行政院國家科學委員會專題研究計畫　成果報告

活化對三磷酸腺甘酸敏感鉀離子通道對缺血前約制誘導出神經性保護在動物狗心臟的研究

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計畫參與人員：

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中　華　民　國　93　年　1　月　20　　日

行政院國家科學委員會專題研究計畫成果報告

活化對三磷酸甘敏感鉀離子通道對缺血前約制誘導出神經性保護在動物狗心臟的研究

Ischemic Preconditioning Induces Neuroprotection by Activation of Myocardial K_{ATP} Channels in Canine Hearts

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一.中文摘要

關健詞：三磷酸甘敏感鉀離子通道(ATP-sensitive K^+ channels)、缺血前約制(IP)、神經性保護、正腎上腺素(Norepinephrine)

活化對三磷酸甘敏感鉀離子通道來解釋缺血前約制(IP)可以減小心肌梗塞面積以及重灌注引起心不整律在交感神經系的機轉，近來報告顯示，缺血前制約除了對心肌細胞保護外對心臟交感神經系統也有控制作用。在缺血時交感神經細胞鈉離子濃度上升會使非細胞外的正腎上腺素分泌。過多的正腎上腺素對缺血性心肌會因細胞內鈣離子超載及細胞骨架分離而惡化，如此心肌梗塞面積會擴大。然而其中有關缺血前約制的交感神經消滅之機轉仍沒有實驗報告，所以我們提出這次實驗計劃其動物模型為成年狗，經麻醉的狗，先將冠狀動脈左前降枝暫時阻斷 60 分鐘後，再有三小時的重灌注為實驗組有缺血前約制處理。實驗結果實驗組對控制組為 15 ± 6%對 43 ± 8% P<0.0001。接著若實驗前將粒線體中三磷酸敏感鉀離子通道拮抗劑 5- hydroxydecanoate 先投予以完全抑制缺血前制約的心臟保護作用。另一實驗使用內質網中三磷酸敏感鉀離子通道拮抗劑 HMR-1098 並不會減小心肌前制約讓心肌梗塞面積縮小的保護作用，同時 5-hydroxydecanoate 單獨使用無論在有無缺氧前約制都不會預防重灌注的心不律的發生。所以缺血前約制可以明顯地減低組織正腎上腺素表現在左心室缺血的心肌邊界效應。無論使用 5- hydroxydecanoate、HMR-1098 都會抑制缺血前約束的心肌保護作用與正腎上腺素在組織表現上升的抑制。以上這些實驗結果可以推論缺血前約制通道不同的三磷酸敏感鉀離子通道，來達到心臟保護作用兩方面，一方面為減小心肌梗塞面積一方面為降低重灌注心律不整發生。從活化不同三磷酸敏感鉀離子通道與正腎上腺素。

二.英文摘要(Abstract)

Abstract

Ischemic preconditioning (IP) reduces myocardial infarct sizes and decreases the severity of reperfusion-induced arrhythmias by activation of different ATP-sensitive K^+ (K_{ATP}) channels. Recent studies have demonstrated that the beneficial effect of IP is not limited to the cardiomyocytes but also can be observed in cardiac sympathetic system. Nonexocytotic release of norepinephrine (NE) during ischemia is a consequence of increased intracellular sodium concentrations of the sympathetic nerve terminal. Excessive release of NE is deleterious to ischemic myocardium by inducing intracellular calcium overload and the degradation of cytoskeletal structure,

leading to expansion of the infarct size and arrhythmia. However, the underlying mechanism of IP-induced sympatholysis remains unknown.

Anaesthetized dogs were subjected to 60 min of the left anterior descending coronary artery occlusion followed by 3 h of reperfusion. Infarct size was markedly reduced in IP-treated dogs compared to controls (15 ± 6% vs. 43 ± 8%, $P < .0001$). Pretreatment with the mitochondrial ATP-sensitive K^+ channel antagonist 5-hydroxydecanoate completely abolished IP-induced cardioprotection. The sarcolemmal ATP-sensitive K^+ channel antagonist HMR 1098 did not significantly attenuate IP-induced infarct size limitation. Additionally, IP reduced the incidence and duration of reperfusion-induced ventricular tachycardia and ventricular fibrillation significantly. Although 5-hydroxydecanoate alone caused no significant effect on the incidence of reperfusion arrhythmias in the presence or absence of IP, the administration of HMR 1098 abolished IP-induced improvement of reperfusion arrhythmias. IP significantly decreased tissue norepinephrine of the left ventricle from the border zone compared with controls. This reduction in the norepinephrine level was blocked by either 5-HD and HMR-1098. These results demonstrate that IP is cardioprotective against infarct sizes and fatal reperfusion arrhythmias by different ATP-sensitive K^+ channels for an IP-related norepinephrine mechanism. The infarct size-limiting and antiarrhythmic effects of IP were abolished by 5-hydroxydecanoate and HMR 1098, suggesting that the effects may result from activation of the mitochondrial and sarcolemmal ATP-sensitive K^+ channels, respectively..

Keywords: ATP-sensitive potassium channel; Ischemic preconditioning (IP); Neuroprotection; Norepinephrine.

三. INTRODUCTION

Ischemic preconditioning (IP) is a cardioprotective phenomenon in which short periods of myocardial ischemia result in resistance by the myocardium to a subsequent stress (1). The phenomenon reduces myocardial infarct sizes (2) and decreases the severity of reperfusion-induced arrhythmias (3). Recent studies have demonstrated that the beneficial effect of IP is not limited to the cardiomyocytes but also can be observed in cardiac sympathetic system (4). Autonomic nerve fibers are integral components of myocardial tissues. During the acute stage of prolonged myocardial ischemia, massive norepinephrine (NE) release into the interstitial space was observed, caused by a nonexocytotic mechanism due to sympathetic nerve injury (5). Nonexocytotic release of NE during ischemia is a consequence of increased intracellular sodium concentrations of the sympathetic nerve terminal, which can be improved by the process of IP (6). Excessive stimulation by NE during myocardial ischemia is deleterious to ischemic myocardium by inducing intracellular calcium overload and the degradation of cytoskeletal structure, leading to expansion of the infarct size (7) and arrhythmia (8). IP prevents malignant arrhythmias such as ventricular tachycardia and ventricular fibrillation (9). This action is speculated to be caused by the depletion of

the NE stores in the sympathetic nerve terminals, since pharmacological depletion of endogenous catecholamines protect the heart against reperfusion-induced arrhythmias (8). These findings suggest the importance of the adrenergic system in the mechanism of the protective action of IP. However, the underlying mechanism of IP-induced sympatholysis remains unknown.

Previous studies have shown that IP is cardioprotective against infarct sizes and fatal reperfusion arrhythmias by activation of different ATP-sensitive K^+ (K_{ATP}) channels (10). Cardiac myocytes contain 2 distinct K_{ATP} channels with one subtype located in the sarcolemma (sar-K_{ATP}) and the other in the inner membrane of the mitochondria (mit-K_{ATP}) (11). Mit-K_{ATP} channels share some pharmacological properties with sar-K_{ATP} channels, whereas possessing a distinct pharmacological response. Sar-K_{ATP} channels are selectively blocked by HMR1098, whereas mit-K_{ATP} channels are specifically inhibited by 5-hydroxydecanoate (11). The infarct size-limiting and antiarrhythmic effects of IP were abolished by 5-hydroxydecanoate and HMR 1098, suggesting that the effects may result from activation of the mitochondrial and sarcolemmal K_{ATP} channels, respectively. However, how the subcellular location of these channels contributes to attenuated sympathetic activities remains unknown.

The existence of K_{ATP} channels on nerves has been demonstrated (12). Since their discovery in cardiac muscle, K_{ATP} channels have been described in a multitude of organs such as pancreas, skeletal and smooth muscle and brain (13). Molecular cloning techniques have established that functional nerve K_{ATP} channels are constituted by heteromeric formation of inwardly rectifying K^+ channel subunits (Kir6.2) and the sulphonyluea receptor isoform SUR1 (12), identical to that revealed for pancreatic β cells (14). Since K_{ATP} conductance is present in neurons, the possibility arises that the release of NE from sympathetic nerves supplying the heart can be modulated via these channels which also might affect cardiac function during physiological and pathophysiological conditions.

Recently, IP has been shown to attenuate the myocardial release of NE after prolonged ischemia via the activation of K_{ATP} channels (4). There are conflicting reports concerning the role of different K_{ATP} channels in NE release. Some studies have shown that nicorandil, a specific agonist of mit-K_{ATP} channels, decreased NE release during prolonged ischemia in dogs, suggesting that mit-K_{ATP} channels play an important role in mediating NE release (4). In contrast, some showed that 5-HD, a specific antagonist of mit-K_{ATP} channels, did not change NE release in guinea-pig model (15), implying that mit-K_{ATP} channels did not relate to NE release. This discrepancy may stem from different species, collateral flow measurements (microspheres vs. others), and model (*in vitro* vs. *in vivo* studies) used in the studies. Thus, the critical question remains to the mechanism of NE release in the ischemic heart. This study will investigate whether IP provides neuroprotection against NE release in a canine model of acute myocardial infarction as it does in myocardial tissue by activation of K_{ATP} channels. To further assess the differential role of K_{ATP} channels in modulating NE release, we will use 5-hydroxydecanoate and

HMR 1098, specific mit- and sar-K_{ATP} channel blockers.

四.材料與方法

Methods

Preparation.

All experiments were conducted on male mongrel dogs, weighing 10-15 kg. The experimental preparation and techniques have been previously described (2). Pentobarbital-anesthetized dogs were instrumented. Fluid replacement, plasma $[K^+]$ and $[Ca^{2+}]$, and basic physiological conditions were controlled as described (2).

Near the base of the heart, the left anterior descending artery proximal to the first diagonal branch was encircled with a 4-0 silk suture. Because the degree of preceding ischemia is one determinant of the severity of infarct size and reperfusion arrhythmias (16), we intended to produce similar ischemia by monitoring collateral blood flow at baseline and during ischemia. Collateral coronary blood flow was detected by intracoronary Doppler flow wire as previously described (2).

Experimental protocol.

The dogs were randomized to one of six groups (**Figure 1**). All animals were subjected to a 60-min coronary occlusion followed by 180 min of reperfusion. The dose of 5-hydroxydecanoate (5 mg/kg, serum level around 100 μM when extracellular space is assumed to be 20% of body weight) was used to selectively block mit-K_{ATP} channels and to avoid to affect sar-K_{ATP} channels, which are insensitive to 500 μM of 5-hydroxydecanoate (17). 5-hydroxydecanoate was administered 5 min prior to prolonged coronary occlusion, which has been shown to be maximally effective in inhibiting cardioprotection (18). HMR1098 was administered at the dose of 3 mg/kg, which was an optimal dosage to determine the effectiveness of inhibiting the sar-K_{ATP} channels in IP (18). Because the sympathetic nerves are distributed in superficial epicardial layers, primarily along the coronary artery pathways (19), it is possible that dissection and ligation of the coronary artery might damage the accompanying nerves. Care will be taken to avoid manipulating tissues adjacent to the artery.

Measurements of Infarct size.

Infarct size was determined using 1% triphenyltetrazolium chloride (Sigma Chemical) in phosphate buffer (pH 7.4) as previously described (2). Left ventricular area at risk and the area of infarcted tissue were measured by an independent, blinded observer using computer planimetry.

To verify the reproducibility of the computer-assisted planimetry, a second measurement was performed by another, blinded investigator.

Arrhythmia analysis.

The acquired single-lead electrocardiographic tracing was continuously displayed. All arrhythmic events were classified by the observer according to the guidelines provided by “the Lambeth conventions” (20). Ventricular tachycardia (VT) was defined as ≥ 4 consecutive ventricular premature beats. Ventricular fibrillation (VF) was defined as a signal that changed from beat to beat in rate and configuration. Reference was made to the blood pressure signal to confirm which type of ectopic activity was occurring, particularly to distinguish between

polymorphic VT and VF. When the former occurs the pressure trace is usually still pulsatile whereas with VF the blood pressure falls rapidly towards zero and is no longer pulsatile. The onset, durations and incidence of VT and VF were measured occurring within the whole reperfusion period. Measurements of all variables were performed in a blinded manner.

Laboratory measurements.

Because of a local release of NE at the border zone, blood samples from the femoral artery and the tissue from the NE and remote interventricular zone were obtained for measurements of systemic and local NE levels at the end of the study. Plasma NE concentration was measured by collecting 4 ml of blood in test tubes containing 2% ethylenediaminetetraacetic acid (80 μl/ml of blood). Blood samples were immediately centrifuges at 3,000*g* for 10 minutes, and the plasmas were stored at -70^0c until further analysis. The myocardiums were homogenized with a kinametic polytron blender in100 mM Tris HCl, pH 7.4, supplemented with 20 mmole/L EDTA, 1 mg/ml pepstatin A, 1 mg/ml antipain, and 1 mmole/L benzamidin. Homogenates were centrifuged at 10,000*g* for 30 minutes to pellet the particulate fractions. The supernatant protein concentration was determined with the BCA protein assay reagent kit (Pierce). NE was measured by enzyme immunoassay (IBL, Hamburg, Germany). Intra-assay and inter-assay coefficients of variation was 7.5% and 15.2%, respectively. The highly sensitive protocol used with this kit has a detection limit of 20 pg/ml. Test samples analyzed with high-performance liquid chromatography showed that the enzyme immunoassay produced comparable results (21).

Exclusion criteria.

Animals were omitted from analysis for infarct size: (1) if intractable VF occurred or arrhythmia needed antiarrhythmic agents to correct; (2) if such severe hypotension was observed that the experiment could not be continued successfully for the duration of the protocol; or (3) presence of heart worms. Because of influence of collateral circulation on infarct sizes (16), we excluded collateral flow >20% of baseline coronary blood flow to make our study animals homogeneous. Dogs with VF during reperfusion were resuscitated and converted to a stable rhythm by internal electric shocks (3x10 W). The low energy did not result in more cell necrosis (22). We calculated survival percentage as (Number of dogs that survived)/(Number of originally assigned dogs- Number of dogs with heart worms or collaterals >20%) x100.

Statistics.

All values were expressed as mean ± SD. Differences among groups in hemodynamics, coronary blood flow, infarct size, area at risk, and NE were compared using one-way analysis of variance followed by Student-Newman-Keuls test. Differences in the incidence of arrhythmias among the groups were determined by the chi-square test and Fisher's exact test if case number < 5. Differences in the VT and VF durations among the groups were tested by the Mann-Whitney test because of no Gaussian distribution. A value of $P < .05$ was

considered to be significant.

五. 結果

Concentration of arterial blood gas, calcium, sodium and potassium were fairly stable throughout the study.

Hemodynamic variables.

Heart rate, mean blood pressure, and rate-pressure product were not significantly different among the 6 groups. Baseline coronary blood flow measured with intracoronary Doppler flow wire was nonsignificantly different among the 6 groups. Five min after occlusion, collateral blood flow in the center of the ischemic region was very low in the 6 groups, and increased only slightly with time. Blood flow of the left anterior descending artery among the 6 groups was similar during coronary occlusion, suggesting that collateral flow to the ischemic region may be not altered by treatment assignment. Coronary blood flow during hyperemic responses and reperfusion was also similar among the 6 groups.

Infarct size and Area at risk.

There was no significant difference in area at risk expressed as a percentage of the left ventricle among the groups, indicating that a comparable degree of ischemic risk. Infarct size in control animals averaged 43 ± 8 % of the risk region, compared with 15 ± 6% of the risk region in IP-treated dogs (P < .0001), indicating an effective dose used in this study. Pretreatment with 5-hydroxydecanoate completely abolished IP-pretreatment (15 ± 6% to 41 ± 6% of the risk region, P < .0001) cardioprotection. HMR 1098 did not significantly attenuate infarct size in the presence of IP. These data suggest a mit-K_{ATP} channel-sensitive, sar-K_{ATP} channel-insensitive mechanism as a mediator of IP-induced infarct size limitation.

Reperfusion arrhythmias.

100% of the control dogs developed reperfusion VT, and 83% of them deteriorated into VF. IP reduced the incidence of VT by 83% and VF by 83%. Duration of VT in the IP-treated heart was significantly shorter than in the control (256 ± 62 vs. 2345 ± 371 sec, P < .0001). Either HMR 1098 or 5-hydroxydecanoate alone did not produce significant increase of fatal reperfusion arrhythmias compared with the control group. However, the antiarrhythmic properties of IP were abolished while the use of HMR 1098 in terms of the incidence and duration of VT and the incidence of VF.

Plasma and tissue NE contents. Circulating NE levels remained similar among the groups. To investigate the possible role of cardiac NE contents, we determined the left ventricular NE levels. LV NE levels were significantly reduced 2.1-fold at the border zone in the IP-treated dogs than in vehicles (4.2 ± 1.9 vs. 7.7 ± 2.6 μg/g protein, P<0.0001). Expression was region dependent with a significant increase at the border zone (7.7 ± 2.6 μg/g protein) compared with that in the interventricular septum (2.9 ± 1.6 μg/g protein, P=0.002) in the vehicle group, consistent with cardiac NE beginning from adjacent to remote non-infarcted myocardium. Compared with dogs treated with IP alone in either 5-HD or HMR-1098-treated dogs, LV ET-1 levels were significantly increased at the border zone.

六．討論

Our present results clearly showed for the first time that IP exerts its beneficial effect on infarct sizes and reperfusion arrhythmias at different subcellular location through a NE-depedent mechanism in anaesthetized dogs. There is a separate mechanism modulating infarct size and reperfusion arrhythmias. The infarct size-limiting effect of IP was abolished by 5-hydroxydecanoate, suggesting that the cardioprotective effect of IP may result from activation of myocardial mit-K_{ATP} channels. IP effectively attenuates the incidence and durations of reperfusion-induced VT and VF. This antiarrhythmic effect was abolished by HMR1098 at the dose to selectively block sar-K_{ATP} channels, implying that beneficial effects appear to be mediated through the opening of myocardial sar-K_{ATP} channels. Our findings imply an important difference in the mechanism of these two channels on cardioprotection.

Mit-K_{ATP} channel activation was involved in the mechanism modulating IP-induced infarct size reduction demonstrated by the fact that infarct sizes increased by administering 5-hydroxydecanoate in the presence of IP. Because 5-hydroxydecanoate alone did not alter infarct size, the abrogation of infarct size limitation can not be ascribed to a detrimental effect of this agent. Opening of mit-K_{ATP} channels results in K^+ influx in to the mitochondrial matrix (23). Mitochondrial membrane depolarization induced by K^+ influx would lead to a reduction in the driving force responsible for Ca^{2+} uptake and/or activation of Ca^{2+} release (24,25). Besides, mitochondrial Ca^{2+} overload during ischemia-reperfusion can be reversed by mit-K_{ATP} channel agonists (26). The attenuated mitochondrial Ca^{2+} overload has been closely correlated with the preservation of cell integrity. Thus, it suggests that opening of mit-K_{ATP} channels may have been associated with reduced infarct sizes by attenuated Ca^{2+} overload. The result was consistent with previous finding (27), showing that mit-K_{ATP} channels, but not sar-K_{ATP} channels, modulate cell viability.

Sar-K_{ATP} channel activation was involved in the mechanism modulating IP-induced reduction of reperfusion arrhythmia demonstrated by the fact that fatal arrhythmias worsened after administering HMR 1098. Although the antiarrhythmic effects of sar-K_{ATP} channel antagonists has been documented during ischemia, there were controversial in vivo data of the literature supporting either proarrhythmic or antiarrhythmic effects (28-30). Different agonists and antagonists including specific and nonspecific agents were used to assess anti- and pro-arrhythmic effects of ATP-sensitive K^+ channels. Because of many other effects in nonspecific agents, it is difficult to compare these studies. For example, glibenclamide was able to prevent accumulation of free arachidonic acid released from the reperfused cell membrane (31). Because accumulated free fatty acids can induce arrhythmias (32), an indirect antiarrrhythmic action of glibenclamide can not be excluded. Besides, prolonged

ischemia results in much greater increases in intracellular calcium concentrations and produces triggered arrhythmias. Shortening of action potential duration by sar-K_{ATP} channel agonists might diminish this type of arrhythmias by decreasing intracellular calcium concentrations.

During acute myocardial ischemia circulating NE is increase (33). The local handling of NE is complex. Three phases of NE release has been characterized following myocardial ischemia (34). During the first 10 minutes, NE release occurs by exocytosis which is dependent on activity of efferent cardiac sympathetic tone (35). Between 10 and 40 minuets of ischemia, massive NE accumulation occurs in the extracellular space, primarily to the energy-dependent failure of reuptake nmechanisms and outward movement of NE via the uptake-1 transport mechanism (35). Beyond 40 minutes of ischemia structural damage of the sympathetic neurons results in a further depletion of NE.

In the context of infarct size and reperfusion arrhythmias, it has also been suggested that release of endogenous NE might contribute to the genesis of infarct extension and arrhythmias and pharmacological depletion of myocardial NE can markedly reduce the severity of infarct extension and arrhythmias. Our results were compatible with previous studies, showing that reduction of NE can provide cardioprotection (36) and inhibition of reperfusion arrhythmias (37).

Conclusions.

This study demonstrates that IP effectively limits infarct sizes and diminishes fatal reperfusion-induced ventricular arrhythmias by different ATP-sensitive K^+ channels for a NE-dependent mechanism in the canine heart. The infarct size-limiting effect of IP was abolished by 5-hydroxydecanoate, suggesting that the effects may result from activation of the mit-K_{ATP} channels. The antiarrhythmic effect of IP during reperfusion was associated with the activation of sar-K_{ATP} channels because IP-mediated antiarrhythmic effect was inhibited by the blocker of sar-K_{ATP} channels, HMR 1098.

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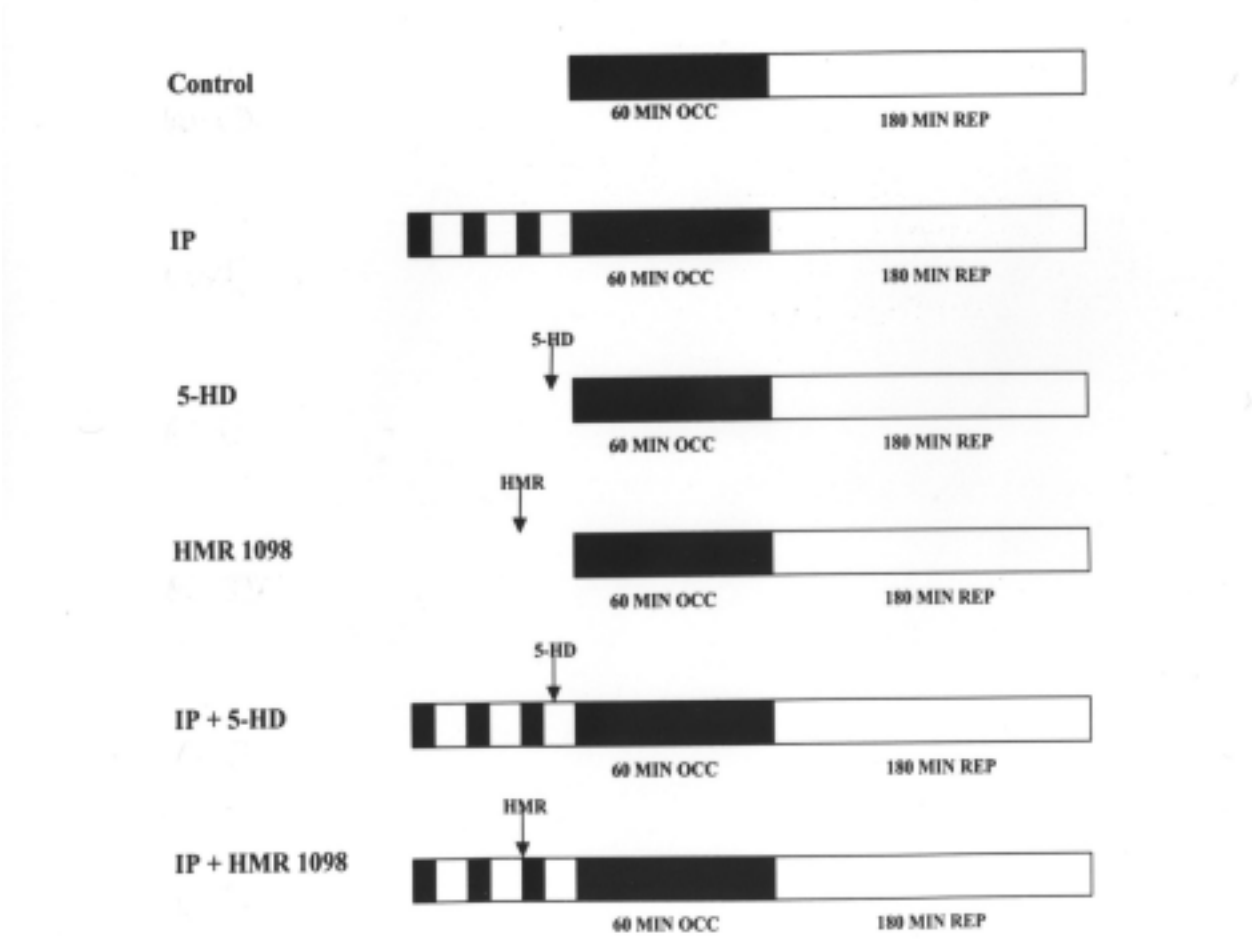

Figure

1. Protocol bars indicate experiments used to study effects of various interventions in a canine ischemia/reperfusion model. *Group I* was the control group, and only vehicle was administered prior to the 60-minute occlusion. In *Group II* (IP), animals were treated with 3 cycles of 5-minute ischemia and 10-minute reperfusion before a 60-minute coronary occlusion. In *Group III* (5-HD), intraventricular 5-hydroxydecanoate (5-HD, 5 mg/kg) was given 5 minutes prior to the 60-minute occlusion. In *Group IV* (HMR 1098), intravenous HMR1098 (3 mg/kg) was given 15 minutes prior to the 60-minute occlusion. In *Groups V-VI*, preconditioned dogs with either 5-HD or HMR1098. IP: ischemic preconditioning; OCC: occlusion; REP: reperfusion.