20-Hydroxyeicosatetraenoic Acid Mediates Isolated Heart Ischemia/Reperfusion Injury by Increasing NADPH Oxidase-Derived Reactive Oxygen Species Production

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**Background:** It has been reported that 20-hydroxyeicosatetraenoic acid (20-HETE) aggravates myocardial ischemia/reperfusion (I/R) injury, but the exact mechanism of action is still unclear.

**Methods and Results:** Experiments were performed in isolated rat hearts subjected to 35 min of ischemia followed by 40 min of reperfusion in Langendorff preparations. Perfusion with HET0016, an inhibitor of 20-HETE production, significantly improved I/R-induced reduction in cardiac contractility, myocardial infarction, and myocardial apoptosis. In contrast, administration of 20-HETE aggravated I/R-induced myocardial injury and enhanced apoptosis. I/R significantly increased production of reactive oxygen species (ROS) and oxidative stress, both of which were significantly inhibited by HET0016 and enhanced by 20-HETE administration. Apocynin, an inhibitor of NADPH oxidase, blocked 20-HETE-induced ROS production in the I/R hearts. 20-HETE increased the expression of gp91phox and p22phox, the subunits of NADPH oxidase; and stimulated NADPH oxidase activity. In addition, GF-109203 significantly attenuated the 20-HETE-induced increases in the NADPH oxidase expression and activity. Finally, in the Langendorff I/R preparation, both apocynin and tempol, ROS scavengers, significantly blocked 20-HETE-induced myocardial dysfunction.

**Conclusions:** All of the results demonstrated that in isolated rat hearts 20-HETE stimulates NADPH oxidase-derived superoxide production, which aggravates I/R-induced myocardial injury via a PKC-dependent mechanism. (Circ J 2013; 77: 1807–1816)

**Key Words:** 20-HETE; Ischemia/reperfusion injury; NADPH oxidase; Protein kinase C; Reactive oxygen species
participates in I/R injury; and exacerbates myocardial I/R injury, the mechanisms by which it contributes to I/R injury in the heart have not been fully explored. Theoretically, 20-HETE may increase infarct size and cardiac I/R injury by decreasing blood flow to the affected area by limiting compensatory vasodilation in the collateral circulation. However, our recent studies also suggest that 20-HETE may act directly on cardiomyocytes, causing apoptosis and production of reactive oxygen species (ROS). Therefore, the present study examined the effects of HET0016, an inhibitor of CYP ω-hydroxylase enzymes, and exogenous 20-HETE on I/R-induced cardiac dysfunction and cardiac infarct size. Furthermore, we also investigated the role of ROS in 20-HETE-mediated I/R cardiac injury and the underlying molecular mechanisms in isolated heart preparations.

**Methods**

**Animals and Drugs**

Male Wistar rats (220±20 g) were fed a standard diet and acclimatized in a quiet quarantine room for 1 week before the experiments. All protocols were approved by Northeast Normal University Institutional Animal Care and Use Committees. 20-HETE, GF-109203x, apocynin and tempol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Dihydroethidium (DHE) was purchased from DCFH-DA (100 μmol/L), a membrane-permeable radical scavenger, or GF-109203 (0.5 μmol/L), a specific inhibitor of NADPH oxidase, were infused 5 min before administration of 20-HETE. Apocynin (100 μmol/L), an inhibitor of NADPH oxidase, tempol (100 μmol/L), a membrane-permeable radical scavenger, or GF-109203 (0.5 μmol/L), a specific inhibitor of NADPH oxidase, were infused 5 min before administration of 20-HETE.

**Determination of Myocardial Infarct Size**

To determine the extent of infarction, after 2h of reperfusion, the hearts were removed from the Langendorff apparatus and frozen at –20°C for 1–2h. They were then sectioned (2–3 mm) and incubated in KH buffer containing 2,3,5-triphenyltetrazolium chloride (TTC, 1%) at 37°C for 15 min, then fixed in 10% formalin. The infarcted area was determined by planimetry measuring the stained (red, live tissue) vs. unstained (white, necrotic) regions.

**Determination of Myocardial Apoptosis**

DNA fragmentation was detected with a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) assay kit according to the manufacturer’s protocol.
Determination of p22phox and gp91phox mRNA Expression
Real-time polymerase chain reaction (qRT-PCR) analysis was used to examine p22phox and gp91phox mRNA expression in myocardial tissue. Total RNA was isolated from cardiac homogenates using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and transcribed into cDNA with a reverse transcription reagent kit (TaKaRa, Dalian, Liaoning, China). Real-time qRT-PCR was performed on the ABI 7000 System (Applied Biosystems, Foster City, CA, USA) using SYBR® Premix Ex TaqTM RT-PCR kit (Takara, Dalian, Liaoning, China). GAPDH served as a loading control. The following primers were used: p22phox sense primer: 5'-ACCGTCTGCTTGGCCATTG-3' and antisense primer: 5'-TCAATGGGAGTCCACTGCTCAC-3'; gp91phox sense primer: 5'-CCTGGAGACCCAGATGCAAGA-3' and antisense primer: 5'-CGTGGTGCACAGCAAAGTGA-3'; GAPDH sense primer: 5'-GGCACAGTCAAGGCTGAGAATG-3' and antisense primer: 5'-ATGGTGAGCAAGAGCAGAAGAATG-3'. Data from the reaction were collected and analyzed by the complementary computer software. Relative quantification of gene expression was calculated using the 2−∆∆Ct data analysis method and normalized to GAPDH in each sample.

Western Blot Analysis
Western blots were performed to evaluate the protein expres-

**Figure 1.** Effect of HET0016, an inhibitor of 20-HETE production, and 20-HETE on myocardial infarct size as determined by TTC staining. The percentage of infarct volume to left ventricular volume was calculated in I/R heart preparations with and without treatment with HET0016 or 20-HETE (3, 10, 30, 50 nmol/L). (Upper) Representative heart sections stained with TTC. (Lower) Bar graphs showing the quantitative analysis of infarct size in each group. Values are mean±SE, from 6–8 hearts/group. *P<0.05 compared with the control group, #P<0.05 compared with the I/R group. 20-HETE, 20-hydroxyeicosatetraenoic acid; I/R, ischemia/ reperfusion; TTC, 2,3,5-triphenyltetrazolium chloride.
cytochrome c reduction. Briefly, myocardial homogenates were incubated with cytochrome c (500 μmol/L) and NADPH (100 μmol/L) in the presence or absence of superoxide dismutase (200U/ml) for 30 min. Cytochrome c reduction was measured at 550 nm wavelength on a microplate reader. Superoxide production was calculated from the difference between absorbance with and without SOD.

Measurement of SOD and Catalase Activities
SOD and catalase activities were assayed using commercially available kits (JianCheng Botech Co), following the protocol provided by the manufacturer. Briefly, SOD activity was based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium by superoxide; and catalase activity was determined by H₂O₂ consumption spectrophotometrically at 240 nm. Re-
20-HETE Mediates Myocardial I/R Injury

Results

Effect of HET0016 and 20-HETE on Myocardial Post-Ischemic Functional Recovery

To test the effect of exogenous and endogenous 20-HETE on I/R-induced myocardial damage, cardiac hemodynamic changes and myocardial contractility were measured in Langendorff heart preparations under control and I/R conditions as described in the Methods. During the initial 20-min equilibration period, cardiac function parameters did not significantly differ among all the hearts (data not shown). At the end of 40-min reperfusion, myocardial functional recovery was measured and statistical comparison between groups was performed. Administration of HET0016 (1 μmol/L) significantly ameliorated the inhibited cardiac function induced by I/R and augmented LVDP from 48.6±3.4% to 71.7±3.5%, +dP/dtmax from 51.9±2.1% to 69±3.2%, –dP/dtmax from 47.1±3.6% to 64.1±3.8%, and RPP from 52.4±1.2% to 71.1±9.2% (P<0.05). Conversely, 20-HETE administered dose-dependently decreased myocardial functional recovery. Administration of 20-HETE (50 nmol/L) significantly decreased LVDP from 48.6±3.4% to 24.1±7.4%, +dP/dtmax from 51.9±2.1% to 29.2±6.3%, –dP/dtmax from 47.1±3.6% to 26.2±7.9%, and RPP from 52.4±1.2% to 18.4±12.4% (P<0.05) (Table 1).

During the equilibration period, treatment with HET0016 (1 μmol/L) or 20-HETE (50 nmol/L) did not significantly alter CF (P>0.05) as shown in Table 1, indicating that 20-HETE has a very weak effect on CF before ischemia. At the end of a 40-min reperfusion, CF was significantly reduced by I/R from 15.0±1.4 ml/min to 8.1±1.1 ml/min (P<0.05). Treatment with HET0016 attenuated I/R-induced reduction in CF by 43.2% (P<0.05). Conversely, exogenous 20-HETE (50 nmol/L) significantly reduced CF from 8.1±1.1 to 4.5±0.7 ml/min in I/R hearts (P<0.05). CK, a marker of myocardial infarction, was...
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Effects of HET0016 and 20-HETE on Myocardial Infarction Induced by I/R

Myocardial infarct size was measured by TTC staining as described in the Methods. The 2h of I/R produced 39.8±2.3% infarction of the heart wall. Treatment of the heart with HET0016 (1 μmol/L) significantly attenuated the I/R-induced increase in cardiac infarct size by 37.2% (P<0.05). Conversely, exogenous administration of 20-HETE dose-dependently increased infarct size as compared with I/R alone. 20-HETE (50 nmol/L) treatment significantly increased infarct size to 58.1±4.4% (P<0.05 as compared with I/R group alone) (Figure 1). These results indicated that inhibition of 20-HETE production with HET0016 attenuated the I/R-induced increase in myocardial infarct size in post-I/R hearts. However, exogenous 20-HETE exacerbated I/R-induced cardiac injury.

Effects of HET0016 and 20-HETE on Myocardial Apoptosis After I/R

TUNEL staining was used to determine myocardial apoptosis after I/R. TUNEL-positive nuclei were significantly increased in the I/R group as compared with the control group (1.69±0.02% in control vs. 18.8±1.3% in I/R group) (P<0.05). HET0016 treatment significantly attenuated the I/R-induced increase in the number of TUNEL-positive nuclei (8.2±0.9% in I/R plus HET0016 group vs. 18.8±1.3% in I/R alone group) (P<0.05). In contrast, administration of 20-HETE increased the TUNEL-positive nuclei by 58.2% compared with the I/R group (P<0.05) (Figure 2). These results suggested that inhibition of 20-HETE production attenuated I/R-induced myocardial apoptosis.

Effects of HET0016 and 20-HETE on I/R-Induced ROS Generation and Protein Peroxidation

It is generally accepted that I/R-induced myocardial damage is attributable to the formation of ROS.11 Therefore, we measured the effects of HET0016 and 20-HETE on I/R-induced ROS generation by DHE fluorescence. After 15-min reperfusion, isolated hearts were removed from the Langendorff apparatus and cardiac ROS production was evaluated. I/R significantly increased ROS production (Figures 3A,B). Compared with the I/R group, HET0016 (1 μmol/L) treatment significantly reduced the I/R-induced increase in DHE fluorescence intensity by 45.8% (P<0.01). In contrast, treatment with 20-HETE (50 nmol/L) further increased DHE fluorescent intensity by 38% (P<0.01 as compared with the I/R group).

To investigate the contribution of NADPH oxidase to the 20-HETE-induced elevation in ROS production in I/R, apocynin (100 μmol/L), an inhibitor of NADPH oxidase, was infused 5 min before 20-HETE administration as described in the Methods. Treatment with apocynin significantly attenuated the 20-HETE-induced increase in ROS production by 28.3% (P<0.05). These data suggest that 20-HETE may enhance ROS in I/R, at least in part through activation of NADPH oxidase. In addition, co-administration of 20-HETE with tempol, a membrane-permeable radical scavenger, attenuated the fluorescence intensity that had been increased by 20-HETE by 43.4% in post-I/R hearts (P<0.05) (Figures 3A,B).

ROS produced by I/R may attack intracellular protein and cause oxidative damage, so we measured the protein carbonyl content using DNPH. I/R caused a significant increase in protein carbonylation compared with control hearts. This effect of I/R was significantly reduced by 34.9% after treatment with HET0016 (P<0.05). Conversely, treatment with 20-HETE (50 nmol/L) significantly enhanced protein carbonylation by 51% (P<0.05 as compared with I/R alone). In addition, co-administration of apocynin (100 μmol/L) or tempol (100 μmol/L) with 20-HETE significantly attenuated 20-HETE-induced protein carbonylation (P<0.05) (Figure 3C).

Effects of HET0016 and 20-HETE on SOD and Catalase Activities After I/R

SOD and catalase are the important enzymes for scavenging O2– and H2O2 in the heart. Therefore, we measured the effects of HET0016 and 20-HETE on the activity of these antioxidant enzymes. Activities After I/R

Effects of HET0016, an inhibitor of 20-HETE production, and 20-HETE on I/R-induced SOD and catalases activities. (A) SOD and (B) catalase activities were measured in I/R hearts treated with control, 20-HETE or HET006. Values are mean±SE, from 4 hearts/group. *P<0.05 compared with the control group. 20-HETE, 20-hydroxyeicosatetraenoic acid; I/R, ischemia/reperfusion; SOD, superoxide dismutase.
expression and activity were assessed as described in the Methods. The mRNA levels of p22<sub>phox</sub> and gp91<sub>phox</sub> were decreased by HET0016 in I/R hearts. However, 20-HETE treatment significantly increased by approximately 2- and 3-fold the p22<sub>phox</sub> and gp91<sub>phox</sub> mRNA levels in I/R hearts (P<0.01, Figure 5A).

Similarly, protein levels of p22<sub>phox</sub> and gp91<sub>phox</sub> were also decreased by HET0016 treatment and enhanced by 20-HETE as shown in Figure 5B. NADPH oxidase activity was evaluated as described in the Methods. Bar graphs summarizing NADPH activity in I/R hearts treated with control, HET0016, 20-HETE, 20-HETE + apocynin (AP), or 20-HETE + GF-1009203 (GF). Values are mean ± SE, from 6–7 hearts/group. *P<0.05 compared with the I/R group, **P<0.01 compared with the I/R group, #P<0.05 compared with the 20-HETE group. 20-HETE, 20-hydroxyeicosatetraenoic acid; I/R, ischemia/reperfusion.

Effects of 20-HETE on NADPH Oxidase Subunits Expression and Activity

Our previous study showed that 20-HETE stimulates NADPH oxidase-derived ROS production in cultured cardiomyocytes. To determine whether this pathway is involved in isolated heart I/R injury, NADPH oxidase subunits (p22<sub>phox</sub> and gp91<sub>phox</sub>)}
Role of PKC on 20-HETE-Induced Increases in NADPH Oxidase Expression and Activity

Our recent studies demonstrated that 20-HETE may act directly on cardiomyocytes and increase NADPH oxidase-derived ROS production via a PKC-dependent mechanism. Thus, we examined the effect of a PKC inhibitor, GF-109203, on 20-HETE-induced increases in both NADPH oxidase expression and activity in I/R hearts. Results are shown in Figure 5, indicating that GF-109203 significantly attenuated 20-HETE-induced increases in NADPH oxidase activity and expression. These data suggested that 20-HETE stimulates NADPH oxidase-derived superoxide production through a PKC-dependent mechanism in I/R hearts.

Role of NADPH Oxidase-Derived ROS in 20-HETE-Mediated I/R Cardiac Injury

To verify whether NADPH oxidase-derived ROS production is involved in 20-HETE-mediated I/R myocardial injury, hearts were infused with apocynin (100 μmol/L) or tempol (100 μmol/L) before 20-HETE administration. Hemodynamics, myocardial

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### Table 2. Effect of Apocynin or Tempol on the Action of 20-HETE in I/R-Induced Cardiac Injury of Perfused Hearts Isolated From Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>LVDP (% of equilibrium)</th>
<th>+dP/dt (% of equilibrium)</th>
<th>−dP/dt (% of equilibrium)</th>
<th>RPP (% of equilibrium)</th>
<th>CF (ml/min)</th>
<th>CK (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td>95.3±2.1</td>
<td>93.1±1.7</td>
<td>89.7±1.2</td>
<td>91.3±4.3</td>
<td>15±1.4</td>
<td>20±1.2</td>
</tr>
<tr>
<td>I/R (n=7)</td>
<td>48.6±3.4*</td>
<td>51.9±2.1*</td>
<td>47.1±3.6*</td>
<td>52.4±1.2*</td>
<td>8.1±1.1*</td>
<td>331.0±12.1*</td>
</tr>
<tr>
<td>20-HETE (50 nmol/L, n=7)</td>
<td>24.1±7.4*</td>
<td>29.3±6.3*</td>
<td>26.3±7.9*</td>
<td>18.5±12.4*</td>
<td>4.5±0.7*</td>
<td>660.6±21.0*</td>
</tr>
<tr>
<td>20-HETE + apocynin (n=6)</td>
<td>45.2±3.2*</td>
<td>44.4±5.2*</td>
<td>42.1±3.8*</td>
<td>41.2±13.2*</td>
<td>6.8±1.4*</td>
<td>492.7±14.2*</td>
</tr>
<tr>
<td>20-HETE + tempol (n=7)</td>
<td>47.1±6.4*</td>
<td>42.3±4.2*</td>
<td>42.3±8.3*</td>
<td>46.4±13.1*</td>
<td>7.1±0.32*</td>
<td>388.2±22.3*</td>
</tr>
</tbody>
</table>

Values are mean±SE, from 6–9 hearts/group. *P<0.05 vs. control groups; †P<0.05 vs. I/R groups; ▲P<0.05 vs. 20-HETE groups. Data were computed at the end of 40-min reperfusion (37°C) and expressed as mean±SE. Abbreviations as in Table 1.
infarct size, CF, and CK level were measured in the I/R hearts. Administration of exogenous 20-HETE significantly decreased LVDP, ±dP/dt and RPP in I/R hearts. The 20-HETE-induced reductions in LVDP, ±dP/dt and RPP were significant abolished by treatment with apocynin or tempol. In addition, administration of 20-HETE (50nmol/L) significantly reduced CF at the end of reperfusion. The 20-HETE-induced reduction in CF was significantly abolished by apocynin or tempol. Similarly, CK levels were also enhanced by 20-HETE administration in I/R heart preparations, which was significantly abolished by apocynin or tempol (25.4% and 41.2%, respectively, P<0.05) (Table 2). In addition, treatment with 20-HETE (50nmol/L) significantly enlarged the I/R-induced infarct, which was significantly attenuated by co-administration with apocynin or tempol (P<0.05) (Figure 6).

**Discussion**

This study provides the first evidence that 20-HETE aggravates myocardial I/R injury via NADPH oxidase-derived ROS production in Langendorf heart preparations.

Our experiments showed that I/R-induced deterioration in cardiac function, myocardial infarction, and CK release was significantly ameliorated by administration of HET0016. Conversely, exogenous 20-HETE administered dose-dependently exacerbated the damaging effects of I/R. It is well known that apoptosis plays an important role in the pathogenesis of I/R injury. Our previous study also demonstrated that 20-HETE acts directly on cardiomyocytes, causing cell apoptosis. Thus, the current study further investigated the effects of HET0016 and 20-HETE on myocardial apoptosis under I/R conditions. Consistent with previous reports, our results demonstrated that I/R markedly increases myocardial cell apoptosis. Furthermore, we also found that inhibition of 20-HETE production with HET0016 significantly attenuated the I/R-induced increase in myocardial apoptosis. These results suggest that the CYP ω-hydroxylases and 20-HETE have a significant detrimental role in myocardial I/R injury, an observation that is consistent with other studies showing that 20-HETE production and CYP ω-hydroxylase enzyme activity are increased during cardiac I/R injury.6.7 Meanwhile, administration of inhibitors of CYP ω-hydroxylase enzymes can ameliorate the inhibited cardiac function induced by I/R, reducing the size of the myocardial infarct.9.16.17 However, the mechanism of 20-HETE exacerbation of myocardial I/R injury needs to be further studied.

The evidence regarding the specific role of 20-HETE in modulating the response in coronary arteries is that, in small porcine coronary arteries, 20-HETE can induce contraction by endothelium-dependent and -independent mechanisms, and the enhanced coronary artery constriction is attributable to increased activity of ω-hydroxylase, which, consequently, increases the synthesis of 20-HETE in vascular smooth muscle. These studies are supported by our observations that CYP ω-hydroxylase enzymes inhibited by HET0016 significantly improves CF and that exogenous 20-HETE treatment aggravated CF in I/R hearts. These findings demonstrated that the CYP ω-hydroxylase inhibitor, HET0016, may decrease infarct size after I/R by increasing blood flow to the affected area and enhancing the compensatory vasodilation in the collateral circulation. In addition, our recent studies also suggest that 20-HETE may act on directly cardiomyocytes causing apoptosis and ROS production.10.11 It suggests that, besides CF, the most important effect of 20-HETE during I/R injury is directly on cardiomyocytes.

It is widely accepted that the generation of ROS in the myocardium is the principal mechanism contributing to the pathogenesis of I/R injury, and that by using antioxidants, ROS are removed in order to improve function post-myocardial I/R and reduce the infarct size. Excessive generation of ROS causes irreversible oxidative damage to most of the cellular proteins, and the contractile-related protein damage may directly cause the decline in myocardial contractility. According to previous studies, after myocardial ischemia, both troponin, the regulatory element of the myofilament, and actin, the structural cytoskeletal protein, are damaged during ischemia, which directly causes the decrease in myocardial systolic function.

It has been reported that CYP enzymes can produce ROS in liver cells and are an important factor in the pathogenesis of Parkinson’s disease, diabetes etc. In studies of myocardial I/R injury, administration of nonspecific CYP enzyme inhibitors, such as chloramphenicol, cimetidine, and sulfaphenazole, prevented I/R-induced myocardial damage, an effect that is associated with reduced ROS production. However, CYP enzymes consist of several subtypes, including CYP2 isoforms and CYP4 isoforms etc, and can metabolize AA to a series of hydroxyicosatetraenoic acids, epoxycosatrienoic acids, and dihydroxyeicosatrienoic acids. The previous research does not clarify the specific subtypes of CYP enzyme and which metabolites of the enzymatic response generated ROS. In the current study, we used HET0016 as a specific inhibitor of CYP ω-hydroxylase enzymes to reduce the generation of 20-HETE induced by I/R, and found that both ROS and protein carbonyl levels were markedly depressed in post-reperfusion treatment with HET0016, which was conversely enhanced by administration of 20-HETE. These data indicate that the effect of 20-HETE on exacerbation of myocardial I/R injury is attributable to promotion of the generation of ROS, leading to contractile-related protein oxidation damage. This conclusion is confirmed by our data obtained from cardiac function measurement in our Langendorf model. We found that treatment with tempol, a ROS scavenger, ameliorated 20-HETE damage to cardiac contractility, CF, CK release, and infarct size in I/R hearts. Furthermore, in this study, we also investigated the effects of 20-HETE on SOD and catalase activities after I/R, and found that treatment with HET0016 or 20-HETE made no significant difference as compared with I/R hearts. These observations demonstrate that SOD or catalase are not involved in the 20-HETE-induced elevation in ROS levels under I/R conditions.

A wide variety of potential ROS sources have been reported in cardiac I/R studies, such as the mitochondrial respiratory chain, NADPH oxidase and nitric oxide synthase. NADPH oxidase has been recently addressed as a major source of superoxide in the myocardium, which is a family of enzymes containing 5 isoforms: Nox1, Nox2, Nox3, Nox4, and Nox5. In the cardiovascular system, the main expressions, gp91phox (Nox2), and with p22phox, are heterodimers and function as the final electron transporter from NADPH to oxygen in generating the superoxide anion. The increased expression of gp91phox and p22phox is related to the lipid peroxidation level after acute myocardial infarction in rats. Therefore, in the present investigation, we examined the effect of HET0016 and 20-HETE on gp91phox and p22phox expressions and NADPH oxidase activity. Both the expression of gp91phox and p22phox and NADPH oxidase activity were inhibited by treatment with HET0016, conversely augmented by 20-HETE after I/R. However, apocynin, a selective inhibitor of NADPH oxidase, blocked the augmented NADPH oxidase activity induced by 20-HETE. In addition, apocynin also reduced the ROS generation induced by 20-HETE after I/R, which indicated that
NADPH oxidase-derived ROS production may play a key role in 20-HETE involved in I/R injury. This result is also confirmed by a study in pulmonary artery endothelial cells, which showed that 20-HETE increases superoxide production, an effect that is blocked by an NADPH oxidase inhibitor and by PEG-SOD. Importantly, in our previous study we observed that 20-HETE increased superoxide production, an effect that is blocked by gp91ds-tat, the inhibitor of NADPH oxidase. However, that study was based at the cellular level, which cannot thoroughly reflect the pathological and physiological conditions. Therefore, the isolated rat heart perfused by Langendorff model was used to verify this mechanism at the organ level. As we predicted, both apocynin and tempol effectively blocked the myocardial dysfunction induced by 20-HETE in I/R hearts. Furthermore, we also observed that 20-HETE-induced increases in NADPH oxidase activity were significantly attenuated by PKC inhibitor, suggesting that a PKC-dependent signaling pathway may be involved in the stimulatory effect of 20-HETE in the I/R heart.

Conclusions
We have provided direct proof of the mechanism of 20-HETE in increasing myocardial I/R injury. In a rat isolated, perfused heart I/R model, 20-HETE stimulated NADPH oxidase-derived superoxide production, which aggravated I/R-induced myocardial injury via a PKC-dependent mechanism. Therefore, agents that interfere with the actions of 20-HETE may have therapeutic benefit in cardiac ischemic disease.

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