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Journal of Molecular and Cellular Cardiology 46 (2009) 452–462

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

PKA rapidly enhances proteasome assembly and activity in in vivo canine hearts

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 16 October 2008
Accepted 3 November 2008
Available online 13 November 2008

Keywords:
Proteasome
PKA
Phosphorylation
Assembly
Ubiquitinated protein
Ischemia/reperfusion

ABSTRACT

Proteasome regulates diverse cellular functions by eliminating ubiquitinated proteins. Protein kinase A (PKA) is a key regulator of proteasome activity. However, it remains unknown how PKA regulates proteasome activity and whether it controls proteasome activity in in vivo hearts. Both the in vitro peptidase assay and the in-gel peptidase assays showed that the treatment with PKA for 30 min dose-dependently activated purified 26S proteasome. Simultaneously, PKA treatment enhanced phosphorylation and assembly of purified 26S proteasome evaluated by non-reducing native polyacrylamide gel electrophoresis, either of which was blunted by the pretreatment with a PKA inhibitor, H-89. In in vivo canine hearts, proteasome assembly and activity were enhanced 30 min after the exogenous or endogenous stimulation of PKA by the intracoronary administration of isoproterenol or forskolin for 30 min or by ischemic preconditioning (IP) with 4 times of repeated 5 min of ischemia. The intracoronary administration of H-89 blunted the enhancement of proteasome assembly and activity by IP. Myocardial proteasome activity at the end of ischemia was decreased compared with the control, however, it did not differ from the control in dogs with IP. IP decreased the accumulation of ubiquitinated proteins in the canine ischemia/reperfusion myocardium, which was blunted by the intracoronary administration of a proteasome inhibitor, epoxomicin. However, proteasome activation by IP was not involved in its infarct size-limiting effects. These findings indicate that PKA rapidly enhances proteasome assembly and activity in in vivo hearts. Further investigation will be needed to clarify pathophysiological roles of PKA-mediated proteasome activation in ischemia/reperfusion hearts.

1. Introduction

The ubiquitin–proteasome system plays a major role in intracellular protein degradation and subsequently regulates cellular functions in various cells [1–4]. 26S proteasome is composed of 20S proteasome as its "core" catalytic unit capped on each end by 19S regulatory complex [5,6]. 26S proteasome is a cylinder-like structure containing 4 concentric rings, each containing 7 subunits. We have previously reported that impairment of proteasome activity may contribute to the progression of cardiac dysfunction along with the accumulation of ubiquitinated proteins in the pressure-overloaded heart of mice [7]. In addition, Bulteau et al. clearly demonstrated the deactivation of proteasome and the subsequent accumulation of ubiquitinated proteins in ischemia/reperfusion myocardium [8]. These findings suggest that impairment of the ubiquitin–proteasome system may be closely associated with cardiac diseases. Therefore, a better understanding the regulation of the ubiquitin–proteasome system may lead to new therapies for cardiac diseases. However, it remains largely unknown how proteasome is regulated in in vivo hearts.

There are several possible mechanisms that could regulate 26S proteasome activity, including 1) changes in protein levels of proteasome subunits, 2) post-translational modification of proteasome subunit such as phosphorylation/dephosphorylation, and 3) assembly/disassembly of proteasome subunits [9,10]. Recently, protein kinase A (PKA) is reported to be one of the key regulators of proteasome activity in the in vitro studies [11,12]. PKA increases proteolytic activities of the cardiac proteasome [11] and phosphorylation of the 19S proteasome subunit by PKA correlates with increased proteasome activity [12]. However, it remains to be resolved whether PKA increases proteasome activity by altering the status of proteasome assembly or by phosphorylating proteasome subunits. Thus, in the present study, we first investigated phosphorylation, assembly and activity of purified proteasome when it was treated with PKA. Next, we investigated proteasome assembly and activity in in vivo canine hearts when cardiac PKA was stimulated endogenously and

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doi:10.1016/j.yjmcc.2008.11.001

exogenously. We also checked the time-course changes in proteasome activity during ischemia/reperfusion period in dogs with and without endogenous PKA stimulation. Finally, we investigated the role of PKA-mediated proteasome activation by IP in the accumulation of ubiquitinated proteins and myocardial infarct size using a proteasome inhibitor.

2. Methods

2.1. Materials

Epoxomicin (a proteasome inhibitor), PKA, isoproterenol, forskolin and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma (St. Louis, MO, USA). A purified 26S proteasome from human erythrocyte and Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (proteasome peptidase substrates) were obtained from Biomol International (Plymouth Meeting, PA, USA). H-89 (a selective PKA inhibitor) and an antibody against serine/threonine phosphorylated proteins were obtained from Upstate (Lake Placid, NY, USA). Antibodies directed against ubiquitinated proteins (clone FK2) and proteasome subunits (Rpt5, α7, and β5) were purchased from Biomol International. Clone FK2 recognizes both mono- and poly-ubiquitinated proteins but not free ubiquitin, so the extent of protein ubiquitination could be determined.

2.2. Measurement of 26S proteasome activity

2.2.1. In vitro peptidase assay

The purified erythrocyte 26S proteasomes treated with various units of PKA with and without 100 μmol/L H-89

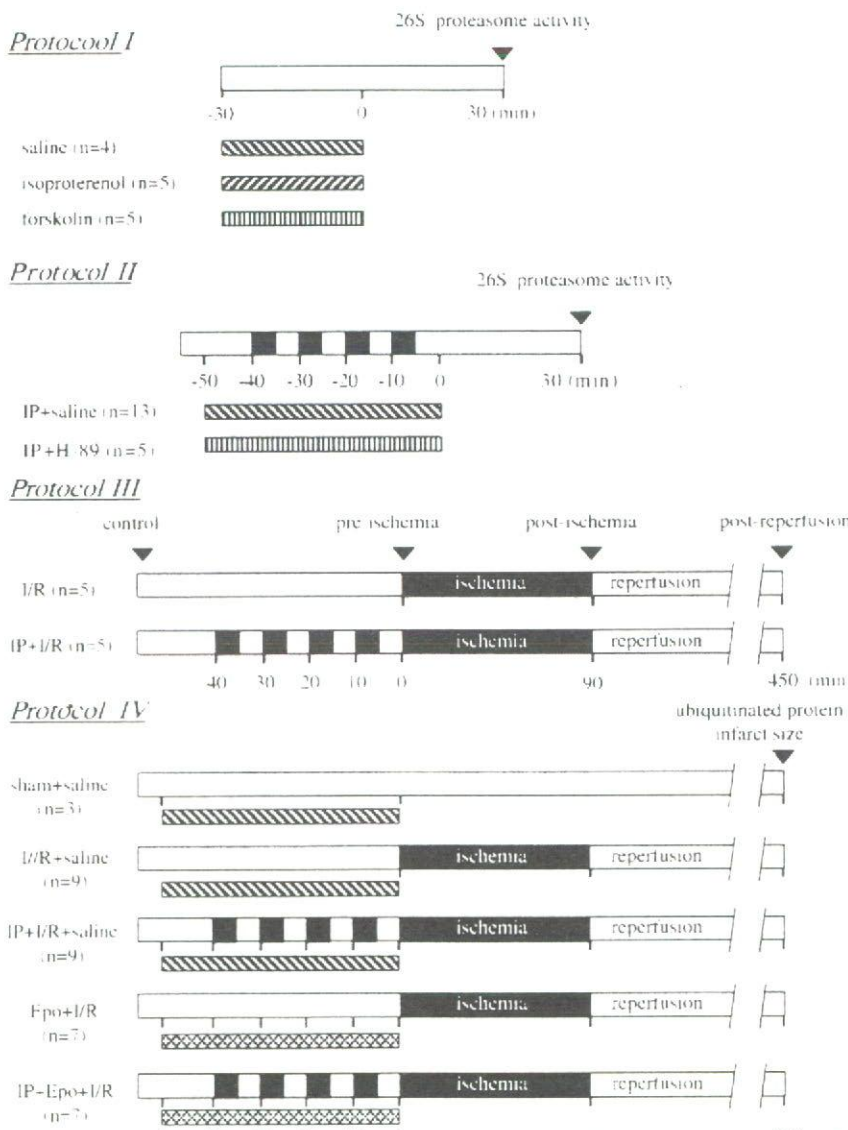


Fig. 1. Experimental protocols in canine model. (Protocol I) Effects of the intracoronary administration of saline ($n=4$), isoproterenol ($n=5$) or forskolin ($n=5$) (an exogenous stimulant of PKA) on proteasome activity in canine hearts. (Protocol II) Effects of ischemic preconditioning (IP) (an endogenous stimulant of PKA) with the intracoronary administration of saline ($n=8$ in LAD-perfused myocardium and $n=5$ in LCx-perfused one) or H-89 ($n=5$) (a PKA inhibitor) on proteasome activity in canine hearts. (Protocol III) Time-course changes in proteasome activity during ischemia/reperfusion period with and without IP ($n=5$ per each group). The triangle indicates the timing for myocardial biopsy. (Protocol IV) Effects of proteasome activation by IP on the accumulation of ubiquitinated proteins and infarct size in canine hearts. Sham operation was performed in 3 dogs. I/R and Epo indicate ischemia/reperfusion and epoxomicin, respectively.

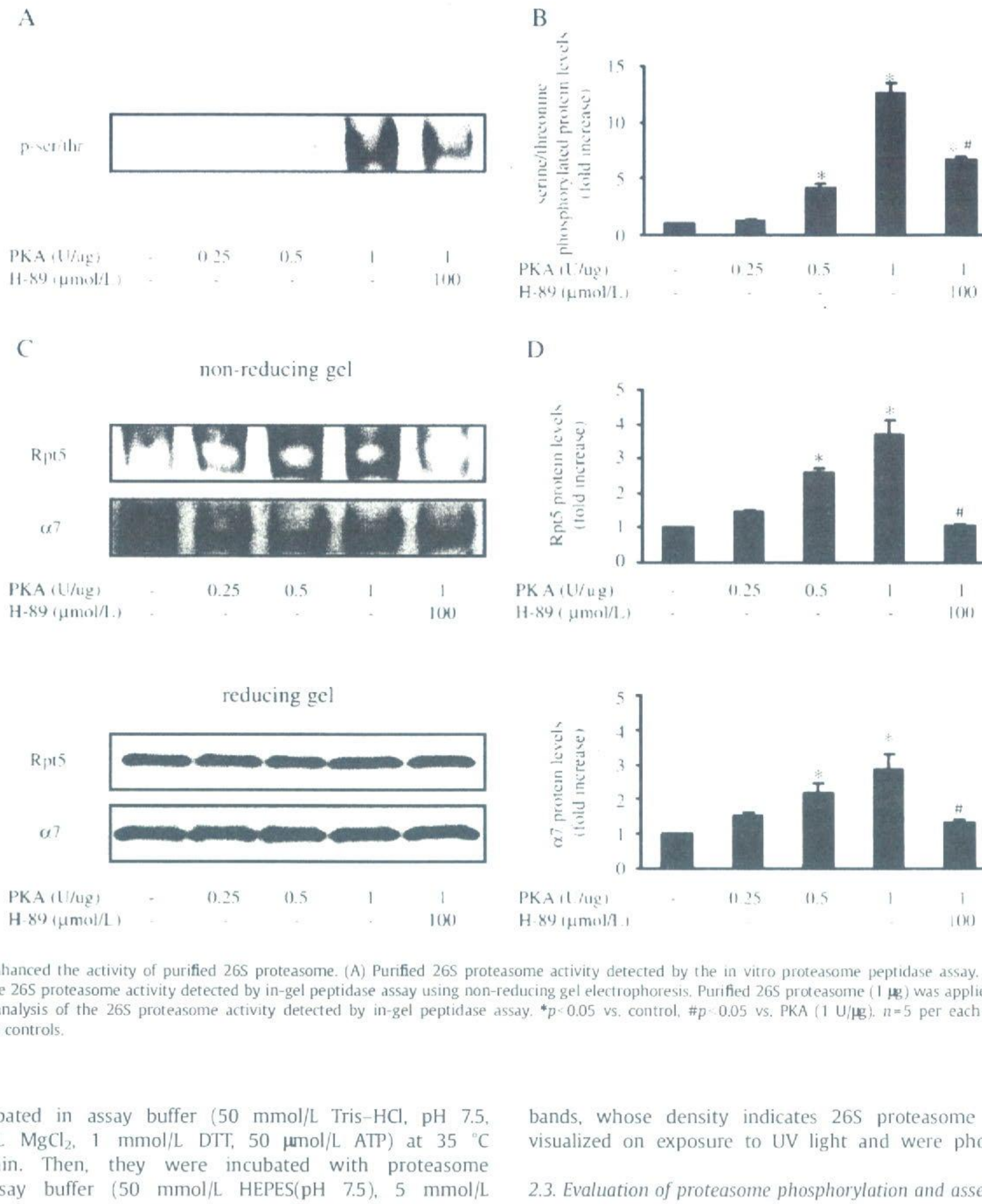


Fig. 2. PKA enhanced the activity of purified 26S proteasome. (A) Purified 26S proteasome activity detected by the in vitro proteasome peptidase assay. (B) Representative example of the 26S proteasome activity detected by in-gel peptidase assay using non-reducing gel electrophoresis. Purified 26S proteasome (1 μg) was applied to each lane. (C) Quantitative analysis of the 26S proteasome activity detected by in-gel peptidase assay. *$p<0.05$ vs. control, #$p<0.05$ vs. PKA (1 U/μg). $n=5$ per each group. Values are normalized to controls.

were incubated in assay buffer (50 mmol/L Tris–HCl, pH 7.5, 20 mmol/L $MgCl_2$, 1 mmol/L DTT, 50 μmol/L ATP) at 35 °C for 30 min. Then, they were incubated with proteasome activity assay buffer (50 mmol/L HEPES(pH 7.5), 5 mmol/L MgCl2, and 1 mmol/L DTT, 50 μmol/L ATP, 40 μmol/L LLVY-AMC) for 2 h at 37 °C. The fluorescence of each solution was measured by spectrophotometry, (HitachF-2000; Hitachi Instruments, Tokyo, Japan) with excitation at 390 nm (Ex) and emission at 460 nm (Em). All readings were standardized relative to the fluorescence intensity of an equal volume of free 7-amino-4-methylcoumarin (Sigma) solution (40 μmol/L).

2.2.2. In-gel peptidase assay

The purified 26S proteasome with different treatments were separated by non-reducing native PAGE using a modification of the method described previously [13]. We used a four gel layer consisting of equal amounts, from the bottom up, of 7.5, 5, 4, and 3% polyacrylamide. Non-reducing gels were run at 125 V for 2.5 h. The gels were incubated on a rocker for 1 h at 37 °C with 15 mL of 0.4 mmol/L Suc-LLVY-AMC in buffer (50 mmol/L Tris–HCl, pH 7.5, 5 mmol/L $MgCl_2$, 50 μmol/L ATP). Proteasome bands, whose density indicates 26S proteasome activity, were visualized on exposure to UV light and were photographed.

2.3. Evaluation of proteasome phosphorylation and assembly in vitro

MThe purified 26S proteasome with different treatments were separated by non-reducing native PAGE described above. Proteins on the non-reducing gels were transferred (110 mA) for 1.5 H onto polyvinylidene difluoride membranes. Western blotting analysis was carried out sequentially for detection of changes in phosphorylation state with anti phospho-serine/threonine antibody and for detection of 26S proteasome with anti Rpt5 or α7-subunit antibody. Antigens were visualized by a chemiluminescent horse-radish peroxidase method with the ECL reagent. A parallel reducing gel was used to confirm the total amount of 26S proteasome.

2.4. Animal instrumentation

Beagle dogs (Oriental Yeast, Osaka, Japan) weighing 8 to 12 kg were anesthetized with sodium pentobarbital (30 mg/kg, intravenously), and were prepared as previously described [14]. Briefly,

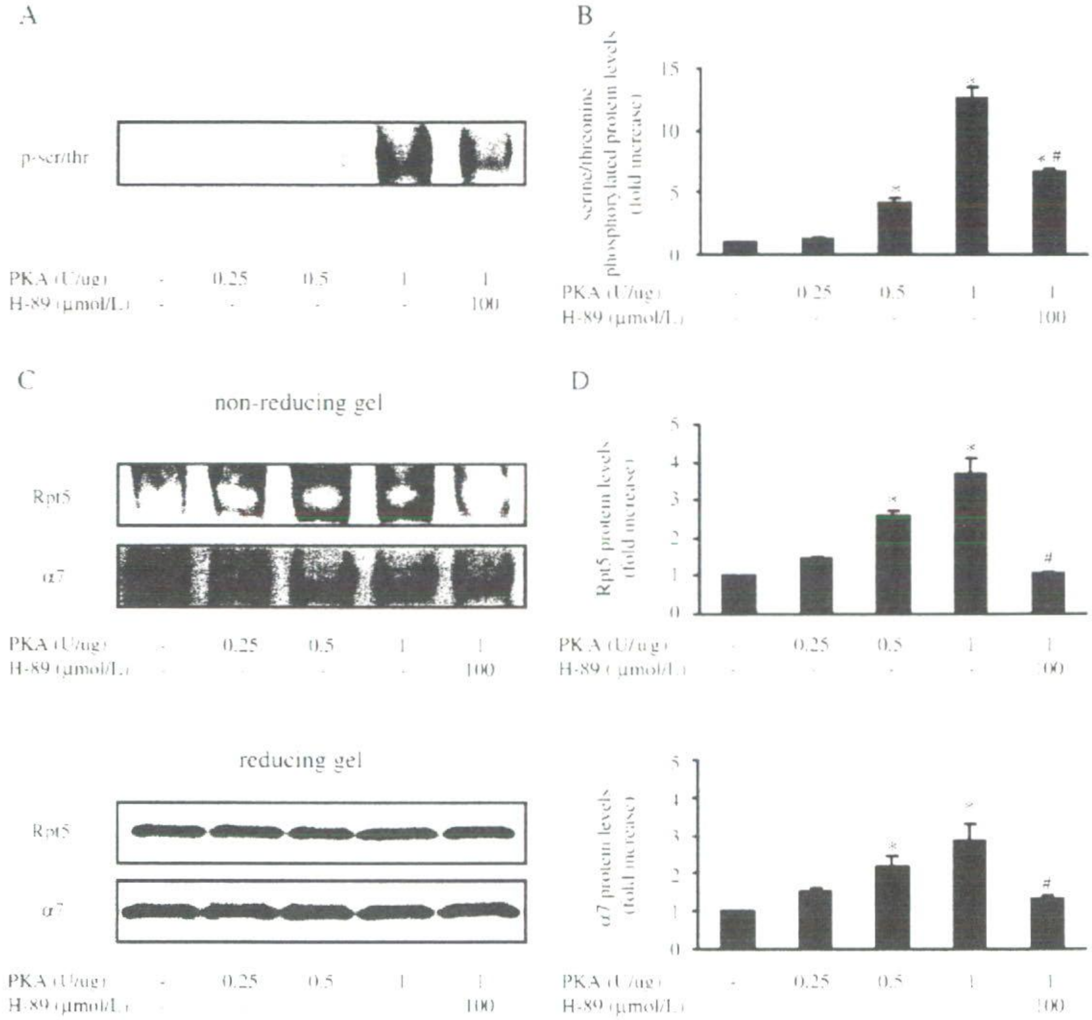


Fig. 3. PKA enhanced the phosphorylation and assembly of purified 26S proteasome. Representative example (A) and quantitative analysis (B) of 26S proteasome phosphorylation by Western blotting analysis with an anti phospho-ser/thr antibody. Representative example (C) and quantitative analysis (D) of Western blotting analysis on non-reducing gels. The status of 26S proteasome assembly was evaluated by Western blotting analysis with an anti-Rpt5 or anti-α7 antibody. Equal amounts of samples were loaded onto reducing and non-reducing gels. *$p<0.05$ vs. control. #$p<0.05$ vs. PKA (1 U/μg). $n=5$ per each group. Values are normalized to controls.

the trachea was intubated and each dog was ventilated by using room air mixed with oxygen. The chest was opened through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After heparinization (500 U/kg), the proximal portion of the left anterior descending coronary artery (LAD) was cannulated and perfused with blood via the carotid artery through an extracorporeal bypass tube. Both the coronary perfusion pressure (CPP) and heart rate (HR) were monitored during the experiments. In all experiments, CPP and HR were set at about 100 mmHg and 130 beats per min, respectively. This model was used to allow selective administration of agents to the LAD and reproduction of ischemia/reperfusion by clamping the bypass tube [15–17]. To examine the effects of PKA on proteasome activity in vivo, we employed isoproterenol or forskolin for exogenous stimulation of PKA and ischemic preconditioning (IP) for endogenous stimulation because PKA was reported to be activated by IP in canine hearts [15]. All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996 revision) and were approved by the Osaka University Committee for Laboratory Animal use.

2.5. Animal study protocols

2.5.1. Protocol I: Effects of isoproterenol or forskolin on proteasome activities in canine hearts

To assess the effects of exogenous PKA stimulation on proteasome activity, we selectively administrated saline ($n=4$), isoproterenol ($n=5$) or forskolin ($n=5$) into the LAD for 30 min in dogs. We preliminarily confirmed that the dose of ISO (10 μmol/L) used increased cAMP levels in the myocardium perfused by the LAD, but not in the myocardium of the left circumflex coronary artery (LCx) (data not shown). We determined the dose of forskolin (0.3 μg/kg/min) that activates PKA in canine hearts according to the previous report [18]. After administration, we rapidly sampled myocardial tissue from the LAD- and LCx-perfused myocardium as saline- or drug-treated myocardium and control one, respectively. Samples were placed into liquid nitrogen and stored at −80 °C (Fig. 1).

2.5.2. Protocol II: Effects of IP on proteasome activity in canine hearts

To assess the effect of endogenous PKA stimulation on the proteasome activity, we performed 4 cycles of 5 min coronary artery occlusion and a subsequent 5-minute period of reperfusion (IP) with

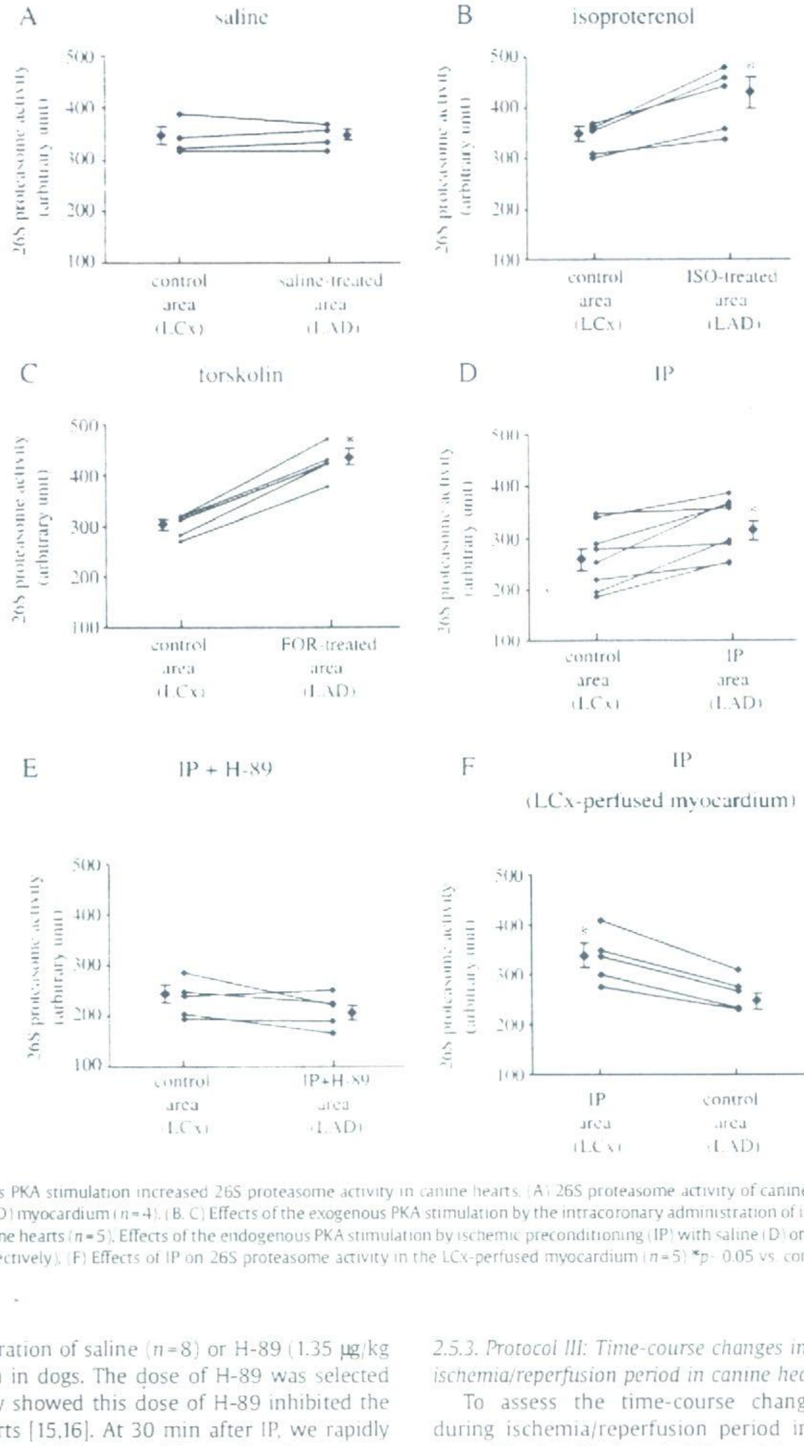


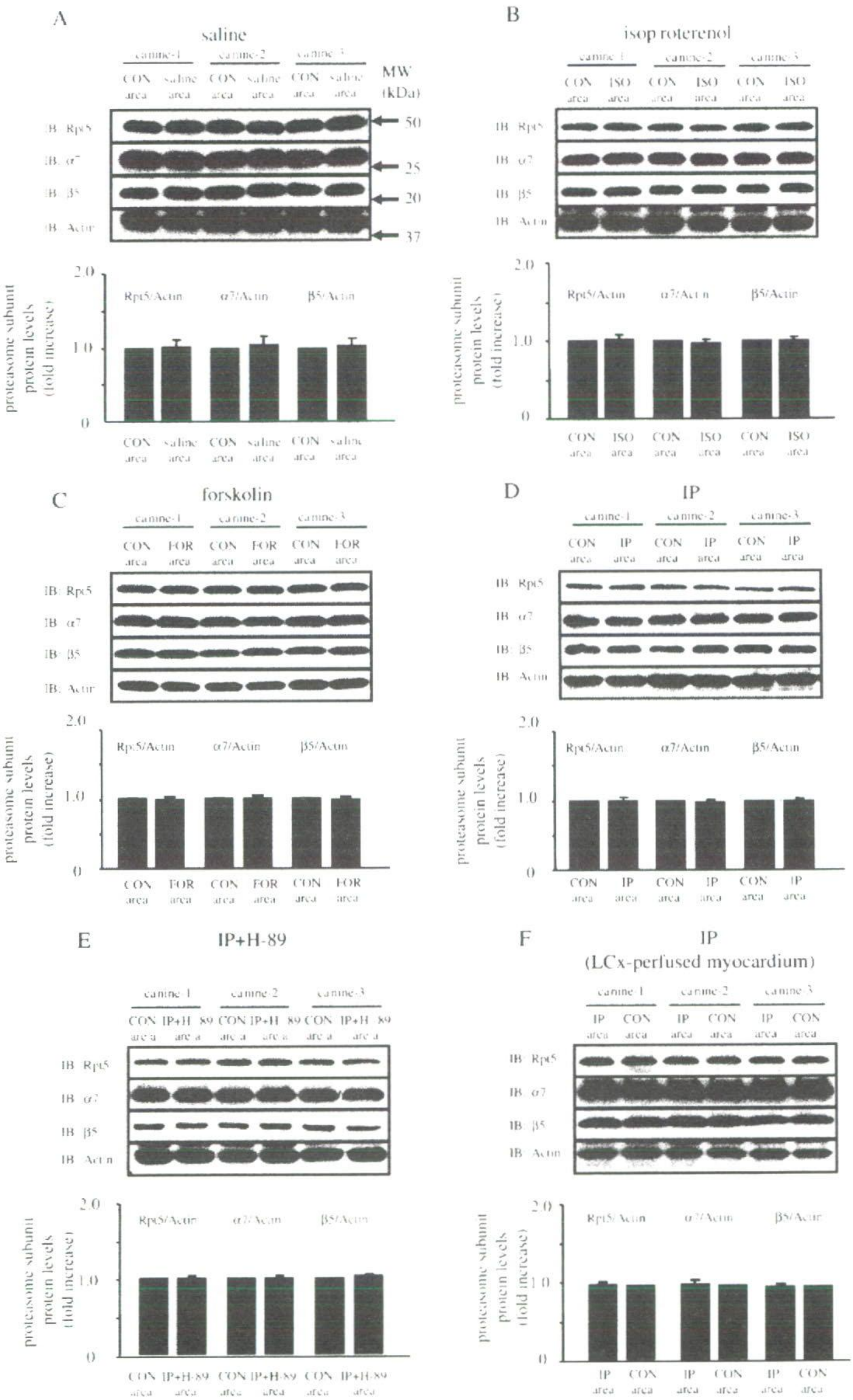
Fig. 4. Exogenous and endogenous PKA stimulation increased 26S proteasome activity in canine hearts. (A) 26S proteasome activity of canine hearts after sham operation in the control (LCx) or saline-treated (LAD) myocardium ($n=4$). (B, C) Effects of the exogenous PKA stimulation by the intracoronary administration of isoproterenol (ISO) or forskolin (FOR) on 26S proteasome activity in canine hearts ($n=5$). Effects of the endogenous PKA stimulation by ischemic preconditioning (IP) with saline (D) or H-89 (E) on 26S proteasome activity in canine hearts ($n=8$ and 5, respectively). (F) Effects of IP on 26S proteasome activity in the LCx-perfused myocardium ($n=5$) *$p<0.05$ vs. control area.

the intracoronary administration of saline ($n=8$) or H-89 (1.35 μg/kg per min) ($n=5$) for 50 min in dogs. The dose of H-89 was selected because the previous study showed this dose of H-89 inhibited the PKA activity in canine hearts [15,16]. At 30 min after IP, we rapidly sampled tissues from the LAD- and LCx-perfused myocardium, placed the samples into liquid nitrogen, and stored them at −80 °C. To confirm that proteasome activation by IP was not dependent on the myocardial area, we also performed the same IP protocol in LCx-perfused myocardium instead of LAD-perfused one in 5 dogs (Fig. 1).

2.5.3. Protocol III: Time-course changes in proteasome activity during ischemia/reperfusion period in canine hearts

To assess the time-course changes in proteasome activity during ischemia/reperfusion period in canine hearts, we underwent 90 min of ischemia followed by 6 h of reperfusion with and without IP in 10 dogs. Myocardial biopsy specimens were taken from LAD-perfused myocardium in each canine at 4 time-points: at the control, just before ischemia (pre-ischemia), at the end of 90 min ischemia (post-ischemia) and 6 h of reperfusion (post-reperfusion) (Fig. 1).

Fig. 5. PKA stimulation did not alter total protein levels of proteasome subunit in canine hearts. Representative example and quantitative analysis of Western blotting analysis of protein levels for 19S proteasome subunit Rpt5 as well as 20S proteasome subunits α7 and β5 in canine hearts after saline treatment (A), isoproterenol (ISO) treatment (B), forskolin (FOR) treatment (C), ischemic preconditioning (IP) (D), IP with H-89 (IP+H-89) (E). IP was performed in the LCx-perfused myocardium (F) instead of LAD-perfused one. CON and MW indicate control and molecular weight, respectively.


A
saline
canine-1 canine-2 canine-3
CON area saline area CON area saline area CON area saline area
MW (kDa)
IB: Rpt5
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50
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proteasome subunit protein levels (fold increase)
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Rpt5/Actin α7/Actin β5/Actin
B
isoproterenol
CON area ISO area
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CON area FOR area
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IP
(LCx-perfused myocardium)
IP area CON area