Effects of Etafenone on Myocardial Energy Metabolism as Studied by an Organ Redoximeter and Biochemical Analyses

Yasuo ETOH, Mikio NAKAZAWA and Shoichi IMAI*
Department of Pharmacology, Niigata University School of Medicine, Niigata 951, Japan
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Abstract—Direct recording of reduced nicotinamide adenine dinucleotide (NADH) fluorescence was conducted with an organ redoximeter in isolated perfused guinea pig heart. Cross-clamping of the aortic inflow line resulted in an increase in NADH fluorescence. After etafenone (10^-6 M), there was a significant prolongation of the time to the detectable or the maximum increase in NADH fluorescence. The magnitude of the increase in NADH fluorescence tended to be reduced (135% as compared with 145% in the control group). Supplemental chemical analyses revealed a significant increase in creatine phosphate, adenosine triphosphate (ATP) and total adenine nucleotide in the etafenone-pretreated group 15 min after postischemic reperfusion, although the ischemia-induced changes were not improved by this compound. It was suggested that the better recovery of myocardial high energy phosphate levels produced by etafenone was brought about by a decrease in oxygen consumption due to a decrease in mechanical performance of the heart and possibly by a better resynthesis of ATP.

As a coronary vasodilator which produces a decrease in the heart rate and myocardial oxygen consumption (1, 2), etafenone has been used in the therapy of ischemic heart disease. With respect to the effect of this compound on the ischemic myocardial energy metabolism, however, there exists only one study conducted with an electron spin resonance method by Suzuki et al. (3), in which the free radical formation by dog myocardial mitochondria was assessed with succinate as substrate. After administration of etafenone, the reduction of the free radical formation produced by occlusion of the coronary artery became smaller, suggesting that etafenone had protected the mitochondrial electron transfer system from ischemic damage.

It is known that reduced nicotinamide adenine dinucleotide (NADH) fluoresces when exposed to ultraviolet light. Making use of this characteristic of the NAD-NADH system as an optimal indicator of the tissue oxidation-reduction state, Chance et al. (4, 5) attempted to assess the energy-linked functions of mitochondria in myocardial tissue and obtained some important information.

In this study, we investigated the effect of etafenone on the myocardial energy status in isolated perfused guinea pig hearts under global ischemia and after posts ischemic reperfusion by directly recording the NADH fluorescence with an organ redoximeter and by determination of high-energy phosphate compounds.

Materials and Methods
Perfusion technique: Guinea pigs of either sex weighing between 360 and 480 g were sacrificed by a blow on the head. Immediately after opening the thorax, the heart was rapidly excised and placed in ice-chilled Krebs-Ringer solution to induce rapid cessation of the heart beat. Within a minute, the aorta was cannulated, and retrograde perfusion (Langendorff's technique) with modified Krebs-Ringer bicarbonate buffer was initiated immediately. The perfusion fluid contained NaCl (125.2 mM), KCl (4.7 mM),
CaCl₂ (2.5 mM), KH₂PO₄ (1.2 mM), NaHCO₃ (24.9 mM), sodium pyruvate (2.0 mM) and glucose (5.5 mM). After passing through a millipore filter in order to remove small particulate substances, the medium was pumped up from a reservoir to an oxygenating chamber devised by Neely et al. (6), which provided the perfusion pressure of 75 cmH₂O and at the same time equilibrated the perfusion fluid with a mixture of 95% oxygen and 5% carbon dioxide to ensure PO₂ values higher than 600 mmHg. A stable temperature of 38±0.3°C was maintained throughout each experiment.

A cannulating type probe (2 mmφ) of an electromagnetic flowmeter (Statham SP 2201) placed just above the aortic cannula measured the mean coronary inflow. The heart rate was counted with a tachometer triggered by the left ventricular pressure pulses. Left ventricular pressure was recorded with a saline filled cannula inserted into the ventricular cavity and connected to a pressure transducer (Statham P 50). The maximum rate of rise of the systolic pressure (dp/dtₘₐₓ) was used as an index of the inotropic state of the heart. According to Furnival et al. (7), this is a more reliable index of inotropic changes in the ventricle than peak pressure in the left ventricle, duration of systole, or stroke work at constant end-diastolic pressure. These measurements were recorded on a linearly recording thermostylus oscillograph (Watanabe Sokki Mark V).

The protocols of the experimental procedures are described in Fig. 1. Etafenone was used in a concentration of 10⁻⁶ M.

**Fluorometric technique:** For the purpose of direct recording of changes in myocardial redox state, an organ redoximeter (Tateishi Electronics Model HEF-4) was used. The coupling of the redoximeter to the heart was accomplished by using a fiber optics light guide made of quartz. The light source was a high-pressure mercury-vapour lamp which illuminated a segment of the light guide at the trifurcated end through filters with maximal transmission at 366 nm. The other common end (3 mmφ) of the light guide, in which the segment of the fibers transmitting the excitation light was randomly distributed, was placed as close as possible to the surface of the left ventricle of the heart with a three dimensional micromanipulator. The pyridine nucleotide fluorescence emission was transmitted through the second segment of the light guide to a photomultiplier equipped with a filter with a maximal transmission at 460 nm. In the preliminary experiments, the fluorescence emission was corrected for an optical artifact by a 1:1 subtraction of reflectance changes at 720 nm from the fluorescence changes. However, significant differences were not noted between the corrected and uncorrected fluorescence. Therefore, the correction was omitted in the actual experiments. The apparatus was shielded from an external light with a blackout curtain during experiments. The recording system for NADH fluorescence was the same as that used for recording the mechanical performance.

The intensity of NADH fluorescence was expressed as a percentage of the initial fluorescence level immediately before the exchange of the perfusion fluid. To further define the development of myocardial ischemia, the following indices were also used: the time from the clamping of the aortic inflow to the beginning of the fluorescence increase (latent period, LP), the time to the maximal fluorescence (T-max) and to 75% of the maximum (T-75).
Biochemical analysis: For the determination of the myocardial content of high energy phosphates, inorganic phosphate (Pi) and protein, the hearts were rapidly excised and immediately frozen with a pair of Wollenberger tongs precooled in liquid N₂. The frozen tissue fragments were crushed into a fine powder in a stainless steel percussion mortar cooled in liquid N₂ and then homogenized with five volumes of 0.6 N perchloric acid at 0°C. After centrifugation at 3,000×g for 15 min, the precipitate was dried to assay protein content by Peterson’s method. After neutralization to pH 7.0 with KOH and removal of the potassium perchlorate precipitate by further centrifugation, the supernatant was used for the determination of the metabolic products. The assay procedures for creatine phosphate, adenine nucleotides and Pi were described in a previous publication. These values were expressed as micromoles per gram of protein.

Statistical analysis: Statistical analysis of the results was made by the non-paired Student’s t-test. When P values were less than 0.05, the difference between the control and etafenone groups was regarded as significant. All results are expressed as the mean±standard error of the mean.

Results

Mechanical performance of the heart: Figure 2 depicts the heart rate, coronary inflow, left ventricular pressure, and dp/dt\textsubscript{max} during preischemic perfusion and postischemic reperfusion, and the effects of 10\textsuperscript{−6} M etafenone on these parameters. This dose of etafenone was chosen because it was

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** Effects of etafenone on the mechanical performances of the heart during a perfusion period of 20 min and during the period of reperfusion following 20 min cross-clamping of the coronary inflow. Closed circles: control hearts. Open circles: etafenone-treated hearts. *P<0.05, **P<0.01, ***P<0.001: Significantly different from control values.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** A representative record of the fluctuations in myocardial NADH fluorescence. Oscillations occur twice, i.e., immediately after ischemia and reperfusion.
estimated to be the effective plasma concentration in humans (11). Significant decreases in heart rate, coronary inflow and \( dp/dt_{max} \) were observed in the etafenone group as compared to the control group. In contrast, as regards to the left ventricular pressure, there was no statistically significant difference between these two groups.

**Studies in myocardial redox state:** A representative example of changes in myocardial NADH fluorescence is illustrated in Fig. 3. Immediately after the occlusion, rapid increase in NADH fluorescence with slight oscillations was observed. NADH fluorescence remained at a high level during the period of global ischemia. Postischemic reperfusion resulted in a decrease in NADH fluorescence within a minute which was accompanied by a damped oscillation.

Although there was no difference in NADH fluorescence between these two groups during preischemic perfusion and postischemic reperfusion, as shown in Fig. 4, the NADH fluorescence during global ischemia tended to be depressed in the etafenone-pretreated group. Similar depression was observed during the 10 and 30 min ischemic period (data not shown).

Furthermore, as Fig. 5 indicates, there was a delay in NADH fluorescence development in response to myocardial ischemia in the

![NADH fluorescence](image)

**Fig. 4.** The effect of etafenone on myocardial NADH fluorescence of the isolated perfused heart during global ischemia and reperfusion. Closed circles: control hearts \((n=4)\). Open circles: etafenone-treated hearts \((n=4)\).

![Effects of etafenone on the indices of increase in NADH fluorescence following cross-clamping of the coronary inflow line.](image)

**Fig. 5.** Effects of etafenone on the indices of increase in NADH fluorescence following cross-clamping of the coronary inflow line. LP: latent period, T-75: time from occlusion of the coronary inflow to 75% of the maximal increase of NADH fluorescence, T-max: time from occlusion of the coronary inflow to the maximal increase of NADH fluorescence.
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Eftafenone-pretreated group. The latent period and T-max were significantly prolonged in the eftafenone group as compared with the control group (6.3 ± 0.28 sec vs. 3.7 ± 0.46 sec, P < 0.001, and 450 ± 98.2 sec vs. 185 ± 65.4, P < 0.05, respectively).

Fig. 6. Effects of eftafenone on creatine phosphate and ATP contents during global ischemia and reperfusion. Closed circles: control hearts (n=3, 4, and 4: before ischemia, after ischemia and reperfusion, respectively). Open circles: eftafenone-treated hearts (n=4, 6 and 4: before ischemia, after ischemia and reperfusion, respectively). *P<0.05, **P<0.01: Significantly different from control values.

Fig. 7. Effects of eftafenone on inorganic phosphate (Pi) and total adenine nucleotide contents during global ischemia and reperfusion. Closed circles: control hearts (n=3, 4 and 4: before ischemia, after ischemia and reperfusion, respectively). Open circles: eftafenone-treated hearts (n=4, 6 and 4: before ischemia, after ischemia and reperfusion, respectively). **P<0.01: Significantly different from control values.
Studies in myocardial energy metabolism:

Changes in myocardial creatine phosphate and ATP contents are shown in Fig. 6. In both the control and etafenone groups, myocardial ischemia induced a reduction of creatine phosphate. However, the creatine phosphate level after reperfusion was significantly higher in etafenone-pretreated hearts as compared with that of the control hearts (76.9±3.12 µmoles/g protein vs. 67.0±1.11 µmoles/g protein, P<0.05). The ATP level was also significantly higher in the etafenone group after reperfusion (45.2±0.92 µmoles/g protein vs. 35.1±2.40 µmoles/g protein, P<0.01), although the level was lower than those in the preischemic period. The time course of changes in myocardial inorganic phosphate and total adenine nucleotide contents are shown in Fig. 7. There was no significant difference in Pi between the two groups throughout the experimental period, while the total adenine nucleotide content behaved in a similar fashion as those of ATP; the recovery of the total adenine nucleotide in the etafenone-pretreated hearts was significantly greater than those achieved with control hearts (57.7±1.33 µmoles/g protein vs. 43.7±2.58 µmoles/g protein, P<0.01).

Discussion

Although etafenone is usually considered to be a coronary vasodilator (1, 2), only a decrease in the coronary flow was observed in the present study in association with a decrease in the heart rate. This may have resulted from the differences in the mode of administration of the agent. In the two papers cited, relatively large doses were administered by a single shot, while in this experiment, the heart was perfused with Krebs-Ringer bicarbonate buffer which contained etafenone. A sustained decrease was also observed by Hashimoto et al. after a transient increase, in association with a sustained decrease in the heart rate.

Chance et al. devised an organ redoximeter based on the fluorescent characteristic of NADH for assessment of the cellular redox state (4, 5). The method is very useful for continuous, non-invasive observations of the myocardial energy state. Yet, there has so far been very few studies with this technique; Kissin and his coworkers used Chance’s method to study the effect of a β-adrenergic blocking agent and a coronary vasodilator on the myocardial energy status during ischemia in isolated perfused rabbit hearts (12, 13). Following their methods, duration of the latent period, the time to peak fluorescence and the time to 75% increase in fluorescence were used in the present study to assess the effects of etafenone on the energy status of the ischemic myocardium. All of these times were prolonged after etafenone, indicating that etafenone delayed the development of energy unbalance in the ischemic myocardium. In addition, the intensity of the NADH fluorescence attained during the global ischemia tended to be lower in the etafenone-pretreated group.

As to the high energy phosphate levels, prominent effects of etafenone were observed not during the period of ischemia, but during the period of postischemic reperfusion. Recently, it was stressed that reperfusion could produce severer injury than the ischemia itself (14–16). A significantly higher level of creatine phosphate, ATP and the total adenine nucleotides observed in the etafenone-treated preparation after reperfusion is consistent with a better preservation of the myocardial redox state during the ischemic period. Marinari and Gastaldi (17) reported that etafenone protected the mitochondria of the rat heart from the impairment of oxidative phosphorylation due to an uncoupling effect caused by an extract of posterior pituitary and dinitrophenol, and Cottalasso et al. (18) found that etafenone produced an improvement in the uncoupled oxidative phosphorylation of enlarged rabbit heart treated with diphteria toxin and loaded with water. Thus, the better preservation of the redox state during ischemia and the better recovery of creatine phosphate and ATP observed after reperfusion in the etafenone-pretreated group may be explained by a decrease in oxygen consumption due to a decrease in the heart rate, although the possibility that the better preservation may have been brought about by an improvement of the ability of the mitochondria to carry out oxidative phosphorylation cannot be ruled
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