Leukocyte-type 12-lipoxygenase-deficient mice show impaired ischemic preconditioning-induced cardioprotection

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Gabel, Scott A., Robert E. London, Colin D. Funk, Charles Steenbergen, and Elizabeth Murphy. Leukocyte-type 12-lipoxygenase-deficient mice show impaired ischemic preconditioning-induced cardioprotection. Am J Physiol Heart Circ Physiol 280: H1963–H1969, 2001.—To investigate the role of 12-lipoxygenase in preconditioning, we examined whether hearts lacking the “leukocyte-type” 12-lipoxygenase (12-LOKO) would be protected by preconditioning. In hearts from wild-type (WT) and 12-LOKO mice, left ventricular developed pressure (LVDP) and 31P NMR were examined during treatment (preconditioning) and during global ischemia and reperfusion. Postischemic function (rate-pressure product, percentage of initial value) measured after 20 min of ischemia and 40 min of reperfusion was significantly improved by preconditioning in WT hearts (78 ± 12% in preconditioned vs. 44 ± 7% in nonpreconditioned hearts) but not in 12-LOKO hearts (47 ± 7% in preconditioned vs. 33 ± 10% in nonpreconditioned hearts). Postischemic recovery of phosphocreatine was significantly better in WT preconditioned hearts than in 12-LOKO preconditioned hearts. Preconditioning significantly reduced the fall in intracellular pH during sustained ischemia in both WT and 12-LOKO hearts, suggesting that attenuation of the fall in pH during ischemia can be dissociated from preconditioning-induced protection. Necrosis was assessed after 25 min of ischemia and 2 h of reperfusion using 2,3,5-triphenyltetrazolium chloride. In WT hearts, preconditioning significantly reduced the area of necrosis (26 ± 4%) compared with nonpreconditioned hearts (62 ± 10%) but not in 12-LOKO hearts (85 ± 3% in preconditioned vs. 63 ± 11% in nonpreconditioned hearts). Preconditioning resulted in a significant increase in 12(S)-hydroxyeicosatetraenoic acid in WT but not in 12-LOKO hearts. These data demonstrate that 12-lipoxygenase is important in preconditioning.

myocardial ischemia; infarction; prostaglandins; nuclear magnetic resonance

PRECONDITIONING with brief intermittent periods of ischemia has been shown to reduce infarct size and postischemic contractile dysfunction after a subsequent sustained period of ischemia (18, 25). The mechanisms responsible for this protection have been debated, but there is strong evidence supporting a role for protein kinase C (PKC) (6, 13, 15, 16, 19, 20, 23) and ATP-sensitive K+ channels (1, 10, 11, 14) in the pathway leading to protection. Lipoxygenase metabolites also have been suggested to be involved in preconditioning (5, 17, 24). Arachidonic acid, which has been shown to be released during ischemia (21), is metabolized by cyclooxygenases, lipoxygenases, and epoxygenases. Previous studies (17, 24) have shown that preconditioning results in the accumulation of 12-lipoxygenase (12-LO) metabolites and that inhibition of lipoxygenase metabolism blocks the accumulation of 12-LO metabolites and the protective effects of preconditioning. Furthermore, addition of the proximal 12-LO metabolite 12(S)-hydroperoxyeicosatetraenoic acid [12(S)-HPETE] mimics the protective effects of preconditioning (5). 12-LO metabolites have been shown to accumulate after activation of PKC, and the PKC inhibitor chelerythrine blocks the preconditioning-induced accumulation of 12-LO metabolites and protective effects, suggesting that 12-LO metabolites are downstream of PKC (5).

12-LO is a member of a family of lipoxygenases that metabolize arachidonic acid (3, 8, 29). 5-, 8-, 12-, and 15-LO have been described. Several 12-LOs have been reported, and they are named on the basis of the tissue from which the lipoxygenase was initially isolated. A platelet-type 12-LO, a leukocyte-type 12-LO, and an epidermal-type 12-LO have all been described in mammalian cells. However, the classification can be confusing as the platelet 12-LO is found in tissues other than platelets, and porcine platelets do not have the platelet-type 12-LO. Rabbits and humans have a 15-LO in leukocytes, whereas other mammals have a related 12-LO, referred to as leukocyte-type 12-LO. Leukocyte 12-LO has a broad distribution in the mouse, including the heart; however, contrary to the name, leukocyte 12-LO is not expressed in leukocytes of mice (7). An epidermal 12-LO has also recently been described (9). The platelet, leukocyte, and epidermal 12-LO all catalyze the formation of 12(S)-HPETE.

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Although previous data have suggested a role for 12(S)-HPETE in preconditioning, these studies are based on the use of inhibitors that may have effects on other pathways. To more directly address the role of 12-LO in preconditioning, we studied mice in which the leukocyte 12-LO gene was disrupted. These studies will also provide information regarding the specific 12-LO responsible for generation of 12(S)-HPETE. To address these questions, we investigated whether the protection afforded by preconditioning could be elicited in mice that were null for the leukocyte 12-LO (12-LOKO) (2, 27). We chose to examine leukocyte 12-LOKO mice because of the broad tissue distribution of leukocyte 12-LO. We found that preconditioned 12-LOKO mice showed reduced protection compared with wild-type (WT) mice and reduced generation of 12-LO metabolites, suggesting that the 12-LO is important in preconditioning.

MATERIALS AND METHODS

Animals. 12-LOKO mice were developed as described previously (2, 27). The 12-LOKO mice are a cross of C57BL/6 × 129Sv mice. C57BL/6 × 129Sv and 129J mice (Jackson Laboratory; Bar Harbor, ME) were used as WT controls. No significant differences were observed between the two groups of controls. All mice were males weighing between 21 and 32 g and were treated in accordance with National Institutes of Health guidelines.

Isolated mouse heart preparation. The mice were anesthetized with 8 mg pentobarbital sodium (diluted and delivered intraperitoneally). Heparin (100 units) was injected intravenously before removal of the heart. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer, and the aortas were cannulated. Retrograde perfusion was from a reservoir suspended 90 cm above the heart. The nonrecirculating perfusate was a Krebs-Henseleit buffer containing (in mmol/l) 120 NaCl, 5.9 KCl, 1.2 MgSO4, 1.75 CaCl2, 25 NaHCO3, and 11 glucose. The buffer was aerated with 95% O2-5% CO2 and maintained at a pH of 7.4 and a temperature of 37°C.

For assessment of contractile function, a latex balloon on the tip of a polyethylene catheter was inserted through the left atrium into the left ventricle. The catheter was connected to a pressure transducer (Argon; Athens, TX) mounted outside the magnet at the same height as the heart. A MacLab system was used to collect and process the left ventricular pressure data (AD Instruments; Milford, MA). Hearts that did not achieve a developed pressure of at least 100 cmH2O were excluded.

31P NMR and left ventricular developed pressure. NMR spectroscopy allows for the continuous monitoring of intracellular pH (pHi) and high-energy phosphates. The NMR studies were carried out on a Varian Unity 500-MHz NMR spectrometer equipped with a variable temperature probe, which was set to maintain the temperature of the hearts at 37°C. A 10-mm broadband Varian NMR probe was tuned to 31P (202.47 MHz). Hearts were bathed in perfusate to improve the resonance line shapes. The magnet was shimmed on the proton/water signal, and we routinely achieved a nonspinning line width at a half height of ~0.15 ppm. Pulses conditioning employed a 70° pulse angle, a 2-s interpulse delay, a 337-ms recycle time, a spectral width of 12,000 Hz, 8,000 data points, and 128 acquisitions, which corresponded to a 5-min block of data collection. The free induction decay was multiplied by an exponential function corresponding to a line broadening of 50 Hz before Fourier transformation.

Hearts from WT and 12-LOKO mice were perfused with Krebs-Henseleit buffer for a 20-min stabilization period, during which time the magnet was shimmed and control NMR spectra were acquired. This was followed by a treatment period (preconditioning or control perfusion), a 20-min period of global ischemia, and a 40-min reperfusion period. The preconditioning protocol consisted of four cycles of 5 min of ischemia and 5 min of reflow. In these studies, we monitored left ventricular developed pressure (LVDP), pHi, and high-energy phosphates [β-ATP and phosphocreatine (PCr)]. The pHi was determined from the chemical shift difference between the PCr and intracellular inorganic phosphate (Pi) resonances.

Necrosis study. Hearts from WT and 12-LOKO mice were given a 20-min stabilization period, followed by a treatment period consisting of either preconditioning or control perfusion, before a 25-min period of global ischemia, which was followed by a 2-h reflow period. We chose a 25-min period of ischemia on the basis of initial studies in which we found that 20 min of ischemia resulted in little or no necrosis in nonpreconditioned WT hearts (~10%) but that 25 min of ischemia resulted in a large infarct (~60% of the total heart cross-sectional area). This would allow us to detect a protective effect of an intervention. Thus we employed a 25-min period of ischemia for measurement of infarct size. At the end of 2 h of reperfusion, hearts were perfused for 8 min (2 ml/min) with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in Krebs-Henseleit buffer. Hearts were removed from the cannula and incubated in 1% TTC at 37°C for 10 min. The hearts were then fixed in formalin. Area of necrosis was measured by taking cross-sectional slices through the ventricles, which were then photographed using an Olympus DP10 digital camera mounted on an Olympus SZX12 stereo microscope. The resulting images were quantitated by measuring the areas of stained versus unstained tissue with the use of the Adobe Photoshop program.

12(S)-Hydroxyeicosatetraenoic acid study. Hearts from WT and 12-LOKO mice were subjected to a 20-min stabilization period, followed by a treatment period consisting of either a control perfusion period or an abbreviated preconditioning protocol, which consisted of two 5-min periods of ischemia separated by a 5-min reflow period. We used this abbreviated preconditioning protocol because it was observed in a previous study (17) to result in increased production of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) compared with baseline. After treatment, the hearts were snap-frozen and stored in liquid nitrogen until extraction. 12(S)-HETE was extracted and assayed as previously described (5). Briefly, hearts were subjected to a Bligh-Dyer extraction, and the chloroform phase was dried under nitrogen gas purified with a C18 column (Waters) and stored at ~70°C until assayed. The recovery was calculated using 12(S)-[3H]HETE. The extracted 12(S)-HETE was quantitated with the use of an enzyme immunoassay kit (Assay Designs; Ann Arbor, MI). The 12(S)-HETE content in the heart is expressed as nanograms per gram of wet weight.

Statistics. Data are expressed as means ± SE. For comparison between two groups, a Student’s t-test was used. For comparison of multiple groups, an ANOVA was used, followed by Tukey’s post hoc test for multiple comparisons.

RESULTS

Contractile function. There were no significant differences in baseline LVDP, heart rate, maximum and

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minimum rates of pressure rise and fall, or coronary flow rate between experimental groups before treatment (see Table 1). After treatment, hearts were subjected to 20 min of global ischemia. The nonpreconditioned hearts (WT and 12-LOKO) started contracture at similar times (~15 min), whereas the preconditioned hearts began contracture earlier (~8 min). The maximum contracture attained was also similar in both groups of nonpreconditioned hearts (~55 cmH2O) and was significantly lower than that obtained in both groups of preconditioned hearts (~90 cmH2O).

Figure 1 shows recovery of the rate-pressure product (percentage of initial preischemic value) for the four groups measured at 40 min of reflow after 20 min of ischemia. As has been observed in rats, preconditioning improved postischemic contractile function in WT mice. WT nonpreconditioned hearts recovered 44 ± 7% of their preischemic function, whereas WT preconditioned hearts recovered 78 ± 12% (P < 0.05). In contrast, preconditioning did not improve postischemic contractile function significantly in the 12-LOKO mice; nonpreconditioned hearts recovered 33 ± 11% of preischemic function, whereas the preconditioned hearts recovered 47 ± 7%.

Necrosis. To further evaluate whether the absence of the leukocyte-type 12-LO reduced the protective effects of preconditioning, we used TTC staining to measure necrosis. Figure 2A shows the area of necrosis (percentage of total area) measured after 25 min of ischemia and 2 h of reperfusion. In WT mice, preconditioned hearts had significantly less necrosis (24 ± 4%) than nonpreconditioned hearts (62 ± 10%, P < 0.05). In the 12-LOKO hearts, preconditioning did not result in a reduction in infarct size (85 ± 3%) compared with nonpreconditioned hearts (63 ± 11%, P > 0.05). Furthermore, preconditioned 12-LOKO hearts had significantly larger infarcts than preconditioned WT hearts (85 ± 3 vs. 24 ± 5%, P < 0.05). Figure 2B shows the recovery of contractile function during the 2 h of reperfusion after the 25 min of ischemia. Consistent with the data in Fig. 1, there was a significant improvement in postischemic function in WT preconditioned hearts compared with the other groups. Also as expected, the recovery of function was poorer after 25 min of ischemia compared with that observed after 20 min of ischemia (Fig. 1).

12(S)-HETE measurements. Because there are multiple 12-LO enzymes, it is important to determine whether the absence of the leukocyte-type 12-LO gene alters the accumulation of 12-LO metabolites observed previously in preconditioned hearts. Figure 3 shows the tissue contents of the stable end product of 12-LO metabolism, 12(S)-HETE, under basal conditions and after preconditioning. In WT hearts, preconditioning led to a significant increase in 12(S)-HETE (404 ± 33 ng/g wet wt) compared with that observed in nonpreconditioned hearts (266 ± 26 ng/g wet wt, P < 0.05). In 12-LOKO hearts, the basal level of 12(S)-HETE was approximately one-half that observed in WT hearts (123 ± 11 ng/g wet wt, P < 0.05 compared with WT nonpreconditioned hearts), and preconditioning did not result in a significant change in 12(S)-HETE (170 ± 24 ng/g wet wt, P > 0.05).

Measurements of pH. It has been shown in rat hearts (24) that preconditioning reduces acid produc-

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Table 1. Hemodynamic measurements at baseline and during ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Heart Rate, beats/min</th>
<th>LVDP, cmH₂O</th>
<th>Flow Rate, ml/min</th>
<th>+dP/dt, cmH₂O/100ms</th>
<th>−dP/dt, cmH₂O/100ms</th>
<th>TOC, min</th>
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<tr>
<td>Non-PC</td>
<td>6</td>
<td>363 ± 15</td>
<td>101 ± 3</td>
<td>1.7 ± 0.1</td>
<td>4.1 ± 0.2</td>
<td>−4.0 ± 0.2</td>
<td>15.7 ± 1.4</td>
<td>51 ± 7</td>
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<tr>
<td>PC</td>
<td>5</td>
<td>380 ± 23</td>
<td>101 ± 3</td>
<td>1.9 ± 0.3</td>
<td>4.3 ± 0.5</td>
<td>−3.7 ± 0.4</td>
<td>8.0 ± 0.4</td>
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<td>12-LOKO</td>
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<tr>
<td>Non-PC</td>
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<td>362 ± 19</td>
<td>109 ± 2</td>
<td>2.2 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>−4.7 ± 0.2</td>
<td>14.3 ± 1.6</td>
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<tr>
<td>PC</td>
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<td>113 ± 4</td>
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<td>4.7 ± 0.4</td>
<td>−4.9 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>84 ± 9</td>
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</table>

Values are means ± SE; n, number of experiments. LVDP, left ventricular developed pressure; +dP/dt and −dP/dt, rates of pressure rise and fall, respectively; TOC, time of start of contracture; 12-LOKO, 12-lipoxygenase (12-LO)-knockout mice; PC and non-PC, preconditioned and nonpreconditioned, respectively.
tion during the sustained ischemic period; however, this reduced acidification does not appear to be sufficient for protection (4, 6, 17). We were interested in determining whether a similar pH effect occurred in mouse hearts. Figure 4 shows that, consistent with previous studies in the rat, WT preconditioned hearts have less acidification during ischemia than nonpreconditioned hearts. After 20 min of ischemia, the pH$_i$ fell to 6.19 ± 0.08 in the nonpreconditioned WT hearts and to 6.60 ± 0.04 in the preconditioned WT hearts (P < 0.05). A similar trend was observed in 12-LOKO hearts (Fig. 4B). In the 12-LOKO mice, pH$_i$ fell to 6.26 ± 0.04 in the nonpreconditioned hearts and to 6.61 ± 0.04 in the preconditioned group (P < 0.05). Interestingly, there was a difference in pH$_i$ between WT and 12-LOKO mice during the preconditioning period. In the WT hearts, the fall in pH$_i$ was attenuated during each successive ischemic period of preconditioning, suggesting that the first cycles of preconditioning affect acid production (metabolism) during subsequent episodes of ischemia. This attenuation of the fall in pH$_i$ was not observed in hearts from the 12-LOKO mice. Thus during the fourth cycle of ischemia in the preconditioning protocol, the pH$_i$ only fell to 6.98 ± 0.03 in WT compared with 6.80 ± 0.05 for the 12-LOKO group (P < 0.05).

NMR measurements of high-energy phosphates. Figure 5 shows the changes in PCr for the four groups as a percentage of initial baseline control values. Similar to the progressive attenuation of the fall in pH$_i$ during preconditioning that occurs in WT but not in 12-LOKO hearts, the recovery of PCr is enhanced during each successive cycle of ischemia in the preconditioning protocol in the WT but not the 12-LOKO hearts. In WT preconditioned hearts (Fig. 5A), at the fourth reflow period of preconditioning, the PCr levels recovered to 156% of control levels versus 100% in the 12-LOKO hearts (Fig. 5B).
PCr levels are not significantly different between any of the groups during sustained ischemia. At the end of the 40-min reperfusion period in WT hearts, there were no significant differences in PCr between preconditioned and nonpreconditioned groups. Similarly, in 12-LOKO hearts, there were no significant differences between preconditioned and nonpreconditioned hearts at the end of reperfusion. However, the recovery of PCr was significantly better in WT preconditioned hearts (157%) than in the 12-LOKO preconditioned hearts (100%).

Figure 6 shows ATP (percentage of initial) levels monitored throughout the study. Consistent with the pH and PCr data, ATP is better preserved during preconditioning in the WT hearts than in the 12-LOKO hearts. In the WT hearts, preconditioning only slightly reduced ATP levels (96% of control), whereas at the end of preconditioning the ATP levels fell to 68% of initial in the 12-LOKO group.

DISCUSSION

Role of 12-LO in preconditioning. Previous studies (5, 17, 24) have shown that lipoxygenase inhibitors block the protective effects of preconditioning and that the preconditioning-induced accumulation of 12-LO metabolites is downstream of PKC. However, the downstream target by which an increase in 12-LO metabolism mediates preconditioning is yet to be elucidated. Interestingly, studies (21) in other tissues have shown that 12-LO metabolites activate a K⁺ channel. Previous studies (5, 17) have also shown an accumulation of 12-LO metabolites in rat and rabbit hearts after preconditioning. Consistent with previous studies, the data in this study show that preconditioning causes an accumulation of 12-LO metabolites in the WT mouse heart. Although these previous studies suggested a role for 12-LO in preconditioning, they relied on the use of inhibitors. To evaluate more directly the role of 12-LO in preconditioning, we employed mice in which the 12-LO gene was disrupted. If 12-LO is important in preconditioning, one would predict that these mice should show reduced preconditioning-induced protection. The data in Figs. 1–3 show that, in contrast to WT hearts, in 12-LOKO hearts, preconditioning does not induce a significant reduction in necrosis, a significant improvement in postischemic contractile function, or a significant increase in 12-LO metabolites. These data demonstrate that leukocyte 12-LO is important to the preconditioning-induced accumulation of 12-LO metabolites and preconditioning-induced protection.

Interestingly, during preconditioning, we find that the recovery of PCr and pH are attenuated during the
of sustained ischemia; however, there was poor reperfusion in the 12-LOKO preconditioned hearts, which lead to incomplete washout of pyridine nucleotides in the 2-h reflow period and therefore variable and inaccurate assessment of infarct size with TTC. We also found that a 2-h reperfusion period was necessary for assessment of infarct size with TTC. We found that infarct size appeared smaller when measured at 1 h of reperfusion compared with that measured after 2 h of reperfusion due to delayed washout of metabolites.

**Leukocyte 12-LO and generation of 12(S)-HETE.** The data in this paper show that hearts lacking 12-LO have a baseline level of 12(S)-HETE ~50% of that found in WT hearts and that preconditioning does not significantly increase 12(S)-HETE in these hearts. The measurable basal levels of 12(S)-HETE in the 12-LOKO hearts could be due to production of 12(S)-HETE by other 12-LOs or it could be due to production of 12(S)-HETE by the cytochrome P-450 pathway, as the cytochrome P-450s generate a racemic mixture of 12(S)-HETE and 12(R)-HETE. The data in the present study, showing that the preconditioning-induced increase in 12(S)-HETE is not observed in the 12-LOKO mice, are consistent our previous study in rats (17). Similar to the data in the present study, a previous study (2) using 12-LOKO mice reported no difference in basal levels of 12(S)-HETE in pancreatic islets between WT and 12-LOKO mice; however, cytokine stimulation caused an increase in 12(S)-HETE in WT islets but not in islets from 12-LOKO mice.

**Preconditioning and mouse hearts.** The data in this study show that preconditioning leads to protection in mouse hearts similar to that reported in other species. A previous study (12) using an in vivo regional ischemia model in mouse hearts reported that preconditioning leads to a reduction in necrosis. Other studies (26, 28) employing a global model of ischemia in perfused mouse hearts have reported a preconditioning-induced reduction in necrosis. However, there is disagreement regarding whether preconditioning in mouse hearts improves contractile function. Sumeray and Yellon (26) report a preconditioning-induced improvement in postischemic contractile function, an effect not observed by Xi et al. (28). Consistent with Sumeray and Yellon (26), we find that preconditioning leads to a significant reduction in necrosis as well as a significant improvement in postischemic contractile function. However, the preconditioning-induced improvement in postischemic contractile function in mouse hearts (177% of control) is not as robust as that observed in rat hearts (typically 200% of control). The smaller percentage improvement in postischemic contractile function observed in mouse hearts might explain why this effect was not observed in a previous study (28). It is worth noting that both our study and the study by Sumeray and Yellon (26), which found that preconditioning improves postischemic contractile function, employed a preconditioning protocol of four cycles of 5 min of ischemia and 5 min of reperfusion, whereas the study of Xi et al. (28), which did not find a protective effect, used one 5-min period of ischemia and reperfusion or two

![Figure 6](http://ajpheart.physiology.org/)

Fig. 6. Time course of changes in ATP (percentage of control) for the PC and non-PC WT hearts (A) and for the PC and non-PC 12-LOKO hearts (B). Values are means ± SE. The number of experiments for each group is given in the legend of Fig. 1.
cycles of 2.5 min of ischemia and reperfusion. These differences in ability to observe a preconditioning-induced improvement in contractile function could also be due to the differences in the extent of injury. The ability to observe a protective effect of preconditioning depends on the extent of injury; if there is too little injury, it is difficult to observe protection, and conversely if the injury is too extensive, it is difficult to observe protection.

In summary, mice deficient in the leukocyte-type 12-LO have impaired protection after preconditioning. Preconditioned hearts from WT mice show a significant improvement in posts ischemic function, a significant reduction in necrosis, and a significant increase in 12(S)-HETE. However, hearts from 12-LOKO mice do not show a significant preconditioning-induced protection, which is consistent with the lack of a significant preconditioning-induced increase in 12-LO metabolites. These conclusions are not based on the use of pharmacological inhibitors, which may have nonspecific mechanisms of action, but rather on genetic disruption to eliminate the putative enzyme responsible for production of 12-LO metabolites in the myocardium. These data suggest that leukocyte 12-LO is an important mediator of preconditioning.

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