Spontaneous Ischemic Events in the Brain and Heart Adapt the Hearts of Severely Atherosclerotic Mice to Ischemia

Shinichi Tokuno, Kazuhiro Hinokiyama, Kumi Tokuno, Christian Löwbeer, Lars-Olof Hansson, Guro Valen

Abstract—To investigate if spontaneous ischemic events in mice with severe multi-organ atherosclerosis could adapt to ischemia, apolipoprotein E/LDL receptor knockout mice were fed an atherogenic diet for 7 to 9 months. Signs of spontaneous ischemia occurred. One to two days later, hearts were excised, Langendorff-perfused with induced global ischemia, and compared with mice without signs of disease. In vivo heart or brain infarctions were verified by heart histology and/or increased serum levels of cardiac troponin T and S100B. Hearts of mice with spontaneous ischemic events had improved function and reduced Langendorff-induced infarctions. To investigate the remote preconditioning effect of brain ischemia, bilateral ligation of the internal carotid arteries was performed in C57BL6 mice. Twenty-four hours later, their isolated hearts were protected against induced global ischemia. A possible role of inducible NO synthase (iNOS) was studied in iNOS knock out mice, who were not preconditioned by induced brain ischemia. Cardiac iNOS was unchanged 24 hours after preconditioning, suggesting that NO is a trigger rather than a mediator of protection. These findings suggest that spontaneous ischemic events in the brain and heart adapt the heart to ischemia. This can be mimicked by induced brain ischemia, with iNOS as a key factor of protection. (Arterioscler Thromb Vasc Biol. 2002; 22:995-1001.)

Key Words: ischemic preconditioning ■ remote preconditioning ■ atherosclerosis ■ brain ischemia ■ inducible nitric oxide synthase

Cardiac adaptation to ischemia can be achieved experimentally by ischemic preconditioning, transient episodes of ischemia and reperfusion before sustained ischemia. Preconditioning offers a profound protection against functional depression and necrosis and is potentially of great clinical interest for treatment of patients with ischemic heart disease. Adaptation can be achieved when preconditioning of the heart takes place <2 hours (classic preconditioning) or 24 to 72 hours (delayed preconditioning) before sustained ischemia. During the last few years, remote preconditioning, which can be either classic or delayed, has been discovered. Thus, short episodes of ischemia and reperfusion in limb, colon, and kidney protects the heart. The mechanisms underlying this endogenous protection are not fully understood.

NO is constitutively produced by endothelial NO synthase (eNOS) and has a variety of biologic actions. On stimuli involving cytokine production and activation of the redox-sensitive transcription factor nuclear factor kappa-B (NFκB), inducible NOS (iNOS) is induced in a variety of cells. In models of preconditioning targeting the heart to protect the heart, NO has been suggested to be a signal molecule initiating adaptation to ischemia. Delayed preconditioning may be mediated by NO produced by iNOS, induced by the preconditioning episode in the heart. Evidence for the latter is provided by studies that pharmacologic and genetic inhibition of iNOS abolishes the preconditioning effect. However, a possible role for iNOS in remote or systemic preconditioning has not been clarified.

Unstable angina may represent a clinical correlate to preconditioning. Several studies indicate that having unstable angina before acute myocardial infarction reduces morbidity and mortality, where unstable angina may cause short episodes of spontaneous ischemia due to intermittent hypoperfusion. It is possible that also spontaneous ischemia in any human organ may influence heart function and necrosis analogous to remote preconditioning, but this is not feasible to investigate.

With the development of recombinant DNA techniques, new animal models of atherosclerosis have emerged, including the apolipoprotein E and LDL receptor double knockout (ApoE/LDLr KO) mouse which has a lesion distribution pattern and composition similar to humans. When ApoE/LDLr KO mice are fed an atherogenic diet for 7 to 9 months to accelerate development of atherosclerosis, severe atherosclerosis with lesions throughout the vasculature develop.
The animals are prone to stress-induced myocardial infarctions. The present study investigates if spontaneous ischemic events in hearts and brains of ApoE/LDLr KO mice with severe atherosclerosis protects their isolated hearts against induced global ischemia, if remote preconditioning could be evoked by brain ischemia in wild-type animals, and searches a possible mechanism of remote preconditioning effect of brain ischemia.

**Methods**

**Animals**
The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health, and was approved by the Ethics Committee for Animal Research at the Karolinska Institute. ApoE/LDLr KO mice were purchased from Bomholtgard (Bomholt, Denmark) and fed an atherogenic diet containing 21% fat and 0.15% cholesterol (R683, AnalyCen) for 7 to 9 months. After this time, some mice developed signs of disease as shown in the Table. The animals were used for experiments 24 to 48 hours after onset of these signs and were compared with age-matched mice without any signs of disease. For induced brain ischemia studies, male C57BL6 mice (20 to 25 g) were purchased from Jackson Laboratories (Bar Harbor, Me). No comparisons were performed between animals from different purchase sites.

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<th>Chest</th>
<th>Head</th>
<th>Fibronectin</th>
<th>Serum CTnT (μg/L)</th>
<th>Serum SB100 (μg/L)</th>
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ApoEHeart, animals 1–6
ApoEBrain, animals 6–11
ApoECon, animals 12–18
C57Brain, n=8
C57Sham, n=7
iNOSBrain, n=8
iNOSSham, n=7

The isolated heart perfusion procedure is described in detail online (please see http://atvb.ahajournals.org). Briefly, hearts were isolated, and the aorta was cannulated and retrogradely perfused with buffer at a constant pressure. A left ventricular balloon recorded systolic (LVSP), end-diastolic (LVEDP), and developed (LVDP) pressures, as well as the maximum (max) and negative (neg) first derivatives of pressures (dP/dt). Coronary flow (CF) was measured, while heart rate and arrhythmias were calculated.

**Induced Brain Ischemia**
To investigate if brain ischemia could precondition the heart, bilateral ligation of the internal carotid arteries 24 to 32 hours before heart isolation was performed in C57BL6 animals or iNOS KO mice to investigate iNOS as a possible mediator. See http://atvb.ahajournals.org for the surgical procedure.

**Protocol**
All hearts were stabilized for 25 minutes. A 40-minute global ischemia was induced by clamping the inflow tubing followed by 60 minutes of reperfusion.

The following groups were investigated. (For ApoE/LDLr KO mice, group allocation was retrospective and a result of signs of...
disease combined with serum analysis and immunohistochemical examination as described below and in the Table):

ApoECon: ApoE/LDLr KO mice without any ischemic events (n=7)
ApoEHeart: ApoE/LDLr KO mice with evidence of recent heart infarction (n=6)
ApoEBrain: ApoE/LDLr KO mice with evidence of recent brain infarction (n=6)
C57Sham: C57BL6 mice with sham operation (n=7)
C57Brain: C57BL6 mice with induced brain ischemia (n=8)
iNOSSham: iNOS KO mice with sham operation (n=7)
iNOSBrain: iNOS KO mice with induced brain ischemia (n=7)

Measurement of Infarct Size
Triphenyl tetrazolium chloride solution 1%, (TTC) was used to calculate infarct size. The procedure as well as measurements of cardiac troponin T (cTnT) and S100B are described online (please see http://atvb.ahajournals.org).

Immunohistochemical Analysis of Recent Heart Infarction
After TTC staining, sectioning, and measurement of infarct size, hearts of all ApoE/LDLr KO mice were investigated for evidence of recent heart infarction in the apex, the middle region, and the aortic root. After paraffin embedding, 5-μm sections were stained with a rabbit anti-fibronectin antibody (Sigma) to find infarctions occurring 24 to 48 hours before the experiments. A biotinylated goat anti-rabbit secondary antibody with an avidin-biotin detection system (Vectorstain Elite ABC Kit), DAB solution (DAB Substrate Kit, Vector Laboratories) and counter staining with hematoxylin was performed for visualization. To differentiate between old and recent infarctions, Masson’s trichrome staining, which stains collagen green at a later stage of scar formation, was used.

Immunoblotting and Real-Time Polymerase Chain Reaction (PCR; TaqMan PCR)
Please see http://atvb.ahajournals.org for details.

Statistical Analysis
Repeated measure ANOVA was used for evaluation of differences in hemodynamics between groups, with a Fisher PLSD post-hoc test. One-factor ANOVA was used to evaluate infarct size, cTnT release, serum S100B, serum cTnT, and optical density of immunoblot bands between groups (Stat View 4.0, Abacus Concepts, Inc). Data are presented as mean±SEM.

Results
Evidence of Spontaneous Cardiac Infarctions

A: Immunohistochemistry
To evaluate possible spontaneous cardiac ischemic events before Langendorff perfusion, hearts of ApoE/LDLr KO mice were sectioned and stained with an anti-fibronectin antibody. Fibronectin is produced during fibrosis formation after tissue necrosis, and it was detected as dark brown deposits (Figure 1a). The areas staining fibronectin-positive were included in the area unstained with TTC (Figure 1b). Areas unstained with TTC and negative for anti-fibronectin antibody were evaluated to be necrotic due to Langendorff-intervention (Figure 1b). Of 11 hearts from animals with signs of disease, 5 had positive anti-fibronectin staining and were allocated to the ApoEHeart group (Table). No heart from the 7 mice without signs of disease stained positive for anti-fibronectin (Table), although there were unstained areas with TTC (Figure 1c and 1d).

B: Serum cTnT
The serum cTnT level in ApoE/LDLr KO mice with suspected in vivo heart infarction was higher than in controls (P=0.03). Serum cTnT was not significantly increased in any other group (Table).

Evidence of Brain Ischemia: Serum S100B
Mice with neurological signs of disease (animals 6, 7, and 8, in Table), or with serum S100B higher than mean ±2SD (>6.1 μg/L) of controls, were allocated into the brain infarction group (animal 9, 10, and 11 in Table). These animals had higher S100B levels than ApoECon (P=0.0002, Table). The serum S100B in the ApoEHeart group was not higher than ApoECon. Inducing brain ischemia increased serum S100B in C57BL6 mice compared with sham-operated
mice \((P<0.03)\), but S100B did not increase in iNOS KO compared with their shams (Table).

Cardiac Performance After Spontaneous Ischemic Events

There were no differences between groups in any parameter before global ischemia (Figure 2). LVDP was depressed during postischemic reperfusion of ApoE/LDLr KO control hearts. Spontaneous ischemic events in heart or brain before Langendorff perfusion attenuated the depression of LVDP \((P=0.02\) and \(P=0.04\), respectively, Figure 2a). LVEDP increased during reperfusion of ApoECon. In hearts of animals with spontaneous heart or brain infarction, the increase of LVEDP was attenuated \((P<0.001\) and \(P=0.003\), respectively, Figure 2b). LVDP and LVEDP pressures, max and neg dP/dt, and CF in Langendorff-perfused hearts of ApoE/LDLr KO mice after 7 to 9 months on an atherogenic diet with spontaneous heart (open circles) or brain (squares) infarctions or without any infarctions (closed circles) subjected to 40 minutes of global ischemia and 60 minutes of reperfusion. Values are mean±SEM of 6 to 7 animals per group. Pre indicates before ischemia; the rest denotes minutes of reperfusion. \(^*P<0.05\) vs controls.

LVDP was depressed during reperfusion of sham-operated C57BL6 mice hearts. Induced brain ischemia 24 to 32 hours before heart isolation attenuated the depression of LVDP \((P=0.02\), Figure 3a). LVEDP increased during reperfusion of sham-operated controls, and this increase was attenuated by induced brain ischemia \((P=0.04\), Figure 3b). Induced brain ischemia also attenuated the depression of max dP/dt \((P=0.01\) and the increase of neg dP/dt \((P=0.02\) during reperfusion (not shown). Induced brain ischemia did not influence CF, heart rate, or occurrence of arrhythmias during reperfusion (data not shown).

Infarct Size and cTnT Release Into the Coronary Effluent

At the end of reperfusion, \(\approx 30\%\) of myocardial tissue was necrotic as judged by TTC staining of control ApoE/LDLr KO mice. Spontaneous ischemic events in heart \((P=0.001)\) or brain \((P=0.003)\) reduced the Langendorff-induced infarct size (Figure 4, top). In sham-operated C57BL6 mice, infarct size was \(\approx 20\%\) and was decreased by induced brain ischemia before heart perfusion \((P=0.002\), Figure 4, top). In hearts of iNOS KO mice, however, no infarct-limiting effect of inducing brain ischemia was found (Figure 4A).

Cardiac TnT release into the coronary effluent increased during reperfusion of ApoE/LDLr KO ischemic control hearts (Figure 3). LVDP (a) and LVEDP (b) pressures in Langendorff-perfused hearts of C57BL6 mice with (squares) or without (closed circles) induced brain ischemia through bilateral internal carotid artery ligation 24 to 36 hours before heart isolation and subjection to 40 minutes of global ischemia and 60 minutes of reperfusion. The same procedure was performed in animals with targeted deletion of the iNOS gene (c, d). Values are mean±SEM of 7 to 8 animals per group. Pre indicates before ischemia; the rest denotes minutes of reperfusion. \(^*P<0.05\) in comparison between groups.

Cardiac Performance After Induced Brain Ischemia

In C57BL6 mice, there were no differences between groups in any parameter before ischemia (Figure 3). LVDP was depressed during postischemic reperfusion of sham-operated C57BL6 mice hearts. Induced brain ischemia 24 to 32 hours before heart isolation attenuated the depression of LVDP \((P=0.02\), Figure 3a). LVEDP increased during reperfusion of sham-operated controls, and this increase was attenuated by induced brain ischemia \((P=0.04\), Figure 3b). Induced brain ischemia also attenuated the depression of max dP/dt \((P=0.01\) and the increase of neg dP/dt \((P=0.02\) during reperfusion (not shown). Induced brain ischemia did not influence CF, heart rate, or occurrence of arrhythmias during reperfusion (data not shown).

In iNOS KO mice, there were no differences between groups in any parameter before ischemia. Induced brain ischemia in iNOS KO mice did not influence the depression of LVDP or the increase of LVEDP during postischemic perfusion (Figure 3c), and did not influence any other parameter during reperfusion (not shown).
hearts (Figure 4, bottom). Both spontaneous heart ($P_{/H11005} 0.02$) and brain ($P_{/H11005} 0.01$) infarction before Langendorff perfusion reduced the release of cTnT. This could be mimicked by induced brain ischemia in C57BL6, but not in iNOS KO mice ($P_{/H11005} 0.01$, Figure 4, bottom).

Expression of iNOS

**A: Protein**

Proteins were extracted from C57BL6 mice hearts 24 hours after induced brain ischemia, with sham-operated animals as controls. When the proteins were separated by electrophoresis, transferred to a membrane, and incubated with a rabbit polyclonal anti-iNOS antibody, a band of 130 kDa corresponding to the size of iNOS appeared (Figure 5a). The bands were related to the protein loading as evaluated with Ponceau solution (not shown). There was no difference in expression of iNOS in hearts of animals with or without induced brain ischemia.

**B: RNA**

RNA was extracted from hearts of C57BL6 mice serially after induced brain ischemia and amplified by real-time PCR, relating iNOS expression to that of $\beta$-actin. No increase of cardiac gene expression of iNOS was detected in a 24-hour time course (Figure 5b).

**Discussion**

The main findings of the present study were that evidence of spontaneous heart or brain infarctions were found in ApoE/LDLr KO mice with severe atherosclerosis. Isolated hearts of these animals had protected function and reduced myocardial necrosis after induced global ischemia. This protection could be mimicked by inducing brain ischemia in C57BL6 mice. Induced brain ischemia did not protect the hearts of iNOS KO mice. As hearts of C57BL6 mice had unchanged cardiac iNOS after induced brain ischemia, the role of iNOS is most likely to be that NO triggers, rather than mediates, the protection. To the authors’ knowledge, this is the first study demonstrating a cardioprotective effect of spontaneous organ infarction and a remote preconditioning effect of brain ischemia.

We have previously investigated atherosclerotic lesions in age- and diet-matched siblings of the presently used ApoE/LDLr KO mice and found advanced fibrofatty lesions distributed throughout the vasculature. Caligiuri et al found that stress in vivo induced myocardial infarctions in these animals, mediated by endothelin receptor signaling. To evaluate if cardiac necrosis observed after reperfusion was due to the Langendorff intervention, recent spontaneous infarction in vivo, or infarction of older age, an approach including different staining techniques and measuring serum cTnT was used. cTnT is a cardiосpecific, structural protein of striated muscle fibers released by myocyte injury. The areas unstained by TTC included all these types of myocardial...
necrosis. Fibronectin is a multifunctional, extracellular matrix glycoprotein, which is present in early wound healing. It has been demonstrated in cardiomyocytes within one day after ischemic onset, and the intensity of staining becomes maximal after 48 hours. At that time, migration of collagen-producing fibroblasts starts in the border zone of myocardial infarction. Masson’s trichrome stains collagen green starting in this later stage of fibrosis formation. We found evidence of spontaneous heart infarctions by anti-fibronectin staining in 5 of 6 hearts in this group, while the sixth heart was allocated to the group based on the very high serum cTnT. Only one of all investigated ApoE/LDLr KO mice hearts had positive staining also for Masson’s trichrome in the border of anti-fibronectin staining area. This particular heart probably had an infarction 48 to 72 hours before Langendorff perfusion, which is within the time course of delayed preconditioning described by others. For the rest of the animals in this group, a combination of signs of disease, high cTnT levels, and/or positive fibronectin staining indicated spontaneous ischemic events 24 to 48 hours before heart isolation.

S100B belongs to a family of intracellular calcium-binding proteins originating from astroglial and Schwann cells, and it leaks to serum if the permeability of the blood-brain barrier is increased. We demonstrated an increased serum level of S100B in 6 ApoE/LDLr KO mice with signs of disease, including 3 paraplegic mice, and in C57BL6 mice with induced brain ischemia. It is therefore indicated that our mice had damage to not only astroglial or Schwann cells but also to the blood-brain barrier, which usually occurs in brain infarction.

Hearts of animals with spontaneous ischemic events in the heart or brain were protected against injury in a manner analogous to ischemic preconditioning. Ischemic preconditioning can be evoked experimentally by brief episodes of ischemia and reperfusion immediately or 24 to 72 hours before a sustained ischemic event. This protects the heart whether the preconditioning is performed on the heart itself or in other organs. However, we have found no publications demonstrating that spontaneously occurring ischemic events may protect the heart. One possible clinical correlate to both ischemic preconditioning and the present findings is evidence of preinfarction or unstable angina protecting the heart. Unstable angina is currently thought to be caused by rupture of an atherosclerotic plaque, and is associated with a systemic inflammatory reaction. Unstable angina activates cardiac heat shock protein 72, eNOS, and the transcription factor NFκB in patients with recent symptoms. We speculate that, from an adaptive point of view, it appears sensible for an organism subjected to an acute ischemic event or inflammation of another etiology to defend itself against a possible next event in the same or another organ through increasing endogenous protection. The present findings pinpoint that organ protection is generated by ischemia rather than by ischemia and reperfusion, and may thus represent a key to the pathway of solving the preconditioning enigma.

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References


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