THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

Ischemia/reperfusion induced-injury: control and mechanisms in the myocardium

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Introduction

Heart disease and cardiac surgery frequently result in periods of myocardial ischemia. Restoration of blood supply and reoxygenation of the myocardial tissue causes characteristic tissue damage, known as reperfusion injury, manifested by symptoms including ventricular arrhythmias and deterioration in cardiac functions. This effect may result primarily from oxidation of myocardial membrane lipids by free radical species produced in a burst during the first minutes of reflow, resulting in impairment of the cell membranes. Supporting this model, are reports that postischemic myocardial function may be improved by administration of antioxidants. Free radicals as major agents of damage in reperfusion injury, were supported by work conducted in our and other laboratories, directly demonstrating postischemic free radical formation as a spin trap adduct; and was further validated by the observation that administration of antioxidants (SOD and catalase), improved postischemic cardiac function and decreased arrhythmias in direct proportion to free radical quenching. EGb 761 (IPSEN, Paris, France), a ginkgolide natural free radical scavenger, was shown to decrease postischemic arrhythmias and improve cardiac function in rat hearts. Platelet activating factor (PAF), an inflammatory mediator produced by arachidonic acid metabolism, by increasing intracellular calcium overload, is also a major contributor to reperfusion injury in cardiac and noncardiac tissues. PAF receptor antagonists diminish reperfusion-induced injury. Hence, EGb 761 and other ginkgolides are expected to diminish reperfusion injury by two mechanisms: one involving neutralization of reperfusion-associated free radicals and a second, dependent on their PAF receptor antagonist properties.

The intracellular calcium-mediated activation of the cytoplasmic phosphatase calcineurin, enhances expression of genes contributing to development of cardiac hypertrophy and ultimately heart failure. Recently, it has been demonstrated that by blocking the activation of calcineurin with the immunosuppressant drug FK 506, the onset of cardiac hypertrophy in mice prone to the disorder may be delayed or abrogated. Calcineurin-dependent events may also contribute to postischemic reperfusion effects, suggesting a possible use for this drug for prevention of both cardiac hypertrophy and ischemia/reperfusion-induced injury. However, sustained treatment of human patients with FK 506 at dosage sufficient to prevent either cardiac hypertrophy, or treat ischemic disease, might result in unacceptably severe side effects due to the drug's toxicity. We hypothesize that combining FK 506 therapy with a ginkgolide, will allow use of the former drug at a subtoxic dosage, an option made particularly attractive by the low toxicity of FK 506.

Adaptation to ischemia or hypoxia is of fundamental importance in developmental, physiological, and pathophysiological processes. Cells, tissues, and organs of humans and other mammals respond to low oxygen tension in part by fine tuning the expression of a group of physiologically relevant genes, proteins, and enzymes. The microsomal heme oxygenase (HO), originally identified by Tenhunen et al, catalyses the oxidative degradation of heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Mammalian heme oxygenase catabolizes cellular heme to biliverdin, carbon monoxide (CO), and free iron, is represented by three isoforms, HO-1, HO-2, and HO-3 encoded by separate genes. HO-3, which in its primary structure resembles HO-2, is marginally active. Evidence has recently accumulated suggesting that CO generated by HO may be a physiological signaling molecule and, on the other hand, HO is thought to provide an antioxidant defense mechanism, on the basis of its marked up-regulation in stressed cells. Furthermore, recent studies suggest that CO could activate guanylyl cyclase and thus regulates cellular cGMP levels, and like nitric oxide, CO may function as a cellular messenger and control vascular tone. In view of increasing evidence that HO-1 confers protection against oxidative injury, much attention has been focused on the antioxidant properties of catalytic byproducts of HO catalysis such as biliverdin and bilirubin. In addition, the concomitant increase in endogenous CO production by heme degradation has also been targeted as a potential mediator of cytoprotection. Although CO is not a reactive free radical, it has been demonstrated to share many common properties with nitric oxide including pharmacological effects, signal transduction, and gene regulation.

The importance of HO system has been implicated in various disorders including asthma, endotoxemia, UV radiation, cardiomyopathy, and ischemia/reperfusion-induced injury. Therefore, it is reasonable to assume that a relationship may exist between the HO system and CO production. Interventions that are known to decrease free radical formation or content, although CO is not a free radical, are found to decrease the vulnerability of tissues to free radical mediated injury. Conversely, agents known to promote free radical production increase the vulnerability to such damage. Keyse and Tyrrell showed in 1989 that HO-1 is also highly induced by a variety of molecules causing oxidative stress. Consequently, many investigators have recently focused their attention on the role, function, and regulation of HO-1 in various in vivo and in vitro models of oxidative tissue injury. Although the function of HO-1 has been recently extensively studied, relatively little attention has been paid on the HO-1 related and directly measured endogenous CO production in the ischemic/reperfused myocardium. In a previous study, we observed a reduction in HO-1 mRNA expression and

enzyme activity in ischemic/reperfused fibrillating hearts but not in nonfibrillating myocardium. The aforementioned finding has led us to speculate that the reduction in HO-1 mRNA expression may change the endogenous CO production in fibrillated myocardium compared to the nonfibrillated heart. If this is so, HO-1 regulated endogenous CO production may play a crucial role in the control of reperfusion-induced myocardial damage.

Apoptosis, a noninflammatory mechanism of cell elimination and death, involves cytoplasmic shrinkage, DNA fragmentation and condensation, and the formation of apoptotic bodies. Prolonged periods of ischemia can cause tissue injury and cell death. Early reperfusion, although essential for the surviving of the previously ischemic tissue, can also cause cell mortality, possible as the result of the inflammatory (necrosis) and noninflammatory (apoptosis) responses. The precise mechanisms of ischemia and reperfusion-induced injury remain to be elucidated. It is well known that a large portion of cell loss during myocardial ischemia and reperfusion occurs through necrosis, and there is currently increasing interest in the possibility that cardiac cell death could also occur through apoptosis. The causes of apoptosis in failing and ischemic/reperfused hearts remain obscure. Moreover, it is not yet clear whether myocardial apoptosis is triggered consequently during heart failure, arrhythmias or ischemia and reperfusion. Many apoptotic mechanisms have been proposed for the development apoptosis, but tumor necrosis factor-α (TNF-α), protein kinase C (PKC), p53, p38 mitogen-activated protein kinase (p38 MAPK), and caspases have been suggested and frequently cited as important signaling mechanisms.

Aims of the studies

I. In the first part of our investigation we would like to determine the effect of combined administration of FK506 and the ginkgolide EGb 761 on reperfusion-induced arrhythmias and cardiac function. Here we show that FK506 reduced postischemic cardiac arrhythmias and attenuated impairment of cardiac function, and EGb 761 potentiated the cardioprotective effect of FK506.

II. The overall aims of the second part of our investigation were to study the (i) role of HO-1 mRNA expression related cellular defense mechanism and endogenous CO production, upon reperfusion, in nonfibrillated and fibrillated hearts, (ii) the effect of PBN, as a spin trap agent, on HO-1 mRNA and protein expression and endogenous tissue CO formation, and (iii) the effect of ZnPPIX, an HO inhibitor, on HO-1 mRNA, protein, and enzyme activity. We have used the technique of gas

chromatography to provide a direct demonstration of endogenous CO production during reperfusion and reoxygenation in the isolated rat myocardium.

III. In the third part of our investigations, as a step toward identifying putative mechanisms of myocardial apoptosis, we ascertained using pharmacological approaches in the present study whether caspase activation is involved in the apoptotic mechanism in isolated rat heart during ischemia/reperfusion. In the present investigation we studied whether the inhibition of caspase activity, using nonselective [acetyl-Tyr-Val-Ala-Asp chloromethylketone (YVAD-cmk)] and specific [Ac-Asp-Glu-Val-Asp-CH_2Cl, (Ac-DEVD-cmk), caspase-3 inhibitor; Z-Leu-Glu-OMe-His-Asp(OMe)-CH_2F (Z-LEHD-fmk), caspase-9 inhibitor] caspase inhibitors could prevent the extension of infarct size, apoptotic cell death, and improve the recovery of postischemic cardiac function in isolated rat hearts.

Male Sprague-Dawley rats (330-380 g) were used for all studies. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985).

Methods

I. The effect of the combined treatment of FK 506 with EGb 761

Isolated "working heart" preparation

Rats were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg body weight) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised and placed in ice cold perfusion buffer. Immediately after preparation, the aorta was cannulated, and the heart was perfused according to Langendorf method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer: 118 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.36 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 10 mM glucose. The left atrium was cannulated and the Langendorff system was switched to the working mode with a left atrial filling pressure of 17 cm of buffer (1.7 kPa) and aortic afterload pressure of 100 cm (10 kPa) of buffer as previously described. Aortic flow was

measured by a calibrated rotameter, and coronary flow rate was measured by a timed collection of the coronary perfusate that dripped from the heart.

Induction of ischemia and reperfusion

After 10 min aerobic perfusion of the heart, the atrial inflow and aortic outflow lines were clamped at a point close to the origin of the aortic cannula. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. To prevent the drying out of the myocardium during global ischemia, the thermostated glassware (in which hearts were suspended) was covered and the humidity was kept at a constant level (90%-95%).

Indices measured

An epicardial ECG was recorded by a polygraph throughout the experimental period by two silver electrodes attached directly to the heart. ECGs were analyzed to determine the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) and whether VF was non-sustained (spontaneously reverting to regular rhythm) or sustained (persisting through the first 3 min of reperfusion). After 3 min of ventricular fibrillation (sustained VF) hearts were defibrillated and myocardial function was recorded. The heart was considered to be in VF if an irregular undulating baseline was apparent on the ECG. VT was defined as 5 or more consecutive premature ventricular complexes; and this classification included repetitive monomorphic VT, which is difficult to dissociate from rapid VT. In each instance, VT spontaneously switched to sinus rhythm or VF, therefore VT was considered non-sustained. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Left ventricular developed pressure (LVDP) which was defined as the difference between left ventricular systolic and end-diastolic pressure, and the first derivative of LVDP ($+LVdp/dt_{max}$) were also recorded (Experimetria, Budapest, Hungary) by the insertion of a catheter into the left ventricle via the left atrium and mitral valve.

Experimental time course

In the first series of the study, rats (n=12 in each group) were orally treated with various doses of FK506 (0, 1, 5, 10, 20, and 40 mg/kg/day) for 10 days. After 24 hours of the last treatment, hearts were excised, isolated, and subjected to 30 min of global ischemia followed by 2 hours of reperfusion. In the second series of the study, rats were orally treated

with 25 mg/kg/day of EGb 761, 25 mg/kg/day of EGb 761 plus 1 mg/kg/day of FK506, 25 mg/kg/day of EGb 761 plus 5 mg/kg/day of FK506 for 10 days, respectively. The concentration of the EGb 761 in the "combination" therapy was selected to our previous study. Twenty four hours following the last treatment, hearts were isolated and the ischemia/reperfusion protocol (30 min. ischemia and 2 hours reperfusion) was conducted. The incidence of arrhythmias (VF and VT) and cardiac function (HR, CF, AF, LVDP, and $+dP/dt_{max}$) were registered.

Exclusion criteria

Preselected exclusion criteria for the present studies demanded that hearts were excluded if: (I) ventricular arrhythmias occurred during the period prior to the induction of global ischemia, (II) coronary flow and aortic flow rates were less than 17 ml/min and 40 ml/min, respectively, prior to the induction of ischemia. These criteria led to exclusion and replacement of 9 hearts in the study.

Statistics

Cardiac function data (HR, CF, AF, LVDP, and $LVdp/dt_{max}$), were expressed as the mean $\pm$ SEM. One-way analysis of variance was first carried out, to test for any differences between the mean values of all groups. If differences were established, the values of the control, drug-free group were compared with those of the drug treated groups by a two-tailed *t*-test with the Bonferroni correction. An analogue procedure was followed for distribution of discrete variables such as the incidence of VF and VT. An overall chi-square test for a 2xn table was constructed followed by a sequence of 2x2 chi-square tests to compare individual groups. A change of $p<0.05$ was considered significant.

II. The role of heme oxygenase related carbon monoxide and ventricular fibrillation in ischemic/reperfused hearts

Isolated "working heart" preparation

The isolation and perfusion of the hearts and the induction of ischemia and reperfusion were the same as earlier.

Measurement of cardiac function and arrhythmias

An epicardial ECG was recorded throughout the experimental period by two silver electrodes attached directly to the heart (Haemosys, Experimetria, Budapest, Hungary). The ECGs were analyzed to determine the development of VF. The heart was considered to be in VF if an irregular undulating baseline was apparent on the ECG. In studies, after 5 min of sustained VF, hearts were defibrillated and myocardial function was recorded in the nonfibrillated myocardium. Before ischemia and during reperfusion cardiac function, including heart rate (HR), coronary flow (CF) and aortic flow (AF) rates, and left ventricular developed pressure (LVDP), was registered (Haemosys, Experimetria, Budapest, Hungary).

Experimental time course

Hearts (n=6 in each group) were perfused for 3 successive 10-minute preischemic periods. During the first 10 minutes, a standard perfusion medium was used in order to remove blood and its elements from the myocardium in Langendorff mode. During the second 10-minute period, the isolated Langendorff preparation was switched to 'working' mode and control drug-free measurements of ECG, HR, CF, AF, and LVDP were recorded. During the third 10-minute period of preischemic perfusion, the composition of the perfusion fluid was changed by switching to a second perfusion reservoir containing perfusion fluid to which 1 μM of PBN had been added. Global ischemia was then induced for 30 min followed by 2 hours of reperfusion using the same PBN-containing solution during the first 10 min of reperfusion. The concentration of PBN was selected according to our previous study. Because the isolated working heart preparation is more sensitive to various interventions compared to the 'nonworking' Langendorff preparation, therefore the concentration of PBN was reduced to 1 μM. Cardiac function was recorded during reperfusion, and tissue CO content was measured by gas chromatography, as described below, at the end of reperfusion. At the end of each experiment, Northern and Western blots were also done for the determination of HO-1 mRNA and protein expression.

Determination of tissue CO content

We measured tissue CO content using gas chromatography as described by Cook et al.. Briefly, at the end of reperfusion period, hearts were removed from the perfusion system and homogenized in 4 volumes of 0.1 M phosphate-buffer (pH: 7.4) using an x520 homogenizer (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The homogenates were centrifuged at 4 ^{o}C for 15 min at 12,800 x g and the supernatant fractions were used for the determination of tissue CO. The reaction mixtures contain: 150 μl of supernatant, 60 μl of

NADPH (4.5 mM) and 50 µl of 3.5/0.35 mM methemalbumin, and for blank samples 60 µl phosphate buffer was used instead of NADPH. The samples were preincubated at 37 ^{o}C for 5 min, then the headspace was purged and the incubation was continued for 1 hour in dark at 37 ^{o}C. The reaction stopped by placing the samples on ice and the headspace gas was analyzed. One thousand µl of the headspace gas from each vial was injected into the gas chromatograph using a gastight syringe (Hamilton Co., USA) in argon gas flow with a speed of 20 ml/min. Analysis took place during the next 90 seconds on a 240 cm stainless-steel column with a 0.3 cm inner diameter. The individual value was expressed in mV, than peak's area was integrated and expressed in arbitrary units. The column was packed with Chromosorb 80/100 mesh (10 % Carbowax 20 M, 3.5 % KOH) and maintained at 120 ^{o}C. The temperature of the injector was controlled and kept at 150 ^{o}C.

Total RNA isolation and Northern blot

Total RNA was isolated from rat heart tissue (100 mg) by homogenization in 1 ml of TRIzol reagent (Gibco BRL, Life Technologies, Eggenstein, Germany), a guanidium thiocianate method, as described in the manual, then Northern blotting was done.

Western blot

Myocardial samples were homogenized in Tris-HCl (13.2 mM/L), glycerol (5.5%), SDS (0.44%), and β-mercaptoethanol. The same amount of soluble protein (50 µg) was fractionated by Tris-glycine-SDS-polyacrylamide gel (12%) electrophoresis, and Western blot was carried out as described by Pellacani et al. with the use of an antibody to recombinant rat HO-1 protein (1:1,000, Stressgen). The relative HO-1 protein expression was densitometry analyzed.

HO activity assay

One hundred milligrams of cardiac tissue were homogenized in 10 ml of 200 mM phosphate buffer and centrifuged at 19,000 x *g* 4 ^{o}C for 10 min. The supernatant was removed and recentrifuged at 100,000 x *g* 4 ^{o}C for 60 min, and the precipitated fraction was suspended in 2 ml of 100 mM potassium phosphate buffer. Biliverdin reductase was crudely purified by the technique of Tenhunen et al.. Heme oxygenase activity was assayed as described by Yoshida et al.. Reaction mixtures consisted of (final volume 2 ml) 100 µM potassium phosphate (pH 7.4), 15 nM hemin, 300 µM bovine serum albumin, 1 mg biliverdin reductase, and 1 mg microsomal fraction of myocardial tissue. The reaction was allowed to proceed for 1

h at 37 ^{o}C in dark in a shaking water bath and was stopped by placing the test on ice. Incubation mixtures were then scanned using a scanning spectrophotometer, and the amount of bilirubin was calculated as the difference between absorbance at 464 nm and 530 nm. Protein content was determined according to Lowry et al. in the microsomal fraction. In some additional studies, hearts were perfused with 5 μM of ZnPPIX and HO enzyme activity, HO-1 mRNA and protein expression were measured.

Statistics

The data were expressed as the mean ± SEM. One-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of nonfibrillated, fibrillated, PBN, and ZnPPIX treated groups were compared by multiple t-test followed by Bonferroni correction. A change of $p<0.05$ between the drug-free control and treated groups was considered to be significant.

III. The role of caspase inhibition in reperfusion induced apoptosis

Isolated heart preparation

The isolation and perfusion of the hearts were the same as earlier.

Induction of ischemia and reperfusion

After a 10-min aerobic perfusion of the heart for occlusion of the left main coronary artery, a suture mounted on a curved needle was placed under the origin of the artery and the ends of the suture were passed through a small plastic tube. Myocardial ischemia was induced by clamping the plastic tube onto the surface of the heart with a surgical clamp. Reperfusion was initiated by releasing the snare. To prevent the myocardium from drying out during ischemia, the thermostated glassware was covered and humidity was kept at a constant level (90%-100%). In order to avoid the high incidence of reperfusion-induced arrhythmias, hearts were initially reperfused in Langendorff mode, and potassium concentration was relatively high in the perfusion buffer. If ventricular fibrillation was registered during the initial phase of reperfusion, the isolated heart was immediately electrically defibrillated using a single 20 V square-wave pulse of 1 ms duration.

Determination of infarct size

Hearts for infarct size measurement were perfused, at the end of each experiment, with 30 ml of 1% triphenyl tetrazolium (TTC) solution in phosphate buffer (Na_2HPO_4 88 mM, NaH_2PO_4

1.8 mM) via the side arm of the aortic cannula then stored at -70˚C for later analysis. Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apico-basal axis into 1 mm thick sections, weighted, blotted dry, placed in between microscope slides and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). Using the NIH Image 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity and distinctness. Risk as well as infarct zones of each slice were traced and the respective areas were calculated in terms of pixels. At the end of each experiment, the risk area determined by Evans blue, and the intact ventricular tissue stained in red by TTC while the infarct area (white) was unstained by TTC. The areas were measured by computerized planimetry software and these areas were multiplied by the weight of each slice, then the results summed up to obtain the weight of the risk zone (g) and the infarct zone (g). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Detection of apoptosis

The detection of apoptotic cell death was evaluated using the terminaldeoxynucleitidyl transferase enzyme for nick end labeling TUNEL method using APOTAG kit (Oncor, Gaithesburg, MD, USA).

Indices measured

An epicardial ECG, for the monitoring of VF, was recorded by a computer acquisition system (Haemosys, Experimetria, Budapest, Hungary) throughout the experimental period by two silver electrodes attached directly to the heart. Aortic flow was measured by an in-line flow rotameter. Coronary flow rate was measured by a timed collection of the coronary effluent that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Left ventricular developed pressure (LVDP) was also recorded by the insertion of a catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were also registered by Haemosys computer acquisition system. Infarct size and TUNEL positive apoptotic cells were measured after 30 min of ischemia followed by 120 min of reperfusion as we described in the previous paragraphs.

Experimental time course

Hearts (n=6 in each group) were randomized to the following: (i) controls: hearts, after the initial stabilization period, were subjected to 30 min of regional ischemia followed by 120

min of reperfusion; (ii) treated hearts received a nonselective or selective caspase inhibitor added in the perfusion buffer for 10 min, starting at the onset of reperfusion. Caspase inhibitors and their doses were as the follows: 0.1 and 0.5 μmol/liter of acetyl-Tyr-Val-Ala-Asp chloromethylketone (YVAD-cmk), a nonselective caspase inhibitor; 0.07 and 0.2 μmol/liter of Ac-Asp-Glu-Val-Asp-CH_2Cl, Ac-DEVD-cmk, a selective caspase-3 inhibitor; 0.07 and 0.2 μmol/liter of Z-Leu-Glu-OMe-His-Asp(OMe)-CH_2F, Z-LEHD-fmk, a selective caspase-9 inhibitor. All caspase inhibitors were dissolved in dimethylsulphoxide, and the aliquots were diluted in perfusion buffer immediately before use. The final concentration of dimethylsulphoxide in the Krebs-Henseleit buffer did not exceed 0.03 %, a concentration which has no effect on cardiac function in this model. Caspase inhibitor-free hearts were perfused with 0.03% of DMSO, started at the moment of reperfusion for 10 min, and the data of this control group served as the caspase inhibitor-free control values.

Exclusion criteria

Pre-selected exclusion criteria for the present studies demanded that working hearts were excluded if: (i) ventricular arrhythmias occurred during the period prior to the induction of regional ischemia, (ii) coronary flow and aortic flow were less than 19 ml/min and 35 ml/min, respectively, prior to the initiation of ischemia. For these reasons, 4 hearts were excluded from the whole study.

Statistical analysis

The values for myocardial functional parameters (HR, CF, AF, LVDP), infarct sizes, and TUNEL positive apoptotic cells were all expressed as the mean ± standard error of the mean (SEM). Differences between data were analyzed for significance by performing a Student's *t*–test. The results were considered to be significant if $p < 0.05$.

Results

I. The effect of the combined treatment of FK 506 with EGb 761

Effects of FK 506 on arrhythmias and cardiac function

I. In the present studies, we required that the control group exhibit a high vulnerability to reperfusion-induced arrhythmias, in order that maximum scope would exist for the demonstration of antiarrhythmic effects of FK 506 and its combination with EGb 761. To

ensure this within the experimental time course and conditions defined for this study, 30 min normothermic global ischemia followed by 2 hours reperfusion was selected. The results demonstrate that in rats treated with different doses of FK 506, the incidence of reperfusion-induced arrhythmias was dose-dependently reduced. Thus, with 1, 5, 10, 20, and 40 mg/kg of FK 506, the incidence of total (irreversible plus reversible) VF was reduced from its drug-free control value of 92% to 92% (NS), 83% (NS), 67% (NS), 33% ($p<0.05$), and 25% ($p<0.05$), respectively. The incidence of irreversible VF and the incidence of VT showed the same pattern. Animals showed excellent tolerance of FK506, with no visible evidence of toxicity observed, even at the high end of the dosage range used.

II. In the drug-free group, the preischemic values of HR, CF, AF, LVDP and $+LVdP/dt_{max}$ were 305±8 beats/min., 27.3±0.8 ml/min., 49.9±1.4 ml/min., 18.2±0.3 kPa and 807±21 kPa/s respectively. No significant changes were observed in these values as a result of FK506 treatment. Thus, a significant recovery of postischemic cardiac function (CF, AF, LVDP, and $LVdP/dt_{max}$), was observed in the groups treated with 20 and 40 mg/kg of FK 506, respectively. Heart rate did not show a significant change in the treated groups in comparison with the drug-free control values, either before the induction of ischemia or during reperfusion.

Combined treatment with FK 506 with EGb 761: effects on arrhythmias and postischemic cardiac function

III. Neither 25 mg/kg of EGb 761 nor 1 and 5 mg/kg of FK 506 significantly reduced the incidence (%) of reperfusion-induced arrhythmias. The combination of 25 mg/kg of EGb 761 with 1 or 5 mg/kg of FK 506 resulted in a significant reduction in the incidence of reperfusion-induced VF and VT. Thus, the coadministration of 25 mg/kg EGb 761 with 1 and 5 mg/kg of FK 506, significantly reduced the incidence of total VF and irreversible VF from their control values of 92% and 92% to 42% ($p<0.05$) and 33% ($p<0.05$), 25% ($p<0.05$) and 8% ($p<0.05$), respectively. This reduction followed the same pattern in the incidence of reperfusion-induced VT.

IV. Cardiac function was not significantly changed in the groups treated with a single intervention during reperfusion. The combination of 25 mg/kg EGb 761 with 1 and 5 mg/kg of FK 506, significantly improved the recovery of cardiac function (CF, AF, LVDP, and $+dP/dt_{max}$) during reperfusion. It is of interest to note that heart rate was not significantly

changed in the combination group either before the introduction of ischemia or during reperfusion.

II. The role of heme oxygenase related carbon monoxide and ventricular fibrillation in ischemic/reperfused hearts

I. The results clearly show that detectable endogenous CO production by GC can be observed in aerobically perfused myocardium, and this peak cannot be seen in ischemic/reperfused-fibrillated hearts. However, in ischemic/reperfused-nonfibrillated myocardium an increase in endogenous CO production was measured, and this peak was further elevated in PBN-treated hearts. Thus, the results show that PBN treatment significantly increases the endogenous CO production in the myocardium from its control value of 306 ±19 arbitrary units to 2228 ± 907 arbitrary units ($p<0.05$). This increase in endogenous CO production could significantly contribute to the antiarrhythmic effect as well as the improvement of postischemic cardiac function of PBN.

II. The expression of HO-1 mRNA (about 3.5-fold) was observed in ischemic/reperfused-nonfibrillated myocardium in comparison with the aerobically perfused nonischemic control heart. In hearts treated with 1 μM of PBN and subjected to 30 min ischemia followed by 2 hours of reperfusion and VF was not developed, the expression of HO-1 mRNA was further increased about 5-fold in comparison with the nonischemic control myocardium. In hearts subjected to 30 min ischemia followed by 2 hours of reperfusion and reperfusion-induced VF was developed, a significant downregulation in HO-1 mRNA expression was observed in comparison with the nonischemic control hearts. Thus, the results clearly show that the stimulation of HO-1 mRNA expression which reflected in an increased endogenous CO production could prevent the development of reperfusion-induced VF.

III. HO-1 protein expression follows the increase in HO-1 mRNA expression, thus, in hearts treated with 1 μM of PBN an increase in protein level was detected in comparison with the nonischemic controls. However, in the fibrillated myocardium, HO-1 protein expression was significantly reduced.

IV. The expression of HO-1 mRNA and its protein can be detected in reperfused/nonfibrillated myocardium. Because ZnPPIX treatment induces the development of reperfusion VF, therefore, the HO-1 mRNA and its protein downregulation was observed, as it was found in the ZnPPIX-free reperfused/fibrillated group.

V. HO enzyme activity was significantly increased in hearts treated with PBN. However, a significant reduction in HO enzyme activity was observed in both

ischemic/reperfused-fibrillated myocardium and in hearts treated with 5 μM of ZnPPIX in comparison with the nonischemic control group.

VI. Preischemic control values of HR, CF, AF, and LVDP were 312 ± 9 beats/min, 26.2 ± 1.3 ml/min, 51.4 ± 2.2 ml/min, and 18.1 ± 0.4 kPa/s, respectively. These preischemic control values were not significantly changed by 1 μM of PBN pretreatment before the induction of 30 min of normothermic global ischemia. However, upon reperfusion, the recovery of postischemic cardiac function was significantly increased in hearts treated with 1 μM of PBN, which reflected in the elevation of CF, AF, and LVDP. A significant improvement in the recovery of postischemic HR was not detected in the PBN treated group in comparison with the drug-free control value.

III. The role of caspase inhibition in reperfusion induced apoptosis

I. YVAD-cmk, a non-specific caspase inhibitor, significantly improved postischemic cardiac function including CF, AF, and LVDP. Thus, after 60 min of reperfusion, hearts treated with 0.1 and 0.5 μmol/liter of YVAD-cmk, AF was significantly increased from its postischemic drug-free control value of 11.1 ± 0.5 ml/min to 17.0 ± 1.9 ml/min ($p<0.05$) and 24.8 ± 1.4 ml/min ($p<0.05$), respectively. The same trend was observed in the postischemic recovery of CF and LVDP after 60 min as well as after 120 min of reperfusion. HR, during reperfusion, did not show any significant changes in the YVAD-cmk treated groups in comparison with the time-matched drug-free control values. Different doses of selective caspase inhibitors (Ac-DEVD-cmk, a caspase-3 inhibitor; Z-LEHD-fmk, a caspase-9 inhibitor) failed to significantly improve postischemic cardiac recovery after 30 min of ischemia followed by 120 min of reperfusion.

II. The percentage of infarct size was markedly reduced from its drug-free control value of 32±5% to 18±3% ($p<0.05$) and 15±5% ($p<0.05$) in hearts treated with 0.1 μmol/liter and 0.5 μmol/liter of the nonselective caspase inhibitor (caspase II), respectively. The selective caspase inhibitors (Ac-DEVD-cmk, a caspase-3 inhibitor; Z-LEHD-fmk, a caspase-9 inhibitor) did not result in a significant protection against the development of infarct size in our model system.

III. An in situ apoptosis detection kit was used to evaluate the extent of apoptosis in the isolated rat hearts subjected to ischemia and reperfusion. In our studies, we measured TUNEL positive apoptotic cells in the infracted area in ischemic/reperfused hearts. We were unable to detect any apoptotic cells in the aerobically perfused nonischemic control hearts. After 30 min ischemia followed by 120 min reperfusion, a substantial number of TUNEL

positive apoptotic cells were detected in the tissue under propidium iodide filter superimposed with a fluorescence filter. In hearts treated with various doses of nonselective caspase II inhibitor, a reduction in apoptotic cells was observed in hearts subjected to ischemia/reperfusion protocol indicating that the application of a nonselective caspase inhibitor is able to reduce the apoptosis-induced cell death in the infarcted area of the myocardium. Thus, in hearts treated with 0.1 μmol/liter and 0.5 μmol/liter doses of caspase II nonselective inhibitor, the TUNEL positive myocytes nuclei were reduced from its ischemic/reperfused drug-free control value of 11.2 ± 2.1% to 6.2 ± 1.6% ($p<0.05$) and 1.2 ± 0.2% ($p<0.05$), respectively. In hearts treated with selective caspase-3 and caspase-9 inhibitors, a significant reduction in TUNEL positive myocyte nuclei was not detected after 30 min ischemia followed by 120 min of reperfusion.

Conclusions

I. In the first part of our investigation, we hypothesized that inhibition of calcineurin with FK506 might also inhibit arrhythmogenesis and improve cardiac function after ischemia/reperfusion. We further reasoned that in addition to the known antioxidant properties of ginkgolides, these compounds also inhibit PAF-dependent rises in intracellular calcium by acting as agonists to the PAF receptor. Their application should contribute cardioprotective properties resulting from calcineurin inhibition due to decreased calcium availability. Finally, we hypothesized that the effect of ginkgolide may potentiate the effect of FK506.

The results of our study support a role for FK506 as an antiarrhythmic agent and additionally demonstrate its capacity to improve cardiac function. A dose-dependent improvement in cardiac function and inhibition of postischemic arrhythmias resulted from pretreatment of rats with FK506. It is likely that this effect is the result of FK506-mediated blockage of events downstream of calcineurin/NFAT3/GATA4, which is known to include expression of immediate early genes such as *c-fos*, and *c-myc*, as well as increased activity by fetal cardiac genes. Nevertheless, since the molecular signaling pathways which couple calcineurin-dependent gene expression to arrhythmias and cardiac function remain to be defined, an accounting for the observed effects cannot be made on the basis of this work

FK506 has been reported to cause kidney damage and adverse reactions to other drugs. Thus, it is clear that combining FK506 with other treatments which lower its dosage requirement without diminishing its cardioprotective properties, may allow clinical use of

FK506 in prevention and therapy of cardiac disorders. The ginkgolide EGb 761 was chosen for use in combination with FK506 due to its demonstrated utility in improvement of post-ischemic cardiac function and reduction in incidence of arrhythmias in rat hearts. In the present investigation, the highest dosage of the ginkgolide was 25 mg/kg, which did not result in significant decreases in either VF or VT. Likewise, FK506 dosages between 1-5 mg/kg failed to affect these parameters. Nevertheless, combined treatment with both drugs synergistically and dose-responsively reduced the incidence of postischemic arrhythmias and additionally resulted in significant improvements in cardiac function. The molecular mechanisms contributing to these effects remain to be defined, but almost certainly include diminished levels of oxygen radical-induced damage to cardiomyocyte membranes, resulting from the demonstrated antioxidant properties of EGb 761, contributing to restoration of stable compartmentalization and transmembrane flow of Na^+, K^+, Ca^{2+} and Mg^{2+}. This conclusion is underscored by our observation in a previous study, that coadministration to rats of EGb 761 plus antioxidant enzymes (SOD or catalase), also produced a dose-dependent reduction in reperfusion-induced arrhythmias, paralleled by decreases in free radical concentrations. Assessment of the degree to which EGb 761 contributed to cardioprotective effects mediated by calcineurin inhibition resulting from blockage of the PAF receptor, are difficult to estimate; nevertheless, this too is undoubtedly a factor in the observed synergism between FK506 and EGb 761. PAF is implicated as a major contributor to a diverse range of pathologies, including cardiac disorders. Moreover, PAF has been demonstrated to be directly contributory to postischemic reperfusion injury of cardiac and other tissue, an effect which may be inhibited by pretreatment with PAF receptor antagonists, including ginkgolides.

In summary, treatment of rats with FK506 resulted in improvement of postischemic cardiac function and decrease in arrhythmias. Corroborated with these findings, this effect was further noted to be synergistic when the two drugs were coadministered. This suggests that pharmaceutical interventions incorporating both classes of drug may provide a new and powerful approach to management of ischemic injury associated with heart disease and surgical intervention. Our findings also raise the possibility that development of cardiac hypertrophy may be prevented by administration of this combined therapy, allowing subtoxic dosage of FK506 by coadministration of a ginkgolide.

II. In the second part of our studies, we have endeavored to obtain more circumstantial evidence for the involvement of HO-1-related endogenous CO production and its direct

measurement in the genesis of reperfusion-induced ventricular fibrillation. We now provide direct evidence by measuring cellular CO production suggesting that this is the case and the protective effect against the development of reperfusion-induced VF is attributable to the generation of endogenous CO through HO-1 mRNA induction. The use of PBN to stimulate HO-1 mRNA expression and endogenous CO production, we demonstrate that endogenous CO production is essential for the prevention of reperfusion-induced VF. The mechanisms by which PBN treatment produces suprainduction of HO-1 gene expression leading to the increase of endogenous CO production could involve the following: First, HO-1 is among various genes whose expression is induced by free radicals. Thus, the first explanation would consider activities of stable PBN-radical adducts formed through the interaction of PBN with reactive oxygen species, which in turn, are known to be generated during the reperfusion component of ischemia leading to the stimulation of HO-1 mRNA. According to this explanation, stable PBN-radical adducts with a prolonged half-life would be more effective in modulating HO-1 gene expression by virtue of their presence in the myocardium resulting in an increased endogenous CO production. The second possible explanation for the suprainduction of HO-1 and increased CO formation may involve the elimination of reactive oxygen species and reducing their direct interaction with cellular components including gene transcription machinery. Therefore, with trapping of powerful oxidizing radical species, the rate of decay in HO-1 mRNA could decrease. The spin trap by itself in the absence of reactive oxygen species may not be an effective modulator of HO-1 gene expression and cardiac function at the concentration studied in the present investigation, because the preischemic cardiac function is unchanged in the presence of PBN, indicating that PBN interaction with reactive oxygen species formed during reperfusion is a necessary step to modulate HO-1 mRNA expression. The mechanism by which reactive radicals transcriptionally regulate HO-1 gene is not known, although involvement of a number of transcription factors such as NFκB and hypoxia inducible factor-1 have been implicated in this process. The expression of HO-1 protein coincides with the expression of HO-1 mRNA in our studies. The results show that in hearts perfused with ZnPPIX, an inhibitor of HO enzyme activity, the incidence of reperfusion-induced VF was detected in all hearts, and consequently, the expression of HO-1 mRNA and its protein was significantly downregulated in the reperfused/fibrillated myocardium. Furthermore, our data show that HO inhibition by ZnPPIX resulted in a significant reduction in HO enzyme activity in the fibrillated myocardium. The results clearly show that a downregulation in HO-1 mRNA and its protein expression, and a reduction in HO enzyme activity could play a crucial role in the development of reperfusion-induced VF.

Thus, it is reasonable to conclude that a significant reduction in HO enzyme activity could be related to the development of reperfusion-induced VF. Our results may also indicate that HO-1-related endogenous CO production is a key factor in the development of reperfusion-induced VF but further studies are necessary to be done in this direction.

The relative contribution of CO and NO to the activation of guanylyl cyclase in the cardiovascular system remain unknown, even under physiological conditions. In many pathological conditions such as hypoxia, thermal injury, and ischemia/reperfusion, CO-mediated effects may be predominant. For instance, it was found that an HO-inhibitor blocked cGMP production, whereas a NOS inhibitor had no effect. Because both NO an CO mediate their effects through the stimulation of guanylyl cyclase, in future investigation, it is necessary to demonstrate that an increased cardiac cGMP level is at least partly due to CO rather than NO in our model system.

In conclusion, our findings suggest that pharmacological stimulation of the expression of HO-1 could prevent the development of reperfusion-induced VF. However, additional studies are needed to resolve the links in the apparent cascade of the up- and downregulation of ischemia/reperfusion-induced gene expression and related protein synthesis, and HO enzyme activity in fibrillated and nonfibrillated myocardium.

III. Coronary artery occlusion/reperfusion results in damage to the myocardium, an important consequence of which is the failure of postischemic cardiac function. It is widely believed that agents modify mechanisms underlying cellular injury may also alter the severity and extent of cardiac function in the myocardium. Apoptosis is a crucial process in development, maintenance of cell homeostasis, and regulation of the immune system.

In the third part of our investigations, we demonstrate that a nonselective caspase inhibitor, YVAD-cmk, when perfused at the onset and initial phase of reperfusion in an experimental model of myocardial infarction, provides substantial protection. The results of this study also demonstrate that the inhibition of apoptosis by specific caspase inhibitors (caspase-3 and -9) was unable to protect against the contractile damage and infarct size that occur during a combined occlusion-reperfusion insult in isolated rat hearts. The protective effect of the general caspase inhibitor, in our study, reflected in a marked reduction in TUNEL positive apoptotic cells. Thus, in hearts treated with 0.1 μmol/liter and 0.5 μmol/liter doses of caspase II nonselective inhibitor, the TUNEL positive myocytes nuclei were significantly reduced. However, the application of selective caspase inhibitors, in our study, failed to reduce the number of TUNEL positive apoptotic cells in our model.

Caspases are a conserved family of cysteine proteases that are universal effectors of apoptosis. Caspase proenzymes are proteolytically cleaved at specific aspartic acid residues in order to generate their active subunits. Activated caspases induce cells to undergo apoptosis by cleaving and altering the function of diverse intracellular proteins. The loss of potentially viable cells through activation of an apoptotic mechanism may profoundly influence cardiac function, given the lack of de novo myocyte proliferation and meager ability of the myocardium for repair after cardiac injury.

It is not clear and is not the aim of the present study to what extent of apoptosis and necrosis individually contribute to the development of myocardial infarction, and probably both of them, a "necro-apoptotic" mechanism contributes to the development of reperfusion-induced injury. However, we demonstrate that under our experimental circumstances the non-specific caspase inhibitor significantly reduces the extent of myocardial infarction and improves cardiac function, providing evidence that one of the key signaling pathways controlling apoptosis could mediate reperfusion-induced injury. Furthermore, the results of this study suggest that, although pro-apoptotic signaling plays an important role in the development of reperfusion-induced damage, the application of a specific caspase inhibitor may not afford alone a significant protection against postischemic damage in our model. There are currently abundant data to indicate that different signal mechanisms contribute to apoptosis leading to postischemic cardiac failure, but it is reasonably to believe that different and multiple mechanisms rather than a single factor could significantly contribute to the development of cardiac apoptosis.

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1. **Bak I.**, Tosaki A. (2001) Caspase inhibition on infarct size and cardiac function in ischemic and reperfused isolated rat hearts. *J. Mol. Cell. Cardiol.* **33:** A7

2. **Bak I.**, Papp G., Joo F., Tosaki A. (2002) Heme oxygenase related carbon monoxide and ventricular fibrillation. *J. Mol. Cell. Cardiol.* **34 (6):** A6

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1. Pataki T., **Bak I**., Csonka C., Kovacs P., Varga E., Blasig I. E., Tosaki A. (2001) Regulation of ventricular fibrillation by heme oxygenase in ischemic/reperfused hearts. *Antioxid. Redox. Signal.* **3:** 125-134; **IF:** 2003-ban várható.

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