

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Keywords: Inflammasome, arglabin, inflammation, cytokines, atherosclerosis.

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Arglabin purification

3H-Oxireno[8,8a]azuleno[4,5-b]furan-8(4aH)-one, 5,6,6a,7,9a,9b-hexahydro-1,4a-dimethyl-7-methylene-, (3aR,4aS,6aS,9aS,9bR)- (CAS 84692-91-1) (C_{15}H_{18}O_3) was purified to chemical homogeneity by reversed-phase high-performance liquid chromatography.$^{1,3}$ An Artemisia glabella raw extract was dissolved and applied onto a semipreparative Reprosil PUR ODS-3 column; it was subsequently eluted with methanol-water 60:80 (v/v) and the fractions containing pure arglabin were detected by a photodiode array detector at 210 and 250 nm. The purity of the isolated compound was checked by an analytical high performance liquid chromatography run on an analytical Reprosil PUR ODS-3 column with methanol-water as a mobile phase and by high performance thin layer chromatographic analyses (C_{18} and SiO_{2}). The purity of the isolated compound was >99.9%. Mass spectrometric analysis performed using a Finnigan MAT SSQ-7000 instrument in the chemical ionization mode confirmed the molecular formula C_{15}H_{18}O_3, through the presence of a peak at m/z 246 [M]+.

Experimental animals

At 6 weeks of age, female ApoE<sub>2</sub>Ki mice (C57Bl/6 background) were randomly divided into two groups. The first group was injected intraperitoneally with 5 µL of DMSO (vehicle), twice a day, during 13 weeks (control group); the second group was injected with arglabin (2.5 ng/g of body weight [b.w.]), twice a day, during 13 weeks (arglabin group). Of note, we used arglabin at 2.5 ng/g of b.w., because in pilot experiments this concentration exerted the maximum effect on IL-1β production in vivo (data not shown). These dosages of DMSO and arglabin have previously been shown to induce no overt toxicity in mice. All mice were fed for 13 weeks a High Fat Western type diet (HFD) (reference E15721-347; ssniff Spezialdiäten, GmbH, Soest, Germany). This HFD is constituted by crude protein 17.1%; crude fat 21.2%; crude fiber 5.0%; ash 4.5%; starch 14.5%; sugar 32.8%; vitamin A 18,000
IU/kg; vitamin D3 1,800 IU/kg; vitamin E 180 mg/kg; vitamin K3 24 mg/kg; vitamin C 1,236 mg/kg and copper 14 mg/kg. For blood collection, mice were food-deprived for 4 hours. After isoflurane anesthesia, blood was collected from the retro-orbital sinus into EDTA tubes, centrifuged and plasma aliquots were kept at -80°C. For flow cytometric experiment, blood was collected in heparin and kept at 4°C. At the end of the study, animals were killed by exsanguination, perfused transcardially with PBS and the hearts and proximal aortas were removed and fixed for lesion analyses. In parallel, similar studies were conducted on Nlrp3-/- mice, kindly provided by Prof. Jürg Tschopp (Lausanne University, Switzerland), which were cross-hybridized with ApoE2.Ki mice (ApoE2.Ki/Nlrp3KO) (n = 6 for each group). All procedures involving animal handling and their care were in accordance with the University of Pierre and Marie Curie Guidelines for Husbandry of Laboratory Mice.

**Plasma lipid quantification**

Plasma was separated by centrifugation at 630xg for 20 min at 4°C. Lipids were determined enzymatically using commercial kits for triglycerides (kit Randox), cholesterol (cholesterol RTU, Biomerieux). For lipid subfraction quantification, we used the Quantimetrix LDL and HDL Lipoprint- System (Quantimetrix Corporation, Redondo Beach, CA, USA) according to the manufacturer’s instructions. LDL was divided in seven subfractions. Subfraction 1 represents large LDL particles; subfraction 2 represents intermediate LDL particles, whereas subfractions 3–7 represent small dense LDL particles. IDL was divided in three subfractions. Subfraction 1 represents large IDL particles, subfraction 2 represents intermediate IDL particles and subfraction 3 represents small IDL particles. HDL was divided in 10 subfractions. Subfractions 1–3 represent the large HDL particles, subfractions 4–7 the intermediate HDL particles and subfractions 8–10 the small HDL particles. Of note, HDL and
LDL were measured directly. Non-HDL cholesterol (non-HDL C) was calculated as total cholesterol minus HDL cholesterol.

**Cytokine determinations**

Blood was collected into EDTA tubes from the retro-orbital sinus of DMSO- or arglabin-treated HFD-challenged ApoE2.Ki and ApoE2.Ki/Nlrp3−/− mice. Plasma samples were separated by centrifugation at 630xg for 20 min at 4°C and frozen in 0.5 mL aliquots at -80°C until tested. For quantification of the various biological parameters, we used the Millipore kit (reference MTH17MAG-47K-04) (Millipore, Billerica, MA, USA) according to the protocol described by the supplier. Briefly, 25 µL from each plasma sample were incubated with 25 µL of beads linked to specific antibodies. This mixture was incubated overnight at 4°C. Thereafter, 25 µL of biotinylated antibody was added for 1 h at room temperature. Finally, 25 µL of streptavidine-phycoerythrine were added for 30 min. Plates were washed twice and 150 µL of buffer and values were determined using a Luminex 200 Millipore apparatus and xPONENT 3.1 software (Millipore, Billerica, MA, USA).

**Evaluation of aortic lesions**

The circulatory system was perfused with 0.9% NaCl by cardiac intraventricular canalization. Hearts and proximal aortas were removed. Hearts were cut directly under and parallel to the leaflet, and the upper portions were embedded in OCT medium. One hundred sections of 10-µm thickness were prepared from the top of the left ventricle, where the aortic valves were first visible, up to a position in the aorta where the valve cusps were just disappearing from the field. The sections were stained for lipids with Oil-Red O and counterstained with Hematoxylin Harris. Ten sections out of the 100, each separated by 90 µm, were used for specific morphometric evaluation of intimal lesions. The intermediate sections were used for
immunohistochemical analyses. In addition, the entire aortic tree was removed and cleaned of adventitia, split longitudinally to the iliac bifurcation, and pinned flat on a dissection pan for analysis by en face preparation. Images were captured with a CAMIRIS video camera (Perfexsciences Toulouse), and the fraction covered by lesions was evaluated as a percentage of the total aortic area with ImageJ software.

**Immunohistochemistry**

Immunohistochemistry was performed in aortic sinus sections. Tissue sections were pre-treated with acetone-methanol (50/50 vol/vol) for 2 min and then rehydrated with PBS for 5 min. After washing with 0.1% Triton X-100 in PBS, sections were incubated with the appropriate antibody for 1 h and then washed three times. All antibodies were used at optimal dilutions. The following monoclonal antibodies were used to detect the presence of macrophages (rat anti mouse F4/80, Serotec, CD206, R&D Systems, and cleaved IL-1β goat polyclonal, Santa Cruz). The sections were counterstained with hematoxilin and digitally recorded with an Axiophot microscope (Carl Zeiss) and a Sony MC-3249 CCD camera.

**Determination of anti-oxLDL antibodies**

- **Lipoprotein isolation**

Human LDLs ($d=1.030-1.053$ g/mL) were isolated from freshly drawn blood from healthy normolipidemic volunteers as previously described, dialyzed against PBS supplemented with 0.01% EDTA to prevent oxidation, sterilized by filtration and stored at 4°C under nitrogen. The relative electrophoretic mobility of LDL was evaluated on Hydragel (Sebia, France).

- **Lipoprotein oxidation**

Prior to oxidation, EDTA was removed by extensive dialysis of the LDL solution against EDTA free PBS. Oxidation was initiated by incubation at 37°C with 5 μM CuSO₄ for 24 h.
Oxidation was stopped by adding 20 µM EDTA. Native and oxLDL were screened for lipopolysaccharide (LPS) contamination by using a limulus amoebocyte lysate assay (Sigma). All the different LDL preparations used in this study contained less than 0.75 IU of LPS/mL. LDL contained 20 ± 4 nmol peroxides/mg protein and 0.163 nmol TBARS (thiobarbituric acid-reactive substances)/mg protein, whereas oxLDL contained 215.2 ± 32.0 nmol peroxides/mg protein and 46 ± 4 nmol TBARS/mg protein.

Before each assay, 96-well microtitration plates were freshly coated with 100 µL of oxLDL (5 µg/mL) in PBS overnight at 4°C. The wells were blocked with 1% bovine serum albumin for 2 h at room temperature. A 100 µL aliquot of diluted sera (1:40) from each group of mice was added in quadruplicate wells and incubated for 2 h at room temperature. After three washes with PBS containing 0.1% Tween-20, a mixture of goat anti-mouse IgM and IgG conjugated with peroxydase (1:1000 dilution, Beckman-Coulter, Paris, France) was added to each well, and the incubation continued for 2 h at room temperature. Plates were washed again, and the alkaline peroxidase activity was determined using ortho-phenylenediamine dichloride (OPD, Sigma) as a substrate and detected at 492 nm.

Cell preparation

Murine peritoneal macrophages were isolated from the peritoneal cavity 4 days after mice were injected intraperitoneally with 4% thioglycollate (Sigma). Cells in serum-free RPMI-1640 medium were seeded in 6-well plates at a density of 0.5x10^6 cells/mL for 3 h. Medium was then removed and adherent cells were washed with PBS and were cultured overnight in RPMI-1640 containing glutamine (2 mM), penicillin (100 U/mL)/streptomycin (100 µg/mL) and 10% heat-inactivated foetal calf serum (FCS). The cells were washed and, serum-free RPMI-1640 medium containing glutamine and antibiotics, was added. Next, the cells were first primed with LPS (10 ng/mL) for 4 h and then treated with vehicle (DMSO) or with
araglin at different concentrations. One hour later, the cells were activated with either CC (1 mg/mL), ATP (5 mM, InvivoGen, France), double-stranded DNA (DS-DNA at 1.25, and 2.5 mg/mL, InvivoGen, France), anthrax (250 and 500 ng/mL, kindly provided by Dr P. Goossens, Pasteur Institute Paris, France) or flagellin (312 and 625 ng/mL, InvivoGen, France). The level of biologically active IL-1β and IL-18, IL-6, and IL-12 were evaluated 24 h later in the supernatant using an ELISA procedure (eBioscience, Paris, France) or Magnetic Luminex Screening Assay (Mouse Premixed Multi-Analyte, IL-1α, IL-6, IL12p70, R&D Systems).

**Autophagy study**

Cells treated for 24 h were analyzed by Western immunoblotting using antibody against LC3 type II, which recognizes primarily the autophagic LC3 type II form (#4108, Cell Signaling Technologies, Danvers, MA). Bafilomycin A1 (30 nM) was used as a positive control because it blocks the fusion of autophagosomes with lysosomes and, therefore, increases the autophagosomes and LC3-II formation. Actin served as loading control (antibody from Chemicon, Temecula, CA).

Cells were pretreated for 2 h with LPS (10 ng/mL), treated with araglin (50 nM) or were left untreated. One h later, CC (1 mg/mL) were added to all samples and cells were incubated for additional 6 h. Earl’s balanced salt solution (EBSS), known to induce autophagy, was used as positive control. To this sample, CC were added in EBSS (positive control). Whole cell lysates were analyzed for anti-LC3II formation (4108, Cell Signaling Technologies), NLRP3 expression (MAB7578 from R&D Systems), pro-IL-1β (3A6, Cell Signaling Technologies), caspase-1 (EPR4321, Abcam).

Activation of autophagy by araglin was also evidenced by formation of cellular autophagosome punctae containing LC3-II. Cells were incubated with araglin for 24 h,
permeabilized, stained with LC3-II antibody (Cell Signaling) and analyzed by confocal microscopy (LSM 710, Zeiss). Nuclei were stained with DAPI. Original magnification 630x.

Flow cytometric analysis

Spleen cells suspensions from each animal were obtained after dilacerations and ACK treatment to remove erythrocytes, and washed three times in PBS. Splenocytes were incubated 1 h on ice with PE-Cy5 coupled anti-CD4, anti-CD8 and anti-B220 antibodies (BD Bioscience, Franklin Lakes, NJ, USA) and FITC conjugated anti-CD11b and PE-conjugated anti Ly6C (Miltenyi Biotec, Germany). After incubation, cells were washed twice in PBS and 500 μL of PBS was added before analysis. At least, 25 000 live splenocytes were acquired on a BD LSRII instrument, and analyzed using FlowJo software (Tree Star’s, Ashland, OR, USA). CD11b/Ly6C expression was determined on CD4, CD8, and B220 negative cells populations.

Statistical analysis

Data are expressed as mean ± S.D or median as indicated. Statistical analyses were performed using either the Wilcoxon rank sum test or the Kruskal-Wallis test when >2 experimental conditions were compared. When the global Kruskall-Wallis test was significant, the specific pairs of outcome were compared by the Wilcoxon rank sum test combined with the Dunn-Sidak correction using MATLAB and the Statistics Toolbox Release 2014b (The MathWorks, Inc., Natick, MA); P<0.05 was considered significant.

Acknowledgments: The authors thank Felicitas Genze and Vimala Diderot for their technical assistance. This work was supported in part by grant from the Nouvelle Société Francophone d’Athérosclérose (NSFA) to A.A.
References


Supplemental results

Supplemental Figure 1. Kinetics of the arglabin effects on IL-1β and IL-18 secretion by murine peritoneal macrophages.

Peritoneal macrophages were isolated from C57BL/6 mice, seeded at a density of 0.5 x 10^6 cells/mL and primed with LPS (10 ng/mL) for 4 h then arglabin (50 nM) was added 1 h before the activation with CC (1 mg/mL). The levels of IL-1β and IL-18 were determined by ELISA at various periods of time. Data are presented as mean of % ± SD of two independent experiments performed in triplicate.
Supplemental Figure 2. Effect of arglabin on the phenotype of monocytes/macrophages in the spleen.

Treatment of ApoE2.Ki mice with arglabin (2.5 ng/g b.w. for 13 weeks) reduces the number of Ly-6C<sup>hi</sup> monocytes associated with hypercholesterolemia and increases the number of Ly-6C<sup>lo</sup>. In contrast, treatment of ApoE2.Ki/Nlrp3<sup>−/−</sup> mice with vehicle or arglabin did not induce any significant change in Ly-6C<sup>hi</sup> population (not shown). Representative plots of spleen leukocytes from untreated (control) or arglabin-treated mice stained with CD11b and Ly-6C, and analyzed by flow cytometry.
Table 1: The effects of Arglabin on different fractions of lipoprotein particles.

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<tr>
<td></td>
<td>Total (mg/mL)</td>
<td>% of Cholesterol</td>
</tr>
<tr>
<td>HDL</td>
<td>308.0 ± 67.5</td>
<td>42.8 ± 2.8</td>
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<tr>
<td>VLDL</td>
<td>179.7 ± 36.2</td>
<td>25.1 ± 2.5</td>
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<tr>
<td>LDL</td>
<td>405.7 ± 51.6</td>
<td>57.1 ± 2.8</td>
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