Synergistic Effects of High Blood Cholesterol and Hypertension on Leukocyte and Platelet Recruitment in the Cerebral Microcirculation

Stephen F. Rodrigues, Lidiana D. Almeida-Paula, Daniel N. Granger

Abstract—Hypertension or hypercholesterolemia can induce a proinflammatory and prothrombogenic phenotype in the microcirculation of the brain; however, less is known about how the combination of these risk factors affects the vasculature. We recently reported that a moderate (60%) increase in plasma cholesterol blunts the recruitment of leukocytes and platelets in the cerebral microcircuits elicited by hypertension. In this study, we examined whether larger increments in blood cholesterol (4-fold) exerts a similar modulating influence on the vasculature in the presence of hypertension. Apolipoprotein E–knockout mice with deoxycorticosterone acetate salt–induced hypertension were placed on a high-cholesterol diet and exhibited exaggerated leukocyte and platelet adhesion responses in cerebral microvessels. Intermittent feeding (every fourth day) with high-cholesterol diet yielded similar phenotypic changes in the vasculature. Once the mice were placed on high-cholesterol diet, 4 days on normal diet (ND) were needed to revert to a normal vascular phenotype. Angiotensin II type 1 receptors and reactive oxygen species seem to contribute to the vascular responses induced by hypercholesterolemia and hypertension. Our findings indicate that the combination of hypertension and large increases in plasma cholesterol concentration results in a severe, but reversible, inflammatory and thrombogenic phenotype in the cerebral microvasculature. (Hypertension. 2014;63:747-752.) • Online Data Supplement

Key Words: hypertension • hypercholesterolemia • inflammation

Cardiovascular disease (CVD) continues to represent the major cause of death worldwide, accounting for >17 million deaths in the past year.1 Extensive research on this problem has led to the identification of several factors that increase the risk for development of CVD. These include hypertension (HTN), aging, obesity, diabetes mellitus, hypercholesterolemia (HCh), smoking, and physical inactivity.2–7 Epidemiological studies have revealed that the risk for CVD increases significantly with the presence of ≥2 risk factors. For example, the combination of HTN and HCh promotes, at a rate that is greater than with either risk factor alone, the development of atherosclerosis, which can ultimately lead to myocardial infarction and stroke.

Although the diverse nature of risk factors for CVD would suggest different underlying mechanisms for the induction of disease, the similarity of responses of the vasculature to these risk factors suggests otherwise. Inflammation, oxidative stress, diminished NO bioavailability, and enhanced thrombogenesis are characteristic features shared by most of the CVD risk factors. In cerebral microcirculation, both HTN and HCh result in enhanced recruitment of adherent leukocytes and platelets,8–11 impair blood–brain barrier (BBB) function,10–12 and alter vaso- motor function.13–15 Although the impact of individual risk factors (eg, HTN versus HCh) on vascular or organ function has been extensively studied, less attention has been devoted to defining how combinations of risk factors influence these target tissues. Given the shared actions of risk factors on the vasculature, it would seem likely that a combination of risk factors should produce additive or synergistic responses. However, we recently demonstrated that diet-induced HCh, with a moderate increase in blood cholesterol concentration (from 70 to 110 mg/dL), blunts, rather than exacerbates, the proinflammatory and prothrombogenic responses of the cerebral microvasculature to HTN.16 Whether higher levels of blood cholesterol would also exert a moderating influence on the proinflammatory and prothrombogenic responses of the cerebral vasculature to HTN remains unclear. A major objective of this study was to address this issue. In addition, we evaluated the effects of angiotensin II type 1 receptor (AT1r) blockade (losartan) and superoxide scavenging (with tempol) on cerebral microvascular responses to the combination of HCh and HTN.

Methods

Animals

Male apolipoprotein E–knockout (ApoE-KO) mice (B6.129P2- Apeo tm1Unc/J) were obtained from Jackson Laboratories, Bar Harbor, ME. The mice (a total of 110) were housed under specific pathogen-free conditions and fed standard laboratory chow and water before entering the study. All the experimental procedures using

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animals were reviewed and approved by the Institutional Animal Care and Use Committee of LSU Health Sciences Center and performed according to the criteria outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Control and Experimental Groups
After 2 days of acclimatization, under ketamine (150 mg/kg) plus xylazine (7.5 mg/kg) intraperitoneal anesthesia (≈100 µL per mouse) the left kidney was removed from all mice (6–8 weeks old), except 1 group (intact group) that was not subjected to any surgical or pharmacological intervention. After surgery, the uninephrectomized (Uni) mice were randomly assigned to the following experimental groups: control mice fed normal chow diet (Uni ApoE-KO); mice fed (3 weeks) a high-cholesterol diet (HCD; Uni ApoE-KO plus HCD); deoxyxycorticosterone acetate (DOCA)-salt hypertensive mice fed normal chow diet (Uni ApoE-KO plus DOCA-salt) or HCD (3 weeks; Uni ApoE-KO plus DOCA-salt plus HCD; n=6–8 per group). A slow-release DOCA pellet (50 mg; 21-day release; Innovative Research of America, Sarasota, FL) was inserted subcutaneously in the DOCA-salt groups, and drinking water was replaced with 1% NaCl/0.2% KCl solution. Nonhypertensive mice received tap water. HCD (TD.94059; Teklad, Indianapolis, IN) contained (in grams per kilogram): casein, 75.0; dextrose, monohydrate, 30.0; sucrose, 16.25; dextrin, 16.25; cocoa butter, 75.0; cholesterol, 12.5; cellulose, 12.5; mineral mix (AIN-76; 170915), 8.75; vitamin mix (Teklad; 40060), 2.5; and choline chloride, 1.25. Total body weight was determined before and at the end of experimental routine. Peripheridymal fat pad weight was measured by the end of experiments. Liquid (consumed >24 hours) and food intake (consumed >24 hours per 100 g body weight) were measured 10 days after uninephrectomy.

Two Uni ApoE-KO plus DOCA-salt plus HCD groups were treated with either AT1 antagonist losartan (Cozaar; Merck & Co, Whitehouse Station, NJ) or membrane-permeable antioxidant 4-hydroxy-TEMPO (tempol; Sigma-Aldrich, St Louis, MO) in drinking solution beginning just after DOCA pellet implantation (n=5–6 per group) for 21 days (see the online-only Data Supplement for details). Additional experiments were performed to assess the influence of intermittent feeding of cholesterol-enriched diet on Uni ApoE-KO plus DOCA-salt mice. Some mice received a ND and cholesterol-enriched diet on alternate days for 3 weeks (Uni ApoE-KO plus DOCA-salt plus 1 HCD/1 ND). In another series of experiments, mice were placed on ND for 3 days followed by 1 day on cholesterol-enriched diet over a period of 3 weeks. Experiments were performed 1 (Uni ApoE-KO plus DOCA-salt plus 1 HCD/3 ND; first day), 2 (Uni ApoE-KO plus DOCA-salt plus 1 HCD/3 ND; second day), or 4 (Uni ApoE-KO plus DOCA-salt plus 1 HCD/3 ND; fourth day) days after the last high-cholesterol intake (n = 4–7 per group). A comparison of these groups allows for an assessment of the reversibility of phenotypic changes induced by HCD.

Blood Pressure Measurement
Blood pressure (BP) was measured in nonanesthetized mice by tail plethysmography using a pressure analysis system (model SC-1000; Hatteras Instruments, Cary, NC). Mice were placed on a heated (40°C) platform and a cuff was placed around the tail and inflated for a period of 60 seconds to record systolic BP. The average of 5 successive measurements was used as systolic BP for each animal. Animals were previously trained for 4 consecutive days before final measurements were taken.

Animal Preparation for Microscopy
Mice were anesthetized with intraperitoneal ketamine (150 mg/kg) and xylazine (7.5 mg/kg; ≈100 µL per mouse). The left femoral vein was cannulated for intravenous administration of Rhodamine 6G, labeled platelets, and supplemental doses of anesthetics. Body temperature was maintained at 36°C during the experiment and monitored with a rectal temperature probe. After skull fixation, a circular skin incision was made, and a craniotomy was created 3 mm lateral and 2 mm posterior to the bregma. The exposed brain tissue was immersed in an artificial cerebrospinal fluid and covered with a glass slide. Cerebral vessels were observed through the dura mater.

Intravital Videomicroscopy
The procedures used to monitor blood cell–vessel wall interactions in murine cerebral venules are described elsewhere in detail. A brief description of this method is available in the online-only Data Supplement.

Brain Water Content
Brain was removed, stripped of dura mater and cerebellum, and divided into 2 hemispheres. Each hemisphere was placed into a 60°C oven for 3 days to achieve complete desiccation. Water content was determined by (wet weight–dry weight)/wet weight, and expressed as percentage.

BBB Dysfunction
BBB permeability was assessed using the Evans blue extravasation method. This procedure is summarized in the online-only Data Supplement.

Serum Cholesterol Levels
At the end of experiments, blood was drawn from the tail vein, centrifuged, and plasma was frozen for subsequent measurement of cholesterol levels, using a spectrophotometric assay kit (Stanbio Laboratory, Boerne, TX).

Statistical Analysis
All data were expressed as mean±SE. Statistical difference between the groups was determined by a 1-way ANOVA with the Tukey post hoc test. All analyses were performed using Prism 5 software (GraphPad Software, Inc). Statistical significance was set at P<0.05.

Results
Body Weight, Peripheridymal Fat Pad Weight, Liquid and Food Intake, BP, and Plasma Cholesterol Concentration Responses to DOCA-Salt HTN, With or Without Placement on a HCD
Although body weight was similar among all groups before surgery (data not shown), it was increased only in HTN plus HCD, compared with HTN plus ND, 3 weeks after nephrectomy (Figure S1A in the online-only Data Supplement). Body weight did not differ statistically between all groups and the Uni group (Figure S1A). However, peripheridymal fat pad weight was reduced in HTN versus normotensive mice, and HCD did not modify this response (Figure S1B). Although food intake was reduced in HTN versus Uni mice, liquid consumption was increased (Figure S1C and S1D). HCD partly reversed these changes in HTN mice. Food intake and liquid consumption were similar in normotensive mice fed HCD or ND (Figure S1C and S1D).

A 40% increase in BP was evidenced in Uni ApoE-KO plus DOCA-salt mice, when compared with control groups (intact ApoE-KO or Uni ApoE-KO; Figure 1A). Placement on a HCD did not change the BP of Uni ApoE-KO mice (Figure 1A). However, BP was significantly reduced in Uni ApoE-KO plus DOCA-salt mice after HCD (Uni ApoE-KO plus DOCA-salt plus HCD; Figure 1A). Plasma cholesterol concentration was increased 4-fold in ApoE mice placed on HCD, compared with their ND counterparts (Figure 1B). Most of the increase in plasma cholesterol resulted from an increase in HDL cholesterol (Figure S2A). No change in plasma non-HDL cholesterol was observed in any group tested (Figure S2B). HTN, per se, was not associated with a change in plasma cholesterol concentration, and the magnitude of increase in plasma cholesterol...
concentration in HCD fed mice was not affected by DOCA-salt HTN (Figure 1B).

Leukocyte and Platelet Adhesion Responses in Cerebral Venules of DOCA-Salt Hypertensive Mice Plus/Minus HCD
Placement of both Uni ApoE-KO and Uni ApoE-KO plus DOCA-salt mice on HCD enhanced the recruitment of adherent leukocytes (Figure 1C) and platelets (Figure 1D) in cerebral venules, compared with controls (intact ApoE-KO, Uni ApoE-KO). When placed on ND, Uni ApoE-KO plus DOCA-salt mice did not exhibit increased blood cell recruitment above that of controls. The most profound changes in blood cell recruitment were noted in Uni ApoE-KO plus DOCA-salt mice placed on HCD (Figure 1C and 1D).

Changes in Brain Water Content and BBB Permeability
Figure S3 summarizes the changes in brain water content (Figure S3A) and Evans blue extravasation (Figure S3B), a measure of BBB permeability, in different experimental groups. The results indicate that neither DOCA-salt HTN, HCh, nor the combination of the 2 risk factors altered brain water content and BBB permeability.

Role of AT1r and Reactive Oxygen Species in BP and Cerebral Microvascular Responses to DOCA-Salt HTN or HCD
Losartan, but not tempol, prevented the reduction of BP observed in mice placed on HCD. However, both losartan and tempol were effective in blunting the exacerbated recruitment of leukocytes and platelets in cerebral venules (Figure 2B and 2C).

Responses to Intermittent Feeding of HCD in ApoE-KO Mice With DOCA-Salt HTN
Uni ApoE-KO plus DOCA-salt mice placed on ND for 3 days followed by 1 day on HCD over a period of 3 weeks showed changes in BP, plasma cholesterol concentration, and recruitment of adherent leukocytes and platelets that are comparable (and not significant from) to the responses noted with daily HCD over the same time period (Figure 3). A reversal of phenotypic changes in these variables was noted 2 to 4 days after removing the mice from cholesterol-enriched diet. A complete
reversal of blood cell adhesion responses was noted on the fourth day of withdrawal from HCD, that is, the adhesion responses did not differ from the responses observed in Uni ApoE-KO mice on normal chow. However, BP was significantly reduced compared with Uni ApoE-KO plus DOCA-salt, except on the fourth day of withdrawal from HCD, and plasma cholesterol levels remained elevated above the levels detected in Uni ApoE-KO mice in all HCD-fed groups.

Discussion
Although it is well known that CVD is more commonly manifested in individuals with ≥2 risk factors, relatively few animal studies have addressed the influence of risk factor combinations on the development of vascular dysfunction and tissue injury. We recently reported that modest increases in plasma cholesterol concentration (from 70 to 110 mg/dL) dampen the proinflammatory and prothrombogenic phenotype that is assumed by the cerebral microvasculature in mice with either angiotensin II- or DOCA-salt–induced HTN. In this study, we addressed whether the protective effect of HCh in hypertensive mice is also evidenced in the presence of a larger increase in plasma cholesterol concentration (=940 mg/dL), achieved by placing ApoE-KO mice on HCD. Our findings reveal that, in the presence of a large increase in plasma cholesterol concentration, the combination of HCh and HTN results in greatly exacerbated inflammatory and thrombogenic responses in cerebral microvasculature. We also demonstrated that intermittent HCD, that is, every fourth day, results in a similar level of cerebral microvascular dysfunction as produced by daily HCD. However, the exaggerated recruitment of leukocytes and platelets noted in these animals subsides <4 days after returning to ND. In addition, we obtained evidence to implicate AT1r and reactive oxygen species (ROS) as mediators of the intense proinflammatory and prothrombogenic responses elicited by the combination of HTN and HCh.

HTN and HCh have been previously shown to independently induce a proinflammatory and prothrombogenic phenotype in cerebral microvasculature. In this study, we observed that plasma cholesterol levels, mostly HDL-cholesterol, increased ≈4-fold when ApoE-KO mice were placed on HCD (compared with ND), and this response was accompanied by an increased recruitment of adherent leukocytes and platelets in cerebral venules. Increases in HDL-cholesterol after HCD have been previously described in ApoE-KO mice. However, although plasma cholesterol was increased to a similar extent in both normotensive and hypertensive ApoE-KO mice after placement on HCD, many more leukocytes and platelets were recruited in the microvasculature in the presence of both risk factors. A similar exacerbation of vascular dysfunction has also been described in reports that address impaired vasoconstrictor function that results from the combination of HTN and HCh, compared with either risk factor alone.

The mechanism(s) that underlie intense leukocyte and platelet recruitment in cerebral microvessels in mice with HTN and severe HCh seems to involve the activation of AT1r and the production of ROS. This assertion is based on our observation that both losartan and tempol were effective in reducing blood cell recruitment responses elicited by the risk factor combination. Both AT1r and ROS have been previously implicated in proinflammatory and prothrombogenic responses observed in cerebral microvessels of different animal models of HTN and HCh. AT1r and ROS have also been implicated in accelerated atherogenesis that results from the combination of HTN and HCh, and vasomotor dysfunction associated with this combination of risk factors. The comparable effectiveness of losartan and tempol in blunting blood cell recruitment elicited by HTN plus HCh is consistent with the view that ROS is a critical component of angiotensin II signaling via AT1r. Hence, our results suggest that the combination of HTN and severe HCh leads to AT1r activation, subsequent generation of ROS, and consequent induction of a proinflammatory and prothrombogenic phenotype in cerebral microvasculature. However, our observation that neither losartan nor tempol treatment afforded complete protection against the blood cell recruitment response to HTN suggests the involvement of other mechanisms and mediators.

An interesting observation in the present study was the more rapid disappearance of blood cell adhesion response than the fall in plasma cholesterol level after removal of cholesterol.
from the diet (Figure 3). This could indicate that vascular responses (eg, adhesion molecule expression) elicited by HCh (or mediators released by it) show an off-response that does not parallel the decline of plasma cholesterol concentration. It could also indicate that a threshold level of elevated cholesterol must be achieved to manifest these changes, and once the cholesterol level falls below the threshold value, then the stimulus for adhesion is dissipated. Finally, it may simply reflect that some other variable related to cholesterol (eg, LDL or HDL concentration), rather than total cholesterol concentration, is driving the response.

Despite the intense recruitment of leukocytes and platelets elicited in normotensive and hypertensive mice by HCh, no alteration of BBB function was detected during either HTN or HCh. We previously reported that hypercholesterolemic C57Bl/6j mice do not exhibit altered BBB function and now demonstrated the same outcome in ApoE-KO mice. However, cholesterol-induced BBB breakdown may depend on the animal model, composition of HCh diet, or duration of HCh diet.

BBB disruption has been detected in New Zealand rabbits fed a HCD,12 in ApoE-KO mice fed a Western diet containing more cholesterol-induced BBB breakdown may depend on the animal model, composition of HCh diet, or duration of HCh diet. The precise mechanism underlying the reduction in BP remains unclear, our finding of a reversal of the BP lowering effect of HCh in mice receiving losartan suggests that AT1r is activated as tumor necrosis factor (TNF)-α in the presence of cholesterol and releases vasodilators, such as nitric oxide. The mechanism of this action remains unclear, but it may be related to the release of AT1r from the endothelial cell surface, which is known to be regulated by TNF release.37

References
What Is Relevant?

- AT1r blockers and antioxidants may reduce the deleterious impact of the combination of HTN and high cholesterol levels.

Summary

These findings indicate that this risk factor combination results in a severe, but reversible, inflammatory and thrombogenic phenotype in cerebral microvasculature, which can be mimicked by intermittent high-cholesterol intake, and that targeting AT1 receptor activation and generation of reactive oxygen species may prove beneficial in reducing the risk of cardiovascular diseases that accompany hypertension and hypercholesterolemia.
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ON LINE SUPPLEMENTAL DATA

Synergistic effects of high blood cholesterol and hypertension on leukocyte and platelet recruitment in the cerebral microcirculation.

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Supplemental Methods-

Tempol and losartan treatments:
Two Uni ApoE-KO + DOCA-salt + HCD groups were treated with a solution containing losartan or tempol. In order to do that, losartan and tempol were dissolved in a 1% saline/0.2% potassium chloride drinking solution to achieve concentrations of 0.5 mmol/L and 1 mmol/L, respectively. The drinking water bottle in each mouse cage was wrapped in aluminum foil to prevent photo-degradation. Fresh losartan or tempol solutions were added to the bottle every other day.

Intravital Videomicroscopy:
The cerebral microcirculation was visualized with an upright fluorescent microscope using a 20x water immersion lens. Color images were captured with a 3 charge coupled device (CCD) color video camera. Randomly selected segments of pial venules (20 to 70 µm diameter, 100 µm long) were chosen for observation. Approximately 100 x 10⁶ platelets were isolated from donor mice, labeled (green) ex vivo with carboxyfluorescein diacetate succinimidyl ester (2), and administered to recipient mice through the left femoral vein (this extracorporeal staining produces minimal activation of platelets) (3). This was followed by the continuous infusion of 0.02% rhodamine 6G, which was used to fluorescently label (red) circulating leukocytes. Adherent leukocytes and platelets were defined as cells remaining stationary within venules for 30 seconds. Cell adhesion data are expressed as number of cells per millimeter squared of venular surface, calculated from venular diameter and length, assuming cylindrical geometry.

Blood–Brain Barrier Dysfunction:
A 2% solution of EB (Sigma-Aldrich) was injected (4 mL/kg) into the femoral vein. Twenty-four hours later, 0.4 mL of blood was obtained by cardiac puncture, and then the mouse was transcardially perfused with phosphate-buffered saline (100 mmHg) for 5 minutes. Brain was removed and separated from the dura mater and cerebellum. The cerebrum was divided into 2 hemispheres, each of which was homogenized and sonicated in 1 mL of 50% trichloroacetic acid (Sigma-Aldrich) and centrifuged at 10,000 rpm for 20 minutes. The supernatant was diluted with ethanol and the concentrations of EB in brain tissue and plasma were measured using a fluorescence spectrophotometer (FLUOstar Optima microplate reader; BMG LABTECH, Inc). BBB permeability was determined by dividing tissue EB concentration (µg/g brain weight) by the EB plasma concentration (µg/g). Hence, the resulting Evans blue value is unitless.
References:


Figure S1. Effects of feeding a cholesterol-enriched diet + DOCA-salt induced hypertension on body weight (panel A), perirenal fat pad weight (panel B), food (panel C) and liquid intake (panel D), Uninucleated ApoE-KO (Uni ApoE-KO, n=5), Uni ApoE-KO fed daily a cholesterol-enriched diet (Uni ApoE-KO + HCD, n=6), Uni ApoE-KO with DOCA-salt treatment (Uni ApoE-KO + DOCA-salt, n=10), and Uni ApoE-KO DOCA-salt fed daily the cholesterol enriched diet (Uni ApoE-KO + DOCA-salt + HCD, n=11) were tested. * P < 0.05 vs. Uni ApoE-KO; ** P < 0.01 vs. Uni ApoE-KO; $ P < 0.05$ vs. Uni ApoE-KO + HCD. ANOVA was used for statistical analysis.
Figure 52. Effects of feeding a cholesterol-enriched diet + DOCA-salt-induced hypertension on plasma HDL cholesterol (panel A) or non-HDL cholesterol (panel B). Intact ApoE-KO (n=5), uninephrectomized ApoE-KO (Uni ApoE-KO, n=3), Uni ApoE-KO fed (daily) a cholesterol-enriched diet (Uni ApoE-KO + HCD, n=3), ApoE-KO with DOCA-salt treatment (Uni ApoE-KO + DOCA-salt, n=3), and Uni ApoE-KO DOCA-salt fed daily the cholesterol-enriched diet (Uni ApoE-KO + DOCA-salt + HCD, n=6) were tested. *P < 0.05 vs. Intact ApoE-KO; **P < 0.05 vs. Uni ApoE-KO; ***P < 0.05 vs. Uni ApoE-KO + DOCA-salt. ANOVA was used for statistical analysis.
Figure 33. Effects of feeding a cholesterol-enriched diet ± DOCA-salt induced-hypertension on brain water content (panel A) and Evans blue extravasation (panel B). Intact ApoE KO (n=6), uninephrectomized ApoE KO (Uni ApoE KO, n=4), Uni ApoE KO fed daily a cholesterol-enriched diet (Uni ApoE-KO + HCD; n=5), Uni ApoE-KO with DOCA-salt treatment (Uni ApoE-KO + DOCA-salt, n=3), and Uni ApoE-KO DOCA-salt fed daily the cholesterol-enriched diet (Uni ApoE-KO + DOCA-salt + HCT, n=5) were tested.