Myocardial infarction mediated by endothelin receptor signaling in hypercholesterolemic mice

(atherosclerosis/cholesterol/gene deletion/hypoxia/stress)

GIUSEPPINA CALIGIURI*, BERNARD LEVY†, JOHN PERNOW‡, PETER THORÉN§, AND GÖRAN K. HANSSON*¶

*Center for Molecular Medicine, Department of Medicine, ‡Cardiology Section, Department of Medicine, and §Department of Pharmacology and Physiology, Karolinska Institutet, SE-17176 Stockholm, Sweden; and †Institut National de la Sante´ et de la Recherche Me ´dicale, Unit 141, Hoˆpital Lariboisie`re, 75475 Paris, France

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ABSTRACT Myocardial infarction is linked to atherosclerosis, yet the sequence leading from silent coronary atherosclerosis to acute myocardial infarction has remained unclear. Here we show that hypercholesterolemic apolipoprotein E −/−/low density lipoprotein receptor−/− mice develop not only coronary atherosclerosis but also myocardial infarction. Exposure of mice to mental stress or hypoxia led to acute ischemia, which, in a large proportion of the mice, was followed by electrocardiographic changes, leakage of troponin T, and loss of dehydrogenase from the mycardium, all indicative of acute myocardial infarction. Apoptotic death of cardiomyocytes was followed by inflammation and fibrosis in the heart. All these pathological changes could be prevented by a blocker of the endothelin type A receptor. Thus, stress elicits myocardial infarction through endothelin receptor signaling in coronary atherosclerosis caused by hypercholesterolemia.

Myocardial infarction remains the most common cause of death in the Western world and is increasing also in the developing world (1). It is almost invariably associated with coronary atherosclerosis, but the mechanisms leading from atherosclerosis to infarction have remained obscure (2, 3). Murine models of atherosclerosis recently have been developed by deletion of genes regulating cholesterol metabolism (4). Deletions of the genes for apolipoprotein E (apoE; refs. 5 and 6) and the low density lipoprotein receptor (7) result in severe atherosclerosis, with the formation of lesions strikingly similar to those found in humans (8–10). Studies using these models show that atherosclerosis is initiated by hypercholesterolemia, which leads to cholesterol accumulation in the artery wall, vascular inflammation with local activation of macrophages and T cells, and a fibrotic response involving smooth muscle cells (8–12).

Abnormalities and cardiac electrophysiology in the laboratory mouse were studied in pilot experiments. All experiments complied with national guidelines and were approved by the regional ethical committee.

ECG. For the mental stress experiments, continuous telemetric ECG monitoring from freely moving mice was obtained as described (16, 19). Briefly, we used a telemetry system (Datasciences, Minneapolis) comprising a transmitter implanted into the peritoneal cavity and a receiver (RA1010) placed underneath the home cage. The signal from the transmitter was acquired digitally, and the electrical activity was computer-analyzed. During hypoxic stress, ECG was recorded by using s.c. bipolar limb leads in anesthetized mice (isoflurane, 1.2 ± 0.2%). Instant ECG was acquired and analyzed digitally (17). The ischemic burden was calculated as the STU area, i.e., the area covered by the STU (repolarization) segment shift from the isoelectric line. The latter was calculated automatically as the mean level in the interval 5–20 msec preceding the Q wave.

Stress Tests. Mental stress. A method developed previously for rats (20) was adopted for mice (17). Mice were implanted 5 days ahead with the telemetric transmitters, and continuous ECG monitoring (ECG averaging and recording every 30 min) lasted 10 days. On days 3 and 9, air was forced into the cage for 30 min (mental stress, ECG averaging and recording every 2 min).

Hypoxic stress. Under isoflurane anesthesia, the mixture of O2 and N2 in the ventilatory mask was modulated and monitored continuously with the help of a spirometer. The percentage of oxygen was modified manually starting at 21% and

Abbreviations: apoE, apolipoprotein E; LDL, low density lipoprotein; LDLR, LDL receptor; E0 × LDLR0, apoE−/− × LDLR−/− mice; TTC, triphenyl tetrazolium chloride; ETA receptor, endothelin type A receptor.

*To whom reprint requests should be addressed at: Center for Molecular Medicine L8:03, Karolinska Hospital SE-17176 Stockholm, Sweden. e-mail: Goran.Hansson@cmm.ki.se.
reducing it to 16, 14, 12, and 10% (2 min each step). Recovery was achieved by reestablishing O₂ at 21%, and ECG was monitored continuously during the acute hypoxia and continued for 20 min after O₂ restoration. A subgroup of mice received the endothelin type A (ETₐ) receptor blocker LU135252 (21) (kindly provided by M. Raschack, Knoll, Ludwigshafen, Germany). A stock solution was dissolved in 1 M NaOH/150 mM NaCl, and the pH was adjusted to 7.5 with 100 mM HCl. The stock solution was diluted further in 150 mM NaCl and injected i.p. at 1 mg/kg of body weight 15 min before hypoxic stress.

**Echography.** During hypoxic stress, anesthetized mice also were monitored by an Echo-Doppler probe (PW, 9 MHz) fixed on the right sternal border. Two-dimensional mode was used to localize the aortic root, whereas PW-Doppler mode was used to measure the aortic pulse peak as an index of cardiac pump function.

**Serum Analyses.** Peripheral blood was obtained at sacrifice. Serum was harvested and analyzed for troponin T by using a standard immunochemical assay (Boehringer Mannheim). Serum cholesterol was determined by using a Cobas Mira (Roche Diagnostics) analyzer. Lipoproteins were fractionated by fast protein liquid chromatography through Superose-6 (Amersham) (22).

**Perfusion and Angiography.** Under anesthesia, animals were sacrificed by exsanguination. The heart was perfused from the apex of the left ventricle with ice-cold PBS for 1 min at a constant pressure of 100 mmHg (1 mmHg = 133 Pa) followed by triphenyl tetrazolium chloride (TTC) (23) perfusion for 1 min. In a subset of mice, barium was injected into the coronary system at constant pressure through a cannula placed in the thoracic aorta and the heart was fixed in PBS/4% formaldehyde. Coronary angiograms were taken with a microangiographic technique by using a highly focused x-ray source and a digital radiologic acquisition system (24).

**Microscopic Analysis.** One-millimeter sections of TTC-stained, formaldehyde-fixed hearts were checked for loss of staining (TTC stains dehydrogenase activity in living tissue) and formaldehyde-fixed hearts were checked for loss of deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) analysis of apoptosis.

### Results

**Coronary Atherosclerosis and Spontaneous Myocardial Infarction in Hypercholesterolemic Mice.** Atherosclerosis-prone E₀ × LDLR₀ male mice (7) were fed a “Western” diet containing 0.15% cholesterol (18). This led to severe hypercholesterolemia with elevated very low density lipoprotein (VLDL) and LDL fractions. Serum cholesterol levels at 8–9 months of age were 26.3 ± 5.2 mmol/liter in E₀ × LDLR₀ mice, which was not significantly different from those in E₀ single-knockout mice (29.3 ± 5.0 mmol/liter). As expected (7, 25), both E₀ and E₀ × LDLR₀ but not wild-type C57BL/6J mice showed prominent lipoprotein peaks in the VLDL range, and E₀ × LDLR₀ mice but not E₀ single-knockout mice had additional peaks in the LDL range (data not shown). Both E₀ and E₀ × LDLR₀ mice showed advanced aortic atherosclerosis (7, 8, 26), and increased mortality was observed in E₀ × LDLR₀ mice older than 7 months. To evaluate whether the coronary tree was affected by disease, we performed postmortem coronary angiography on E₀ × LDLR₀ mice (Fig. 1a and b). It showed severe reduction in the lumen diameter of all three main coronary arterial branches in all mice and total occlusion in 28 of 36 mice. Typically, stenoses were most severe in the proximal segments of the coronary arteries (Fig. 1b).

Histologic analysis of heart cross sections (Fig. 1c) confirmed the presence of advanced atherosclerotic plaques in the coronary arteries of E₀ × LDLR₀ mice (Fig. 1d) as well as E₀ mice (data not shown). Myocardial scars were detected around occluded coronary arteries, suggesting that they were caused by myocardial infarction (Fig. 1e). These findings suggest that myocardial infarction can occur as a consequence of coronary atherosclerosis in genetically hypercholesterolemic mice.

**Mental Stress Elicits Myocardial Infarction in Mice with Coronary Atherosclerosis.** To test whether myocardial infarction could be induced experimentally in freely moving atherosclerotic mice, we subjected awake mice to a mental stress test. Air was forced into the cage, causing a mental stress that leads to increased heart workload in mice (17). The ECG was recorded through a s.c. transmitter implanted 1 week in advance (19). E₀ × LDLR₀ mice older than 7 months showed small deviations of the repolarization ECG segment that were suggestive of myocardial ischemia (27) already at rest (Fig. 2). These deviations were increased substantially during the stress test (Fig. 2). Pathological ECG patterns occurred within 1 min...
after initiation of the stressful stimulus and remained for 12 ± 7 min. They were never found in younger E0 × LDLR0 mice or age-matched wild-type controls.

We also analyzed serum levels of cardiac troponin T, a contractile protein that leaks out of injured cardiomyocytes (28) and is used commonly as a sensitive assay to detect myocardial infarction in humans (29). Troponin T levels were elevated significantly after mental stress in atherosclerotic mice and not in controls, suggesting that the transient ECG changes observed in the atherosclerotic mice during the stress test were due to myocardial infarction (Fig. 2). A subset of mice (14/32, 44%) with more prolonged ECG changes (>20 min) showed large infarcted zones with loss of dehydrogenase activity from the myocardium (Fig. 2). Together, the results of these experiments show that stress can cause myocardial infarction in mice with coronary atherosclerosis.

**Hypoxic Stress Induces Myocardial Ischemia Followed by Infarction.** A fully standardized induction of myocardial infarction was deemed necessary to dissect the pathogenetic mechanism, and, therefore, we exposed anesthetized, artificially ventilated mice to global hypoxia by reducing the oxygen supply. Hypoxia causes reflex coronary vasoconstriction (30), which may result in dynamic coronary occlusion in the presence of severe atherosclerotic stenoses. Whereas age-matched wild-type mice did not show ECG changes or echographic signs of reduced myocardial contractility even at very low oxygen levels (down to 8%), atherosclerotic mice showed typical ECG deviations within 2 min after reduction of O2 below 12% (Fig. 3). Echocardiographic analysis revealed reduced (22 ± 7%) peak aortic blood flow velocity during the hypoxic stress, probably reflecting depressed left ventricular function caused by massive ischemia (31).

In most mice, ECG deviations disappeared upon cessation of hypoxia, and only 33% of them exhibited persistent ECG changes at 8 h after reoxygenation. However, a total of 31 of 57 mice (54%) developed altered QRS complexes in the myocardium, which is diagnostic for myocardial infarction in humans. Box-and-whisker plot analysis of plasma troponin T, which was elevated (>0.2 μg/liter) at 72 h through to 14 days after hypoxia (Fig. 3). At autopsy, 22 of 31 E0 × LDLR0 mice with persistent ECG changes (71%) had morphological signs of infarction, i.e., loss of dehydrogenase activity in the myocardium. The structural development of infarcted hearts was analyzed in mice killed at day 1 (n = 6), 3 (n = 7), 7 (n = 6), 14 (n = 6), and 21 (n = 6) after hypoxia. Histologic analysis revealed an early phase of apoptosis (day 1–3), followed by inflammatory infiltration (day 3), removal of dead myocytes, development of granulation tissue (day 7), and subsequent healing by a dense collagenous scar (day 21) (Fig. 4). Thus, ECG, biochemical, and histologic data show that hypoxic stress elicits myocardial infarction in E0 × LDLR0 mice. Hypoxic stress also induced myocardial infarction in 12-month-old but not in 7-month-old E0 single-knockout mice (data not shown).

**ETα Receptor Mediates Hypoxia-Induced Myocardial Infarction.** The sudden but transient development of ECG changes and signs of depressed pump function during hypoxic stress in atherosclerotic mice suggested that myocardial ischemia might develop through a vasoconstrictor reflex response to hypoxia (30) in the atherosclerotic coronary artery (32, 33). Hyperlipidemia and hypoxia increase endothelium-dependent vasoconstriction (34). Among vasoactive molecules, endothelin plays a pivotal role as a vasoconstrictive peptide that is present in endothelial cells and macrophages of active coronary atherosclerotic plaques (35). It is released upon hypoxic stress (36) and elevated in patients with myocardial infarction because of increased secretion and/or reduced clearance (37, 38).

Because the vasoconstrictor effects of endothelin are mediated by the ETα receptor, which is expressed by vascular smooth muscle cells (39), we exposed atherosclerotic mice to...
hypoxic stress before and after administration of an ET<sub>A</sub> receptor blocker, LU135252 (1 mg/kg of body weight). Such treatment dramatically attenuated the ECG signs of myocardial ischemia and also reduced the extent of subsequent myocardial infarction as assessed by troponin T leakage (Fig. 3). To rule out that the first exposure to hypoxia preconditioned the heart to make it resistant to a second hypoxic stress, we repeated the experiment but exposed the mice to LU135252 during the first rather than the second hypoxic episode. Again, ET<sub>A</sub> receptor blockade prevented the ECG signs of myocardial ischemia. One week later, when hypoxia was performed without the ET<sub>A</sub> blocker, the ECG alterations reappeared.

**DISCUSSION**

These studies demonstrate, through combined use of electrophysiological, biochemical, radiological, and morphological methods, that (i) hypercholesterolemia induces coronary atherosclerosis that leads to myocardial infarction, (ii) infarction can be precipitated by mental or hypoxic stress in mice with coronary atherosclerosis, and (iii) ET<sub>A</sub> receptor signaling mediates the ischemic response to hypoxia in the atherosclerotic heart.

Although the correlation between hypercholesterolemia and myocardial infarction is well established by a large series of epidemiological studies, no previous experimental data have demonstrated that hypercholesterolemia causes myocardial infarction. Mice deficient in apoE develop severe atherosclerosis involving the coronary arteries (8), and the occurrence of occasional fibrotic scars in their myocardium (8) suggests that they may develop ischemic heart disease. In the present study, we extend these observations by showing that E<sup>0</sup> × LDLR<sup>0</sup> mice develop angiographic stenosis and occlusions, which are indicative of severe coronary heart disease, ECG changes characteristic for myocardial damage, and elevated plasma troponin T levels, which are diagnostic for myocardial infarction. Thus, hypercholesterolemia induces coronary atherosclerosis, which, in turn, elicits myocardial infarction.

Both E<sup>0</sup> and E<sup>0</sup> × LDLR<sup>0</sup> mice developed coronary atherosclerosis, myocardial scars, and myocardial infarction upon hypoxic stress. However, the atherosclerotic process was accelerated in the latter model, which also became sensitive to stress at an earlier stage. This is in line with the observation that E<sup>0</sup> × LDLR<sup>0</sup> mice develop more extensive atherosclerosis than E<sup>0</sup> mice (25). The two strains differ significantly in lipoprotein profiles but not in serum cholesterol levels (refs. 7 and 25; this study), suggesting that LDL-like lipoproteins and/or the lack of LDL receptors add to the atherogenicity of hypercholesterolemia and increased chylomicrons and remnant particles, which are common to both models. The finding that myocardial infarction could be elicited only when coronary atherosclerosis was present indicates that atherosclerosis rather than hyperlipoproteinemia per se was needed for the development of ischemic heart disease.

The role of stress in ischemic heart disease has been controversial. Although clinicians have observed that patients suffering from acute coronary syndromes often have experienced stressful stimuli in their recent past, experimental studies have failed to clarify the role of stress in coronary disease. We exposed atherosclerotic mice to two different types of stressful stimuli: a mental stress that may increase heart workload and a hypoxic stress that reduces oxygen delivery to the heart muscle. Both stimuli led to acute myocardial infarction in atherosclerotic mice but did not affect infarction in wild-type mice. This experimental evidence shows that stress causes myocardial infarction in hyperlipidemia-induced atherosclerosis. It confirms the importance of an imbalance between oxygen demand and the supply through an atherosclerotic coronary artery for development of acute ischemia. That endothelin receptor blockade completely prevented myocardial infarction during hypoxic stress shows the importance of vasodynamic responses in the sequence of events leading from silent coronary atherosclerosis to acute myocardial infarction.

Clinical studies previously have suggested that endothelin may be important in myocardial infarction (37), and ETA receptor blockade can restore endothelial function in E<sup>0</sup> mice (40) and protect the myocardium from neutrophil injury during ischemia/reperfusion (41). We now provide direct experimental evidence that coronary vasoconstriction through ETA receptor signaling plays a pivotal role in the development of stress-induced myocardial infarction in the atherosclerotic mouse. It is likely that endothelin, increased rapidly upon hypoxia (34, 39), induces smooth muscle contraction, which, in turn, causes vasospasm of the atherosclerotic coronary arteries (32, 33). This may explain the sudden but reversible ECG deviations observed at the time of provocative tests in the E<sup>0</sup> × LDLR<sup>0</sup> mice. Stress may, by inducing endothelin-dependent vasoconstriction in the atherosclerotic coronary artery, initiate a process that results in irreversible myocardial injury, which is detectable as apoptosis, loss of dehydrogenase from cardiomyocytes, leakage of troponin T, and, eventually, inflammation and formation of a fibrotic scar in the myocardium. It should now be possible to dissect all the critical steps involved...
in this sequence of events by using specific blockers and compound mutant mice.

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