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Inhibition of the Inflammasome NLRP3 by Arglabin Attenuates Inflammation, Protects Pancreatic β-Cells from Apoptosis, and Prevents Type 2 Diabetes Mellitus Development in $ApoE_2Ki$ Mice on a Chronic High-Fat Diet

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ABSTRACT

Intraperitoneal injection of arglabin (2.5 ng/g of body weight, twice daily, 13 weeks) into female human apolipoprotein E_2 gene knock-in ($ApoE_2Ki$) mice fed a high-fat Western-type diet (HFD) reduced plasma levels of glucose and insulin by ~20.0% ± 3.5% and by 50.0% ± 2.0%, respectively, in comparison with vehicle-treated mice. Immunohistochemical analysis revealed the absence of active caspase-3 in islet sections from $ApoE_2Ki$ mice fed a HFD and treated with arglabin. In addition, arglabin reduced interleukin-1β (IL-1β) production in a concentration-dependent manner in Langerhans islets isolated from $ApoE_2Ki$ mice treated with lipopolysaccharide (LPS) and with cholesterol crystals. This inhibitory effect is specific for the inflammasome NOD-like receptor family, pyrin domain-containing 3 (NLRP3) because IL-1β production was abolished in Langerhans islets isolated from $Nlrp3^{-/-}$ mice. In the insulin-secreting INS-1 cells, arglabin inhibited, in a concentration-dependent manner, the maturation of pro-IL-1β into biologically active IL-1β probably through the inhibition of the maturation of procaspase-1 into active capsase-1. Moreover, arglabin reduced the susceptibility of INS-1 cells to apoptosis by increasing Bcl-2 levels. Similarly, autophagy activation by rapamycin decreased apoptosis susceptibility while autophagy inhibition by 3-methyladenin treatment promoted apoptosis. Arglabin further increased the expression of the autophagic markers Bcl2-interacting protein (Beclin-1) and microtubule-associated protein 1 light chain 3 II (LC3-II) in a concentration-dependent manner. Thus, arglabin reduces NLRP3-dependent inflammation as well as apoptosis in pancreatic β-cells in vivo and in the INS-1 cell line in vitro, whereas it increases autophagy in cultured INS-1 cells, indicating survival-promoting properties of the compound in these cells. Hence, arglabin may represent a new promising compound to treat inflammation and type 2 diabetes mellitus development.

Introduction

A body of evidence indicates that interleukin-1β (IL-1β) and IL-18, are involved in obesity-associated inflammation and insulin resistance (Tack et al., 2012). Thus, mice fed a high-fat Western-type diet (HFD) have increased IL-1β protein levels in adipose tissue in comparison with mice fed a low fat diet (Koenen et al., 2011) suggesting that adipose tissue-derived IL-1β is associated with obesity and insulin resistance. Moreover, plasma IL-18 has also been positively associated with increased risk of insulin resistance and type 2 diabetes mellitus (T2DM) (Ahmad et al., 2013; Lindegaard et al., 2013).

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) (Naik and Dixit, 2010). They are involved in innate immune recognition of pathogen-associated molecular patterns as well as intracellular and extracellular damage-associated molecular patterns. Several members of the NLR family, such as NOD-like receptor family, pyrin domain-containing 1, 3, and 4 (NLRP1, NLRP3, and NLRC4), have

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ABBREVIATIONS: ApoE, murine apolipoprotein E; $ApoE_2$, human $ApoE_2$ isoform; $ApoE_2Ki$, human $APOE_2$ gene knock in; ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; Beclin-1, Bcl2-interacting protein; BSA, bovine serum albumin; BW, body weight; CC, cholesterol crystals; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; HFD, high-fat Western-type diet; IL-1β, interleukin-1β; IL-18, interleukin-18; INS-1, insulin-secreting cell line; LC3, microtubule-associated protein 1 light chain 3; LDL, low-density lipoprotein; LPS, lipopolysaccharide; $Nlrp3^{-/-}$, Nlrp3-deficient mice; NLR, nucleotide-binding domain and leucine-rich repeat-containing receptor; NLRP3, NOD-like receptor family, pyrin domain-containing 3; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus.

been shown to assemble into large multiprotein complexes named inflammasomes, which activate procaspase-1 to caspase-1; the latter, in turn, can convert pro-IL-1β and pro-IL-18 into IL-1β and IL-18 forms (Chen and Nunez, 2011; Gross et al., 2011). Among inflammasomes, the NLRP3 inflammasome can be activated by a variety of molecules such as cholesterol crystals (CC) (Duewell et al., 2010).

It is well known that murine apolipoprotein E (ApoE) can bind low-density lipoprotein (LDL) receptor family proteins and mediate clearance of triglyceride-rich lipoproteins from circulation (Mahley et al., 2009). $ApoE_2$ is defective in binding to the LDL receptor (Mahley et al., 2009), and almost all heterozygous and homozygous ε2 carriers have elevated triglyceride levels due to impaired hepatic clearance of triglyceride-rich lipoproteins (Hui et al., 1984; Mahley et al., 2009).

Several pieces of evidence indicate that high plasma triglyceride levels promote systemic inflammation (Ceriello et al., 2004; Harbis et al., 2004; Alipour et al., 2008), which increases the risk of metabolic diseases (Harbis et al., 2004). In addition, after being fed a lipid-rich meal, C57Bl/6 mice, in which the endogenous APOE mouse gene has been replaced by the human APOE2 gene ($ApoE_2$Ki mice), display elevated fasting plasma lipid and insulin levels and prolonged postprandial hyperlipidemia accompanied by increased granulocyte number and inflammation (Kuhel et al., 2013). $ApoE_2$Ki mice also showed increased adiposity when maintained on a Western-type high-fat, high-cholesterol diet (HFD) (Kuhel et al., 2013). Moreover, adipose tissue dysfunction with increased macrophage infiltration and inflammation corresponded with hyperinsulinemia observed in $ApoE_2$Ki mice after being fed the HFD (Kuhel et al., 2013).

Arglabin, mainly synthesized by *Artemisia glabella,* shows anti-inflammatory and antitumor activity, yet only the latter is being explored in Kazakhstan in form of a rather unstable dimethylamino hydrochloride derivative for the treatment of breast, colon, ovarian, and lung cancer (Shaikenov and Adekenov, 1997; Abil'daeva et al., 2004). Previous studies from our laboratory with lipopolysaccharide (LPS)-primed macrophages exposed to CC provided evidence for inhibition of the NLRP3 inflammasome by arglabin (Abderrazak et al., 2015). Therefore, we tested, whether arglabin might protect pancreatic β-cells against apoptosis and T2DM development in $ApoE_2$Ki mice fed a HFD.

Materials and Methods

Arglabin Isolation and Purification

Arglabin (CAS 84692-91-1) ($C_{15}H_{18}O_3$), 3*H*-oxireno[8,8a]azuleno [4,5-*b*]furan-8(4a*H*)-one,5,6,6a,7,9a,9b-hexahydro-1,4a-dimethyl-7-methylene-(3a*R*,4a*S*,6a*S*,9a*S*,9b*R*), was purified to chemical homogeneity by reverse-phase high-performance liquid chromatography (Büchele and Simmet, 2003; Büchele et al., 2003; Morad et al., 2011). An *Artemisia glabella* raw extract was dissolved and applied onto a semipreparative Reprosil PUR ODS-3 column (Dr.Maisch GmbH, Ammerbuch-Entringen, Germany); it was subsequently eluted with methanol-water, 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 analytic high-performance liquid chromatography on a Reprosil PUR ODS-3 column with methanol-water as a mobile phase and by high-performance thin-layer chromatographic analysis (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 (Thermo Fisher Scientific, Waltham, MA) 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]+.

Cell Culture

Rat Insulinoma INS-1 Cells. Rat insulinoma INS-1 cells were cultured in RPMI 1640 medium buffered with 10 mM HEPES containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 100 units/ml penicillin/streptomycin (all from Invitrogen, Paris, France). Cells were cultured in six-well plates until reaching 80% confluence. The cells were washed, and serum-free RPMI-1640 medium containing glutamine and antibiotics was added. The cells were first treated with LPS (10 ng/ml) (Sigma, Paris, France) for 4 hours, then incubated with vehicle (dimethylsulfoxide [DMSO]) or with arglabin at different concentrations. One hour later, the cells were activated with CC (1 mg/ml), and the level of biologically active IL-1β was evaluated 24 hours later in the supernatants using an enzyme-linked immunosorbent assay (ELISA) procedure (eBioscience, Paris, France).

Isolation of Langerhans Islets. $ApoE_2Ki/Nlrp3^{+/+}$ mice or $ApoE_2Ki/Nlrp3^{-/-}$ mice were injected into the bile duct with 4 ml of a collagenase solution at 1 mg/ml (Sigma-Aldrich, St. Louis, MO). After pancreatic digestion at 37°C for 20 minutes and washing with cold Hanks' balanced salt solution, the Langerhans islets were collected and counted microscopically.

Experimental Animals and Diet

The $ApoE_2$Ki mice used in this study were genetically engineered from C57Bl/6 mice in which the ApoE gene was replaced by the human $ApoE_2$ isoform. At 6 weeks of age, female $ApoE_2$Ki mice were randomly divided into two groups. The first group was treated intraperitoneally with either 5 μl of diluted DMSO (1/100) (vehicle, control group, $n = 6$) or arglabin (2.5 ng/g of body weight [BW]), twice a day, during 13 weeks (arglabin group, $n = 6$). Of note, we used arglabin at 2.5 ng/g of BW because in pilot experiments this concentration exerted the maximum effect on IL-1β production in vivo (data not shown). These doses of DMSO and arglabin have previously been shown to induce no overt toxicity in mice.

All mice were fed for 13 weeks a HFD (reference E15721-347; ssniff Spezialdiäten, GmbH, Germany). This HFD is composed of 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 1800 IU/kg, vitamin E 180 mg/kg, vitamin K3 24 mg/kg, vitamin C 1236 mg/kg, and copper 14 mg/kg. For weight measurement, age-matched $ApoE_2$Ki and $ApoE_2Ki/Nlrp3^{-/-}$ mice fed either a chow diet or HFD and treated or untreated with arglabin were housed according to their genotype with one mouse per cage. Food consumption was monitored daily. No difference in the average amounts of food consumed per animal was observed.

After isoflurane anesthesia, blood samples were collected from the retro-orbital sinus in EDTA tubes then centrifuged; plasma aliquots were kept at −80°C. At the end of the study, animals were killed by exsanguination, and pancreatic tissues were removed and fixed for apoptosis analyses.

In parallel, similar studies were conducted on C57Bl/6/Nlrp3-deficient mice ($Nlrp3^{-/-}$), kindly provided by Prof. Jürg Tschopp (Lausanne University, Switzerland), which were cross-hybridized with $ApoE_2$Ki mice ($ApoE_2Ki/Nlrp3^{-/-}$) ($n = 6$ 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, Insulin, and Blood Glucose Levels

Lipids. Plasma was separated by centrifugation at 630*g* for 20 minutes at 4°C. Lipids were determined enzymatically using commercial kits for triglycerides (Randox Laboratories, Crumlin, United

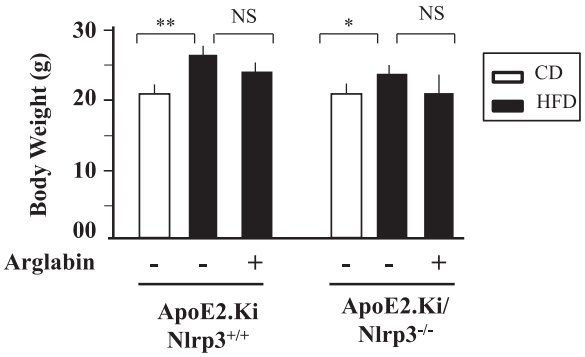

Fig. 1. Effects of arglabin on BW of ApoE$_2$Ki/Nlrp3$^{+/+}$ and ApoE$_2$Ki/Nlrp3$^{-/-}$ mice fed a chow diet or HFD. At 6 weeks of age, female ApoE$_2$Ki/Nlrp3$^{+/+}$ and ApoE$_2$Ki/Nlrp3$^{-/-}$ mice were randomly divided into three groups. One group of each mouse strain (n = 6) was fed a regular chow diet (CD) and the two others were fed a HFD for 13 weeks. Two groups (one of each strain) fed a HFD were treated twice daily with arglabin (2.5 ng/g BW) for 13 weeks. The two others groups (one of each strain) were treated with vehicle. After isoflurane anesthesia, the BW of each mouse was determined. *$P < 0.05$; **$P < 0.01$; NS, not statistically significant.

Kingdom) and cholesterol (cholesterol RTU; bioMérieux, Marcy l'Etoile, France).

Insulin. Plasma insulin levels were measured with a rat insulin ELISA kit (Crystal Chem, Chicago, IL).

Glucose. Blood was collected after an overnight fast. Plasma glucose levels were measured with an automatic glucometer (Accu-chek; Roche, Burgess Hill, United Kingdom).

Immunohistochemistry

Pancreatic tissues were removed, fixed in 10% buffered formalin and frozen for apoptosis studies. Frozen serial sections (10 μm) of pancreatic tissues were treated with 0.3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase activity, followed by blocking in 4% bovine serum albumin (BSA; Sigma). Slides were incubated (2 hours at 20°C) with purified rabbit polyclonal antibody specific to mouse active caspase-3 (ab2302; Abcam, Cambridge, MA) at a 1:20 dilution. A horseradish peroxidase–conjugated goat polyclonal was used as the secondary antibody.

Western Blotting Analyses

The INS-1 cells, washed and incubated in serum-free media, were first treated with LPS (10 ng/ml) for 4 hours, then were treated with vehicle (DMSO) or arglabin at different concentrations. One hour later, the cells were stimulated with CC (1 mg/ml) for priming and activating the cleavage process of pro-IL-1β. The cells were lysed in Tris buffer containing a mixture of protease inhibitors (Sigma). Protein concentrations were measured by Peterson's method with BSA as standard.

The samples were denatured with SDS-loading buffer and subjected to SDS-PAGE (Invitrogen SARL, Cergy-Pontoise, France). The samples were transferred to nitrocellulose membranes and blocked with 5% nonfat dried milk. The blots were incubated with monoclonal rabbit anti-rat Bcl2-interacting protein (Beclin-1) antibody, rabbit anti-rat microtubule-associated protein 1 light chain 3 (LC3) antibody, rabbit anti-Bax antibody, or rabbit anti-rat Bcl2 antibody. All antibodies were from Cell Signaling Technology (Beverly, MA) and were used at a dilution of 1:1000. After washing, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:500 dilution; Bio-Rad Laboratories, Hercules, CA). Rat anti-actin (1:1000; Santa Cruz Biotechnology, Dallas, TX) served as loading control. The blots were visualized with a chemiluminescence kit (Amersham Biosciences, Little Chalfont, United Kingdom).

Statistical Analysis

Data were expressed as mean ± S.D. Statistical analyses were performed using Student's t test, Welch corrected. Differences at $P < 0.05$ were considered statistically significant.

Results

This study was designed to evaluate the impact of the inhibition of inflammasome NLRP3 by the natural product, arglabin, on inflammatory response and T2DM in ApoE2Ki mice fed a HFD. In a previous study we showed that arglabin inhibits the NLRP3 activation in mouse macrophages with EC_{50} values of ~10 nM. Intraperitoneal injection of arglabin into ApoE$_2$Ki mice fed a HFD resulted in a decrease of IL-1β, total cholesterol, and triglycerides plasma levels (Abderrazak et al., 2015). In this study we extended our study on the effect of arglabin on cultured Langerhans islets and INS-1 cells as well as its effect on ApoE$_2$Ki mice fed a HFD.

Arglabin Slightly Reduced Diet-Induced Body Weight in ApoE$_2$Ki. Female ApoE$_2$Ki mice were placed on a HFD at 6 weeks of age and treated either with arglabin or with vehicle twice daily, and BW was determined after

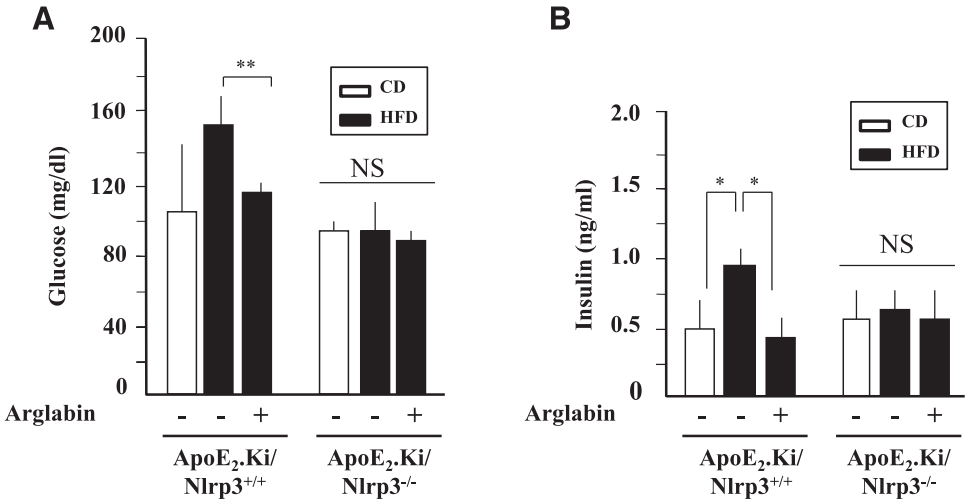

Fig. 2. Effects of arglabin on plasma glucose and insulin levels of ApoE$_2$Ki/Nlrp3$^{+/+}$ and ApoE$_2$Ki/Nlrp3$^{-/-}$ mice. At 6 weeks of age, female ApoE$_2$Ki/Nlrp3$^{+/+}$ and ApoE$_2$Ki/Nlrp3$^{-/-}$ mice were randomly divided into six groups. Two groups of each mouse strain (n = 6) were fed a regular chow diet (CD) and the other four were fed a HFD for 13 weeks. Among these four groups, two groups (one of each strain) were treated twice daily with arglabin (2.5 ng/g BW) for 13 weeks, and the other two groups were treated twice daily with vehicle. After isoflurane anesthesia, blood was collected in EDTA tubes, and plasma glucose (A) and insulin (B) were determined enzymatically or by using ELISA, respectively. Data are presented as the mean ± S.D. of one experiment performed in duplicate *$P < 0.05$; **$P < 0.01$; NS, not statistically significant.

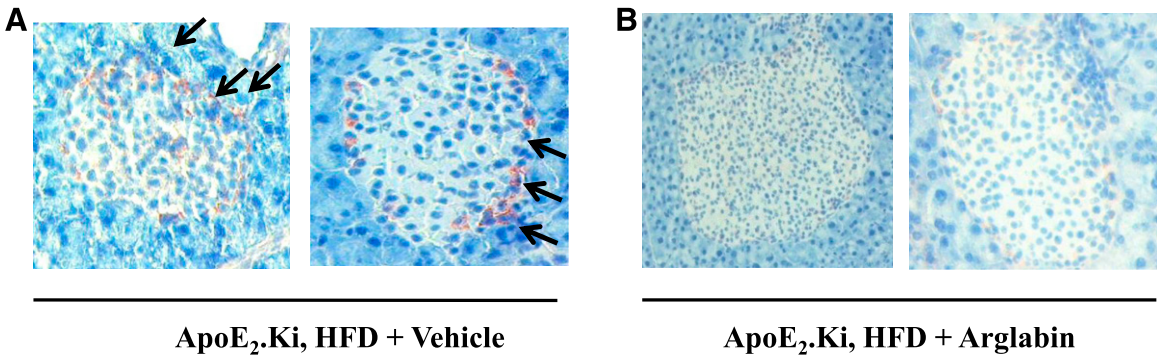

Fig. 3. Arglabin counteracts apoptosis in Langerhans islets. Frozen serial sections (10 μm) of pancreas were treated with 0.3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase activity, followed by blocking in 4% BSA. The slides were incubated for 2 hours at 20°C with purified rabbit polyclonal antibody specific to mouse-active caspase-3. Horseradish peroxidase–conjugated goat polyclonal was used as secondary antibody. Sections were counterstained with hematoxylin (blue). Active caspase-3 (red) is indicated by arrows. Representative sections of $ApoE_2Ki$ mice fed a HFD and treated either with (A) vehicle or (B) arglabin (2.5 ng/g BW) for 13 weeks as specified in Fig. 2.

13 weeks. Consistent with previous findings, mean BW of $ApoE_2Ki$ mice fed a HFD was significantly increased compared with $ApoE_2Ki$ mice fed a regular chow diet (27 ± 2 g versus 22 ± 2 g; $P < 0.01$) (Fig. 1). Compared with vehicle-treated animals, arglabin treatment slightly reduced the BW of HFD-fed $ApoE_2Ki$ mice (24 ± 3 g versus 27 ± 2 g; not significant) (Fig. 1). Likewise, in $ApoE_2Ki/Nlrp3^{-/-}$ mice, HFD-fed animals exhibited an increased BW compared with those fed a regular chow diet (24 ± 2 g versus 21 ± 1 g; $P < 0.05$) (Fig. 1). HFD-fed $ApoE_2Ki/Nlrp3^{-/-}$ mice treated with arglabin displayed approximately the same BW as vehicle-treated animals (Fig. 1).

Arglabin Reduces the Plasma Levels of Glucose and Insulin in $ApoE_2Ki$ Mice Fed a HFD. The plasma levels of glucose and insulin were increased in $ApoE_2Ki$ mice fed a HFD compared with those fed regular chow. Arglabin treatment reduced the glucose and insulin levels of the HFD mice by 20.0% ± 3.5% and by 50.0% ± 2.0%, respectively: 6.0 ± 0.2 versus 7.5 ± 0.6 mM for glucose ($P < 0.01$, Fig. 2A) and 0.7 ± 0.28 ng/ml versus 1.0 ± 0.4 ng/ml for insulin ($P < 0.05$, Fig. 2B). In $ApoE_2Ki/Nlrp3^{-/-}$ mice, either HFD-fed or not, plasma glucose and insulin levels remained low (Fig. 2, A and B). Arglabin treatment did not further affect those parameters in these animals (Fig. 2, A and B).

Arglabin Inhibits Caspase-3 Activation in Islets of Langerhans of $ApoE_2Ki$ Mice Fed a HFD. Because elevated systemic IL-1β has been reported to affect Langerhans cell viability, we investigated the expression of active caspase-3 in Langerhans islets of arglabin-treated or vehicle-treated $ApoE_2Ki$ mice fed a HFD. Immunohistochemical analysis of tissue sections showed clearly the presence of active caspase-3 in mice treated with vehicle (Fig. 3A), whereas no such staining was found in mice that had been treated with arglabin (Fig. 3B).

Arglabin Inhibits the Production of IL-1β in Murine Langerhans Islets. As shown in Fig. 4A, arglabin inhibits IL-1β secretion in cultured mouse Langerhans islets in a concentration-dependent manner. The viability of cells was >95% during all the treatments (not shown). To investigate the specificity of CC as activator for the inflammasome NLRP3, we compared the levels of IL-1β in the supernatants of cultured Langerhans islets isolated from $Nlrp3^{+/+}$ or $Nlrp3^{-/-}$ mice, which were stimulated with LPS and CC. As shown in Fig. 4B, levels of IL-1β were completely abolished in $Nlrp3^{-/-}$ cells.

Arglabin Inhibits the Maturation of pro-IL-1β into IL-1β in INS-1 Cells. As shown in Fig. 5, arglabin inhibits IL-1β production in cultured INS-1 cells in a concentration-dependent manner. Using Western immunoblot analysis, we

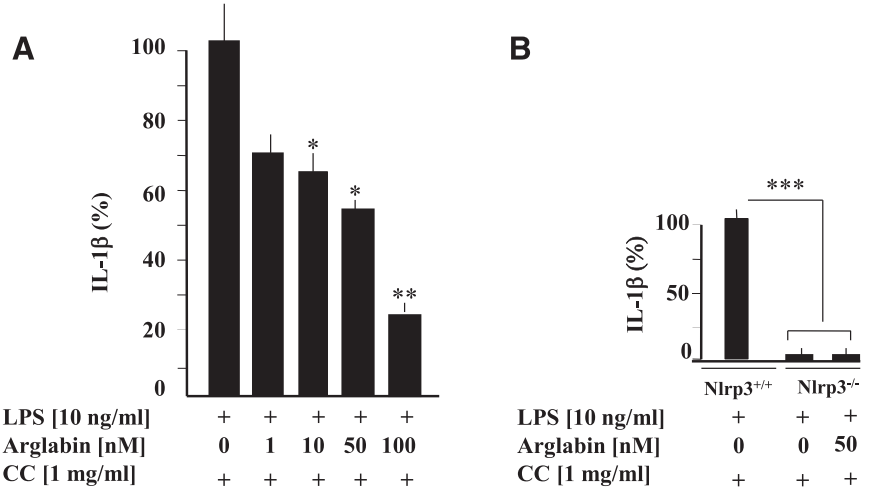

Fig. 4. Arglabin reduces IL-1β production in Langerhans islets. Langerhans islets were isolated from $ApoE_2Ki/Nlrp3^{+/+}$ as indicated in the *Materials and Methods* section. (A) Langerhans islets were pretreated with LPS (10 ng/ml) for 4 hours, then arglabin was added at final concentrations of 0, 1, 10, 50, or 100 nM. After 1 hour, the islets were activated with CC (1 mg/ml) for 24 hours. IL-1β levels in the supernatants were determined by ELISA. (B) Langerhans islets were isolated from $ApoE_2Ki/Nlrp3^{-/-}$ and treated with LPS/CC as above, and arglabin was added or not at 50 nM. IL-1β levels in the supernatants were determined by ELISA and compared with those obtained from Langerhans islets isolated from $ApoE_2Ki/Nlrp3^{+/+}$ and activated with LPS/CC. Data are mean ± S.D., $n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

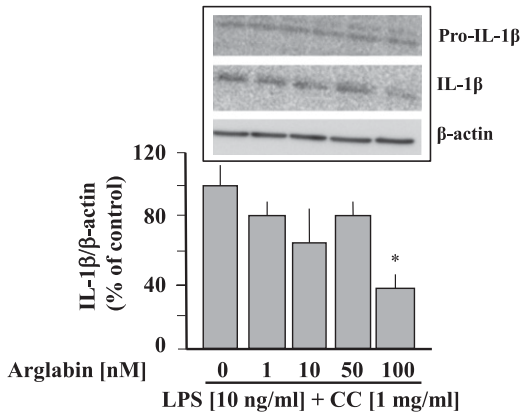

Fig. 5. Arglabin inhibits the maturation of pro-IL-1β into IL-1β in INS-1 cells. INS-1 cells were pretreated with LPS (10 ng/ml) for 4 hours, then arglabin was added at final concentrations of 0, 1, 10, 50, or 100 nM. After 1 hour, the cells were activated with CC (1 mg/ml) for 24 hours. Pro-IL-1β and IL-1β levels in cell lysates were determined by Western immunoblotting using specific antibodies. Actin immunoblotting was performed for normalization. Western blots were quantified densitometrically and the results for IL-1β are presented as a graph. Data are mean ± S.D., $n = 3$; $^*P < 0.05$.

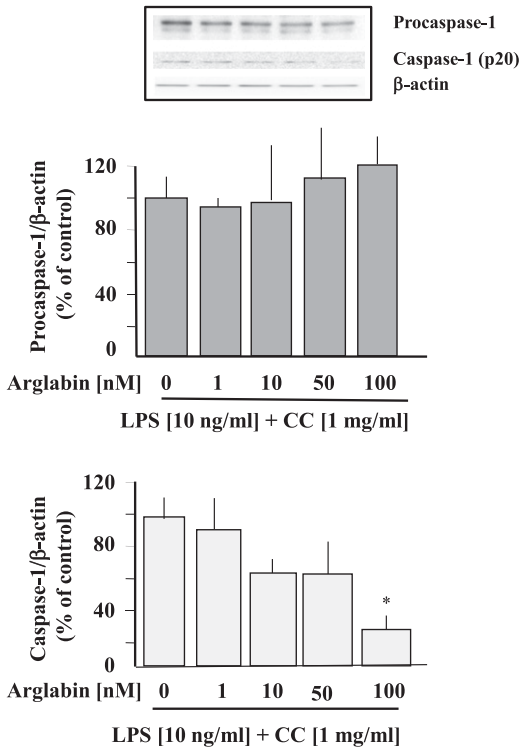

Fig. 6. Arglabin inhibits the maturation of procaspase-1 into caspase-1 in INS-1 cells. INS-1 cells were pretreated with LPS (10 ng/ml) for 4 hours, then arglabin was added at final concentrations of 0, 1, 10, 50, or 100 nM. After 1 hour, the cells were activated with CC (1 mg/ml) for 24 hours. Procaspase-1 and caspase-1 levels were determined in cell lysates by Western immunoblotting using specific antibodies. Actin immunoblotting was performed for normalization. Western blots were quantified densitometrically, and the results are presented as a graph. Data are mean ± S.D., $n = 2$; $^*P < 0.05$.

show that this inhibition resulted from the inhibition of the maturation of pro-IL-1β into IL-1β.

Arglabin Inhibits the Maturation of Procaspase-1 into Caspase-1 in INS-1 Cells. As shown in Fig. 6, arglabin inhibits the maturation of biologic active caspase-1 in cultured INS-1 cells in a concentration-dependent manner.

Arglabin Increases the Expression of Antiapoptotic Bcl-2 and Reduces the Expression of Proapoptotic Bax in Cultured INS-1 Cells. We analyzed the role of arglabin on apoptosis-relevant proteins in cultured INS-1 cells. After LPS (10 ng/ml) treatment of 4 hours, the cells were treated with arglabin (50 nM) or with vehicle. One 1 hour later, we activated the INS-1 cells with CC (1 mg/ml), and the protein levels of Bax and Bcl-2 were determined using Western immunoblot analysis. The results showed a slight but not statistically significant increase in the protein levels of Bax after normalization with β-actin (Fig. 7). In contrast, an increase of the antiapoptotic Bcl-2 level was observed after treatment with arglabin (Fig. 7). As a consequence, the Bax/Bcl-2 ratio was decreased (1.0 ± 0.07 in controls, 0.62 ± 0.13 for 50 nM of arglabin) (Fig. 7).

A decreased ratio of Bax/Bcl-2 is consistent with a decreased susceptibility to apoptosis. Treatment of INS-1 cells with 3-methyladenine, an autophagy inhibitor, before the treatment with arglabin showed a significant increase in the Bax/Bcl-2 ratio (1.77 ± 0.55; $P < 0.01$) (Fig. 7). Supplementation of arglabin counteracted the inhibitory effect due to 3-methyladenin treatment and antagonized apoptosis by increasing Bcl-2 expression (0.89 ± 0.12; $P < 0.05$). In contrast, autophagy activation using rapamycin decreased apoptosis because the ratio of Bax/Bcl-2 significantly decreased (0.50 ± 0.21; $P < 0.05$). Moreover, addition of arglabin slightly enhanced this phenomenon.

Arglabin Increases Autophagy in Cultured INS-1 Cells. T2DM is characterized by insulin resistance, a decreased pancreatic β-cell mass, and failure of β-cells to secrete adequate amounts of insulin (Kitamura, 2013). Recent studies have shown that autophagy, which is involved in both cell survival and cell death, is not only crucial for T2DM-associated inflammation but is also essential for proper insulin secretion, β-cell survival, and insulin sensitivity (Marselli et al., 2013). In fact, transgenic mice lacking autophagy in their β-cells exhibit decreased β-cell mass and an aberrant response to glucose stimulation. Moreover, inhibition of autophagy increases the vulnerability of β-cells to cytotoxic effects (Yin et al., 2012). It has therefore been suggested that autophagy is essential to maintain the structure, mass, and function of β-cells. To that effect, dysregulated autophagy in β-cells has been proposed as a precipitating factor in the progression from obesity to diabetes due to inappropriate response to obesity-induced endoplasmic reticulum stress (Quan et al., 2012).

With this background, we examined whether arglabin treatment might affect autophagy in the pancreatic β-cell line INS-1. To address this question, we examined the protein levels of Beclin-1 and LC3, which had previously been shown to promote autophagy (Rami et al., 2008), using LPS and CC-preactivated INS-1 cells. Our results indicate a concentration-dependent increase of Beclin-1 and LC3-II protein levels in INS-1 cells treated with arglabin (Fig. 8) indicating autophagy induction.

Discussion

Chronic inflammation has long been associated with metabolic disorders such as T2DM. Inflammatory cytokines such as

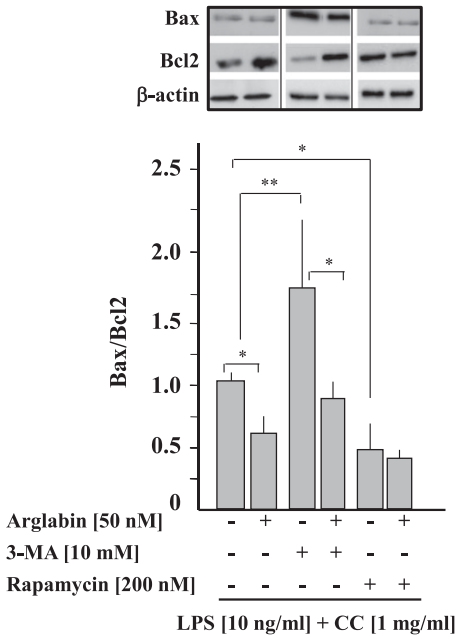

Fig. 7. Arglabin reduces the susceptibility of cultured INS-1 cells to apoptosis. Cells were pretreated with LPS (10 ng/ml) for 4 hours, then arglabin was added at final concentrations of 0 or 50 nM. After 1 hour, the cells were activated with CC (1 mg/ml) for 24 hours. Bax and Bcl2 expression were evaluated by Western immunoblotting using specific antibodies. To verify whether the effect of arglabin was mediated by autophagy, we inhibited autophagy with 3-methyladenin (3-MA, 10 mM) or activated it with rapamycin (200 nM) and determined its effect on Bax/Bcl-2 ratio in the presence or absence of arglabin. Actin immunoblotting was performed for normalization. Western blots were quantified densitometrically and are presented as a graph. Data are mean ± S.D., $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$.

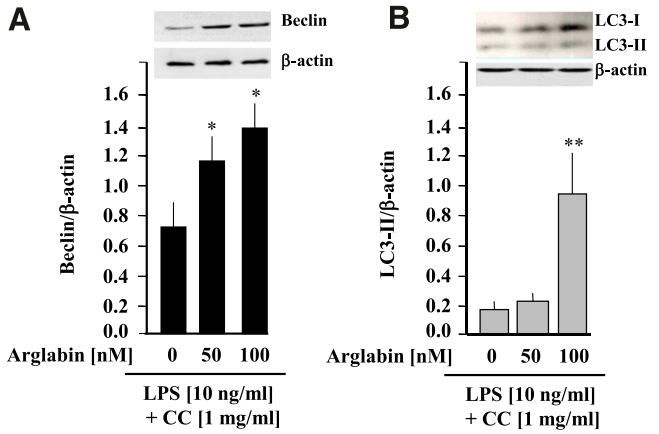

Fig. 8. Arglabin induces autophagy in INS-1 cells. (A) Cells were pretreated with LPS (10 ng/ml) for 4 hours, then arglabin was added at concentrations of 0, 50, or 100 nM. After 1 hour, the cells were activated with CC (1 mg/ml) for 24 hours. Beclin-1 expression was evaluated by Western immunoblotting using specific antibodies. Actin immunoblotting was performed for normalization. (B) Cells were treated as in (A). Whole cell lysates were analyzed for LC3 expression. Western blots were quantified densitometrically and normalized to actin, and are presented as a graph. Data are mean ± S.D., $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$.

IL-1β and tumor necrosis factor-α have been implicated in the development of several metabolic disorders. Because inflammasome has a central role in the generation of active IL-1β, several laboratories have studied the role of inflammasomes in metabolic disorders.

In mice lacking components of the inflammasome such as NLRP3, caspase-1, or ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain), glucose tolerance and insulin sensitivity were improved in spite of a HFD (Zhou et al., 2010). In addition, a direct association between NLRP3 activation and the development of insulin resistance has been reported (Vandanmagsar et al., 2011; Wen et al., 2011). Moreover, activation of inflammasomes in macrophages has been reported to induce insulin resistance in other cell types such as hepatocytes or T cells (Vandanmagsar et al., 2011; Wen et al., 2011). Therefore, identification of specific inhibitors of inflammasomes might be of great importance for the treatment of metabolic disorders such as T2DM.

We have recently shown that arglabin inhibits specifically the NLRP3 inflammasome without affecting the activity of NLRP1, NLRPC4, or AIM2 inflammasomes (Abderrazak et al., 2015). Indeed, arglabin reduced in a concentration- and dose-dependent manner the production of IL-1β and IL-18 in murine peritoneal macrophages and in plasma of $ApoE_2Ki$ mice fed a HFD. We have also reported that arglabin oriented the polarization of tissue macrophages toward the M2 anti-inflammatory phenotype. In addition, mice fed a HFD have total plasma cholesterol and triglycerides concentrations approximately 3 times greater than those of mice fed a regular chow diet. We observed that the treatment of mice by arglabin significantly reduces total plasma cholesterol and triglycerides in $ApoE_2Ki$ mice fed a HFD, probably by inhibition of intestinal lipid absorption.

In our present study, we extended our investigation of the effect of arglabin on β-cell protection against apoptosis and on glucose and insulin normalization in $ApoE_2Ki$ mice fed a HFD. It is interesting to note that $ApoE_2Ki$ mice, when maintained on a Western-type high-fat, high-cholesterol diet, exhibit elevated fasting plasma lipid and insulin levels. In addition, they display prolonged postprandial hyperlipidemia, which is accompanied by increased granulocyte number and inflammation as well as adiposity (Kuhel et al., 2013). Moreover, adipose tissue dysfunction with increased macrophage infiltration, abundant crown-like structures, and inflammation correspond to hyperinsulinemia observed in these mice (Kuhel et al., 2013). These data indicate that chronic high-fat feeding of $ApoE_2Ki$ mice accentuates inflammation and leads to T2DM development.

Arglabin reduced significantly the plasma glucose and insulin levels that were induced by HFD in $ApoE_2Ki$ mice. Interestingly, in NLRP3-deficient $ApoE_2Ki$ mice we found, similar to arglabin treatment, reduced glucose and insulin plasma levels, even when the mice were fed a HFD. Both arglabin and NLRP3 deficiency tended to reduce the BW of the mice, although these changes did not reach statistical significance. In addition, arglabin reduced active caspase-3 in islets of Langerhans of these mice as well as the apoptosis susceptibility in cultured INS-1 cells. In contrast, it increased autophagy in the latter cell line. Taken together, our results indicate that lowering the activity of the inflammasome NLRP3 may play a role in the resistance of arglabin-treated mice to diet-induced obesity and their improved insulin sensitivity.

The ability of arglabin to induce autophagy is of special interest because autophagy is a catabolic pathway for bulk

turnover of long-lived proteins and organelles via lysosomal degradation. A growing body of evidence suggests that autophagy plays a role in many diseases by promoting or preventing their progression (De Meyer and Martinet, 2009). Thus, defective autophagy was found to contribute to the pathogenesis in T2DM (Li et al., 2013). Indeed, depletion of the autophagic proteins LC3B and Beclin-1 increases NLRP3 activation through enhancing caspase-1 activation and IL-1β and IL-18 secretion (Nakahira et al., 2011). Of note, our results show that arglabin enhances the expression of Beclin-1 and LC3, especially of LC3-II, indicating that autophagy is a protective mechanism in INS-1 cells. Hence, autophagy is believed to control the magnitude of the inflammatory responses by reducing the amount of reactive oxygen species (ROS) and by targeting the inflammasome components to autophagosomes (Latz et al., 2013).

Autophagy may limit the NLRP3 activity by sequestering and facilitating the degradation of inflammasome components, such as pro-IL-1β, NLRP3, caspase-1, or ASC (Abderrazak et al., 2015) and (Nakahira et al., 2011). Therefore, arglabin might control the inflammasome activation and the levels of plasma glucose and insulin by the induction of autophagy. Of note, expression of LC3-II correlates with the increased levels of autophagic vesicles. However, LC3-II itself is subject to autophagic degradation in lysosomes, and overall low levels of LC3-II could be a sign of autophagosomal turnover, so-called autophagic flux, a process of autophagy including the delivery of cargo to the lysosomes (Barth et al., 2010).

In addition, autophagy activation promotes the expression of Bcl2. This is supported by our findings that demonstrate that inhibition of autophagy by 3-methyladenine decreases the expression of Bcl2, whereas activation of autophagy by rapamycin, similar to arglabin, increases the expression of Bcl2. The mechanism of autophagy-mediated effects on the Bcl2 could be related to the autophagic clearance of cells from damaged DNA, which is known to activate p53 (Lakin and Jackson, 1999) and to induce its expression via Nrf2 (Filomeni et al., 2015). P53, in turn, induces expression of Bcl2 (Dashzeveg and Yoshida, 2015). Likewise, ROS negatively regulate the Bcl2 expression (Hildeman et al., 2003). Therefore, reduction of ROS levels by mitophagy will lead to increased Bcl2 expression.

In conclusion, our findings indicate that the NLRP3 inflammasome is an interesting target to treat T2DM and that arglabin is a promising natural molecule that almost normalizes plasma glucose and insulin levels in the ApoE$_2$Ki mice model.

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Authorship Contributions

Participated in research design: Rouis, Slimane, Simmet.

Conducted experiments: Abderrazak, El Hadri, Bosc, Büchele, Couchie, Blondeau.

Contributed new reagents or analytic tools: Büchele.

Performed data analysis: Couchie, Rouis.

Wrote or contributed to the writing of the manuscript: Rouis, Simmet.

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