Canadian Journal of Physiology and Pharmacology
Revue canadienne de physiologie et pharmacologie

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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2017-0170.R1
Manuscript Type:	Article
Date Submitted by the Author:	26-Apr-2017
Complete List of Authors:	Kamtchueng Simo, Olivier; Universite de Sherbrooke Faculte des Sciences Ikhlef, Souade; Universite de Sherbrooke Faculte de medecine et des sciences de la sante Berrougui, Hicham; Universite Sultan Moulay Slimane de Beni-Mellal, Department of biology Khalil, Abdelouahed; University of Sherbrooke
Is the invited manuscript for consideration in a Special Issue?:	IACS Sherbrooke 2016 special issue Part 1
Keyword:	Advanced glycation end products, HDL, Reverse cholesterol transport, Aging

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Advanced glycation end products affect cholesterol homeostasis by impairing ABCA1 expression on macrophages

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Abstract

Reverse cholesterol transport (RCT), which is intimately linked to high-density lipoproteins (HDLs), plays a key role in cholesterol homeostasis and the prevention of atherosclerosis. The goal of the present study was to investigate the effect of aging and advanced glycation end products (AGEs) on RCT as well as on other factors that may affect this anti-atherogenic property of HDLs. The transfer of macrophage-derived cholesterol to the plasma and liver and then to the feces for elimination was significantly lower in aged mice than in young mice. Chronic injection of D-galactose (D-gal) or AGEs also significantly reduced RCT (65.3% reduction in [^3H]-cholesterol levels in the plasma of D-gal-treated mice after 48 h compared to control mice, $P<0.01$). The injection of both D-gal and aminoguanidine hydrochloride (AMG) increased [^3H]-cholesterol levels in the plasma, although the levels were lower than those of control mice. The *in vitro* incubation of HDLs with dicarbonyl compounds increased the carbonyl and conjugated diene content of HDLs and significantly reduced PON1 paraoxonase activity (87.4% lower than control HDLs, $P<0.0001$). Treating J774 macrophages with glycated FBS increased carbonyl formation (39.5% increase, $P<0.003$) and reduced ABCA1 protein expression and the capacity of macrophages to liberate cholesterol (69.1% decrease, $P<0.0001$). Our results showed, for the first time, that RCT is altered with aging and that AGEs contribute significantly to this alteration.

Keywords: Advanced glycation end products, HDL, reverse cholesterol transport, aging

Introduction

A number of epidemiological and interventional studies have shown that there is an inverse relationship between high-density lipoprotein (HDL)-cholesterol plasma levels and the risk of cardiovascular disease (CVD), whereas low-density lipoprotein (LDL)-cholesterol levels are directly related to the rate at which CVD events occur (Assmann et al. 1992; Bennet et al. 2007; Castelli et al. 1986; Jukema et al. 2004). However, there is no causal relationship between HDL-cholesterol per se and atheroprotection, but rather HDL functionality, which cannot be reliably estimated by simply measuring HDL-cholesterol levels. HDLs play a pivotal role in the prevention of CVD through their antioxidant and anti-inflammatory activities, but the major antiatherogenic property of HDLs is their capacity to promote reverse cholesterol transport (RCT).

RCT is a process by which excess cholesterol is transported from peripheral cells to the liver and feces for elimination (Cuchel et al. 2006). The first and rate-limiting step of RCT is the efflux of cholesterol from macrophages. This step depends on the predisposition of cells to release cholesterol and on the capacity of HDLs to transport the free cholesterol (Yancey et al. 2003), which is mediated by an interaction between the ATP-binding cassette (ABCA1) transporter and lipid-poor or lipid-free apoA1 (Phillips 2014). This leads to the lipidation of apoA-1 and the generation of pre- β HDLs, which in turn are gradually transformed into mature HDLs (Haghighpassand et al. 2001). We recently showed that paraoxonase 1 (PON1), a protein exclusively associated with HDLs, significantly stimulates cholesterol efflux from macrophages via an apoA-1-like mechanism (Berrougui et al. 2012). Tward et al. showed that the overexpression of PON1 protects against atherosclerosis (Tward et al. 2002) while Ikhlef et al. recently showed that the overexpression of PON1 significantly increases RCT, indicating that RCT plays a major role in reducing the risk of atherosclerosis (Berrougui et al. 2012; Ikhlef et al. 2017). However, the impact on RCT on the age-related decrease in PON1 activity and the capacity of HDLs to mediate cholesterol efflux has never been investigated (Berrougui et al. 2007; Seres et al. 2004).

Furthermore, aging is accompanied by the accumulation of AGEs, which may have an impact on several physiological functions and induce oxidative stress conditions (Peppas et al. 2008). Elevated serum levels of advanced glycation end products (AGEs) have been associated with increased arterial stiffness and thickness (Yoshida et al. 2005) and the acceleration of atherosclerosis (Basta et al. 2004). Hedrick et al. studied the effect of glycation on the antiatherogenic properties of HDLs, especially in patients with type II diabetes in which hyperglycemia accelerates atherogenesis by increasing lipoprotein oxidation, and showed that the glycation of proteins involved in HDL metabolism may result in abnormal HDL functionality and contribute to the acceleration of atherogenesis (Hedrick et al. 2000).

Glycation is a complex series of non-enzymatic Schiff base and Amadori reactions between the carbonyl groups of reducing sugars and proteins, lipids, and nucleic acids (Goldin et al. 2006) that generate AGEs. Glycation can promote atherogenesis by oxidizing lipoproteins that can be deleterious to the integrity and function

of vessel walls (Basta et al. 2004). This process may occur on the proteinaceous and phospholipidic components of lipoproteins, increasing their susceptibility to oxidative damage (Basta et al. 2004; Bucala et al. 1995). It has also been reported that the accumulation of AGEs can cause circulating blood cells to adhere to the endothelium of the vessel wall and that AGEs can cross-bridge vessel wall macromolecules. The engagement of the receptor for AGEs (RAGE) with AGEs generates intracellular reactive oxygen species (ROS) and activates mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF- κ B) signaling and, ultimately, the production of several inflammatory factors, resulting in the progression of atherosclerosis (Fukami et al. 2014).

Little is known about the role that AGE proteins play in accelerating atherosclerosis, or about the impact of D-galactose-induced glycation on HDL functionality, especially their capacity to modulate RCT *in vivo*, the effect of aging on RCT, or the mechanisms by which age-related oxidative damage affects the ability of HDLs to maintain cholesterol homeostasis *in vivo*.

Materials and methods

Chemicals

Methanol, chloroform, n-butanol, ethanol, n-isopropanol, toluene, hexane, ethyl acetate, aqueous ammonia, Tris-HCl, calcium chloride, sodium hydroxide, glycine, glycerol, bromophenol blue, D-galactose, L-lysine, aminoguanidine hydrochloride (AMG), butylated hydroxytoluene (BHT), 2,4-dinitrophenylhydrazine (DNPH), and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (USA). J774.A1 macrophages were purchased from the American Type Culture Collection (ATCC). Cholesterol ((1, 2- ^{3} H (N) (^{3} H-cholesterol)), and Western Lightning® Plus-ECL kits were from Perkin Elmer (Canada). Dulbecco's Modified Eagle's Medium (DMEM), bovine serum albumin (BSA), fetal bovine serum (FBS), sodium pyruvate, phosphate buffered saline or PBS (NaCl 0.9%, pH 7.4), and penicillin/streptomycin were from Wisent (Canada).

Animals and treatments

Male wild-type 4-month-old (young) C57/BL6 mice were obtained from Jackson laboratories (Canada) while 24-month-old (aged) mice were obtained from the Quebec Network for Research on Aging. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Université de Sherbrooke. The mice were handled in strict accordance with Canadian Council on Animal Care guidelines. The 4-month-old mice were randomly divided into four groups of five mice. Five 24-month-old mice made up the fifth group. The mice were all given standard chow and water *ad libitum*. After a 1-week adaptation period, they were given one of the following subcutaneous injections daily for 8 weeks: 4-month-old mice (young): **a-** 0.4 mL of PBS (control group), **b-** 50 mg/kg of D-gal, **c-** 50 mg/kg of D-gal + 100 mM AMG in drinking water, **d-** 50 mg/kg of AGE-lysine (tail vein); 24-

month-old mice (aged): e- 0.4 mL of PBS. Visual examinations of the mice throughout the experiment did not reveal any health problems, and no significant differences in the weights of the mice were noted.

To measure *in vivo* RCT, the mice were placed in metabolic cages for the duration of the experiment (48 h). Feces were collected during the 48-h period. The mice were then euthanized, and serum and tissue samples were collected and were stored at -70°C until used.

Cell cultures

J774.A1 macrophages (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 10% pyruvate sodium, and 100 U/mL of penicillin/streptomycin, and were maintained in a 5% CO_2 atmosphere at 37°C .

HDL isolation

Blood samples were collected in EDTA vacuum tubes from young healthy volunteers after overnight fasting. All procedures involving human subjects were approved by the Ethics Committee of the University Institute of Geriatrics of Sherbrooke (no. 2009/19). Written informed consent was obtained from all subjects. The plasma was separated by low speed centrifugation (1000 $\times g$). EDTA (0.4 mg/mL) was added to the serum, and HDLs were isolated by ultracentrifugation using a Beckman Optima TLX ultracentrifuge equipped with a TLA-100.4 rotor as previously described (Ikhlef et al. 2016; Sattler et al. 1994). HDL concentrations were measured using protein assay kits (Bio-Rad) and were expressed as total protein concentrations.

Formation of dicarbonyl compounds

The formation of dicarbonyl compounds (Dic-C) was induced by incubating 50 mM glucose in 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 1 week under aseptic condition as previously described (Thornalley et al. 1999).

Glycation was induced by incubating HDLs and FBS in N_2 -saturated PBS containing 40 mM BHT and 50 mM Dic-C for 1 week at 37°C (Thornalley et al. 1999). FBS was glycated in the presence or absence of 100 mM aminoguanidine (AMG) followed by extensive dialysis. HDL and FBS glycation was confirmed by measuring the carbonyl content (Loued et al. 2013). Briefly, trichloroacetic acid-precipitable materials were derivatized using dinitrophenylhydrazine, and the carbonyl content was determined by measuring the absorbance at 370 nm ($\epsilon = 22\,000$ per M cm).

Measurement of paraoxonase 1 activity

PON1 paraoxonase activity was measured by recording the increase in the absorbance at 412 nm using paraoxon (O, O-diethyl-O-p-nitrophenylphosphate; Sigma) as the substrate as previously described (Loued et al. 2012). Briefly, 10 μ L of plasma was mixed with 250 μ L of 100 mM Tris-HCl buffer (pH 8.0) containing 2 mM CaCl_2 and 5.5 mM paraoxon at 25°C. The rate of 4-nitrophenol release was measured at 412 nm, and enzymatic activity was calculated using a molar extinction coefficient of 13893.75 $\text{M}^{-1}\text{cm}^{-1}$. One unit of paraoxonase activity was defined as 1 nmole 4-nitrophenol formed per minute.

Assessment of *in vitro* cholesterol efflux

Impact of glycation on HDL cholesterol efflux

J774 macrophages were labeled with [^3H]-cholesterol for 24 h, washed twice with PBS, equilibrated for 12 h in DMEM containing 0.2% BSA, and incubated with 50 mg/mL of native HDL (Control-HDL), 50 mg/mL of glycated-HDL, or 50 mg/mL of glycated-HDL + 100 mM AMG to assess cholesterol efflux.

Effect of glycation on the capacity of macrophages to liberate cholesterol

Unstimulated and stimulated J774 macrophages were incubated with 8-Br cyclic AMP to enrich them with ABCA1 transporter. They were then cultured overnight in DMEM containing non-glycated FBS (Control), glycated FBS, glycated-FBS + AMG, or AMG alone (100 mM). They were then incubated for 4 h with apoA-1 to measure ABCA1-dependent cholesterol efflux.

Cholesterol efflux was determined by measuring the radioactivity in the cells and the culture medium. The percentage of cholesterol efflux was calculated using the following formula: (radioactivity (cpm) in medium/radioactivity (cpm) in cells + medium) (Berrougui et al. 2015)

Impact of glycation on ABCA1 protein expression by J774 macrophages

To determine whether FBS glycation affects ABCA1 protein expression, ABCA1-enriched J774 macrophages were incubated for 7 h with control-FBS, glycated-FBS, glycated-FBS + AMG, or AMG alone (100 mM). The macrophages were then lysed in RIPA buffer. Identical amounts of protein were separated on 10% SDS/PAGE gels and were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free powdered milk in TBST (15 mM Tris/HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) and were incubated with ABCA1 or β -actin primary antibodies (Abcam, USA). The membranes were washed three times with TBST and were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG H&L (Abcam). Immunoreactive bands were visualized using the ECL Western blotting system (GE Healthcare, Canada). Carbonyl formation in the J774 macrophages was also measured as described above for HDLs.

***In vivo* RCT measurements**

J774 macrophages were loaded with 100 µg/mL of oxLDL and 4 µCi/mL of [³H]-cholesterol as described previously (Berrougui et al. 2012) and were equilibrated overnight. They were carefully harvested and were injected intraperitoneally into mice (~ 3.5 10⁶ dpm/mouse). Blood samples were drawn at 0 h, 6 h, 24 h, and 48 h, and [³H]-cholesterol levels in the plasma were determined by liquid scintillation counting. Feces were collected for 48 h. The mice were euthanized after 48 h, and their livers were collected. [³H]-cholesterol in 50-mg pieces of liver was extracted using the Folch method as previously described (Ikhlef et al. 2016). Fecal [³H]-cholesterol and [³H]-bile acids were extracted as previously described (Ikhlef et al. 2016). The radioactivity in the samples was counted using a liquid scintillation counter.

Macrophage RCT was assessed by monitoring [³H]-cholesterol transfer from labeled intraperitoneally injected macrophages to the plasma, liver, and feces. The results are expressed as the percentage of radioactivity recovered in the plasma, liver, and feces. The plasma volume was estimated at 3.85% of body weight.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (Graph Pad Software, Inc., USA). Data are expressed as means ± SEM. Statistical differences between two groups were assessed using the unpaired t-test. An ANOVA followed by Dunnett's multiple comparisons test was used to compare more than two groups. Statistical significance for all comparisons was set at $P < 0.05$.

Results

RCT decreases with aging

We first investigated the impact of aging on cholesterol homeostasis by measuring RCT in young (4 months old) and aged mice (24 months old) and compared our results to those obtained with the D-gal-induced aging model (8-week chronic injections of D-gal). RCT was determined by injecting [^3H]-cholesterol-labeled J774 macrophages into the peritoneal cavities of the mice in each group and measuring the [^3H]-cholesterol counts in the plasma, liver, and feces 6, 24, and 48 h later. Aged mice displayed lower plasma and liver [^3H]-cholesterol counts than the control young mice (**Fig. 1A and 1B**). The fecal excretion of macrophage-derived cholesterol by aged mice was also significantly lower than that of young mice (**Fig 1C**). [^3H]-cholesterol levels in the plasma of the D-gal-treated mice were significantly lower at all time points than those of the control mice (50.2%, 53.4%, and 65.3% lower at 6, 24, and 48 h, respectively, $P<0.008$ and $P<0.01$). [^3H]-cholesterol levels in the liver and feces were also significantly lower in the D-gal-treated mice. Interestingly, the decrease in RCT induced by D-gal was comparable to that observed in aged mice (**Fig. 1A, 1B, and 1C**).

AGE formation significantly affects cholesterol homeostasis *in vivo*

Chronic injection of D-gal, which is considered a model of accelerated aging, induced a physiological alteration similar to that seen with normal aging (Parameshwaran et al. 2010). D-gal is a physiological nutrient that reacts with the free amines of amino acids to form AGEs by non-enzymatic glycation (Parameshwaran et al. 2010). When compared to glucose, D-gal reacts rapidly with extracellular and intracellular matrix to form AGEs and induce oxidative stress through its metabolism (Zhang et al. 2005). To investigate the mechanism by which D-gal might affect cholesterol homeostasis, we measured RCT in mice that received chronic injections of D-gal alone or combined with AMG (AGE formation inhibitor) or chronic injections of AGEs alone. [^3H]-cholesterol levels in the plasma of D-gal-treated mice were significantly lower at all time points than those of control mice (50.2%, 53.4%, and 65.3% lower at times 6, 24, and 48 h, respectively, than control mice, $P<0.008$ and $P<0.01$) (**Fig. 2A**). While the combined treatment of D-gal and AMG increased [^3H]-cholesterol levels in the plasma, they remained lower than those of the control mice. Interestingly, the AGE-lysine-treated mice had lower [^3H]-cholesterol plasma levels than the control, D-gal-treated, and D-gal + AMG-treated mice (**Fig. 2A**).

[^3H]-cholesterol counts in the livers of the D-gal and AGE-lysine-treated mice were also significantly lower than those of the control mice (75% and 87.5% lower, respectively, $P<0.001$). While AMG increased [^3H]-cholesterol counts in the livers by approximately 56% ($P<0.01$), the counts were 46% lower than those of the control mice ($P<0.03$).

These results were confirmed by the [^3H]-cholesterol counts in the feces, which showed that chronic injection of D-gal or AGEs significantly reduced the fecal excretion of macrophage-derived [^3H]-cholesterol by 66.3% and 57.2%, respectively ($P < 0.03$), compared to the control mice (**Fig. 2C**). The impact of D-gal on the fecal excretion of [^3H]-cholesterol was completely reversed in the presence of AMG, which brought the levels of excreted cholesterol back to those of the control mice (**Fig. 2C**).

Hyperglycemic conditions induce oxidative modifications of HDLs

The first and rate-limiting step of RCT is cholesterol efflux from macrophages, which can be influenced by two parameters: (1) the cholesterol efflux capacity of HDLs and (2) the capacity of macrophages to liberate cholesterol (Yancey et al. 2003). We first investigated the effect of D-glucose on chemical modifications of HDLs and the extent to which this might affect HDL functionality, that is, their capacity to mediate cholesterol efflux.

Human HDLs were incubated in an N_2 -saturated solution of BHT supplemented with 50 mM Dic-C alone or 50 mM Dic-C + 100 mM AMG for 1 week. Biochemical modifications to HDLs were determined by measuring the carbonyl content of the HDLs. Incubating HDLs with 50 mM Dic-C significantly increased the carbonyl content of the HDLs (64% increase, $P < 0.001$), while AMG significantly decreased the carbonyl content of the HDLs (78.2% decrease compared to Dic-C alone, $P < 0.001$) (**Fig. 3A**).

We also measured conjugated diene formation in control HDLs, HDLs incubated with Dic-C, and HDLs incubated with Dic-C + AMG. Incubating HDLs with Dic-C resulted in a significant formation of conjugated dienes compared to control HDLs (67.3% increase in the O.D.₂₃₄, $p < 0.005$). This effect was significantly reduced in the presence of AMG (45.7% decrease, $P < 0.001$) (**Fig. 3B**).

Hyperglycemic conditions affect the functionality of HDL

To determine the impact of hyperglycemic conditions on HDL functionality, we measured HDL-associated PON1 paraoxonase activity after a 1-week incubation of HDLs with Dic-C. PON1 paraoxonase activity was reduced in the presence of Dic-C (87.36% decrease compared to control HDLs, $P < 0.0001$). This effect was significantly prevented in the presence of 100 mM AMG (50.9% decrease in PON1 paraoxonase activity compared to control HDLs, $P < 0.003$) (**Fig. 4A**). We measured the effect of D-glucose on the capacity of HDLs to mediate cholesterol efflux. J774 macrophages were radiolabeled with [^3H]-cholesterol and were incubated for 24 h with control HDLs, HDLs pretreated with Dic-C, or HDLs pretreated with Dic-C + AMG (100 mM). Surprisingly, the Dic-C treatment had no significant impact on the cholesterol efflux capacity of the HDLs (**Fig. 4**).

The glycation of macrophages affects their capacity to liberate cholesterol

The capacity of macrophages to liberate cholesterol is the second parameter that might regulate cholesterol efflux and thus RCT. To investigate the effect of glycation on the capacity of macrophages to liberate cholesterol, J774 macrophages were incubated with glycated-FBS in the absence or presence of AMG, and cholesterol efflux to apoA-1 was measured. There was a significant decrease in the capacity of macrophages treated with glycated-FBS to liberate cholesterol compared to control macrophages (69.1% reduction, $P<0.0001$) (**Fig. 5A**). This effect was significantly reduced in the presence of AMG (39.5% increase compared to glycated-FBS alone, $P<0.003$).

There was a significant increase in the carbonyl content of J774 macrophages when they were incubated with glycated-FBS (**Fig. 5B**). However, this effect was significantly reduced in the presence of AMG ($P<0.0006$). There was also a significant decrease in the expression of ABCA1 when J774 macrophages were incubated with glycated-FBS (**Fig. 5C**), and this effect was reduced in the presence of AMG (**Fig. 5C**).

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Discussion

The severity of atherosclerosis and the subsequent cardiovascular complications, including myocardial infarction, stroke, and ischemic heart failure, increase significantly with aging (Bilato et al. 1996). Atherosclerosis is now defined as a disease of aging, and aging is considered an independent risk factor for the development of atherosclerosis (Wang et al. 2012). Atherosclerosis is a progressive disease characterized by subendothelial accumulations of cholesterol-engorged macrophage foam cells that contribute to atherosclerotic plaque formation. This pathological process can be inhibited or reduced by HDLs, which possess several anti-atherogenic properties, including the capacity to mediate the transport of cholesterol from macrophages to the liver for excretion via RCT.

During aging, HDLs undergo several biochemical changes that affect their anti-atherogenic properties (Berrougui et al. 2007; Berrougui et al. 2009; Loued et al. 2013). *In vitro* studies have shown that with aging there is a reduction in cholesterol efflux, which is the first and rate-limiting step of RCT (Berrougui et al. 2007). This alteration of HDL functionality has been attributed to oxidative stress conditions that characterize the process of aging (Berrougui et al. 2007). Although the capacity of HDLs to mediate cholesterol efflux has been demonstrated *in vitro*, the impact of alterations in cholesterol efflux on *in vivo* cholesterol homeostasis, particularly on RCT, has not been elucidated. We showed, for the first time, that there is a significant reduction in all components of the RCT process with aging, including cholesterol efflux from macrophages to the plasma, the transfer of cholesterol to the liver, and its elimination in the feces.

Aging is a complex process characterized by the accumulation of oxidative damage that induces a progressive alteration of macromolecules, tissues, and organ systems (Sohal et al. 2002). AGEs accumulate with aging and have been implicated in cardiovascular risk factors such as diabetes, inflammation, and renal failure (Wu 1993). It has even been proposed that they be used as aging biomarkers (Gkogkolou et al. 2012). The accumulation of AGEs, a group of modified proteins and/or lipids, causes carbonyl stress with aging, which can trigger a series of cellular events that play a role in the acceleration of atherosclerosis (Basta et al. 2004). Previous studies have shown that chronic intravenous administration of AGEs in animals' results in vascular complications resembling those seen with aging (Vlassara et al. 1992). AGEs are formed during hyperglycemia in diabetic patients and contribute to the pathogenesis of diabetic complications (Brownlee 2001; Negre-Salvayre et al. 2009). Increasing evidence has implicated AGEs in the development of chronic degenerative diseases of aging such as Alzheimer's and cardiovascular diseases (Luevano-Contreras et al. 2010).

AGE-induced damage is due, on the one hand, to the capacity of AGEs to contribute to the formation of free radicals (Ukeda et al. 1997) and, on the other, to the capacity of AGEs to cross-bridge with proteins, altering their function (Gkogkolou et al. 2012). However, the effect of AGEs on cholesterol homeostasis and, in particular, on

RCT, a process by which HDLs protect against atherosclerotic cardiovascular disease, has not been studied. In the present study, we used the D-gal-induced aging mice model to determine the extent to which AGEs contribute to the initiation of atherosclerosis.

Chronic injection of D-gal significantly reduced the fecal elimination of macrophage-derived [^3H]-cholesterol compared to control mice. This reduction was comparable to that observed in aged mice. The inhibitory effect of D-gal on RCT could be due to the action of AGEs and/or the generation of reactive oxygen species (ROS) through the oxidative metabolism of D-gal (Song et al. 1999). AMG significantly reduced the inhibitory effect of D-gal, confirming that the alteration to RCT is due, in part, to ROS-related oxidative modifications. AGEs alone also impair RCT, which may be due to the capacity of AGEs to cross-bridge with proteins, altering their function (Gkogkolou et al. 2012). However, the results of previous studies on uremic mice, which have high plasma levels of AGEs, indicated that AGEs likely do not contribute significantly to the impairment of RCT (Low et al. 2012). In order to resolve this contradiction, we measured the impact of D-gal and AGEs on each component of the RCT process *in vitro*. We showed that the capacity of glycated HDLs to mediate cholesterol efflux is not significantly impaired compared to control HDLs. This is counterintuitive given that oxidative modifications to HDLs, as determined by the measurement of their carbonyl group and conjugated diene content, should affect their functionality and especially their capacity to mediate cholesterol efflux. However, these results were in agreement with a study by Dullaart et al., who reported that there are no significant changes in the capacity of HDLs to mediate cholesterol efflux (Dullaart et al. 2008) and a study by de Vries et al., who reported that HDLs from diabetic patients display an increased capacity to mediate cholesterol efflux (de Vries et al. 2008). On the other hand, Zhou et al. reported that the capacity of HDLs from diabetic patients to mediate cholesterol efflux is impaired (Zhou et al. 2008). It should be noted that *in vitro* measurements of cholesterol efflux depend on the type of HDLs used (purified apoA-1, total HDLs, prebeta-HDLs, or apo-B-depleted plasma) and on the cell type used (fibroblasts, THP1, Fu5AH, J774) (Rothblat et al. 1999). On the one hand, the different types of HDL do not react in the same way with membrane transporters and receptors and, on the other, the various cell types express different levels of the membrane proteins involved in cholesterol exchanges (ABCA1, ABCG1, SR-BI) (Rothblat et al. 2010). The fact that glycation had no impact on HDL cholesterol efflux in our conditions can be explained by the fact that J774 macrophages interact much less with mature HDL particles than with lipid-poor apoA-1 (Oram et al. 2000). However, HDL-associated paraoxonase activity was reduced significantly by glycation. PON1 is associated exclusively with HDLs and contributes to its antioxidant and anti-inflammatory activities (Shih et al. 1998). Recent studies have shown that PON1 also stimulates the cholesterol efflux capacity of HDLs (Berrougui et al. 2012) and that a decrease in PON1 paraoxonase activity is a marker of altered HDL functionality (Bounafaa et al. 2015). Although our results did not reveal any changes in the capacity of HDLs to mediate cholesterol efflux, the increase

in their carbonyl group content and the decrease in their paraoxonase activity confirmed that HDL functionality is altered in mice receiving chronic injections of D-gal or AGEs.

We also investigated the effect of glycation on macrophages. Pretreating J774 macrophages with D-gal had a significant impact on their capacity to liberate cholesterol via the ABCA1-apoA-1 pathway. J774 macrophages also expressed significantly less ABCA1 in the presence of D-gal. Although we did not investigate the mechanisms by which D-gal decreased ABCA1 protein expression, the results we obtained with AMG suggested that this effect is associated with ROS that are produced in the presence of high concentrations of D-gal. Our results were in agreement with those of a previous study showing that hyperglycemia down-regulates ABCA1 mRNA and protein expression and that this effect can be adduced to ROS that activate the extracellular signal-regulated kinase (ERK) signaling pathway (Chang et al. 2013). It should be noted that the decrease in ABCA1 protein expression might explain in great part the significant decrease in RCT in mice receiving chronic injections of D-gal or AGEs. Indeed, AGEs isolated from poorly controlled diabetic patients alter the ABCA1 transporter and stimulate intracellular lipid accumulation, which may contribute to the acceleration of the atherosclerosis process (Machado-Lima et al. 2013). In poorly controlled diabetics patients, AGEs impair cholesterol efflux from macrophages *in vitro* (Traldi et al. 2015). The beneficial effect of AMG in our conditions could thus be attributed to its antioxidant effect.

Our results showed that the RCT process is altered with aging, contributing to the accumulation of cholesterol and the formation of foam cells, one of the first steps in the atherosclerosis process. The alteration of RCT with aging is comparable to that induced by chronic injections of D-gal, suggesting that the alteration could be due to AGEs. AGEs affect RCT by inducing the glycation of plasma and cell proteins, including membrane cholesterol transporters, which reduces the efflux of cholesterol from macrophages and its transport to the liver and feces for elimination. The accumulation of AGEs with aging could contribute significantly to the progression of atherosclerosis and the onset of its clinical manifestations. Our results should be confirmed *in vitro* using diabetic mouse model compared to aging mice. The demonstration of the effect of AGEs on cholesterol homeostasis in diabetes and during aging should lead us to focus on strategies for reducing the formation of AGEs or inhibiting their effects.

Acknowledgment

This work was supported by grants from the Canadian Institutes of Health Research (MOP-89912 and IAO-134212).

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Figure legends

Figure 1. Reverse cholesterol transport decreases with aging

The RCT process in aged and young mice was compared by giving the mice daily injections of 0.4 mL of PBS (0.9% NaCl) (Control) or 50 mg/kg of D-Gal. J774 macrophages were radiolabelled with [^3H]-cholesterol and were injected into the intraperitoneal cavities of the mice. Radioactivity was measured in the (A) plasma, (B) liver, and (C) feces (sterols and bile acids). Data are expressed as means $\pm$ standard error of the mean (SEM). $n = 5$ mice/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. AGE formation significantly affects cholesterol homeostasis *in vivo*

Mice were injected daily with 0.4 mL of PBS (Control), 50 mg/kg of D-Gal, 50 mg/kg of D-Gal + 100 mM AMG, or 100 mM AMG alone in drinking water. J774 macrophages were radiolabelled with [^3H]-cholesterol and were injected into the intraperitoneal cavities of the mice. [^3H]-cholesterol plasma levels were measured after 6, 24, and 48 h. Feces were collected over 48 h. (A) Time course of ^3H -cholesterol distribution in the plasma. (B) ^3H -cholesterol recovery from the liver and (C) from the feces (sterols and bile acids). Data are expressed as means $\pm$ SEM. $n = 5$ mice/group. * $P < 0.05$, ** $P < 0.01$.

Figure 3. Hyperglycemic conditions induce oxidative modifications of HDL

(A) HDL carbonyl and (B) conjugated diene levels were measured following Dic-C -induced HDL glycation in the presence or not of AMG. Data are expressed as means $\pm$ SEM. $n = 5$ mice/group. *** $P < 0.001$, **** $P < 0.0001$.

Figure 4. Hyperglycemic conditions affect HDL functionality

(A) Plasma paraoxonase 1 activity and (B) cholesterol efflux from loaded J774-macrophages to HDLs were measured in glycosylated HDLs in the absence or presence of AMG. Data are expressed as means $\pm$ SEM of at least three distinct experiments *** $P < 0.001$.

Figure 5. Glycation of macrophages affects their capacity to liberate cholesterol

J774 macrophage cells were cultured with glycosylated FBS in the presence or not of AMG. (A) J774 macrophages loaded with [^3H]-cholesterol were used to assess cholesterol efflux to apo-AI for 6 h. Non-radiolabeled J774 macrophages were used to (B) to measure protein carbonyl levels and (C) to measure ABCA1 protein expression by Western blotting. Data are expressed as means $\pm$ SEM of at least three independent experiments. * $P < 0.03$, ** $P < 0.003$, *** $P < 0.0006$, **** $P < 0.0001$.

Figure 1

Figure 2