Adiponectin Receptor 2 Deficiency Results in Reduced Atherosclerosis in the Brachiocephalic Artery in Apolipoprotein E Deficient Mice

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Abstract

Adiponectin has been shown to have beneficial cardiovascular effects and to signal through the adiponectin receptors, AdipoR1 and AdipoR2. The original aim of this study was to investigate the effect of combined AdipoR1 and AdipoR2 deficiency (AdipoR1⁻/⁻AdipoR2⁻/⁻) on atherosclerosis. However, we made the interesting observation that AdipoR1⁻/⁻AdipoR2⁻/⁻ leads to embryonic lethality demonstrating the critical importance of the adiponectin signalling system during development. We then investigated the effect of AdipoR2-ablation on the progression of atherosclerosis in apolipoprotein E deficient (ApoE⁻/⁻) mice. AdipoR2⁻/⁻ApoE⁻/⁻ mice fed an atherogenic diet had decreased plaque area in the brachiocephalic artery compared with AdipoR2⁺/+ApoE⁻/⁻ littermate controls as visualized in vivo using an ultrasound biomicroscope and confirmed by histological analyses. The decreased plaque area in the brachiocephalic artery could not be explained by plasma cholesterol levels or inflammatory status. However, accumulation of neutral lipids was decreased in peritoneal macrophages from AdipoR2⁻/⁻ApoE⁻/⁻ mice after incubation with oxidized LDL. This effect was associated with lower CD36 and higher ABCA1 mRNA levels in peritoneal macrophages from AdipoR2⁻/⁻ApoE⁻/⁻ mice compared with AdipoR2⁺/+ApoE⁻/⁻ controls after incubation with oxidized LDL. In summary, we show that adiponectin receptors are crucial during embryonic development and that AdipoR2-deficiency slows down the progression of atherosclerosis in the brachiocephalic artery of ApoE-deficient mice.

Introduction

Adiponectin is one of the most abundant factors secreted from adipocytes [1–4]. Hypoadiponectinemia is observed in patients with obesity [5], type 2 diabetes [6] and coronary artery disease [7,8] and high levels of adiponectin reduce the risk of developing type 2 diabetes [9] and myocardial infarction [10].

Several studies in experimental animals have demonstrated anti-diabetic [11–13] and anti-atherosclerotic [12–15] effects of adiponectin. In addition, adiponectin can protect the heart from ischemia-reperfusion injury [16] and can act as an endogenous anti-thrombotic factor [17]. The anti-atherosclerotic effects of adiponectin have been demonstrated at several stages in plaque development, ranging from endothelial dysfunction and plaque initiation to plaque progression and rupture (reviewed in 18). In vitro studies have shown that adiponectin suppresses monocyte adhesion and expression of endothelial cell adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [7]. Furthermore, adiponectin inhibits foam cell formation by downregulating macrophage scavenger receptor A, resulting in reduced lipid accumulation in macrophages [19,20]. Studies have also shown that adiponectin can induce cholesterol efflux from macrophages via upregulation of the ATP-binding cassette transporter ABCA1 [20,21].
Adiponectin has been proposed to mediate its effects via at least two transmembrane receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) [22]. We and others have demonstrated the physiological importance of these receptors using gene knock-out mice [23–25]. AdipoR1+/− mice showed decreased glucose tolerance [23,25]. In contrast, AdipoR2−/− mice were resistant to high-fat diet induced obesity and exhibited improved glucose tolerance and decreased plasma cholesterol levels [23,24]. In addition, knock-down of AdipoR2 using antisense oligonucleotides reduced plasma glucose levels in insulin resistant leptin-deficient ob/ob mice [24]. Thus, AdipoR1 and AdipoR2 are clearly involved in glucose and energy metabolism but have opposing roles [23].

In this study, we could show that mice lacking both AdipoR1 and AdipoR2 were embryonically lethal, i.e. found dead in utero at day 16.5 with macroscopical swelling and microscopical autolysis and tissue disintegration. We then hypothesized that the favourable metabolic phenotype of AdipoR2−/− mice we and others have observed previously [23,24] also could be associated with a protective effect against developing atherosclerosis. We generated mice deficient in both AdipoR2 and ApoE (AdipoR2−/−ApoE−/−) and littermate control mice lacking only ApoE (AdipoR2+/−ApoE−/−) in order to study the impact of AdipoR2 deficiency on the atherosclerosis process. Interestingly, the progression of atherosclerosis was attenuated in the brachiocephalic artery of AdipoR2 deficient mice.

**Methods**

**Ethics Statement**

All experiments were approved by the Gothenburg Ethics Committee for Experimental Animals.

**Generation of AdipoR2−/−ApoE−/− mice**

AdipoR1+/− and AdipoR2+/− mice obtained from Deltagen (San Carlos, CA) have been described previously [23]. AdipoR2−/− mice were backcrossed with C57BL/6J mice for eight generations before they were inter-crossed with ApoE−/− mice (Taconic Europe, C57BL/6J). AdipoR2−/− mice were cross-bred with ApoE−/− mice to generate offspring that were heterozygous for both AdipoR2 and ApoE (AdipoR2+/−ApoE−/−). These heterozygous mice were then inter-crossed to generate AdipoR2−/−ApoE−/− mice. Finally, AdipoR2−/−ApoE−/− mice were inter-crossed to produce AdipoR2−/−ApoE−/− mice and AdipoR2+/−ApoE−/− littermate controls used in the study.

**Diets, body weight and termination**

Male AdipoR2−/−ApoE−/− mice and AdipoR2+/−ApoE−/− littermate controls were given standard laboratory chow diet (R3; Lactamin, Kimstad, Sweden) for 8 weeks. Thereafter, they received a high-fat diet (Lard diet) containing 21% swine Lard and 0.15% cholesterol (Diet 821424; Special Diets Services, Witham, England) for 14 weeks until termination. The lard diet contained 0.03% C14:1, 0.11% C16:1, 6.69% C18:1, 1.95% C18:2, 0.11% C18:3, 0.03% C20:4, 0.03% C12:0, 0.37% C14:0, 4.56% C16:0, and 2.04% C18:0, resulting in an w6/w3 ratio of 18. Body weights were registered every other week. At termination, blood was collected via heart puncture from isoflurane-anaesthetized (Forene Isoflurane, Abbott Scandinavia AB, Sweden) mice. Liver and gonadal white adipose tissue (WAT) were dissected and weighed. The cardiovascular system was perfused with NaCl, followed by dissection of the aorta and brachiocephalic artery for histological analyses.

**Ultrasound measurements**

The mice were anaesthetized with isoflurane gas and maintained anaesthetized during the ultrasound measurement with an isoflurane dose of 1.5% in room air. Ultrasound measurements were performed at 8, 15 and 22 weeks of age. Ultrasound biomicroscopy equipment (Vevo 770, Visual Sonics, Toronto, Canada) with a transducer frequency of 40 MHz was used for vascular imaging. The brachiocephalic artery was visualized as described previously [26].

**Blood samples, plasma and liver analyses**

Blood samples were collected from Vena Saphena at 7 weeks of age (before switched to the Lard diet), at 11 and 18 weeks of age and at termination at 22 weeks of age. Lipids were extracted from plasma samples using the Folch method [27]. Triglycerides in total lipid extracts were separated using normal phase liquid chromatography, NPLC, and measured by evaporative light scattering detection, ELSD [28]. A calibration curve was obtained by injecting commercial lipid standard. Total plasma cholesterol levels were measured with an enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany). Cholesterol distribution profiles were measured on 10 µl pooled plasma from 10 mice in each group, using a size-exclusion high performance liquid chromatography system, SMART (GE Healthcare, Waukesha, WI). Plasma adiponectin levels were measured using a radioimmunoassay from Linco Research (St. Charles, MO) and plasma cytokines were determined using bead based multiplex suspension array kits with the Luminex technology on a BioPlex (Bio-Rad, CA). Liver triglyceride content was measured on an ABX Pentra 400 (HORIBA Medical, Kyoto, Japan) after homogenization in isopropanol (1 ml/50 mg), incubation at 4°C for 1 h, and centrifugation at 2,500 rpm for 5 min.

**Histological analyses**

Paraffin sections (4 µm) from the proximal brachiocephalic artery and the aortic arch were stained with Miller’s Elastin and Picrosirius Red (Histolab Products AB, Gothenburg, Sweden) and used for measurement of plaque area and collagen content, respectively. A Mac-2 antibody (CL8942AP clone M3/38; Cedarlane, Hornby, Canada) at a dilution of 1:20,000 was used for quantification of macrophages and macrophage-derived foam cells. For the en face analysis, the thoracic part of the descending aorta was cut open longitudinally and mounted in Tissue-Tek O.C.T compound (Sakura, Torrance, CA). All plaque analyses were done using BioPix IQ 2.1 Software (BioPix, Gothenburg, Sweden). Plaque area in the descending aorta is expressed as percentage of total vessel area.
Protein extraction and western blot

Liver protein was extracted by homogenization in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with added protease inhibitors according to the manufacturer’s protocol. Protein concentrations were determined with the Pierce BCA Protein Assay kit (Thermo Scientific). 30 µg protein was separated on a 4-12% Bis-Tris gel and subsequently transferred onto a nitrocellulose membrane. The membrane was incubated with affinity purified primary antibodies against human AdipoR2 (1 µg/ml in phosphate-buffered saline with 0.05% Tween and 5% milk powder). The AdipoR2 antibodies were generated by immunizing rabbits with the peptide CSRTPEPDRLRKGHLQDG (94% sequence homology with the corresponding mouse sequence). The secondary HRP-conjugated goat anti-rabbit IgG antibody (Novus Biologics, Littleton, CO) was used at 1:1 000. The bands were visualized using the Novex ECL Chemiluminescent substrate reagent kit (Invitrogen, Carlsbad, CA).

Analyses of lipid accumulation, inflammatory response and gene expression in peritoneal macrophages

Primary peritoneal macrophages were isolated by flushing the peritoneum of 8 weeks old male AdipoR1−/−ApoE−/− and AdipoR2−/−ApoE−/− littermate controls with PBS. Cells were collected and allowed to adhere to a cell culture dish for 2 h in serum-free RPMI 1640 media supplemented with sodium-pyruvate (2mM), non-essential amino acids, sodium bicarbonate (1.5 g/l), penicillin (100 U/ml) and streptomycin (100 mg/l) at 37°C. Cells not attached to the plastic after 2 h were removed by washing three times with PBS and adherent cells were cultured in supplemented RPMI 1640 media containing 10% FCS. For analysis of inflammatory response, peritoneal macrophages were incubated with or without oxidized LDL (oxLDL) (50 µg/ml) or LPS (0.1 mg/ml) for 24 h and cytokine levels in the culture medium were determined as described above.

Probe 5´-AAATGAGAAAAAGCTTGTGCAATGGCAATTCTG-3´, IL-10, Forward primer 5´-CCAGAGCCACATGCTCCTAGA-3´, Probe 5´-CTGCGGACTGCCTTCAGCCAGG-3´. All TaqMan primer and probe sequences are available in Table 2. Relative expression levels were calculated according to the formula 2−∆CT, where ∆CT is the difference in cycle threshold (CT) values between the target and the 18S rRNA internal control.

Statistics

Values are expressed as means ± SEM. Comparisons between groups were made by Kruskal-Wallis test and Mann-Whitney U test. P<0.05 was considered statistically significant.

Results

Generation of AdipoR1 AdipoR2 double deficient (AdipoR1−/−AdipoR2−/−) mice

The generation of AdipoR1−/− and AdipoR2−/− mice and their respective phenotypes in energy and glucose homeostasis have been described before [23]. In order to study the effects of combined AdipoR1 and AdipoR2 deficiency on atherosclerosis during ApoE deficiency, we first wanted to produce AdipoR1−/−AdipoR2−/− mice. Mice heterozygous for the two receptors (AdipoR1+/−AdipoR2−/−) were generated using AdipoR1+/− males and AdipoR2−/− females (backcrossed for eight generations towards C57Bl/6J). We used this strategy since AdipoR2−/− males in contrast to AdipoR1−/− males did not produce any offspring (Table 1). This is probably due to an atrophy of the seminiferous tubules and aspermia associated with reduced testes weight as we have described before [23]. Next, AdipoR1−/−AdipoR2−/− mice were inter-crossed in order to generate AdipoR1−/−AdipoR2−/− mice. Interestingly, in spite that three wild type mice were generated in this inter-cross, not a single AdipoR1−/−AdipoR2−/− mouse was detected (Table 2). In order to investigate this further, we set up another breeding using offsprings from the AdipoR1−/−AdipoR2−/− inter-cross. Male

Table 1. Litter sizes produced by AdipoR1−/− or AdipoR2−/− males.

|                  | Females | MAles |
|------------------|---------|-------|
| AdipoR1−/−       | n.d.    | 22    |
| AdipoR2−/−       | 0       | n.d.  |
| AdipoR1−/−AdipoR2−/− | n.d. | 15    |

AdipoR1−/− and AdipoR2−/− males were inter-crossed in order to generate AdipoR1−/−AdipoR2−/− mice. Interestingly, in spite that three wild type mice were generated in this inter-cross, not a single AdipoR1−/−AdipoR2−/− mouse was detected (Table 2). In order to investigate this further, we set up another breeding using offsprings from the AdipoR1−/−AdipoR2−/− inter-cross. Male
mice homozygous for AdipoR1 deficiency and heterozygous for AdipoR2 deficiency (AdipoR1+/AdipoR2−/− mice) were bred with AdipoR1−/−AdipoR2−/−, AdipoR1−/−AdipoR2−/−, or AdipoR1−/−AdipoR2−/− females. Again, none of these breeding produced any AdipoR1−/−AdipoR2−/− mice (Table 3). Next, we set up an identical breeding using the same parent mice analyzing the resulting embryos at embryonic day 16.5 (Table 4). At this time point, AdipoR1−/−AdipoR2−/− mouse embryos were found dead in utero with a swollen appearance macroscopically (Figure 1), and microscopical examination at this stage showed autolysis with accompanying early disintegration of organ and tissue structures. As a specific finding we noted an intravascular accumulation of immature nucleated blood cells (data not shown). We conclude that knocking out both AdipoR1 and AdipoR2 in mice results in embryonic lethality.

**Generation of AdipoR2 ApoE double deficient AdipoR2−/−ApoE−/− mice**

Based on the previous finding by us and Liu et al. [23,24] that AdipoR2−/− mice are protected from high-fat diet-induced dyslipidemia and insulin resistance, we then hypothesized that AdipoR2 deficiency could potentially have a protective role also against atherosclerosis. Mice deficient in both AdipoR2 and ApoE (AdipoR2−/−ApoE−/−) and littermate control mice lacking only ApoE (AdipoR2+/−ApoE−/−) were generated to study the effect of AdipoR2 deficiency on the progression of atherosclerosis. The absence of AdipoR2 protein in AdipoR2−/−ApoE−/− mice was confirmed by western blot analysis of liver protein extracts (Figure 2).

**Effect of AdipoR2 deficiency on plaque area and morphology**

Male AdipoR2−/−ApoE−/− mice and corresponding AdipoR2+/−ApoE−/− littermate controls were examined by ultrasound scanning to follow the progression of atherosclerotic plaques in the brachiocephalic artery. Up to 8 weeks of age, the mice were fed standard laboratory chow diet. At 8 weeks of age, no lesions could be detected by ultrasound scanning (data not shown). Thereafter the mice were fed a high-fat (Lard) diet. At

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**Table 2.** Genotypes of offspring following intercross of 4 heterozygous AdipoR1+/AdipoR2−/+ males with 8 heterozygous AdipoR1−/AdipoR2−/+ females.

| Genotypes | AdipoR1++ | AdipoR1+/− | AdipoR1−/− |
|-----------|-----------|------------|------------|
| AdipoR2++ | 3         | 7          | 2          |
| AdipoR2+/−| 11        | 1          | 6          |
| AdipoR2−/−| 4         | 0          | 0          |

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**Table 3.** Genotypes of offspring following intercross of AdipoR1−/AdipoR2−/+ males with AdipoR1+/AdipoR2−/+ (n=6) or AdipoR1+/AdipoR2−/+ (n=2) or AdipoR1−/AdipoR2−/+ (n=1) females.

| Genotypes | AdipoR1++ | AdipoR1+/− | AdipoR1−/− |
|-----------|-----------|------------|------------|
| AdipoR2++ | 6         | 7          | 1          |
| AdipoR2+/−| 7         | 2          | 3          |
| AdipoR2−/−| 7         | 0          | 0          |

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Figure 1. **AdipoR1+**/**AdipoR2+** and AdipoR1−/AdipoR2− mouse embryos at development stage 16.5 post coitum. AdipoR1−/AdipoR2− mouse embryo was found dead in utero, with macroscopical swelling corresponding to the beginning autolysis and organ disintegration at the microscopical level. Scale bar: 3 mm.

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significant trend towards a decreased plaque burden in controls (Figure 3A). This effect on plaque area was not related to vessel size, since the diameter of the brachiocephalic artery was similar between the genotypes (AdipoR1+/+ vs. AdipoR2−/− littermate controls (Figure 3A). Following an additional 7 weeks of high-fat diet, the plaques had more than doubled in size. At this timepoint, AdipoR2−/− mice had significantly smaller plaque area compared with AdipoR2+/− mice in most cases were smaller and contained a high degree of macrophages, although cholesterol clefts and fibrous caps were also present in some of these plaques (Figure 3E-G). The Mac2-immunopositive area (i.e. area of stained macrophages and/or macrophage-derived foam cells) was larger in plaques from AdipoR2−/− mice compared with AdipoR2+/− controls (AdipoR2+/− vs. AdipoR2−/−; 12.5 ± 2.2 % vs. AdipoR2+/−, 27.1 ± 4.4 % of the total plaque area, *P*=0.002, Mann-Whitney U test). In contrast, picrossirus staining demonstrated a lower collagen content in plaques from AdipoR2−/− mice compared with AdipoR2+/− controls (AdipoR2+/− vs. AdipoR2−/−, 43.6 ± 3.2 % vs. AdipoR2+/−, 32.3 ± 4.1 % of the total plaque area, *P*=0.049, Mann-Whitney U test). No obvious calcifications were noted in any of the genotypes, thus no difference could be detected between the groups in this respect.

### Table 4. Genotype of E16.5 embryos following intercross of AdipoR1+/+AdipoR2−/− males with AdipoR1+/+AdipoR2−/− or AdipoR1+/+AdipoR2−/− females.

| Males | Females | AdipoR1+/+AdipoR2−/− (n=4) | AdipoR1+/+AdipoR2−/− (n=1) |
|-------|---------|-----------------------------|-----------------------------|
| AdipoR1+/+ AdipoR2−/− | 3 AdipoR1+/+AdipoR2+/+ | 3 AdipoR1+/+AdipoR2−/− |
| | 14 AdipoR1+/+AdipoR2−/− | 1 AdipoR1+/+AdipoR2−/− |
| 2 AdipoR1+/+AdipoR2−/− | 2 AdipoR1+/+AdipoR2−/− |
| 5 AdipoR1+/+AdipoR2−/− | 5 AdipoR1+/+AdipoR2−/− |
| 2 AdipoR1+/+AdipoR2−/− | 2 AdipoR1+/+AdipoR2−/− |
| 1 AdipoR1+/+AdipoR2−/− | 1 AdipoR1+/+AdipoR2−/− |

*P*=0.049, Mann-Whitney U test.

15 weeks of age (7 weeks of high-fat diet), distinct plaques were found in the brachiocephalic artery and there was a nonsignificant trend towards a decreased plaque burden in AdipoR2−/−ApoE−/− mice compared with AdipoR2+/−ApoE−/− littermate controls (Figure 3A). Following an additional 7 weeks on high-fat diet, the plaques had more than doubled in size. At this timepoint, AdipoR2−/−ApoE−/− mice had significantly smaller plaque area compared with AdipoR2+/−ApoE−/− littermate controls (Figure 3A). This effect on plaque area was not related to vessel size, since the diameter of the brachiocephalic artery was similar between the genotypes (AdipoR2+/−ApoE−/−; 0.85 ± 0.02 mm vs. AdipoR2−/−ApoE−/−; 0.83 ± 0.02 mm, *P*=0.90, Mann-Whitney U test). After the final ultrasound scan, all mice were terminated and the brachiocephalic arteries and descending aortas were dissected for histological analyses of plaque size and morphology. The plaque area in AdipoR2−/−ApoE−/− mice was 40% smaller compared with AdipoR2+/−ApoE−/− controls (Figure 3B). In contrast to the brachiocephalic artery, the plaque area measured by histology in the aortic arch and en face analysis of the descending aorta showed a similar plaque burden between the genotypes (Figure 3C and D).

In addition to plaque area measurements, the histological samples were used to study plaque morphology in the brachiocephalic artery. Plaques in AdipoR2+/−ApoE−/− mice were generally larger and characterized by cholesterol clefts and a clear fibrous cap, whereas plaques in AdipoR2−/−ApoE−/− mice in most cases were smaller and contained a high degree of macrophages, although cholesterol clefts and fibrous caps were also present in some of these plaques (Figure 3E-G). The Mac2-immunopositive area (i.e. area of stained macrophages and/or macrophage-derived foam cells) was larger in plaques from AdipoR2−/−ApoE−/− mice compared with AdipoR2+/−ApoE−/− controls (AdipoR2+/−ApoE−/− vs. AdipoR2−/−ApoE−/−; 12.5 ± 2.2 % vs. AdipoR2+/−ApoE−/−, 27.1 ± 4.4 % of the total plaque area, *P*=0.002, Mann-Whitney U test). In contrast, picrossirus staining demonstrated a lower collagen content in plaques from AdipoR2−/−ApoE−/− mice compared with AdipoR2+/−ApoE−/− controls (AdipoR2+/−ApoE−/− vs. AdipoR2−/−ApoE−/−, 43.6 ± 3.2 % vs. AdipoR2+/−ApoE−/−, 32.3 ± 4.1 % of the total plaque area, *P*=0.049, Mann-Whitney U test). No obvious calcifications were noted in any of the genotypes, thus no difference could be detected between the groups in this respect.

### Effects of AdipoR2-deficiency on body weight and plasma biochemistry

Body weights, body weight gain, WAT, liver weights and liver triglyceride content were similar between AdipoR2+/−ApoE−/− mice and AdipoR2+/−ApoE−/− controls (Table 5). Total cholesterol levels were followed during the study and measured at four time points (Table 6). Cholesterol levels were similar between the genotypes, except for the measurement at termination when AdipoR2−/−ApoE−/− mice had higher plasma cholesterol levels compared with AdipoR2+/−ApoE−/− controls (Table 6). Since the plasma cholesterol levels were equal, or at termination even higher in AdipoR2−/−ApoE−/− mice, the smaller plaque size in these mice must be explained by mechanisms other than reduction in total cholesterol levels. In addition, the cholesterol distribution profiles were almost identical between
Figure 3. Plaque area in the brachiocephalic artery of AdipoR2⁺⁺/⁻⁻ and AdipoR2⁻⁻/⁻⁻ littermate controls after 7 and 14 weeks on Lard diet. (A) Plaque area in the brachiocephalic artery measured by ultrasound at 15 and 22 weeks of age (AdipoR2⁺⁺/⁻⁻ n = 25, AdipoR2⁻⁻/⁻⁻ n = 14). (B) Plaque area in the brachiocephalic artery measured by histology at termination at 22 weeks of age (AdipoR2⁺⁺/⁻⁻ n = 24, AdipoR2⁻⁻/⁻⁻ n = 14). (C) Plaque area in the aortic arch measured by histology at termination at 22 weeks of age (AdipoR2⁺⁺/⁻⁻ n = 25, AdipoR2⁻⁻/⁻⁻ n = 11). (D) Plaque area (% of total vessel area) in the descending aorta measured by en face analysis at termination at 22 weeks of age (AdipoR2⁺⁺/⁻⁻ n = 22, AdipoR2⁻⁻/⁻⁻ n = 14). Miller’s elastin staining (E), Mac2 staining (F), and Picrosirius Red staining (G) of representative brachiocephalic artery plaques from AdipoR2⁺⁺/⁻⁻ mice (I-II) and AdipoR2⁻⁻/⁻⁻ mice (III-IV). Values are means ± SEM. *P < 0.05, **P < 0.01 for comparison between AdipoR2⁻⁻/⁻⁻ and AdipoR2⁺⁺/⁻⁻ mice (Mann-Whitney U test).

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The mice were fed Lard diet from 8 weeks of age until termination at 22 weeks of age. Values are means ± SEM. *P< 0.05 (Mann-Whitney U test).

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Effects of AdipoR2 deficiency on inflammation

Plasma levels of the inflammatory cytokines IL-1α, IL-1β, IFN-γ and TNF-α were similar between the genotypes and the plasma IL-6 levels were higher in AdipoR2-/-ApoE-/- mice compared with AdipoR2+/+ApoE-/- controls (Figure 4B). AdipoR2 deficiency had no effect on total plasma adiponectin levels (Table 6). Thus, neither plasma levels of lipids or adiponectin could help to explain the protective effect of AdipoR2 deficiency against atherosclerosis.

Effects of AdipoR2 deficiency on inflammation

Plasma levels of the inflammatory cytokines IL-1α, IL-1β, IFN-γ and TNF-α were similar between the genotypes and the plasma IL-6 levels were higher in AdipoR2-/-ApoE-/- mice compared with AdipoR2+/+ApoE-/- controls (Table 7). Interestingly, adiponectin treatment has been shown to enhance insulin sensitivity by increasing IRS-2 expression in the liver via an IL-6 dependent pathway [29]. However, liver IRS-2 mRNA levels were similar between the genotypes (AdipoR2-/-ApoE-/- = 100±27 vs. AdipoR2+/+ApoE-/- = 65±21 % of control, n = 10, P=0.2, Mann-Whitney U test) indicating that the elevated IL-6 levels observed in AdipoR2-/-ApoE-/- mice was not sufficient to drive an enhanced IRS-2 expression in the liver.

To investigate if there was a different inflammatory response in macrophages from AdipoR2-/-ApoE-/- mice and AdipoR2+/+ApoE-/- controls, peritoneal macrophages were incubated with or without oxLDL or LPS for 24 h and the secretion of IL-1α, IL-1β, IL-6 and TNF-α into the medium was analyzed. As expected, LPS induced a strong inflammatory response, but there was no difference in the response between the genotypes (Table 8). Incubation of AdipoR2-/-ApoE-/- macrophages with oxLDL increased the level of IL-1α in the culture medium whereas this was not seen in culture medium from AdipoR2-/-ApoE-/- control macrophages (Table 8). Thus, the decreased progression of atherosclerosis in AdipoR2-/-ApoE-/- mice was likely due to other mechanisms than decreased inflammation.

Table 5. Body and organ weights of AdipoR2+/+ApoE-/- mice and AdipoR2-/-ApoE-/- littermate controls.

|                     | AdipoR2+/+ApoE-/- (n=25) | AdipoR2-/-ApoE-/- (n=14) |
|---------------------|--------------------------|--------------------------|
| Body weight at termination (g) | 30.3 ± 0.63 | 30.8 ± 0.81 |
| Body weight gain (g) | 5.20 ± 0.56 | 5.40 ± 0.57 |
| Body weight gain (g/day) | 0.05 ± 0.01 | 0.06 ± 0.01 |
| Reproductive WAT (g) | 0.86 ± 0.09 | 0.90 ± 0.11 |
| Reproductive WAT (% of bw) | 2.74 ± 0.22 | 2.67 ± 0.27 |
| Liver (g) | 1.39 ± 0.04 | 1.34 ± 0.04 |
| Liver (% of bw) | 4.59 ± 0.09 | 4.35 ± 0.05 |
| Liver triglycerides (g TG/100g liver) | 7.6 ± 1.7 (n = 10) | 5.1 ± 0.7 (n = 10) |

The mice were fed Lard diet from 8 weeks of age until termination at 22 weeks of age. Values are means ± SEM.

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Table 6. Plasma cholesterol and adiponectin levels in AdipoR2+/+ApoE-/- mice and AdipoR2-/-ApoE-/- littermate controls.

|                     | AdipoR2+/+ApoE-/- (n=25) | AdipoR2-/-ApoE-/- (n=14) |
|---------------------|--------------------------|--------------------------|
| Cholesterol, 7 w (mmol/l) | 9.5 ± 0.35 | 10.6 ± 0.48 |
| Cholesterol, 11 w (mmol/l) | 49 ± 1.40 | 54 ± 3.30 |
| Cholesterol, 18 w (mmol/l) | 52 ± 1.40 | 51 ± 3.20 |
| Cholesterol, 22 w (mmol/l) | 36 ± 1.01 | 41 ± 2.03 * |
| Adiponectin, 7 w (nmol/l) | 361 ± 40 | 308 ± 24 |
| Adiponectin, 11 w (nmol/l) | 360 ± 18 | 359 ± 20 |
| Adiponectin, 18 w (nmol/l) | 321 ± 23 | 361 ± 21 |
| Adiponectin, 22 w (nmol/l) | 312 ± 17 | 308 ± 16 |

The mice were fed Lard diet from 8 weeks of age until termination at 22 weeks of age. Plasma levels of cholesterol and adiponectin were measured during the study as described in Methods. Values are means ± SEM. *P< 0.05 (Mann-Whitney U test).

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the groups (Figure 4A). Furthermore, the plasma triglyceride levels were higher at termination in AdipoR2-/-ApoE-/- mice compared with AdipoR2+/+ApoE-/- controls (Figure 4B). AdipoR2 deficiency had no effect on total plasma adiponectin levels (Table 6). Thus, neither plasma levels of lipids or adiponectin could help to explain the protective effect of AdipoR2 deficiency against atherosclerosis.
Discussion

In this study in male mice, we could for the first time show that knocking out both AdipoR1 and AdipoR2 results in embryonic lethality demonstrating the critical importance of these receptors and the adiponectin signalling system during embryonic development. We could also demonstrate the novel finding that AdipoR2 deficiency has a protective effect on the progression of atherosclerosis in ApoE^-/- mice. AdipoR2^-/-ApoE^-/- mice had smaller plaque area in the brachiocephalic artery compared with AdipoR2^+/+ApoE^-/- littermate controls. The protective effect was unrelated to changes in plasma cholesterol levels, inflammatory status or differences in the distribution of cholesterol in lipoprotein fractions. However, the protective effect of AdipoR2 deficiency in the brachiocephalic artery could be associated with decreased lipid accumulation in macrophages as demonstrated in intraperitoneal macrophages upon incubation with oxLDL.

At embryonic day 16.5, an AdipoR1^-/-AdipoR2^-/- embryo was found dead in utero with macroscopical swelling and microscopical autolysis and tissue disintegration and intravascular accumulation of immature nucleated blood cells. Interestingly, adiponectin has been shown to stimulate angiogenesis both in vitro and in vivo [30,31], and it would be of interest for future studies to explore the contribution of the two adiponectin receptors in this process. Our finding that AdipoR1^-/-AdipoR2^-/- mice die during embryonic development is in contrast to a study by Yamauchi et al [25] in which viable AdipoR1^-/-AdipoR2^-/- mice were generated. The AdipoR2^-/- mice described by Yamauchi et al [25] was shown to express an aberrantly spliced AdipoR2 mRNA and the AdipoR2^-/- mice studied by us and Liu et al has been shown to express exon 3 and exon 7 [24] surrounding the deleted exon 5 (corresponding to several transmembrane parts of this seven membrane receptor). Importantly, using an antibody directed against a peptide sequence in exon 2 containing the ATG (outside the targeting construct), we could confirm lack of AdipoR2 protein in livers of AdipoR2^-/-ApoE^-/- mice in this study and in AdipoR2^-/- mice in our previous study [23], indicating that complete lack of adiponectin receptors results in embryonic lethality. However, the non viable AdipoR1^-/-AdipoR2^-/- embryos generated in this study were on a C57Bl/6J genetic background while the

Table 7. Plasma levels of inflammatory mediators in AdipoR2^-/-ApoE^-/ mice and AdipoR2^-/+ApoE^-/ littermate controls.

|         | AdipoR2^-/+ApoE^-/ (n=25) | AdipoR2^-/-ApoE^-/ (n=14) |
|---------|-------------------------|--------------------------|
| IL-1α (pg/ml) | 19 ± 2.1 | 20 ± 1.7 |
| IL-1β (pg/ml) | 226 ± 49 | 245 ± 35 |
| IFN-γ (pg/ml) | 87 ± 13 | 108 ± 49 |
| TNF-α (pg/ml) | 488 ± 215 | 373 ± 52 |
| IL-6 (pg/ml) | 81 ± 12 | 122 ± 21* |

The mice were fed Lard diet from 8 weeks of age until termination at 22 weeks of age. Plasma levels of inflammatory mediators were measured at termination as described in Methods. Values are means ± SEM. *P< 0.05 (Mann-Whitney U test).

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Figure 4. Plasma lipids in 22 weeks old AdipoR2^-/-ApoE^-/ mice and AdipoR2^-/+ApoE^-/ littermate controls after 14 weeks on Lard diet. (A) Cholesterol distribution profiles were measured by size exclusion HPLC on pooled plasma from 10 mice in each group. (B) Plasma triglycerides were measured in lipid extracts from plasma after separation by normal phase liquid chromatography, NPLC (AdipoR2^-/+ApoE^-/ n = 25, AdipoR2^-/-ApoE^-/ n = 14). Values are means ± SEM. *P< 0.05 (Mann-Whitney U test).

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Atherosclerosis, whereas areas of laminar flow are more resistant [33]. The brachiocephalic artery (innominate artery) is comparison between oxLDL or LPS

Thus, the fact that there is a difference in plaque area between the descending aorta may have been affected. Site specific

AdipoR1−/− AdipoR2−/− mice generated by Yamauchi et al were on a more mixed 129/Sv x C57Bl/6 genetic background [25]. This may be the reason for the different results concerning generation of viable double receptor deficient mice between our laboratories.

The difference in plaque area between AdipoR2−/−ApoE−/− mice and AdipoR2−/−ApoE−/− littermate controls, and cultured in supplemented RPMI 1640 media containing 10% FCS. Inflammatory mediators in the culture medium were analyzed after incubation with or without oxidized LDL (oxLDL) (50µg/ml) or LPS (0.1 mg/ml) for 24 h. Values are means ± SEM. *P<0.05, **P<0.01, ***P<0.001 for comparison between oxLDL or LPS vs. Basal within each genotype (Kruskal-Wallis U test).

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AdipoR1−/− AdipoR2−/− mice were generally smaller and often rich in macrophages, whereas the majority of the plaques from AdipoR2−/−ApoE−/− mice were larger and characterized by cholesterol clefts and a clear fibrous cap. Plaques from AdipoR2−/−ApoE−/− mice contained less collagen and more macrophages compared with AdipoR2−/−ApoE−/− controls. Therefore, it cannot be ruled out that plaque stability was affected. However, macrophage infiltration and foam cell formation is an early process in plaque development, whereas extracellular matrix, including collagen, is produced in more mature lesions [40]. Thus, these compositional data indicate that plaques in AdipoR2−/−ApoE−/− mice represent earlier lesions with a higher content of macrophages, whereas more mature lesions are seen in the AdipoR2−/−ApoE−/− control mice.

We and Liu et al have previously shown that AdipoR2−/− mice are resistant to high-fat diet induced obesity [23,24], lasting at least until 15 weeks of high-fat diet [23]. The fact that AdipoR2−/−ApoE−/− mice and AdipoR2−/−ApoE−/− controls were comparable in terms of body weight gain or obesity in this study is however not surprising since ApoE-deficiency has been shown to attenuate diet-induced obesity in mice [41]. Further, we and Liu et al have shown that AdipoR2 deficiency leads to hypercholesterolemia [23,24]. AdipoR2−/− male mice have a plasma cholesterol level of ~3 mmol/l [23], while AdipoR2−/−ApoE−/− males have a plasma cholesterol level of ~40 mmol/l when fed a high-fat diet. Thus, the severe hypercholesterolemia in ApoE−/− mice attenuated the hypercholesterolemic phenotype seen in AdipoR2−/− mice [23,24]. Therefore, the decreased atherosclerosis in AdipoR2−/−ApoE−/− mice compared with AdipoR2−/−ApoE−/− controls must be explained by other mechanisms than reduced plasma cholesterol levels. Indeed, AdipoR2−/−ApoE−/− mice had even higher plasma cholesterol levels compared with control mice at termination. Although high-fat diet fed ApoE−/− mice are regularly used in preclinical atherosclerosis studies, the cholesterol levels obtained are extremely high. Thus, it will be important for future studies to investigate the long-term consequences of AdipoR2-deficiency on atherosclerosis under more moderately elevated cholesterol levels.

| Stimuli | AdipoR2+/−ApoE+/− (n=7-9) | AdipoR2+/−ApoE+/− (n=7-8) |
|---------|--------------------------|--------------------------|
| IL-1α  | 219 ± 47                | 162 ± 52                 |
| oxLDL  | 307 ± 58                | 512 ± 165*               |
| LPS    | 663 ± 66**              | 633 ± 102**              |
| IL-1β  | 15 ± 0.7                | 21 ± 4.7                 |
| oxLDL  | 15 ± 0.7                | 31 ± 9.3                 |
| LPS    | 228 ± 48**              | 215 ± 43**               |
| TNF-α  | 15 ± 1.4                | 19 ± 2.7                 |
| oxLDL  | 13 ± 1.4                | 105 ± 90                 |
| LPS    | 2852 ± 440***           | 2938 ± 823***            |
| IL-6   | 1247 ± 251              | 2224 ± 633               |
| oxLDL  | 807 ± 187               | 2451 ± 1457              |
| LPS    | 20555 ± 2864***         | 24759 ± 4400***          |

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Table 8. Secretion of inflammatory mediators from peritoneal macrophages isolated from AdipoR2−/−ApoE−/− mice and AdipoR2−/−ApoE−/− littermate controls.
Figure 5. Lipid accumulation and gene expression in primary macrophages from 8 weeks old AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} mice and AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} littermate controls. (A) Lipid accumulation in primary macrophages was analyzed after incubation with or without 50 μg/ml oxLDL for 24 h (Basal; AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} n = 18, AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} n = 15. OxLDL; AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} n = 14, AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} n = 13). Lipid accumulation is expressed as total oil red O area per cell. (B) CD36, (C) ABCA1, (D) IL-6, and (E) IL-10 mRNA expression in primary macrophages incubated with or without 50 μg/ml oxLDL for 24 h (Basal; AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} n = 8, AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} n = 6. OxLDL; AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} n = 8, AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} n = 5). Values are means ± SEM. *P < 0.05 for comparison between AdipoR2\textsuperscript{-}ApoE\textsuperscript{-} and AdipoR2\textsuperscript{+}ApoE\textsuperscript{-} macrophages within each treatment group (Mann-Whitney U test).

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Since macrophages play an important role in atherogenesis as modulators of both lipid metabolism and immune responses, we isolated primary macrophages to study whether there was a difference in their capacity to accumulate lipids or secrete inflammatory mediators. The inflammatory response was similar in macrophages from \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/+} and \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/-} controls. On the other hand, primary macrophages from \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/-} mice accumulated less neutral lipids compared with macrophages from \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/-} mice upon incubation with oxLDL. Interestingly, macrophages from \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/-} mice had lower expression levels of CD36 mRNA and higher expression levels of ABCA1 mRNA compared with control macrophages. Studies have demonstrated that CD36-deficiency dramatically inhibits lesion development in \textit{ApoE}^{+/-} mice [42,43], and can also have a protective effect in late stages of atherosclerosis [44]. The protective effect of CD36-deficiency was shown to be macrophage-dependent, since mice lacking CD36 selectively in macrophages were protected against atherosclerosis, whilst re-introduction of CD36 expressing macrophages resulted in an increased lesion area [45]. Furthermore, macrophage-specific overexpression of ABCA1 inhibits the progression of atherosclerosis [46], whereas macrophage-specific inactivation of ABCA1 increased foam cell formation and atherosclerosis [47]. Thus, downregulation of CD36 and upregulation of ABCA1 in macrophages may have contributed to the slower progression of atherosclerosis in the brachiocephalic artery of \textit{AdipoR2}^{-/-} \textit{ApoE}^{+/-} mice in the present study.

Interestingly, homozygotes and heterozygotes of the \textit{ADIPOR2} gene polymorphism rs767870 major allele were recently shown to have lower flow mediated dilatation, increased intima-media thickness and increased \textit{ADIPOR2} protein levels in peripheral monocytes compared with homozygotes of the minor allele [48]. The same gene polymorphism has previously been associated with elevated liver fat content and fasting triglyceride levels [49] and type 2 diabetes [50]. Thus, high \textit{ADIPOR2} levels may be associated with an increased risk of developing cardiovascular disease in humans.

In summary, we could for the first time show that combined genetic deletion of both adiponectin receptors results in embryonic lethality demonstrating the crucial importance of the adiponectin signalling system during embryonic development. We could also show that genetic ablation of \textit{AdipoR2} slows down the progression of atherosclerosis in the brachiocephalic artery in atherosclerosis prone male mice lacking \textit{ApoE} indicating that antagonizing \textit{AdipoR2} or activating \textit{AdipoR1} may be an attractive therapeutic approach for the treatment of atherosclerosis.

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Author Contributions

Conceived and designed the experiments: AL ML MB-Y JB L-MG DL. Performed the experiments: AL ML SRB JW AA CM DL. Analyzed the data: AL ML SRB JW GB DL. Wrote the manuscript: AL ML DL.

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