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Linoleic Acid Metabolite DiHOME Decreases Post-ischemic Cardiac Recovery in Murine Hearts

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Abstract

Cardiac ischemia/reperfusion injury is associated with the formation and action of lipid mediators derived from polyunsaturated fatty acids. Among them, linoleic acid (LA) is metabolized to epoxyoctadecanoic acids (EpOMEs) by cytochrome P450 (CYP) epoxygenases and further to dihydroxyoctadecanoic acids (DiHOMEs) by soluble epoxide hydrolase (sEH). We hypothesized that EpOMEs and/or DiHOMEs may affect cardiac post-ischemic recovery and addressed this question using isolated murine hearts in a Langendorff system. Hearts from C57Bl6 mice were exposed to 12,13-EpOME, 12,13-DiHOME, or vehicle (phosphate buffered sodium; PBS). Effects on basal cardiac function and functional recovery during reperfusion following 20 min of ischemia were investigated. Electrocardiogram (ECG), left ventricular (LV) pressure and coronary flow (CF) were continuously measured. Ischemia reperfusion experiments were repeated after administration of the sEH-inhibitor 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA). At a concentration of 100 nM, both EpOME and DiHOME decreased post-ischemic functional recovery in murine hearts. There was no effect on basal cardiac parameters. The detrimental effects seen with EpOME, but not DiHOME, were averted by sEH inhibition (AUDA). Our results indicate that LA-derived mediators EpOME/DiHOME may play an important role in cardiac ischemic events. Inhibition of sEH could provide a novel treatment option to prevent detrimental DiHOME effects in acute cardiac ischemia.

Keywords Epoxyoctadecanoic acid (EpOME) · Dihydroxyoctadecanoic acid (DiHOME) · Soluble epoxide hydrolase (sEH) · Linoleic acid · Langendorff perfused heart · Ischemia/reperfusion injury · 12-(3-Adamantan-1-yl-ureido) dodecanoic acid (AUDA)

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Abbreviations

AUDA	12-(3-Adamantan-1-yl-ureido)dodecanoic acid
CF	Coronary flow
CYP	Cytochrome P450
DiHOME	Dihydroxyoctadecanoic acid
EET	Epoxyeicosatrienoic acid
EpOME	Epoxyoctadecanoic acid
HR	Heart rate
LA	Linoleic acid
LV	Left ventricle
LVdia	Left ventricular diastolic pressure
LVDP	Left ventricular developed pressure
PBS	Phosphate-buffered sodium
sEH	Soluble epoxide hydrolase

Introduction

Cardiovascular disease is a major cause of death in the world [1]. Acute heart failure and cardiac arrest is a fatal complication of myocardial infarction [2]. Post-ischemic recovery is crucial for the outcome and prognosis of patients with myocardial ischemia and cardiac arrest [3]. Lipid-derived molecular mediators play an important role in apoptosis and inflammatory myocardial damage [4]. Polyunsaturated fatty are bound to phospholipids of cell membranes. During ischemia, when cell injury occurs, LA is released. At a physiological pH fatty acids build lamellar or crystalline aggregates that can diffuse via plasma membranes or be incorporated into these [5]. Free LA can further be metabolized via different pathways: Lipoxygenases generate hydroperoxides, serving as precursors for leukotriens; cyclooxygenases form peroxides, which can be converted to potent mediators such as prostaglandins, thromboxanes and prostacyclins, and CYP epoxygenase isoenzymes (*CYP-1A2, -3A4, -2C*, and *-2J)* produce their corresponding epoxides [6]. The latter can further be metabolized to diols by soluble epoxide hydrolase (sEH) [7]. The gene encoding sEH (*EPHX2*) is expressed in different tissues; among them are atria and ventricles of the heart [8]. Inhibition of sEH has been associated with beneficial cardiovascular effects, which have been mainly attributed to higher levels of epoxyeicosatrienoic acids (EETs) [9]. However, linoleic acid (LA)-derived epoxyoctadecanoic acid (EpOME) has also been described as important substrate of the sEH. In contrast to the EETs, the role of EpOMEs in the cardiovascular system and in ischemia–reperfusion is less clear. In a number of experimental models, increased EpOME levels have been associated with detrimental effects on cardiac function [10–15]. Human studies on EpOMEs and DiHOMEs revealed plasma levels of 10–100 µM in burn patients with consecutive organ failure and shock [16, 17]. In animal models much lower concentrations showed to have detrimental effects on organ function and in isolated cells [18–21]. Noteworthy, EpOMEs are metabolized to dihydroxyoctadecanoic acids (DiHOMEs) by sEH in situ, which needs to be considered when studying and interpreting EpOME effects. As a matter of fact, increased DiHOME tissue levels have been recently observed in porcine models of cardiac ischemia–reperfusion injury, which have been discussed to cause effects on ion channel kinetics and mitochondrial dysfunction [22–26]. In addition, during the past years, sEH inhibitors have been developed as novel pharmacological drugs in cardiovascular disorders [9]. Both pharmacological inhibition and genetic deletion of the sEH were effective in reducing cardiac ischemia/reperfusion injury [27–29]; however, whether these beneficial effects were due to the stabilization of protective epoxy-metabolites, like the EETs, or prevention of potentially toxic diol-metabolite accumulation remained largely elusive. Considering the high intake of LA in people following Westernized dietary patterns, in particular, the effects of EpOMEs and DiHOMEs on cardiac function in ischemic and post-ischemic states could be of high clinical relevance. Accordingly, the present study has been focussed to evaluate the differential effects of EpOMEs and DiHOMEs, especially on postischemic recovery.

Materials and Methods

Chemicals, Equipment, and Animals

12,13-EpOME and 12,13-DiHOME were purchased from Cayman Chemicals, German reseller Biomol GmbH, Hamburg, Germany. Aliquots of the corresponding ethanolic solutions were evaporated under a stream of nitrogen and the compounds were re-dissolved in PBS, before being administered to the isolated perfused heart preparations via infusion at 10% of total flow.

Experiments were performed on male C57Bl6 wild type mice aged between 8 and 12 weeks (25–30 g). They were held under a 12-h/12-h day/night cycle with free access to food and water. The investigation was approved by the local government authorities, conforms to the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and the ethics policies of Charité University and the Land Berlin.

Isolated Perfused Hearts

For Langendorff perfusion, hearts were rapidly excised and perfused with albumin-free modified Krebs-Henseleit buffer (in mM): $MgSO_4$ 2.1, NaCl 118, KCl 4.7, $NaHCO_3$ 24.7, KH_2PO_4 0.23, $CaCl_2$ 1.5, glucose 11, and oxygenated with 95% O_2 and 5% CO_2. Epicardial ECG and coronary flow (CF) were traced, a balloon connected to a pressure transducer was inserted into the left ventricle (LV) and LV-developed pressure (LVDP), LV diastolic pressure (LVdia) and dP/dt_{max} as well as dP/dt_{min} were monitored. Buffer and chamber temperatures were 37 °C. All hearts were allowed to stabilize for 20 min before recordings were started. Experiments were only performed when hearts showed systolic LVP > 50 mmHg and heart rate > 200/min. Perfusion pressure was set 70 mmHg.

Basal Cardiac Function (A)

Baseline parameters were recorded for 20 min. Mean cardiac parameters calculated from the final 200 s of this period were used as baseline values. 12,13-EpOME and 12,13-DiHOME both at concentrations of either 1 nM or 100 nM, or phosphate buffer sodium (PBS) vehicle were administered for 40 min. From the final 200 s of this period mean cardiac parameters were used as intervention values (Fig. 1a), $n=6$ hearts in the vehicle, DiHOME 1 nM and 100 nM group, $n=5$ hearts in the EpOME 1 nM and 100 nM group.

Ischemia/Reperfusion (B)

Here, the mean of each parameter from the final 200 s prior to intervention were used as baseline values. Hearts were then subjected to 20 min of global no-flow ischemia followed by a total period of 40 min of reperfusion. Means from the last 200 s served as intervention values. 12,13-EpOME 100 nM ± sEH-inhibitor 2-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA), 12,13-DiHOME 100 nM ± AUDA, or vehicle, respectively, were administered until the end of the experiments (Fig. 1b), $n=5$ hearts in each of the five groups.

To characterize the effect on LVDP, LVdia, HR, and CF baseline values were set 100%. To investigate changes in the parameters listed above throughout the experiment relative values were used and expressed in per cent.

Metabolite Concentrations

Concentrations of 12,13-EpOME and 12,13-DiHOME were measured with rapid resolution high-performance liquid chromatography using triplequad tandem spectroscopy by Lipidomix GmbH Berlin. Perfusate was collected directly after no-flow ischemia.

Statistical Analysis

Values are expressed as mean ± standard deviation (SD). Statistics were performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM). Differences between groups (i.e. baseline: PBS vehicle, EpOME 1 nM/100 nM, DiHOME 1 nM/100 nM and I/R: PBS vehicle, EpOME 100 nM ± AUDA, DiHOME 100 nM ± AUDA) were analyzed using analysis of variance (ANOVA) with Least significance difference post hoc test. Values of $p<0.05$ were considered significantly different.

Results

Effects of 12,13-EpOME/DiHOME on Basal Cardiac Function

Cardiac function was assessed in Langendorff isolated-perfused hearts. Cardiac parameters including heart rate (HR), CF, LVdia and LVDP were examined. At the final concentrations (1 nM and 100 nM), 12,13-EpOME and 12,13-DiHOME did not affect any of the cardiac parameters compared to vehicle over the course of 40 min (Table 1).

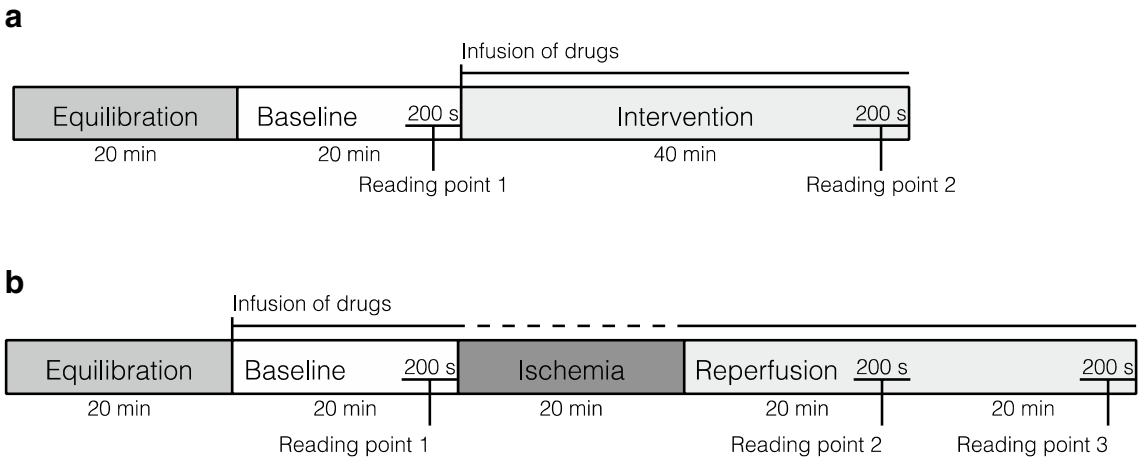

Fig. 1 Schematic illustration of Langendorff heart perfusion. **a** Hearts were allowed to equilibrate for 20 min, followed by 20 min of baseline perfusion, and were then subjected to 12,13-EpOME/DiHOME 1 nM/100 nM for 40 min. **b** Hearts were allowed to equilibrate for 20 min, followed by 20 min of perfusion with 12,13-EpOME 100 nM ± AUDA 1 µM, 12,13-DiHOME 100 nM ± AUDA 1 µM, and PBS vehicle, respectively, and were then subjected to 20 min of global no-flow ischemia followed by 40 min of reperfusion

Table 1 Effects of 12,13-EpOME/DiHOME on cardiac functional parameters under non-ischemic conditions

Parameters	Control	EpOME 1 nM	EpOME 100 nM	DiHOME 1 nM	DiHOME 100 nM
n	6	5	5	6	6
HR [%]	89 ± 17	124 ± 79	113 ± 55	113 ± 42	96 ± 28
LVDP [%]	84 ± 10	95 ± 28	75 ± 18	89 ± 25	106 ± 26
LVdia [%]	89 ± 15	99 ± 22	82 ± 20	84 ± 15	99 ± 16
CF [%]	83 ± 13	102 ± 22	93 ± 20	96 ± 22	124 ± 46

Values are percentages ± SD. Increase/decrease of parameters after 40 min of administration of 12,13-EpOME/DiHOME at different concentrations. The corresponding baseline values were set 100%, (compare Fig. 1a)

Control PBS vehicle, *HR* heart rate, *LVDP* left ventricular developed pressure, *LVdia* left ventricular diastolic pressure, *CF* coronary flow, *n* number of hearts

Table 2 Effects of 12,13-EpOME/DiHOME ± AUDA on post-ischemic functional recovery

Parameters	Control	EpOME 100 nM	EpOME 100 nM + AUDA 1 µM	DiHOME 100 nM	DiHOME 100 nM + AUDA 1 µM
n	5	5	5	5	5
HR [%]	87 ± 10	94 ± 52	80 ± 13	184* ± 79	160* ± 94
[min^{-1}]	348.7 ± 83.1	412.8 ± 386.5	305.5 ± 103.7	653.1 ± 198.6	415.8 ± 238.9
LVDP [%]	89 ± 30	49* ± 24	83 ± 20	31* ± 28	45* ± 30
[mmHg]	54.6 ± 24.6	45.5 ± 29.1	41.4 ± 11.6	24.1 ± 19.4	30.7 ± 23.9
LVdia [%]	60 ± 31	96 ± 38	109 ± 24	134 ± 92	179 ± 141
dP/dt$_{max}$ [%]	85 ± 25	43* ± 20	74 ± 16	33* ± 28	41* ± 27
[mmHg/s]	2976.95 ± 1181.6	2232.4 ± 1189.4	2062.7 ± 588.8	1467.0 ± 1180.9	1452.8 ± 1072.7
d*P*/dt$_{min}$ [%]	83 ± 24	44* ± 19	71 ± 15	34* ± 24	44* ± 27
[mmHg/s]	1965.9 ± 967.7	1669.5 ± 867.5	1412.5 ± 481.1	1114.2 ± 776.4	1156.1 ± 767.9
CF [%]	84 ± 21	62 ± 17	71 ± 28	78 ± 21	105 ± 26
[ml/min]	1.3 ± 0.4	1.5 ± 0.4	0.8 ± 0.4	1.8 ± 0.5	1.0 ± 0.6

*$p < 0.05$ versus. Control

Values are percentages and absolute values ± SD. Increase/decrease of parameters after 40 min of reperfusion compared to before ischemia, (compare Fig. 1b)

Control PBS vehicle, *HR* heart rate, *LVDP* left ventricular developed pressure, *CF* coronary flow, *n* number of hearts

Effects of 12,13-EpOME/DiHOME on Post-ischemic Cardiac Recovery

HR, CF, LVDP, d*P*/dt$_{max}$, and d*P*/dt$_{min}$ as shown in Table 2, were measured to investigate cardiac functional recovery after 20 min of no-flow ischemia.

Administration of 12,13-EpOME 100 nM resulted in a significant decrease of LVDP recovery (89 ± 30% vs. 49 ± 24%; $p = 0.029$) as well as lower d*P*/dt$_{max}$ and d*P*/dt$_{min}$ (85 ± 25% vs. 43 ± 20%; $p = 0.009$ and 83 ± 24% vs. 44 ± 19%; $p = 0.012$, respectively) after 40 min of reperfusion.

12,13-DiHOME 100 nM significantly decreased LVDP recovery after reperfusion (89 ± 30% vs. 31 ± 28%; $p = 0.003$) and lowered d*P*/dt$_{max}$ and d*P*/dt$_{min}$ (85 ± 25% vs. 33 ± 28%; $p = 0.002$ and 83 ± 24% vs. 34 ± 24%; $p = 0.003$, respectively), as shown in Fig. 2a. In addition, it led to an increase in post-ischemic heart rate (87 ± 10% vs. 184 ± 79%; $p = 0.018$), as shown in Fig. 2b.

sEH inhibitor AUDA 1 µM protected against all the detrimental effects of 12,13-EpOME. There were no differences between 12,13-EpOME + AUDA on cardiac parameters compared to vehicle regarding all above characteristics.

In contrast, AUDA 1 µM did not avert the 12,13-DiHOME effects. Hearts treated with the combination of 100 nM 12,13-DiHOME + 1 µM AUDA showed lower LVDP recovery after reperfusion (89 ± 30% vs. 45 ± 30%; $p = 0.015$). The combination also resulted in lower d*P*/dt$_{max}$ and d*P*/dt$_{min}$ (85 ± 25% vs. 41 ± 27%; $p = 0.007$ and 83 ± 24% vs. 44 ± 27%; $p = 0.012$, respectively). Of note, there was no statistically significant difference between 12,13-DiHOME and 12,13-DiHOME + AUDA effects regarding all parameters (Fig. 2).

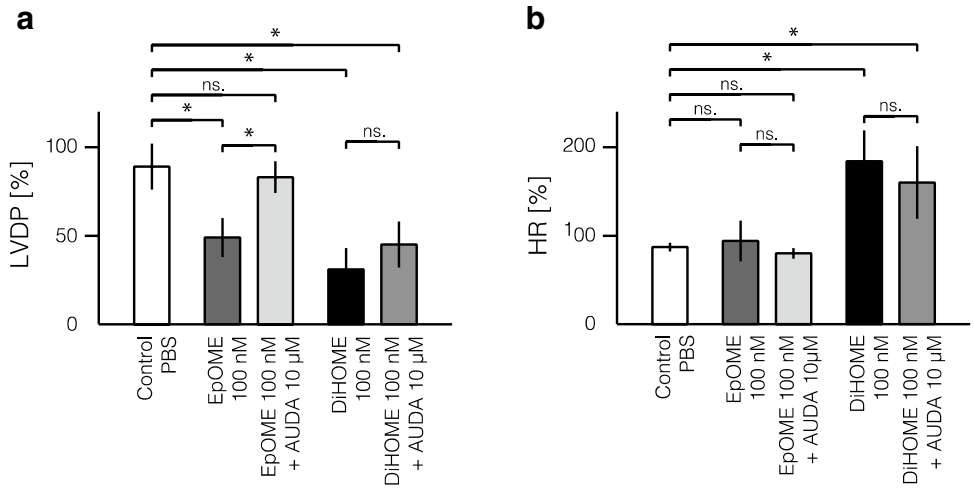

Fig. 2 Left ventricular functional recovery after ischemia. **a** Left ventricular developed pressure (LVDP) recovery after 40 min of reperfusion. Data show diminished LVDP recovery in hearts subjected to 12,13-EpOME (100 nM), 12,13-DiHOME (100 nM), and 12,13-DiHOME (100 nM) + AUDA (1 µM). AUDA protected against 12,13-EpoME-, but not 12,13-DiHOME induced impairment of functional recovery. **b** Heart rate (HR) after 40 min of reperfusion. Data show increased HR in hearts subjected to 12,13-DiHOME and 12,13-DiHOME + AUDA. $n = 5$ in each group; Control = PBS vehicle, $*p < 0.05$ versus control, *ns* non significant

DiHOME concentrations in the perfusate correlated with decreased post-ischemic LVDP ($n = 22$; $r = -0{,}644$; $p < 0{,}001$). We found significantly higher concentrations of 12,13-DiHOME in the DiHOME (0.06 ng/ml ± 0.03 vs. 11.5 ng/ml ± 4.61; $p < 0{,}001$) and DiHOME + AUDA group (0.06 ng/ml ± 0.03 vs. 11.8 ng/ml ± 6.40; $p < 0{,}001$) as well as in EpOME group EpOME (0.06 ng/ml ± 0.03 vs. 1.02 ng/ml ± 0.85; $p = 0.002$) compared to vehicle.

Upon metabolite analysis there was a ratio of 34:1 regarding *cis–trans* isomers of EpOME.

Discussion

This study aimed to clarify the role of 12,13-EpOME and 12,13-DiHOME in post-ischemic myocardial recovery. Our results show that 12,13-EpOME and 12,13-DiHOME-treated hearts exhibit diminished cardiac functional recovery after ischemia. While AUDA protected against the detrimental 12,13-EpOME effects, it did not ameliorate the 12,13-DiHOME effects. Both LA metabolites had no effect on basal cardiac function.

In the previous studies, EpOME and DiHOME were reported as putative toxic mediators in cardiac and lung failure [10–13]. DiHOME was shown to be increased in pigs after ischemia, decrease contractility of cat papillary muscles, and was associated with mitochondrial dysfunction and failure of Na^+/K^+-ATPase [12, 22, 25, 30]. Edin et al. reported that sEH-deficient mice exhibit increased recovery of LVDP after ischemia and that this effect was not only associated with higher concentrations of EETs but also with lower concentrations of LA diols [18]. Moreover, these authors attributed the negative effect of endothelial CYP2C8 overexpression on post-ischemic functional recovery to CYP2C8-mediated increased 9,10-DiHOME formation [18]. In our study, we found diminished contractility of murine hearts treated with 12,13-EpOME or 12,13-DiHOME after ischemia compared to hearts treated with PBS vehicle. DiHOME additionally led to an increased post-ischemic heart rate.

When LA is released, e.g. in ischemia as result of an inflammatory response, CYP epoxygenases generate the corresponding epoxides. The resulting epoxides can then be converted to its diols by sEH. Noteworthy, LA itself and its metabolites have the ability to cross plasma membranes and reach high intracellular concentrations when not bound to albumin [31]. In our study, administration of sEH inhibitor AUDA protected against all of the cardiac EpOME effects in ischemia–reperfusion, but had no impact on the detrimental effects of DiHOME. These results suggest the importance of a sEH-dependent pathway in cardiac ischemia–reperfusion injury in mice, with DiHOME being an active detrimental metabolite in this process. Our results support previous reports suggesting beneficial effects of sEH inhibition in cardiac ischemia and reperfusion [29, 32].

The underlying mechanisms for the deleterious effects of DiHOME have been widely discussed and different possible pathways identified in the past. It seems likely, that the DiHOME effects are not attributable to a single mechanism, but a variety of different mechanisms:

In our study, synergistic effects of DiHOME and ischemia on release and action of oxygen radicals pose a putative

cause for LV depression [33]. Since energy supply via ATP regeneration and membrane potential stability is crucial for myocardiocyte function, also mitochondrial and Na^+/K^+-ATPase dysfunction are likely to contribute the effects we observed [25, 26, 30]. Further research is needed to confirm our hypothesis.

In addition, mitochondrial function plays a major role in I/R injury by several mechanisms [34]. DiHOME acts as inhibitor of mitochondrial function [26]. Therefore, disturbed mitochondrial function may in part explain our results. Yet, this question has not been addressed in cardiomyocytes or cardiac I/R models.

Furthermore there is evidence for DiHOME to alter ion channel kinetics in ventricular myocytes [23]. In our experiments, DiHOME increased post-ischemic heart rate significantly. As Stimers et al. proposed, early after-depolarizations due to sodium channel inhibition can result in tachycardia. These effects may account for our findings. Noteworthy, incidence of cardiac arrhythmias was low in our study and did not differ significantly between study groups.

As stated above plasma levels ranged from up to 100 µM in burn patients to nano-molar concentrations in rodents. Concentrations of 1 nM and 100 nM seemed, therefore, reasonable for our experiments. Nevertheless, it remains unclear whether DiHOME exhibits a dose-dependent manner.

Chaudhary et al. furthermore pointed out a regioisomeric effect of 9,10- and 12,13-DiHOME and saw the latter responsible for cardiac damage in their experiments [29]. To complicate matters further, the enantiomeric distribution and susceptibiltiy of the same regioisomer can differ and thus functional effectiveness or potential damage, as reported for arachidonic acid- and some linoleic acid-derived metabolites before [35–38].

Studies need to be conducted in the future to investigate our findings regarding dose dependency as well as regio- and stereoselectivity of EpOME- and DiHOME-induced effects on cardiac function and damage.

Conclusion

Our experiments revealed an important role for EpOMEs and DiHOMEs to decrease post-ischemic functional recovery of LVDP in murine hearts. Noteworthy, the EpOME, but not the DiHOME effects, are sensitive to sEH inhibition. Thus, DiHOME appears to be the active metabolite and sEH inhibition deserves scrutiny as a possible therapeutic target for the treatment of cardiovascular disorders, especially in ischemic cardiogenic shock or cardiac arrest.

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Compliance with Ethical Standards

Disclosure All authors have nothing to disclose.

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