g）黄疸

肝うっ血により，肝機能障害をきたし，時には黄疸を生じることがある．そのような患者では，皮膚や眼球結膜の黄染が認められる．

h）貧血

近年，心血管危険因子として慢性腎臓病 chronic kidney disease（CKD）が注目されており，両者の病態には共通点が少なからず存在することが指摘されている（心腎連関）．腎性貧血はCKD ステージ 3 程度の比較的早期の段階で発症し，さらに貧血が CKD の要因となる．Silverberg らは，CKD-貧血-慢性心不全がお互いに悪影響を及ぼし，悪循環を形成する cardio-renal anemia（CRA）症候群という新しい概念を提唱した[1]．心不全症例において貧血を合併している割合は，15～60％と幅広い報告があるが，心不全の重症化に伴い，貧血の合併頻度も高率となる．これらのことから，眼瞼結膜で貧血の有無を確認することも重要と考えられる．

i）ばち指 clubbed finger

爪床部を中心とした線維組織の増生により，指の先端が太鼓のばちのように局所的に膨らんだ状態で，通常 160°以下である皮膚と爪のなす角度が，慢性心不全患者では 190°を超える場合がある．線維組織の増生した爪床部は軟らかく，押すとくぼむが，離すと元に戻る．足の指よりも，手の指に認められやすい．

## 2 バイタルサインの診察

日常診療では一評価項目でしかないが，救急医療ではバイタルサインはしばしば診察の方向性を与える重要な初期情報となるため，まず最初に測定するものである．

a）血圧

心原性ショックでは，少なくとも 30 分以上血圧が 90 mmHg 未満か，普段の血圧より 30 mmHg 以上低下しており，そのような症例では頻脈を呈することが多い．血圧が低下している場合の血圧の目安として，頸動脈が触知可能であれば 60 mmHg 以上，大腿動脈が触知可能であれば 70 mmHg 以上，橈骨動脈が触知可能であれば 80 mmHg 以上とされる．一方，過度の後負荷に対する代償機能不全 afterload mismatch による心不全では，血圧は高値である．血圧測定は，初診時には左右差の有無を確認する必要がある．

b）脈拍

① 脈拍数

著しい徐脈の場合，洞不全症候群や II 度もしくは III 度房室ブロックの存在を考慮する．このような時は，一過性の意識障害や失神（Adams-Stokes 症候群）をきたすことがある．高度徐脈の持続により，心拍出量が低下し，心不全や臓器循環不全から活動性の低下をきたすことがある．一方，著しい頻脈の場合も徐脈と同様に一過性の意識障害や失神，心不全をきたすことがある．頻脈は心不全の原因にもなりうるが，心原性ショックのように心不全の結果としても起こる．

② リズム

リズムが規則正しい場合を「整」，乱れている場合を「不整」と表現し不整脈とよぶ．上室

性・心室性期外収縮では脈が一つ抜けたように感じる（欠滞）．全く規則性がない不整脈を絶対性不整脈とよび，その原因として心房細動が多い．絶対性不整脈の時，心音の聴診やモニター心電図の心拍数に比し，脈拍数が少ない場合があり，脈拍欠損 pulse deficit とよばれるが，脈拍欠損も大脈と小脈が交互に繰り返す交互脈とともに心不全の兆候として重要である．

③ 大きさ

脈拍の大きさは，収縮期と拡張期の血圧差である脈圧を反映する．脈圧が大きいものを大脈，小さいものを小脈とよぶ．収縮期-拡張期（脈圧）/収縮期が 25 %未満の場合，心係数が $2.2l/\min/m^2$ 未満であることが多いとされる[2]．

c）動脈血酸素分圧（$PaO_2$）とヘモグロビン酸素飽和度（$SaO_2$）

酸素は血漿中の物理的に溶解している量（$PaO_2$）よりも，赤血球のヘモグロビンと結合している量（$SaO_2$）のほうがはるかに多い．$PaO_2$ と $SaO_2$ とは平衡しており，両者には一定の関係がある（図 2-1）．全身の酸素化の指標として，$PaO_2$ は動脈血液を採取して分析する必要があるが，$SaO_2$ はパルスオキシメータにより非侵襲的に，リアルタイムに測定することが可能である（$SpO_2$）．ただし，$SpO_2$ が 90 %でも $PaO_2$ は約 60 mmHg に低下していることから，$SpO_2$ のわずかな変化を見逃してはならない．

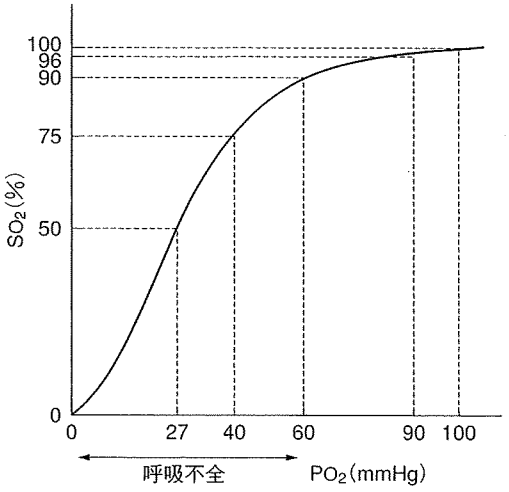


図 2-1 $PaO_2$ と $SaO_2$ の関係（37 ℃，pH 7.4，$PaCO_2$ 40 mmHg におけるヘモグロビン酸素解離曲線）

## 3 心不全の身体所見

心不全には，「急速に心ポンプ機能の代償機転が破綻し，それに基づく症状や徴候が急速に出現した状態」である急性心不全と「慢性の心ポンプ機能の低下により末梢主要臓器の酸素需要量に見合うだけの血液量を拍出できず，肺または体静脈系にうっ血をきたし生活機能に障害を生ずる状態」である慢性心不全がある．心不全の罹患期間の違いはあるものの，慢性心不全の急性増

悪を含んだ急性心不全も慢性心不全も，身体所見においては類似する点も多い．表2-2に臨床で広く用いられている心不全基準であるFramingham criteriaを示す[3]．心不全の程度や重症度の分類は，自覚症状による分類であるNYHA（New York Heart Association）心機能分類，他覚所見による分類であるKillip分類[4]や血行動態による分類であるForrester分類[5]などがある．Killip分類（表2-3）とForrester分類は，元来急性心筋梗塞症の重症度分類であるが，今日では心不全の重症度の指標として用いられることも少なくない．Nohriaらは胸腹部と四肢を触診

表2-2 うっ血性心不全の診断基準（Framingham criteria）

大基準2つか，大基準1＋小基準2つ以上で心不全と診断する

[大基準]
- 発作性夜間呼吸困難または起坐呼吸
- 頸静脈怒張
- ラ音
- 心拡大
- 急性肺水腫
- III音奔馬調律
- 静脈圧上昇（16 $cmH_2O$以上）
- 循環時間延長（25秒以上）
- 肝・頸静脈逆流

[小基準]
- 下腿浮腫
- 夜間咳嗽
- 労作時呼吸困難
- 胸水貯留
- 肺活量の減少（最大量の1/3以下）
- 頻脈（120/分以上）

[大または小基準]
5日間の治療に反応して4.5kg以上の体重減少があった場合，それが心不全治療であれば大基準，心不全治療以外であれば小基準として扱う

表2-3 Killip分類

| | 身体所見 | 1カ月後の院内死亡率 |
|---|---|---|
| Class I | 心不全徴候なし | 6％ |
| Class II | 軽度～中等度心不全<br>ラ音聴取領域が全肺野の50％未満 | 17％ |
| Class III | 重症心不全<br>肺水腫，ラ音聴取領域が全肺野の50％以上 | 38％ |
| Class IV | 心原性ショック<br>血圧90 mmHg未満，尿量減少，チアノーゼ<br>冷たく湿った皮膚，意識障害 | 81％ |

（注）1カ月後の院内死亡率は，再灌流療法が普及する以前のデータであり，現在は大きく改善している．

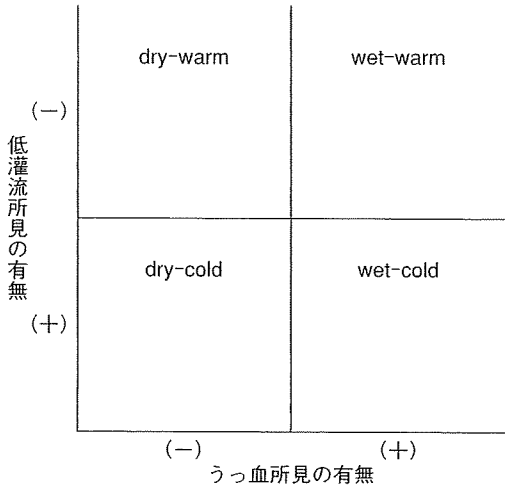


図2-2 触診による急性心不全の臨床病型
dry-warmが最も予後がよく，wet-coldが最も予後が悪い．
低灌流所見：脈圧狭小化，交互脈，症候性低血圧，四肢冷感，傾眠傾向
うっ血所見：起坐呼吸，頸静脈怒張，ラ音，肝・頸静脈逆流，腹水，浮腫

し，胸腹部の温度に比べ四肢が温かいか冷たいか，乾燥しているか湿っているかによって，心不全を4つの病型（図2-2）に分類する方法を提唱した[6]．本法は，救急現場での触診だけで，患者の血行動態だけでなく，1年後の予後（死亡率や緊急移植のイベント）によく相関するため，大変有用と考えられる．

## 4 視診

### a）頸静脈の視診

右房との間に発達した弁がなく，右房や上大静脈から直線的な右内頸静脈は中心静脈を反映しやすいため，右内頸静脈で行う．内頸静脈は患者を仰臥位にすると心臓と同じ高さになり観察できる．上半身を45°にすると胸骨角（胸骨柄関節）の高さは，右房から約5cmの高さになる．この位置から右内頸静脈の拍動が確認できる最高点の高さを測定し，右房から胸骨角の高さである5cmを加えることで，中心静脈圧を推定することができる（図2-3）．座位で怒張している場合は右心不全によるうっ血が考えられる．一方，仰臥位でも頸静脈が観察できない場合（虚脱）は脱水が考えられるが，肥満者では観察できないこともあるため，評価には注意を要する．

### b）心尖拍動

通常は，座位において，第5肋間で胸骨中線から左側10cm以内の部位で観察できる．しかし，心尖拍動が，その外側にある場合は，左室拡大（容量負荷）が考えられる．また，心尖拍動が2峰性の場合は，聴診でIVの存在が示唆され，心肥大など左室コンプライアンスが低下する病態が考えられる．

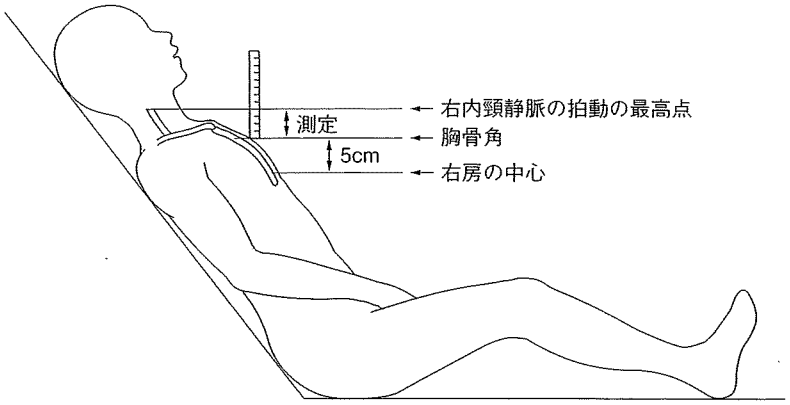


図2-3 中心静脈圧の推定

胸骨角から右内頸静脈の拍動が確認できる最高点の高さを測定して，その数値に5cmを加えた値が中心静脈圧に近似する．正常は8～9cm$H_2O$までである（1.36cm$H_2O$＝1.0mmHg）．

## 5 胸部の聴診

### a）呼吸音 respiratory sounds

正常で聴取しうる狭義の呼吸音 breath sounds と，正常では聴取できない副雑音 adventitious sounds に分類される．

#### ① 狭義の呼吸音 breath sounds

正常で聴取しうる呼吸音には，気管呼吸音，気管支呼吸音，気管支肺胞呼吸音，肺胞呼吸音がある．気管呼吸音，気管支呼吸音，気管支肺胞呼吸音は，それぞれ前胸部の気管や気管支に沿った部位で聴取される．気管・気管支では気流速度が速く，空気の流入出で乱流が生じやすいために強い呼吸音が聴取され，末梢気道になるほど呼吸音は低音で弱くなる．肺うっ血が生じると，肺実質の含気量が低下し呼吸音の伝播が亢進することで，肺胞呼吸音が聴取される末梢領域で気管支肺胞呼吸音や気管支呼吸音が聴取されることがある．また，胸水貯留では呼吸音の減弱～消失が認められることがあり，左右差の有無をチェックする．

#### ② 副雑音 adventitious sounds

肺内から発生するラ音 pulmonary adventitious sounds と肺外から発生する胸膜摩擦音や血管性雑音などその他の副雑音がある．ラ音には，①断続性ラ音，②連続性ラ音に分類される．断続性ラ音には，吸気初期に聴取される水泡音 coarse crackles と吸気終末に聴取される捻髪音 fine crackles がある．水泡音は，気道分泌物で形成された液体膜が破裂するときに発生する低調音で，心不全により肺うっ血が生じ，さらに肺水腫をきたした場合に聴取される．捻髪音は，閉塞した末梢気道の急激な開放に伴う圧較差の解消と気道壁張力の急激な変化によって発生する．捻髪音が聴取される代表的な疾患として，間質性肺炎があげられるが，心不全の初期で肺うっ血が認められる頃に，肺底部で聴取されることもある．一方，連続性ラ音は呼気に聴取されやすく，

高調の笛(様)音 wheezes と低調のいびき(様)音 rhonchi があり，ともに気道の攣縮，粘膜浮腫・腫脹，気道分泌物，腫瘍などにより，気道が狭窄したときに発生する．連続性ラ音が聴取される代表的な疾患として，気管支喘息があげられるが，心不全で肺うっ血や肺水腫をきたした場合，気管支の浮腫や気道分泌物による気道狭窄で聴取されることがあるため，注意を要する（心臓喘息）．心不全の時に発生する副雑音は，うっ血が生じやすい下肺野で聴取されやすい．

b）心臓の聴診

心臓の聴診は，主に4つの弁から発生する心音，心雑音，過剰心音を，① 大動脈弁領域（第2肋間胸骨右縁），② 肺動脈弁領域〔第2，3（Erb 領域）肋間胸骨左縁〕，③ 三尖弁領域（第4肋間胸骨左縁），④ 僧帽弁領域（心尖部）の4箇所の聴診部位にて行う．

**① 心音**

心音には，I音，II音，III音，IV音があり，常に聴取されるのは，I音とII音だけである．表2-4 に心音の異常と病態を示す．

(i) I音，II音

I音は房室弁（僧帽弁と三尖弁）の閉鎖により発生し，僧帽弁成分（$I_M$）と三尖弁成分（$I_T$）からなり，通常 $I_M \rightarrow I_T$ の順で聴取される．II音は半月弁（大動脈弁と肺動脈弁）閉鎖により発生し，大動脈弁成分（$II_A$）と肺動脈弁成分（$II_P$）からなり，通常 $II_A \rightarrow II_P$ の順で聴取される．

(ii) III音，IV音

III音は拡張早期の急速充満期の終了する付近で急速に心室の伸展が停止することで心臓や大血管が振動するために発生すると考えられている．III音は，弱い低調の音であるため，患者を左側臥位にしてベル型を軽く触れる程度で聴取する．若年者で聴取される生理的III音と病的III音があり，病的III音は心不全で認められる重要な聴診所見である．一方，IV音は拡張後期の心房収縮期付近でI音の直前に発生する音で，心室コンプライアンスが低下する病態で聴取される．I音，II音とIII音のリズム関係は「おっかさん」，IV音とI音，II音のリズム関係は「おとっつぁん」のリズムに似たものとなり，奔馬調律 gallop rhythm とよばれる．

**② 心雑音**

心血管の構造的な異常により，血液の乱流が生じ心血管が振動するために発生することで聴取される．収縮期に出現する収縮期雑音，拡張期に出現する拡張期雑音，収縮期・拡張期にまたがり出現する連続性雑音がある．心雑音は，一般に心音よりも持続時間が長く，音量の程度により，6段階に分類される〔Levine 分類（表2-5)〕．表2-6に心雑音の種類と病態を示す．

**③ 過剰心音**

(i) 駆出音 ejection sound

半月弁の開放により発生し，I音の後で聴取される．① 大動脈や肺動脈の拡大，② 半月弁狭窄，③ 半月弁を通過する血流増大（相対的半月弁狭窄），④ 圧負荷などの存在により発生する．

表 2－4　心音の異常と病態

| | | | |
|---|---|---|---|
| I 音 | 亢進 | 房室弁の硬化（MS，TS）<br>心収縮力の増大（甲状腺機能亢進症，貧血，発熱，脚気）<br>PQ 時間短縮（WPW 症候群など）<br>cannon sound（完全房室ブロック）<br>右室容量負荷（ASD など） | |
| | 減弱 | MR<br>心収縮力の低下（心不全，甲状腺機能低下症など）<br>PQ 時間延長<br>胸水，心嚢液貯留 | |
| II 音 | 亢進 | $II_A$ | 高血圧，AR，高度の動脈硬化 |
| | | $II_P$ | 肺高血圧，PR，MS，ASD |
| | 減弱 | $II_A$ | 低血圧，AS |
| | | $II_P$ | PS |
| | 分裂 | 生理的分裂（吸気時に $II_P$ が遅れ II 音が分裂し，呼気時に分裂が消失する） | |
| | | 病的分裂（$II_A$ ～ $II_P$ の間隔が呼気・吸気ともに幅広く分裂する） | |
| | | | $II_A$ が早くなる　MR，VSD |
| | | | $II_P$ が遅れる　右脚ブロック，PS |
| | | 固定性分裂（$II_A$ ～ $II_P$ の間隔が呼吸の影響を受けずに一定）　ASD | |
| | | 奇異性分裂（$II_P$ が $II_A$ に先行し，呼気時に分裂が出現し，吸気時に分裂が消失する） | |
| | | | $II_A$ が遅れる　AS，左脚ブロック |
| | | | $II_P$ が早くなる　PDA，TR |
| III 音 | | 生理的 | 30 歳以下では 50 %以上で聴取でき，40 歳以上では聴取できない |
| | | 病的 | 病的心拡大による拡張早期の急速充満障害（拡張型心筋症，MR，AR）<br>心室コンプライアンスの低下（肥大型心筋症） |
| IV 音 | | | 心室コンプライアンスの低下（肥大型心筋症） |

MS：僧帽弁狭窄症，TS：三尖弁狭窄症，ASD：心房中隔欠損症，MR：僧帽弁閉鎖不全症，
VSD：心室中隔欠損症，AR：大動脈弁閉鎖不全症，PR：肺動脈弁閉鎖不全症，
AS：大動脈弁狭窄症，PS：肺動脈弁狭窄症，PDA：動脈管開存症，TR：三尖弁閉鎖不全症

表 2－5　Levine 分類

| |
|---|
| Levine I 度：きわめて微弱で，注意深く聴診することにより，はじめて聴取できる |
| Levine II 度：弱いが，聴診器を当てることにより聴取できる |
| Levine III 度：中等度の雑音だが，振戦を伴わない |
| Levine IV 度：高度の雑音で，振戦を伴う |
| Levine V 度：著しく強いが，聴診器を胸壁から離すと聴取できなくなる |
| Levine VI 度：聴診器を胸壁から離しても聴取できる |

表 2-6 心雑音の種類と病態

| | | |
|---|---|---|
| 収縮期雑音 | 駆出性 | AS，PS，HOCM，ASD，ECD，機能性雑音<br>(hyperdynamic state) |
| | 逆流性（全収縮期） | MR，TR，VSD，僧帽弁逸脱症候群 |
| 拡張期雑音 | 灌水性（逆流性） | AR，PR |
| | 拡張期ランブル | MS，TS，重症の ASD ・ VSD |
| | 前収縮期 | MS |
| 連続性雑音 | PDA，Valsalva 洞動脈瘤破裂，<br>A-P window（大動脈中隔欠損症），AV fistula（冠/肺動静脈瘻） | |
| to and fro（往復）雑音 | AR，AS ＋ AR，PS ＋ PR，VSD に合併した AR | |
| Rivero-Carvallo 徴候<br>Graham Steell 雑音<br>Carey Coombs 雑音<br>Austin Flint 雑音 | 右心系雑音が吸気時に，胸腔内圧低下→心肺血流量増加により増強する<br>MS などにより著明な肺高血圧となると機能的 PR を生じる<br>相対的 MS による拡張期ランブル<br>AR により拡張期に僧帽弁前尖が左房側に押し上げられて生じる MS 様ランブル | |

AS：大動脈弁狭窄症，PS：肺動脈弁狭窄症，HOCM：閉塞性肥大型心筋症，ASD：心房中隔欠損症，ECD：心内膜床欠損症，VSD：心室中隔欠損症，AR：大動脈弁閉鎖不全症，PR：肺動脈弁閉鎖不全症，MS：僧帽弁狭窄症，TS：三尖弁狭窄症，PDA：動脈管開存症

(ii) 開放音 opening snap

房室弁の開放により発生し，II 音の後で聴取される．① 房室弁狭窄，② 房室弁を通過する血流増大（相対的房室弁狭窄），などの存在により発生する．

(iii) 収縮期クリック

僧帽弁逸脱症候群に特有の鋭い高調の過剰心音で，収縮中期から後期にかけて，僧帽弁が左室内圧により左房に押し出される時に発生する．

(iv) 心膜摩擦（ノック）音

収縮性心膜炎で聴取される高調の過剰心音で，II 音の後で，III 音より早い拡張早期の急速流入期に聴取される．炎症により肥厚，癒着，石灰化した心膜により，心室の拡張が制限されることで心臓や大血管が振動するために発生すると考えられている．III 音に比し，広範囲で聴取され，ベル型を胸壁に押し当てても音が消失しない．

## 6 触診

急性心不全では，肝臓が腫大し，肝被膜が急速に伸展されるために右季肋部の圧痛が出現することがある．右心不全では，中心静脈圧の上昇により，上腹部中央を 30 秒程度強く圧迫すると，頸静脈怒張が認められることがあり，この現象は肝・頸静脈逆流 hepatojugular reflux とよばれる．

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Original article

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

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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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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

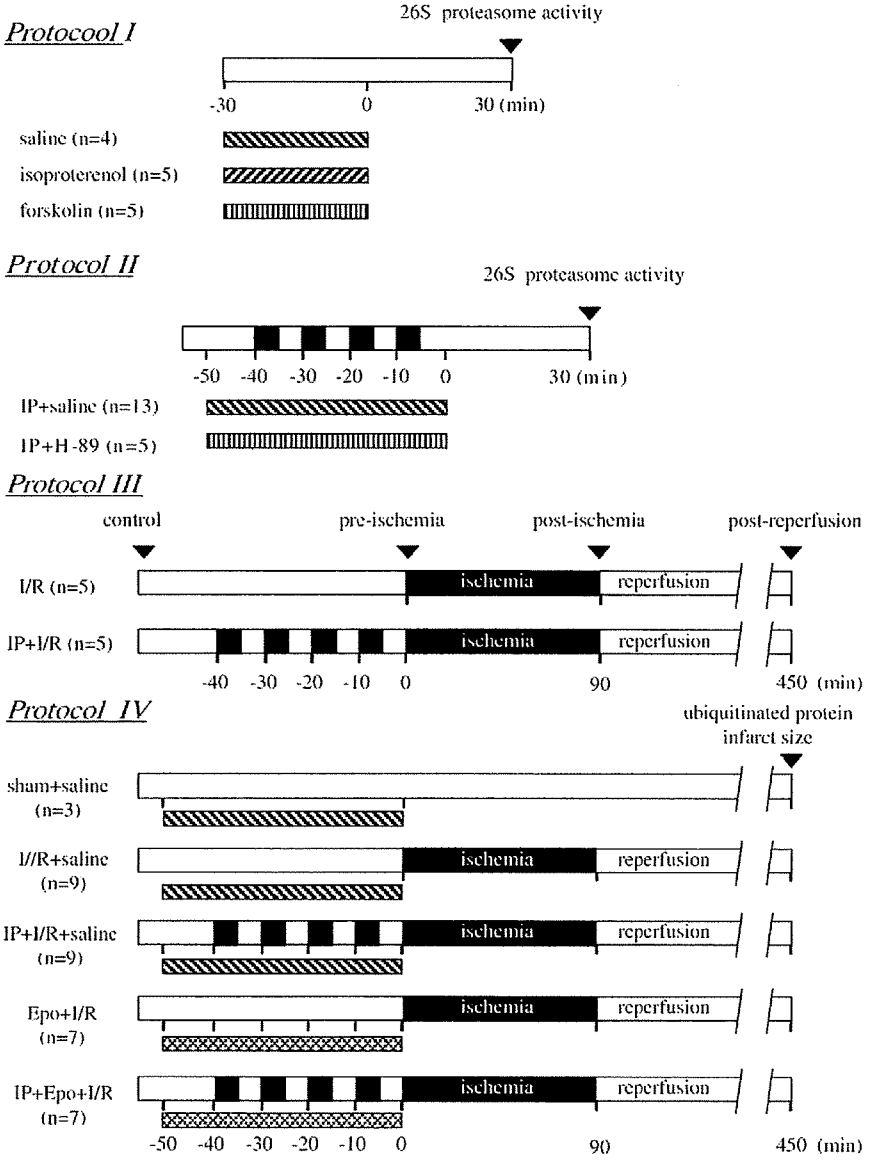



**Fig. 1.** Experimental protocols in canine model. (Protocol I) Effects of the intracoronary administration of saline ($n=4$), isoproterenol ($n=5$) or forskolin ($n=5$) (an exogenous stimulant of PKA) on proteasome activity in canine hearts. (Protocol II) Effects of ischemic preconditioning (IP) (an 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.

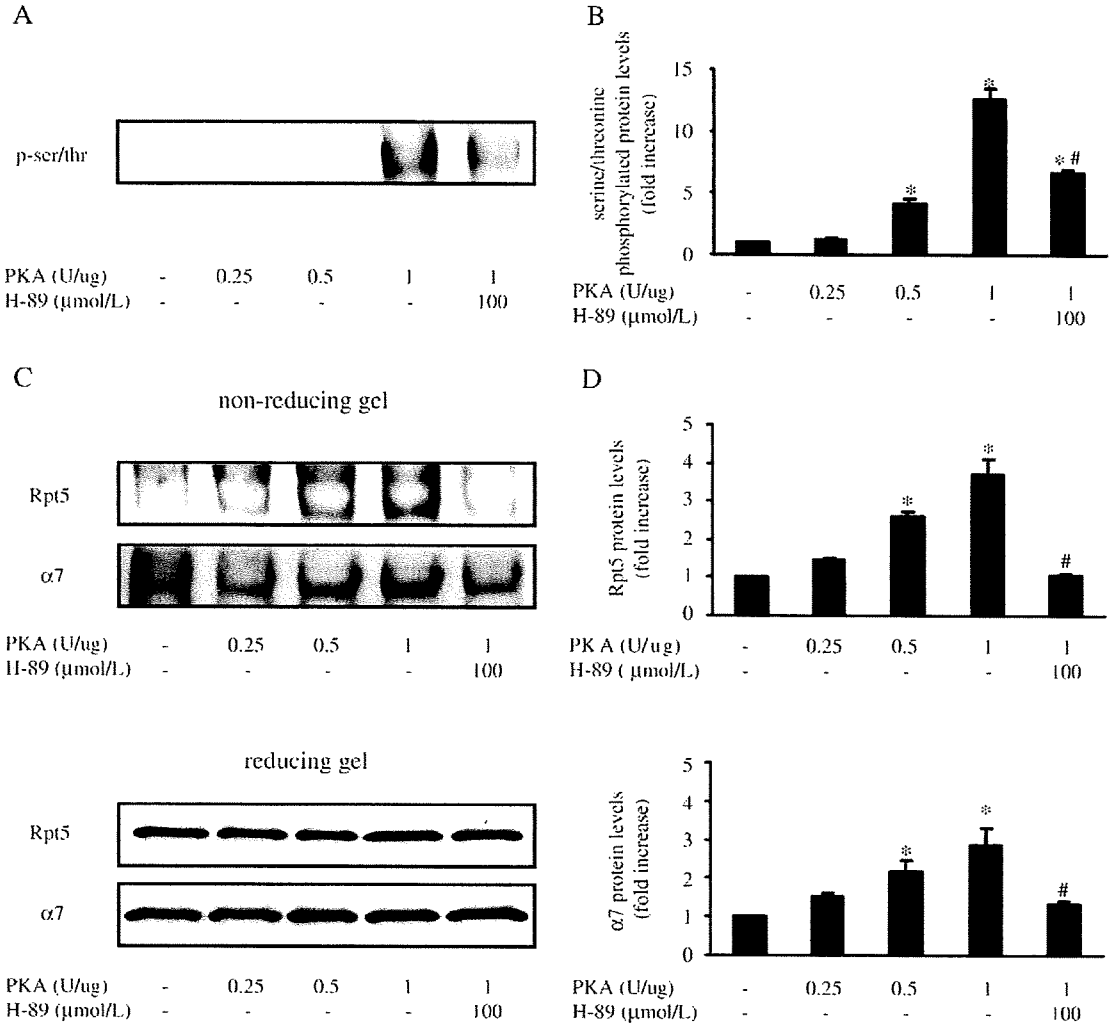



**Fig. 2.** PKA enhanced the activity of purified 26S proteasome. (A) Purified 26S proteasome activity detected by the in vitro proteasome peptidase assay. (B) Representative example of the 26S proteasome activity detected by in-gel peptidase assay using non-reducing gel electrophoresis. Purified 26S 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,

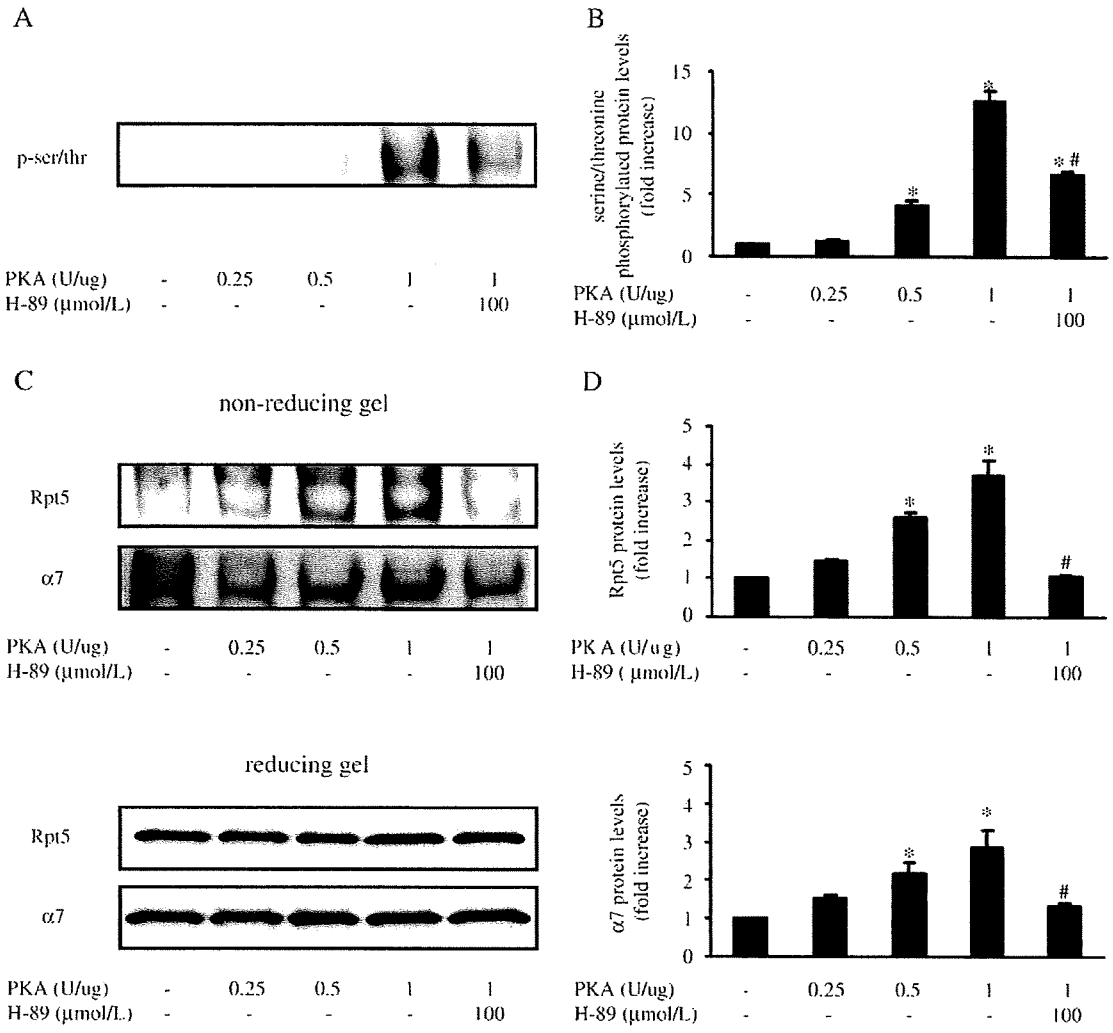



**Fig. 3.** PKA enhanced the phosphorylation and assembly of purified 26S proteasome. Representative example (A) and quantitative analysis (B) of 26S proteasome phosphorylation by Western blotting analysis with an anti phospho-ser/thr antibody. Representative example (C) and quantitative analysis (D) 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

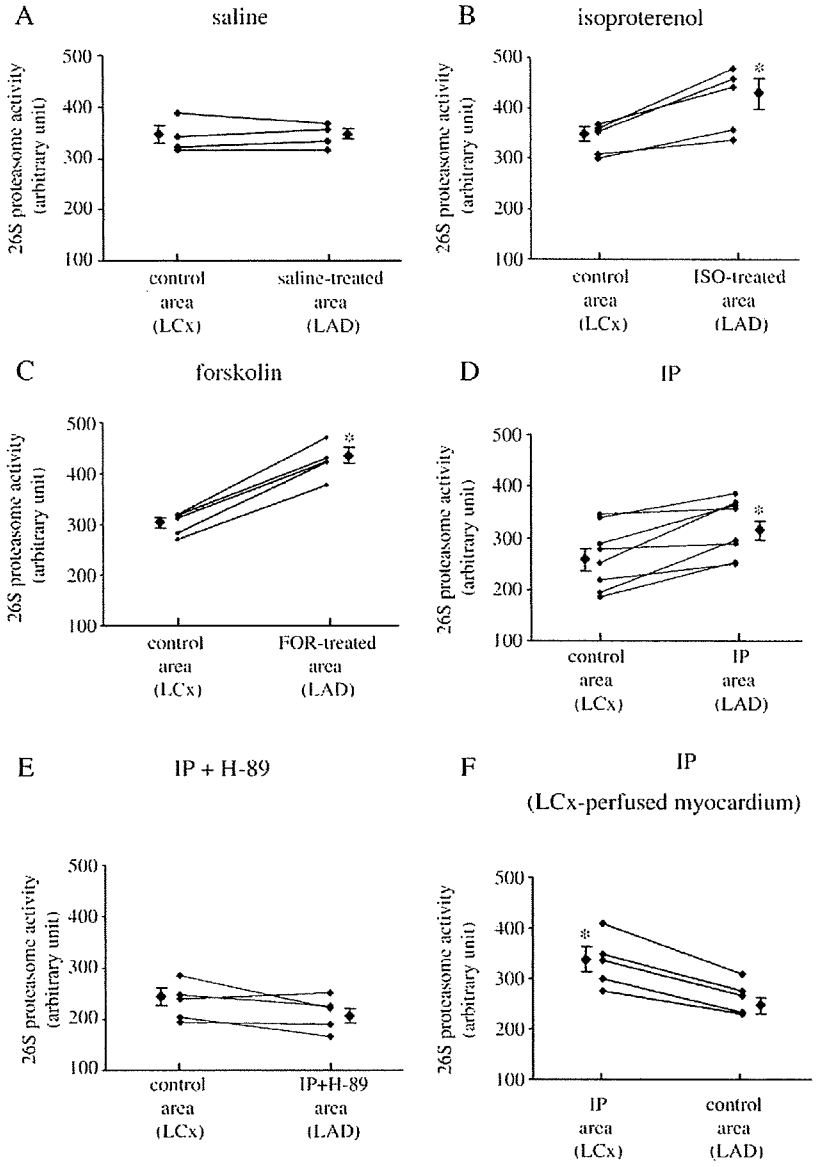


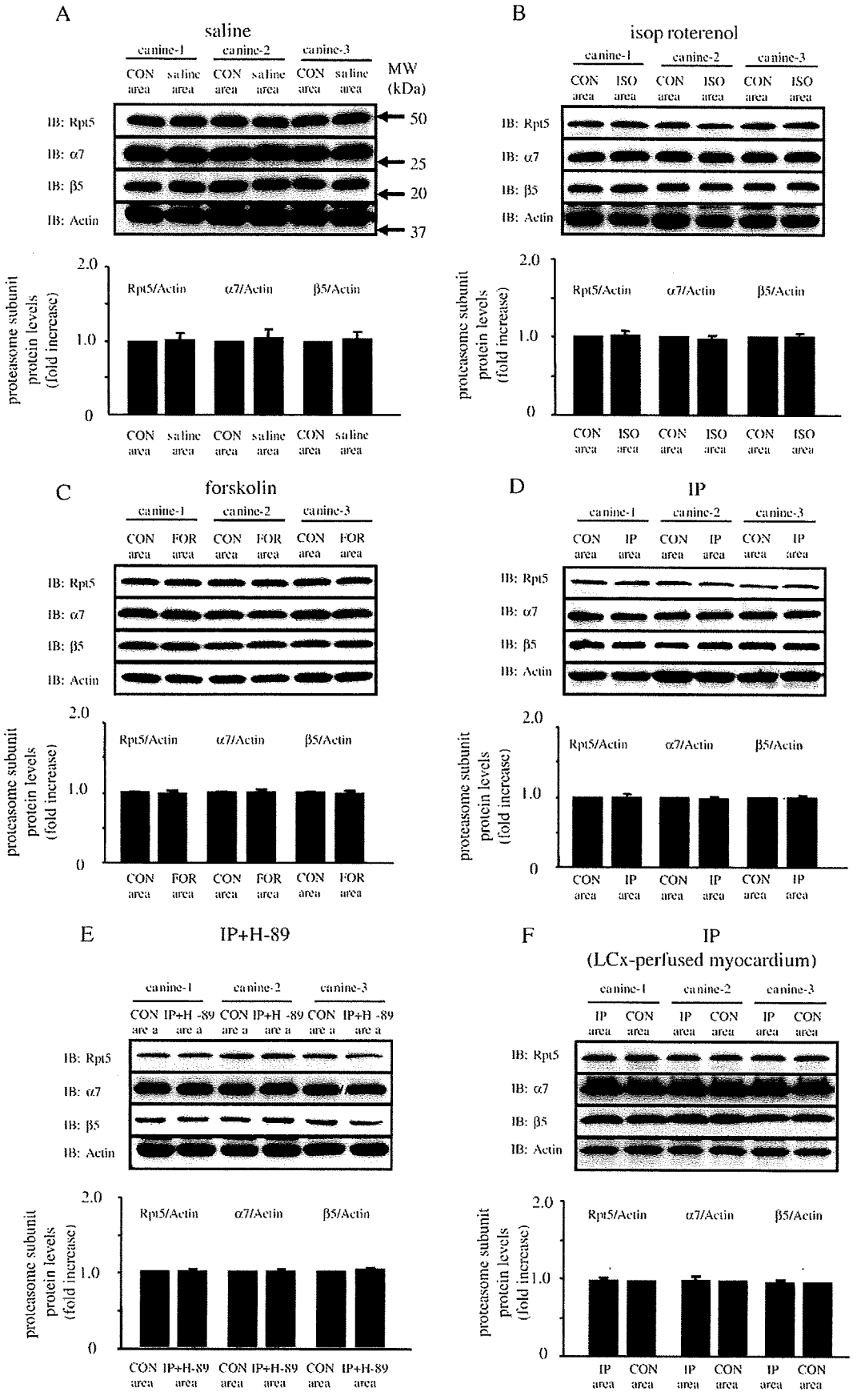

**Fig. 4.** Exogenous and endogenous PKA stimulation increased 26S proteasome activity in canine hearts. (A) 26S proteasome activity of canine hearts after sham operation in the control (LCx) or saline-treated (LAD) myocardium ($n=4$). (B, C) Effects of the exogenous PKA stimulation by the intracoronary 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
MW (kDa)
IB: Rpt5
IB: α7
IB: β5
IB: Actin
50
25
20
37
proteasome subunit protein levels (fold increase)
Rpt5/Actin
α7/Actin
β5/Actin
2.0
1.0
0
B
isop roterenol
ISO area
C
forskolin
FOR area
D
IP
IP area
E
IP+H-89
IP+H -89 area
F
IP
(LCx-perfused myocardium)

*2.5.4. Protocol IV: Effects of proteasome activation by IP on the accumulation of ubiquitinated proteins and infarct size in canine hearts*

To assess pathophysiological roles of the proteasome in the ischemia/reperfusion myocardium, we intracoronarilly administered saline ($n$=9) or a proteasome inhibitor (epoxomicin at 2.5 μg/kg) ($n$=7) for 50 min and then we performed 90 min of ischemia followed by 6 h of reperfusion in dogs. To assess pathophysiological roles of the proteasome activation by IP in the ischemic/reperfused myocardium, we intracoronarilly administered a proteasome inhibitor (epoxomicin at 2.5 μg/kg) for 50 min with ($n$=7) and without IP ($n$=9) and then we performed 90 min of ischemia followed by 6 h of reperfusion in dogs. We preliminarily confirmed that this dose of epoxomicin reduced 26S proteasome activity by 43.0±6.2% ($n$=3) in the LAD-perfused myocardium compared with that in the LCx-perfused one. After 6 h of reperfusion, we rapidly sampled LAD-perfused myocardium, stored the samples at −80 °C, and investigated the level of the ubiquitinated proteins. We also evaluated the area at risk and the necrotic area after 6 h of reperfusion by Evans blue/TTC staining as described previously [19]. Myocardial infarct size was expressed as the necrotic area/area at risk (Fig. 1).

*2.6. Purification of cardiac proteasome*

Proteasome was purified from canine hearts according the method reported previously [20]. The peptidase assay was performed using the cytosolic fraction from the LAD- and LCx-perfused myocardium of canine hearts or the fractions containing 26S proteasomes separated on a 10–40% glycerol gradient centrifugation according to the method described above.

*2.7. Western blotting analysis*

Western blotting analysis was performed as described previously [21]. Immunoreactive bands were quantified by densitometry (Molecular Dynamics).

*2.8. Statistical analysis*

Data are expressed as the mean±SEM. Proteasome activities in LAD- and LCx-perfused myocardium were compared by the paired $t$-test. The time-course changes in proteasome activity during ischemia/reperfusion myocardium were analyzed by the two-way repeated analysis of variance (ANOVA) followed by Fisher's test. Other results were compared by the one-factor ANOVA followed by Fisher's test. In all analyses, $p<0.05$ was accepted as statistically significant.

## 3. Results

*3.1. PKA enhanced the activity of purified 26S proteasome*

The in vitro peptidase assay (Fig. 2A) and the in-gel peptidase assays (Figs. 2B, C) showed that the treatment of purified 26S proteasome with PKA enhanced 26S proteasome activity in a dose-dependent manner, while this effect was blocked by the pretreatment with H-89.

*3.2. PKA enhanced the phosphorylation and assembly of purified 26S proteasome*

Western blotting analysis of non-reducing gels probed with the antibody against serine/threonine phosphorylated proteins showed that PKA dose-dependently enhanced the phosphorylation of purified 26S proteasome (Figs. 3A, B). The phosphorylation of 26S proteasome was blocked by the pretreatment with H-89 (Figs. 3A, B). Interestingly, Western blotting analysis of non-reducing gels probed with the antibody against Rpt5 or α7 revealed that PKA dose-dependently increased either protein level that corresponded to 26S proteasome, which was blunted by H-89 (Figs. 3C, D upper panel). Western blotting analysis of reducing gels showed that the purified 26S proteasome were equally loaded to each lane (Figs. 3C, D lower panel). These results suggest that PKA enhanced the phosphorylation and assembly of proteasome, which may lead to the increase in proteasome activity.

*3.3. PKA stimulation increased 26S proteasome activity in canine hearts*

We found no differences in the proteasome activity in the saline-treated (LAD-perfused) and the control (LCx-perfused) myocardium (Fig. 4A). In contrast, exogenous and endogenous PKA stimulation by the selective intracoronary administration of isoproterenol or forskolin and IP, respectively, significantly increased 26S proteasome activity in LAD-perfused myocardium compared with that in LCx-perfused one (Figs. 4B, C, D). The selective intracoronary administration of a PKA inhibitor, H-89, blocked proteasome activation by IP (Fig. 4E). We confirmed that proteasome activation by IP in LCx-perfused myocardium was the same as that in LAD-perfused one (Fig. 4F). These results suggest that exogenous and endogenous PKA stimulation increased 26S proteasome activity in canine hearts.

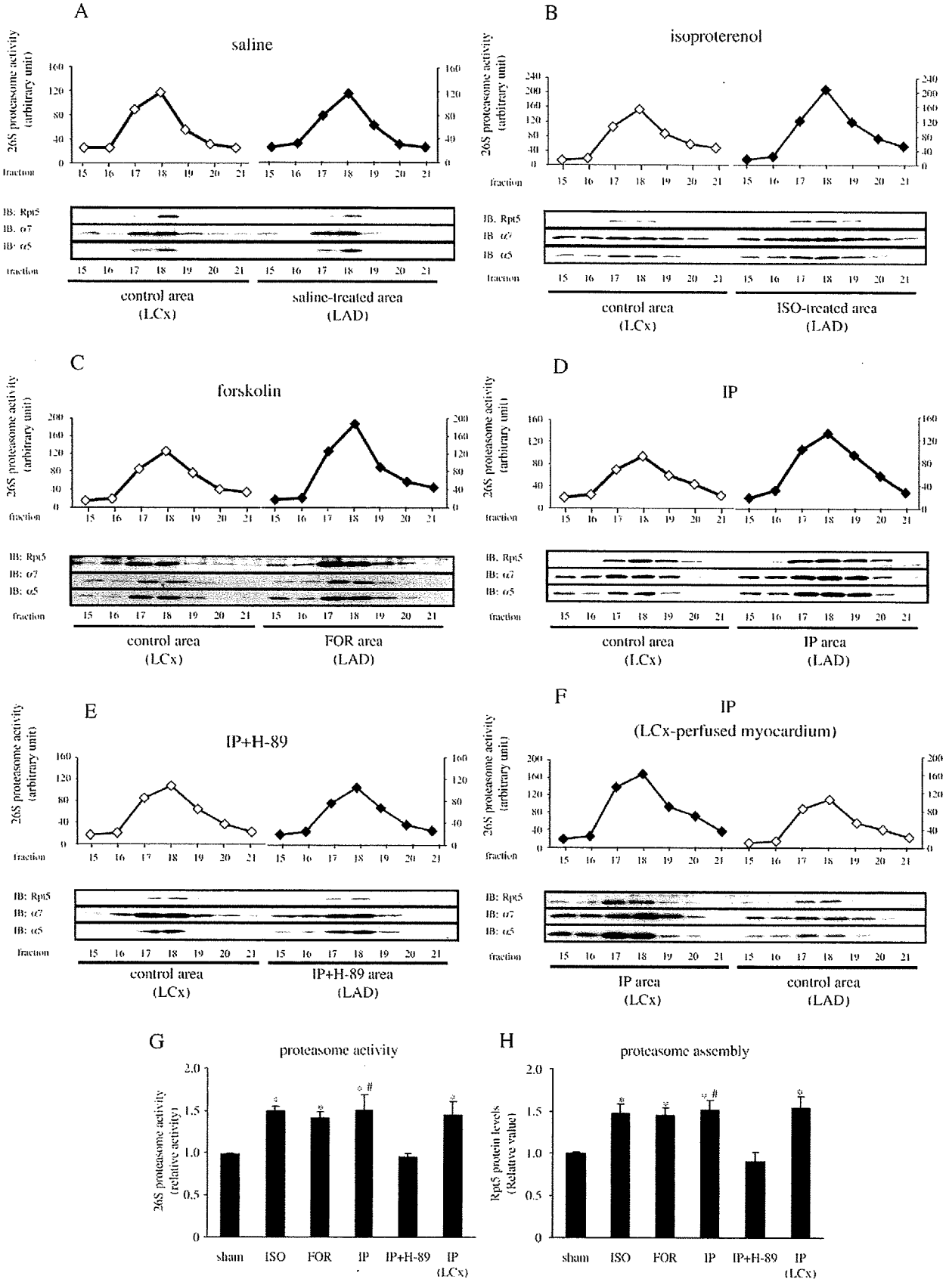
*3.4. PKA stimulation did not alter total protein levels of proteasome subunits in canine hearts*

We found no differences in the total protein levels of proteasome subunits in the saline-treated (LAD-perfused) and the control (LCx-perfused) myocardium (Fig. 5A). Then, we examined the changes in protein levels of the proteasome subunits such as Rpt5, α7 and β5 in LAD- and LCx-perfused myocardium when proteasome was activated by exogenous and endogenous PKA stimulation in canine hearts. Importantly, there were no differences in the total protein levels of 3 proteasome subunits (Rpt5, α7, β5) in groups tested (Figs. 5B–F). These results suggest that exogenous and endogenous PKA stimulation did not alter total protein levels of proteasome subunits in the in vivo canine hearts.

*3.5. PKA stimulation enhanced 26S proteasome activity and assembly in canine hearts*

Since we found 26S proteasome activity of canine hearts mainly in the fractions 17–19 after glycerol gradient centrifugation (Figs. 6A–F, upper panels), samples from fractions 15–21 in the LCx- and LAD-perfused myocardium were immunoblotted with antibodies against Rpt5, α7 or β5 (Figs. 6A–F, lower panels). Consistently, Western blotting analysis with SDS-PAGE gel showed that proteasome subunit Rpt5, α7 or β5 was found mainly in fractions 17–19 (Figs. 6A–F, lower panels).

**Fig. 6.** Exogenous and endogenous PKA stimulation enhanced 26S proteasome assembly in canine hearts. Representative analysis of 26S proteasome activity (upper panel) and Western blotting analysis of proteasome subunits (lower panel) in the control and treated myocardium. The number indicated fractions separated on a 10–40% glycerol gradient centrifugation. (A) saline, (B) isoproterenol (ISO), (C) forskolin (FOR), (D) ischemic preconditioning (IP), (E) IP with H-89 (IP+H-89), (F) IP in LCx-perfused myocardium. (G) Quantitative analysis of 26S proteasome activity in canine hearts. Proteasome activity was expressed as the summation of proteasome activity in fractions 17–19 in the treatment myocardium which were divided by that in the same fractions in the control one ($n$=4 to 8 each). (H) Quantitative analysis of proteasome assembly in canine hearts. Proteasome assembly was expressed as the summation of Rpt5 protein levels in fractions 17–19 in the treatment myocardium which were divided by that in the same fractions in the control ones ($n$=4 to 8 each). *$p<0.05$ vs sham. #$p<0.05$ vs IP+H-89.


A
saline
26S proteasome activity (arbitrary unit)
fraction
IB: Rpt5
IB: α7
IB: α5
control area (LCx)
saline-treated area (LAD)
B
isoproterenol
control area (LCx)
ISO-treated area (LAD)
C
forskolin
control area (LCx)
FOR area (LAD)
D
IP
control area (LCx)
IP area (LAD)
E
IP+H-89
control area (LCx)
IP+H-89 area (LAD)
F
IP
(LCx-perfused myocardium)
IP area (LCx)
control area (LAD)
G
proteasome activity
26S proteasome activity (relative activity)
sham
ISO
FOR
IP
IP+H-89
IP (LCx)
H
proteasome assembly
Rpt5 protein levels (Relative value)

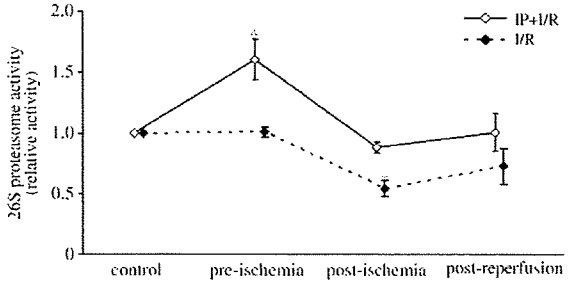



**Fig. 7.** Time-course changes in proteasome activity during ischemia/reperfusion period. Proteasome activity during ischemia/reperfusion period with and without IP. Myocardial biopsy specimens were taken at the control, just before ischemia (pre-ischemia), at the end of 90 min of ischemia (post-ischemia) and 6 h of reperfusion (post-reperfusion). Proteasome activity in IP+I/R and I/R groups was normalized to the corresponding control ones. IP+I/R and I/R indicate ischemia/reperfusion with and without IP, respectively. *$p<0.05$ vs control at the corresponding group.

Fig. 6 showed the representative proteasome activity and assembly using the myocardial sample of canine-1 in each groups in Fig. 5. Since we confirmed that the total amount of proteasome subunits were same in the control and treated myocardium, the alteration in protein levels of proteasome subunits in proteasome enriched fractions 17–19 indicate the alternation in the status of proteasome assembly. No differences were found in protein levels of proteasome subunits in the saline-treated (LAD-perfused) and the control (LCx-perfused) myocardium (Fig. 6A). Importantly, along with the increase in proteasome activity, the exogenous and endogenous PKA stimulation by isoproterenol, forskolin and IP significantly increased the protein levels of 3 different proteasome subunits in fractions 17–19 in the LAD-perfused myocardium compared with those in LCx-perfused one (Figs. 6B–D). Moreover, the selective administration of H-89 blunted the increases in protein levels of 3 different proteasome subunits and proteasome activation by IP in fractions 17–19 in the LAD-perfused myocardium (Fig. 6E). We also confirmed that the same findings were induced by IP in LCx-perfused myocardium (Fig. 6F). Quantitative analysis also showed that PKA enhanced proteasome activity and assembly, both of which were expressed as the summation of proteasome activity and Rpt5 protein levels in fractions 17–19 in the treatment myocardium which were divided by that in the same fractions in the control one, respectively (Figs. 6G, H). These results suggest that PKA stimulation enhanced 26S proteasome assembly and activity in canine hearts without alteration of total protein levels of proteasome subunits.

### *3.6. Time-course changes in proteasome activity during ischemia/reperfusion period with and without IP*

The analysis of consecutive myocardial biopsy samples also revealed that IP increased the proteasome activity in the LAD-perfused myocardium in the same dog (Fig. 7). In canine hearts with IP, the proteasome activities at the post-ischemia and post-reperfusion were significantly lower than that at the pre-ischemia (=just after IP), but they did not differ from the control. In canine hearts without IP, the proteasome activity at the post-ischemia was significantly decreased compared with that at the control or pre-ischemia (Fig. 7). Myocardial proteasome activity at the post-reperfusion did not differ from that at the post-ischemia in groups with and without IP (Fig. 7).

### *3.7. IP blunted the accumulation of ubiquitinated proteins in ischemia/reperfusion myocardium*

To examine the pathophysiological role of proteasome activation by IP, we investigated effects of IP on the accumulation of ubiquitinated proteins, which may predict recovery of postischemic cardiac function [22], in the ischemia/reperfusion myocardium in canine model. Western blotting analysis revealed that ubiquitinated proteins were increased in ischemia/reperfusion myocardium, while their accumulation was attenuated by IP (Figs. 8A, B). The reduction in

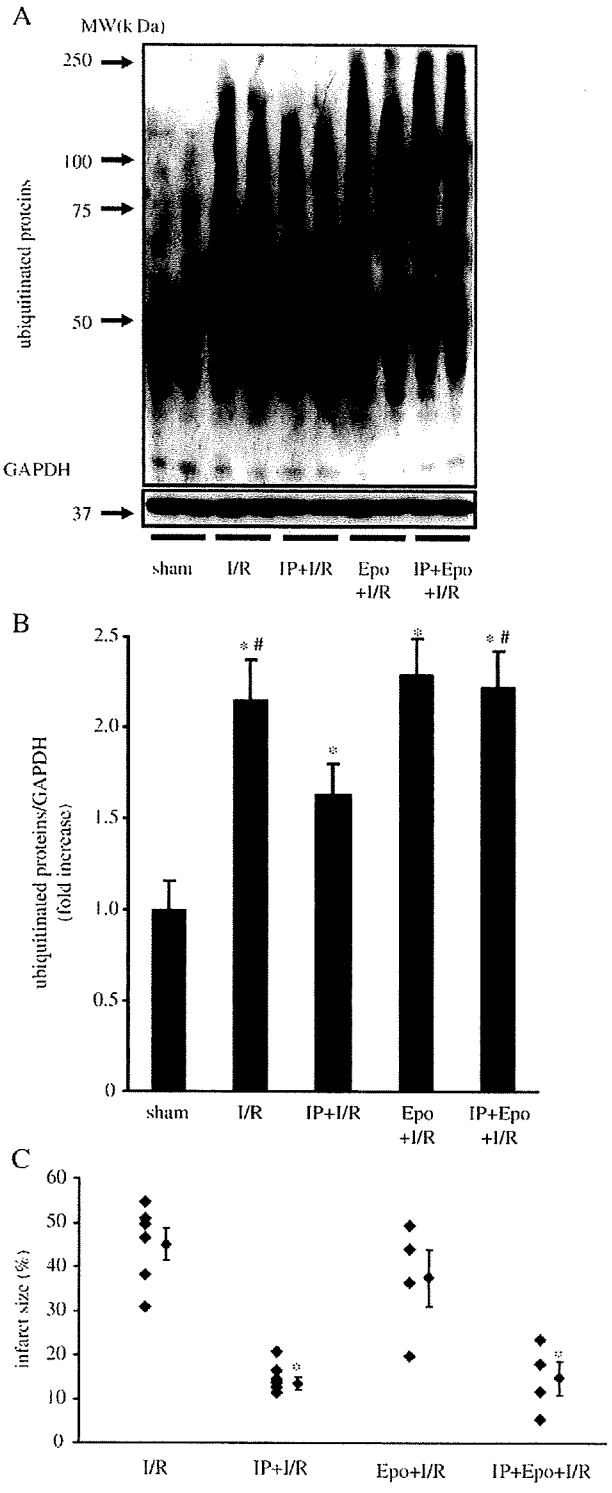



**Fig. 8.** Pathophysiological role of the enhancement of proteasome activity by IP. Representative example (A) and quantitative analysis (B) of Western blotting analysis of ubiquitinated proteins in canine ischemia/reperfusion (I/R) myocardium. *$p<0.05$ vs. sham, #$p<0.05$ vs. IP+I/R. $n=3$ per group. Values were normalized to sham. (C) Myocardial infarct size. *$p<0.05$ vs. I/R. MW, IP and Epo indicate molecular weight, ischemic preconditioning and epoxomicin (a proteasome inhibitor), respectively.

accumulated ubiquitinated proteins by IP was blunted by a proteasome inhibitor, epoxomicin. These results indicate that proteasome activation by IP alleviated the accumulation of ubiquitinated proteins in canine ischemia/reperfusion hearts.

### 3.8. Proteasome inhibition did not alter infarct size in canine hearts with and without IP

Fig. 8C showed the infarct size for each group in protocol IV. The intracoronary administration of epoxomicin before ischemia did not alter infarct size in this canine model. Consistent with the previous reports [15], we found that IP reduced myocardial infarct size. The infarct size-limiting effects of IP were not affected by the intracoronary administration of epoxomicin during IP procedure.

## 4. Discussion

### 4.1. PKA rapidly enhances phosphorylation, assembly and activity of 26S purified proteasome

Proteasome regulates cellular functions by eliminating ubiquitinated proteins [1–4]. Proteasome activity is enhanced by an increase in the levels of proteasome subunit proteins and their assembly, as well as by the post-translational modification of proteasome subunit through processes such as phosphorylation/dephosphorylation [9,10]. Recent studies have shown that PKA can phosphorylate several sites and increase proteasome activity in vitro [11,12]. Furthermore, although the involvement of PKA is not shown, the phosphorylation of proteasome subunits alters the status of proteasome assembly [23,24]. In the present study, we firstly showed that PKA activation enhanced the proteasome assembly, which contributed to the increase in proteasome activity. These findings suggest that altering proteasome subunit configuration through directed assembly by PKA may lead to the increase in proteasome activity, although we cannot exclude the possibility that PKA-mediated phosphorylation of proteasome subunits directly activates proteasome. Further investigation will be needed to clarify what subunit of proteasome is phosphorylated by PKA and the direct association between phosphorylation and assembly.

### 4.2. PKA stimulation enhances assembly and activity in in vivo canine hearts without affecting the total protein levels of proteasome subunits

Since proteasome activity is regulated by the multiple factors such as intracellular ATP levels and post-translational modification of proteasome [9,10], the in vitro findings of proteasome regulation are not always applied in the vivo model. Thus, to clarify whether the in vitro findings were also valid in vivo, we examined whether PKA stimulation could modulate proteasome assembly and activity in canine hearts. We employed two maneuvers to activate PKA in vivo, which were isoproterenol and forskolin as an exogenous stimulant of PKA [18,25] and IP as an endogenous stimulant [15]. We confirmed that both exogenous and endogenous stimulation of PKA enhanced 26S proteasome activity at 30 min after administration without changing the total protein levels of proteasome subunits in in vivo canine hearts. To our knowledge, the present study is the first to show the intervention to increase proteasome activity in vivo, suggesting that the pathophysiological conditions due to proteasome dysfunction in hearts could be treated.

We have found both 20S ($\alpha$7 and $\beta$5) and 19S (Rpt5) subunit proteins in fractions where proteasome activity was detected, indicating that 26S proteasome was indeed eluted in these fractions. We confirmed that exogenous and endogenous PKA stimulation increased the protein levels of proteasome subunits in these fractions without changing total amount of proteasome subunits. These findings suggest that PKA stimulation enhanced 26S proteasome assembly as well as activity in in vivo canine hearts.

### 4.3. Time-course changes in proteasome activity during ischemia/reperfusion period with and without IP

To examine the time-course changes in proteasome activity during ischemia/reperfusion period, we performed myocardial biopsy at 4 time-points during ischemia/reperfusion period: at the control, just before ischemia (pre-ischemia), at the end of 90 min ischemia (post-ischemia) and 6 h of reperfusion (post-reperfusion). Previous study indicated that proteasome activity was decreased after ischemia/reperfusion [8]. Consistent with the previous report, the proteasome activity at the post-ischemia was significantly decreased compared with that at the control or pre-ischemia in groups without IP. Meanwhile, the proteasome activity at the post-ischemia was significantly lower than that at the pre-ischemia (=just after IP), however, it did not differ from the control in groups with IP (Fig. 7). These findings suggest that stimuli during ischemic period decreased myocardial proteasome activity and that proteasome activation by IP during ischemic period may play an important role in the protein turnover and cellular functions in ischemia/reperfusion hearts. Myocardial proteasome activity at the end of reperfusion did not differ from that at the end of ischemia in groups with and without IP, suggesting that stimuli during reperfusion did not significantly affect proteasome activity. Unfortunately, due to the small volume of biopsy samples, we could not check the time-course changes in the status of proteasome assembly.

### 4.4. IP blunts the accumulation of ubiquitinated proteins in ischemia/reperfusion myocardium

Recently, the ubiquitination of proteins is important post-translational processes that modify the functions of many proteins. We and others have reported that the accumulation of ubiquitinated protein in hearts was associated with the progression of cardiac dysfunction due to apoptosis [7,26]. In addition, the injured myocardium by ischemia/reperfusion is concomitant with a reduced proteasome activity [8]. Consistent with these previous reports, we found the decreased proteasome activity and the accumulation of ubiquitinated proteins in the ischemia/reperfusion myocardium. Interestingly, we found the less accumulation of ubiquitinated proteins in ischemia/reperfusion myocardium, which may be attributable to the 40% increase in proteasome activity by endogenous PKA stimulation. Since the accumulation of ubiquitinated proteins may predict recovery of postischemic cardiac function [21], the removal of damaged proteins due to proteasome activation by IP may contribute to improve postischemic cardiac function.

### 4.5. Proteasome inhibition did not alter infarct size in canine hearts with and without IP

Finally, we examined whether proteasome activation by IP contributed to its infarct-size limiting effects in the canine model. The infarct-size limiting effects of IP were not prevented by the intracoronary administration of epoxomicin, a proteasome inhibitor, at the dose that reduces proteasome activity by 43%. These findings suggest that proteasome activation by IP was not involved in the infarct-size limiting effects of IP in the acute phase. Future studies will be required about the pathophysiological role of proteasome activation in the chronic phase after myocardial infarction. Moreover, the intracoronary administration of epoxomicin itself could not reduce the infarct size in this model. This data was inconsistent with the previous ones that the inhibition of proteasome could reduce myocardial infarct size in rats and pigs [27,28]. The discrepancy between the previous and our studies might be due to the differences in animals used, experimental protocols and the drugs used. Further investigation will be needed to clarify whether reduced proteasome activity is beneficial or detrimental in the ischemia/reperfusion injury in the acute phase.

In conclusion, the present study demonstrated that PKA rapidly enhances proteasome activity and assembly in the in vivo heart. Modulation of proteasome activity and assembly might be a promising new therapeutic approach for cardiovascular diseases.

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