AMPK-mediated HMGCR regulation of cholesterol metabolism in macrophages

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Abstract

Atherosclerosis, the accumulation of cholesterol-loaded macrophages (foam cells) in the arteries, leads to cardiovascular disease, which is the leading cause of mortality in the developed world. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is the rate-liming step in cholesterol biosynthesis and is regulated by several mechanisms, including inhibition by reversible phosphorylation by the energy-sensing AMP-activated protein kinase (AMPK). AMPK activators are under investigation for their potential as anti-atherogenic agents. The purpose of this project was to test the physiological significance of AMPK regulating HMGCR activity in macrophages. Using bone marrow-derived macrophages from HMGCR knock-in (KI) mice, where the phosphorylation site had been eliminated, this project investigated the impact that AMPK signaling to HMGCR has on cholesterol synthesis, accumulation of cholesterol, and cholesterol efflux. Since atherosclerosis is tightly linked with inflammation in plaques, cytokine expression was also assessed after treating macrophages with a pro-inflammatory stimulus. While there was increased cholesterol synthesis in macrophages from HMGCR KI mice than wild-type (WT) mice, there were no differences between WT and HMGCR KI macrophages in all the other aspects examined. This suggests that the regulation of HMGCR through phosphorylation by AMPK is not physiologically significant to the development of atherosclerosis. Further investigation is required *in vivo* to confirm this finding. To further understand how AMPK activators may be a potential drug strategy for treating atherosclerosis, other pathways and mechanisms need to be investigated.

Introduction

Atherosclerosis is when cholesterol-loaded macrophages (foam cells) accumulate in the arteries and form plaques, which leads to cardiovascular disease[1]. These macrophages have an increased cholesterol content due to an increase in lipid scavenging from low-density lipoprotein[2] and a reduction in cholesterol efflux through the ATP-binding cassette (ABC) transporters to extracellular receptors. Cholesterol can be delivered to the lipid-poor apolipoprotein A-1 (ApoA1) through ABCA1[3] or high-density lipoprotein (HDL) through ABCG1[4].

Atherosclerosis is also associated with inflammation, possibly due to increased levels of proinflammatory immune cells. Compared to lean individuals, a larger portion of macrophages from obese individuals are M1 (classically activated) macrophages, which produce proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6)[5].

3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is the rate-liming step in cholesterol biosynthesis[6] and is regulated by several mechanisms, including inhibition by reversible phosphorylation by AMP-activated protein kinase[7] (AMPK). AMPK is a highly conserved, serine/threonine kinase that plays an essential role in energy homeostasis by activating catalytic processes and inhibiting anabolic pathways[8].

While it is known that AMPK phosphorylates and inhibits HMGCR, the physiological significance of this regulation and its role in the pathogenesis of atherosclerosis are not known. We investigated this regulation by using A-769662, a small molecule that specifically and directly activates AMPK by binding to the β1 subunit[9].

Objectives

Using HMGCR Ser871Ala knock-in (HMGCR KI) mice where the phosphorylation site had been eliminated, we investigated the physiological significance of AMPK-mediated HMGCR inhibition using bone marrow derived macrophages (BMDMs) on the following aspects:

- cholesterol synthesis
- accumulation of cholesterol
- cholesterol efflux
- expression of the proinflammatory cytokines TNF-α and IL-6

Methods

- **Macrophage culture**: Bone marrow was isolated from the tibia and femur, and left to differentiate into macrophages in the presence of L929 (a source of macrophage colony stimulating factor) for 7 days. Cells were then scraped, counted and seeded for subsequent experiments.
- **Lipogenesis assay**: BMDMs from WT and HMGCR KI mice were treated with 1μCi/mL [^{3}H] sodium acetate for 4 hours. Lipids were extracted using the Bligh and Dyer method[10]. Radioactivity in lipids was determined by liquid scintillation counting (LSC). Cholesterol was separated from total lipids using thin-layer chromatography. Radioactivity in cholesterol was determined by LSC.
- **Cholesterol efflux**: BMDMs were treated with 0.5 μCi/mL [^{3}H] cholesterol for 30 hours, then with either A-769662 (100 μM), T0901317 (100 μM) or DMSO for 24 hours, and then with cholesterol accepts ApoA1 (5 μg/mL) or HDL (50 μg/mL) for a final 24 hours. The radioactivity in the media and in the cells were determined by LSC.
- **Cholesterol accumulation**: BMDMs were incubated in acetylated LDL (50 μg/mL) for 30 hours, with and without A-769662 (100 μM). Cholesterol was quantified using the Amplex Red Cholesterol Assay Kit.
- **Proinflammatory cytokine expression**: BMDMs were incubated with lipopolysaccharide (LPS; 100 ng/mL), with and without A-769662 (100 μM), for 6 hours. After RNA isolation and cDNA synthesis, quantitative PCR was performed to determine the expression of TNF-α and IL-6. ELISAs were performed to quantify cytokine secretion.

Results

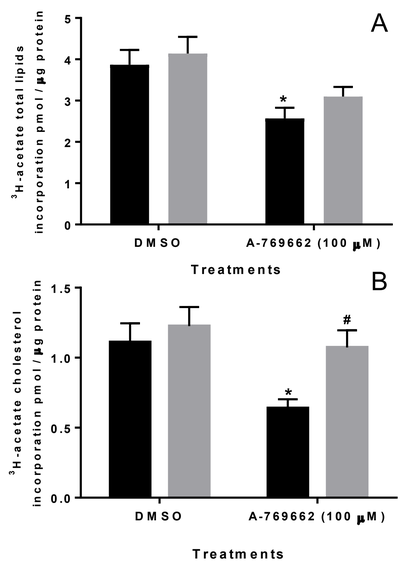

Figure 1. Lipid and cholesterol synthesis in BMDMs from WT and HMGCR KI mice. The incorporation of [^{3}H] acetate into the total lipid fraction (**A**) and cholesterol only (**B**) after isolation by TLC. Treatment of BMDMs from WT and HMGCR KI mice in the absence or presence of A-769662 (100 μM). Data represents mean ± SEM and are from at least ten separate bone marrow isolations per genotype, performed in triplicate, where *P < 0.05 are differences compared within genotype to the vehicle control and #P < 0.05 are differences within a treatment compared to the WT.

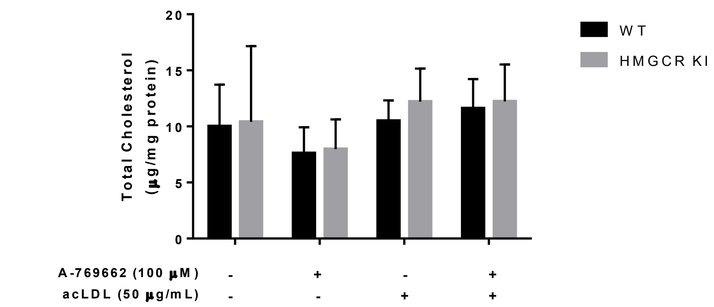

Figure 2. Cholesterol accumulation in BMDMs from WT and HMGCR KI mice. BMDMs were lipid-loaded with acLDL (50 μg/mL) for 30 hours in the presence or absence of the AMPK activator A-769662 (100 μM), after which cholesterol content was determined. Data represents mean ± SEM and are from five separate bone marrow isolations per genotype, performed in triplicate.

Results

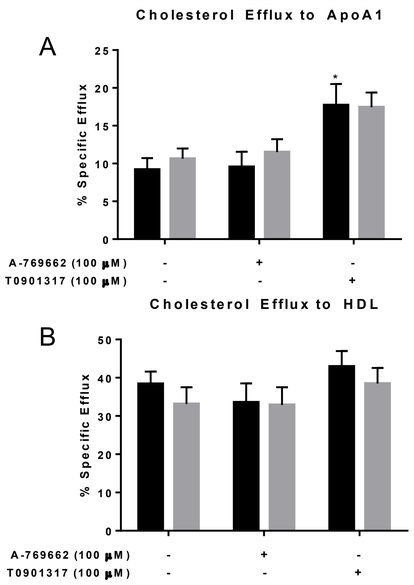

Figure 3. Cholesterol efflux in BMDMs from WT and HMGCR KI mice. BMDMs were treated with acLDL (50 μg/mL) loaded with [^{3}H] cholesterol (0.5 μCi/mL), in the presence or absence of A-769662 (100 μM). Percent specific efflux was measured to two different extracellular receptors: ApoA1 **(A)** and HDL **(B)**. Data represents mean ± SEM and are from five separate bone marrow isolations per genotype for efflux to ApoA1 and from six separate bone marrow isolations per genotype for efflux to HDL, performed in triplicate, where *P < 0.05 are differences compared within genotype to the vehicle control.

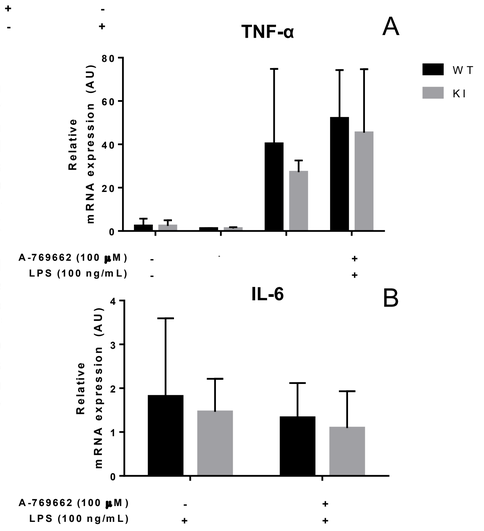

Figure 4. Gene expression of proinflammatory cytokines TNF-α and IL-6 in BMDMs from WT and HMGCR KI mice. BMDMs were treated with LPS (100 ng/mL) in the presence or absence of the AMPK activator A-769662 (100 μM). After mRNA extraction and cDNA synthesis, quantitative PCR was performed to quantify the expression of TNF-α **(A)** and IL-6 **(B)** transcripts. Data represents mean ± SEM and are from three separate bone marrow isolations per genotype, performed in duplicate.

Conclusions

- Cholesterol synthesis was higher in macrophages derived from HMGCR KI mice than WT mice when AMPK was activated.
- There were no significant differences between the between the WT and HMGCR KI macrophages in terms of total lipid synthesis, cholesterol accumulation, cholesterol efflux or proinflammatory cytokine expression.
- Therefore, the phosphorylation of HMGCR by AMPK is not physiologically significant to the development of atherosclerosis.
- Future work could examine this regulation *in vivo*.

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