ABC Study ケースカードⅢ（2）（2年時）

ABC No. A_____　　イニシャル（名・姓）_____・_____

心血管事故・糖尿病薬変更等のイベント：

□あり（未記入の場合、イベントカードをご記入下さい）　□なし

投薬内容

ボグリボース □ 0.6mg/day　□変更_____mg/day（理由：□消化器症状　□その他_____）
内服コンプライアンス（平均服用率：患者様への問診より、推定してください）
□ 100-80%　□ 80-60%　□ 60-40%　□ 40-20%　□ 20%以下

糖尿病関係追加薬剤：　□アマリール　□オイグルコン（ダオニール）　□グリミクロン
□ その他：_____　投与量_____mg

ACE：　□インヒベース　□エースコール　□コバシル　□タナトリル　□レニベース
□プレラン　□ その他：_____　投与量_____mg

ARB：　□ディオバン　□ニューロタン　□ブロプレス　□オルメテック　□ミカルディス
投与量_____mg

抗高脂血症薬：□メバロチン　□リピトール　□リポバス　□ローコール　□ベザトール
□その他：_____　投与量 _____mg

Ca blocker: □アダラート　□カルブロック　□コニール　□ノルバスク　□ヘルベッサー
□その他：_____　投与量_____ mg

β blocker:　□アーチスト　□ケルロング　□セレクトール　□セロケン　□メインテート
□その他：_____　投与量_____ mg

利尿剤：　□アルダクトンA　□ダイアート　□ラシックス　□ ルプラック　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗血小板薬： □バイアスピリン　□バファリン　□パナルジン　□プレタール　□その他：_____
投与量 _____mg　_____mg　_____mg　_____mg　_____mg

抗凝固薬：　□ワーファリン　投与量_____mg

血管拡張薬： □アイトロール　□ニトロール　□ニトロダーム　□フランドル
□その他：_____　投与量_____mg

抗潰瘍薬：　□ガスター　□ザンタック　□タケプロン
□その他：_____　投与量_____mg

アデノシン増強薬：　□ペルサンチン　投与量_____mg

K_{ATP} チャンネル開口薬：　□シグマート　投与量_____mg

その他の投薬：（心血管病薬以外も）

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ABC Study ケースカードIV（3年時）

ABC No. A_____　　イニシャル（名・姓）_____・_____

心血管事故・糖尿病薬変更等のイベント：

□あり（未記入の場合、イベントカードをご記入下さい）　□なし

投薬内容

ボグリボース □ 0.6mg/day　□変更_____mg/day（理由：□消化器症状　□その他_____）
内服コンプライアンス（平均服用率：患者様への問診より、推定してください）
□ 100-80%　□ 80-60%　□ 60-40%　□ 40-20%　□ 20%以下

糖尿病関係追加薬剤：　□アマリール　□オイグルコン（ダオニール）　□グリミクロン
□ その他：_____　投与量_____mg

ACE：　□インヒベース　□エースコール　□コバシル　□タナトリル　□レニベース
□プレラン　□ その他：_____　投与量_____mg

ARB：　□ディオバン　□ニューロタン　□ブロプレス　□オルメテック　□ミカルディス
投与量_____mg

抗高脂血症薬：□メバロチン　□リピトール　□リポバス　□ローコール　□ベザトール
□その他：_____　投与量_____mg

Ca blocker: □アダラート　□カルブロック　□コニール　□ノルバスク　□ヘルベッサー
□その他：_____　投与量_____mg

β blocker:　□アーチスト　□ケルロング　□セレクトール　□セロケン　□メインテート
□その他：_____　投与量_____mg

利尿剤：　□アルダクトンA　□ダイアート　□ラシックス　□ ルプラック　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗血小板薬： □バイアスピリン　□バファリン　□パナルジン　□プレタール　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗凝固薬：　□ワーファリン　投与量_____mg

血管拡張薬： □アイトロール　□ニトロール　□ニトロダーム　□フランドル
□その他：_____　投与量_____mg

抗潰瘍薬：　□ガスター　□ザンタック　□タケプロン
□その他：_____　投与量_____mg

アデノシン増強薬：　□ペルサンチン　投与量_____mg

K_{ATP} チャンネル開口薬：　□シグマート　投与量_____mg

その他の投薬：（心血管病薬以外も）

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ABC Study ケースカードV（4年時）

ABC No. A_____　　イニシャル（名・姓）_____・_____

心血管事故・糖尿病薬変更等のイベント：

□あり（未記入の場合、イベントカードをご記入下さい）　□なし

投薬内容

ボグリボース □ 0.6mg/day　□変更_____mg/day（理由：□消化器症状　□その他_____）
内服コンプライアンス（平均服用率：患者様への問診より、推定してください）
□ 100-80%　□ 80-60%　□ 60-40%　□ 40-20%　□ 20%以下

糖尿病関係追加薬剤：　□アマリール　□オイグルコン（ダオニール）　□グリミクロン
□ その他：_____　投与量_____mg

ACE：　□インヒベース　□エースコール　□コバシル　□タナトリル　□レニベース
□プレラン　□ その他：_____　投与量_____mg

ARB：　□ディオバン　□ニューロタン　□ブロプレス　□オルメテック　□ミカルディス
投与量_____mg

抗高脂血症薬：□メバロチン　□リピトール　□リポバス　□ローコール　□ベザトール
□その他：_____　投与量 _____mg

Ca blocker: □アダラート　□カルブロック　□コニール　□ノルバスク　□ヘルベッサー
□その他：_____　投与量_____ mg

β blocker:　□アーチスト　□ケルロング　□セレクトール　□セロケン　□メインテート
□その他：_____　投与量_____ mg

利尿剤：　□アルダクトンA　□ダイアート　□ラシックス　□ ルプラック　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗血小板薬： □バイアスピリン　□バファリン　□パナルジン　□プレタール　□その他：_____
投与量 _____mg　_____mg　_____mg　_____mg　_____mg

抗凝固薬：　□ワーファリン　投与量_____mg

血管拡張薬： □アイトロール　□ニトロール　□ニトロダーム　□フランドル
□その他：_____　投与量_____mg

抗潰瘍薬：　□ガスター　□ザンタック　□タケプロン
□その他：_____　投与量_____mg

アデノシン増強薬：　□ペルサンチン　投与量_____mg

K_{ATP} チャンネル開口薬：　□シグマート　投与量_____mg

その他の投薬：（心血管病薬以外も）

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ABC Study ケースカードVI（5年時）

ABC No. A_____　　イニシャル（名・姓）_____・_____

心血管事故・糖尿病薬変更等のイベント：

□あり（未記入の場合、イベントカードをご記入下さい）　　□なし

投薬内容

ボグリボース □ 0.6mg/day　□変更_____mg/day（理由：□消化器症状　□その他_____）
内服コンプライアンス（平均服用率：患者様への問診より、推定してください）
□ 100-80%　□ 80-60%　□ 60-40%　□ 40-20%　□ 20%以下

糖尿病関係追加薬剤：　□アマリール　□オイグルコン（ダオニール）　□グリミクロン
□ その他：_____　投与量_____mg

ACE：　□インヒベース　□エースコール　□コバシル　□タナトリル　□レニベース
□プレラン　□ その他：_____　投与量_____mg

ARB：　□ディオバン　□ニューロタン　□ブロプレス　□オルメテック　□ミカルディス
投与量_____mg

抗高脂血症薬：□メバロチン　□リピトール　□リポバス　□ローコール　□ベザトール
□その他：_____　投与量_____mg

Ca blocker: □アダラート　□カルブロック　□コニール　□ノルバスク　□ヘルベッサー
□その他：_____　投与量_____mg

β blocker:　□アーチスト　□ケルロング　□セレクトール　□セロケン　□メインテート
□その他：_____　投与量_____mg

利尿剤：　□アルダクトンA　□ダイアート　□ラシックス　□ ルプラック　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗血小板薬：　□バイアスピリン　□バファリン　□パナルジン　□プレタール　□その他：_____
投与量 _____mg　_____mg　_____mg　_____mg　_____mg

抗凝固薬：　□ワーファリン　投与量_____mg

血管拡張薬：　□アイトロール　□ニトロール　□ニトロダーム　□フランドル
□その他：_____　投与量_____mg

抗潰瘍薬：　□ガスター　□ザンタック　□タケプロン
□その他：_____　投与量_____mg

アデノシン増強薬：　□ペルサンチン　投与量_____mg

K_{ATP} チャンネル開口薬：　□シグマート　投与量_____mg

その他の投薬：（心血管病薬以外も）

臨床研究支援センター　〒560-0082　大阪府豊中市新千里東町 1-4-1-9F
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ABC Study ケースカードⅦ（6年時）

ABC No. A_____　　イニシャル（名・姓）_____・_____

心血管事故・糖尿病薬変更等のイベント：

□あり（未記入の場合、イベントカードをご記入下さい）　□なし

投薬内容

ボグリボース □ 0.6mg/day　□変更_____mg/day（理由：□消化器症状　□その他_____）
内服コンプライアンス（平均服用率：患者様への問診より、推定してください）
□ 100-80%　□ 80-60%　□ 60-40%　□ 40-20%　□ 20%以下

糖尿病関係追加薬剤：　□アマリール　□オイグルコン（ダオニール）　□グリミクロン
□ その他：_____　投与量_____mg

ACE：　□インヒベース　□エースコール　□コバシル　□タナトリル　□レニベース
□プレラン　□ その他：_____　投与量_____mg

ARB：　□ディオバン　□ニューロタン　□ブロプレス　□オルメテック　□ミカルディス
投与量_____mg

抗高脂血症薬：□メバロチン　□リピトール　□リポバス　□ローコール　□ベザトール
□その他：_____　投与量_____mg

Ca blocker: □アダラート　□カルブロック　□コニール　□ノルバスク　□ヘルベッサー
□その他：_____　投与量_____mg

β blocker:　□アーチスト　□ケルロング　□セレクトール　□セロケン　□メインテート
□その他：_____　投与量_____mg

利尿剤：　□アルダクトンA　□ダイアート　□ラシックス　□ ルプラック　□その他：_____
投与量_____mg　_____mg　_____mg　_____mg　_____mg

抗血小板薬：　□バイアスピリン　□バファリン　□パナルジン　□プレタール　□その他：_____
投与量 _____mg　_____mg　_____mg　_____mg　_____mg

抗凝固薬：　□ワーファリン　投与量_____mg

血管拡張薬：　□アイトロール　□ニトロール　□ニトロダーム　□フランドル
□その他：_____　投与量_____mg

抗潰瘍薬：　□ガスター　□ザンタック　□タケプロン
□その他：_____　投与量_____mg

アデノシン増強薬：　□ペルサンチン　投与量_____mg

K_{ATP} チャンネル開口薬：　□シグマート　投与量_____mg

その他の投薬：（心血管病薬以外も）

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ABC Study イベントカード

ABC No. A＿＿＿＿＿＿　イニシャル（名・姓）＿＿＿・＿＿＿

死亡、入院、糖尿病薬の追加、腎機能の悪化のイベントが発生した際、記載して下さい

（複数イベントがあった場合はイベント毎にカードをご記入下さい。）

死亡　（２０＿＿年＿＿＿月＿＿＿日）

☐ 心臓死（☐心筋梗塞 ☐心不全 ☐不整脈 ☐その他＿＿＿＿＿＿＿＿＿＿）

☐ その他の死因

☐ 脳血管障害 （☐脳梗塞 ☐脳出血 ☐くも膜下出血）
☐ 大血管障害 （☐大動脈瘤破裂 ☐大動脈解離）
☐ その他（病名： ）

死亡経過の詳細＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

入院　（２０＿＿年＿＿＿月＿＿＿日）

☐ 虚血性心疾患（☐心筋梗塞（非致死性）☐不安定狭心症 ☐安定狭心症）

病変： ☐新規病変 ☐再狭窄病変
治療法：☐PTCA（ステント） ☐CABG ☐その他の手術 ☐薬物療法のみ

☐ 心不全

☐ その他の心臓血管イベントによる入院

☐ 脳血管障害（ＴＩＡ以外）（☐脳梗塞 ☐脳出血 ☐くも膜下出血）

☐ 閉塞性動脈硬化症

☐ その他（病名： ）

入院経過の詳細＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

糖尿病薬の追加

☐ 有り （　　年　　月　理由：HbA1c ＿＿＿＿%　　）

追加薬剤名＿＿＿＿＿＿＿＿＿＿　投与量＿＿＿＿＿＿＿＿

腎機能の悪化

☐ 有り （　　年　　月　理由：血清クレアチニン ＿＿＿mg/dl　　）

上記の内容を速やかにご登録下さい http://www.csscj.com 又は FAX：06-6836-5211

ABC
α -glucosidase-inhibitor Blocks Cardiac events

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ABC Study 中止報告書

ABC No. A_____　　イニシャル（名・姓）_____・_____

登録病院名 _____　　登録医師名 _____

中止理由

□ 患者様側より、辞退の申し入れ

□今までの登録データ抹消を希望

□今後のデータ登録を希望しない （これまでのデータ登録は了承）

□ 医療側による、薬剤投与の中止

□有害事象の発生により、薬剤の継続投与困難

□肝機能障害 （トランスアミアラーゼが正常上限の 2.5 倍以上）

□低血糖発作

□他の有害事象：_____

□その他：_____

状況をお教え下さい。

_____

_____

_____

上記の内容を速やかにご登録下さい http://www.csscj.com 又は FAX：06-6836-5211

ABC

α -glucosidase-inhibitor Blocks Cardiac events

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研究成果の刊行に関する一覧表

主任研究者氏名 ： 北風 政史

所属機関名 ： 国立循環器病センター 職名 ： 部長

課題名 ： 食後血糖上昇の抑制による心筋梗塞二次予防に関する大規模薬剤介入臨床研究
（臨床研究実施チームの整備）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	頁	出版年
Hirata A, Tomoike H(13 人省略 15 番目), Hori M, **Kitakaze M**.	Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in canine hearts.	Cardiovasc Drugs Ther	19(1)	33-40	2005
Liao Y, Tomoike H(6 人省略 8 番目), Hori M, **Kitakaze M**.	Amlodipine ameliorates myocardial hypertrophy by inhibiting EGFR phosphorylation.	Biochem Biophys Res Commun	327(4)	1083-7	2005
Liao Y, Tomoike H(6 人省略 8 番目), Hori M, **Kitakaze M**.	Benidipine, a long-acting calcium channel blocker, inhibits cardiac remodeling in pressure-overloaded mice.	Cardiovasc Res	65(4)	879-88	2005
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Liao Y, **Kitakaze M**(8 人省略 last author).	Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism.	Cardiovasc Res	67(4)	705-13	2005
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BASIC PHARMACOLOGY

Erythropoietin Just Before Reperfusion Reduces Both Lethal Arrhythmias and Infarct Size via the Phosphatidylinositol-3 Kinase-Dependent Pathway in Canine Hearts

Akio Hirata[1], Tetsuo Minamino[1], Hiroshi Asanuma[1], Shoji Sanada[1], Masashi Fujita[1], Osamu Tsukamoto[1], Masakatsu Wakeno[2], Masafumi Myoishi[2], Ken-ichiro Okada[1], Hidekazu Koyama[1], Kazuo Komamura[3], Seiji Takashima[1], Yoshiro Shinozaki[4], Hidezo Mori[3], Hitonobu Tomoike[3], Masatsugu Hori[1], and Masafumi Kitakaze[3]

[1]*Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan;* [2]*Department of Bioregulatory Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, Japan;* [3]*Cardiovascular Division of Internal Medicine, National Cardiovascular Center, Suita, Osaka, Japan;* [4]*Department of Physiological Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan*

Summary. **Although recent studies suggest that erythropoietin (EPO) may reduce multiple features of the myocardial ischemia/reperfusion injury, the cellular mechanisms and the clinical implications of EPO-induced cardioprotection are still unclear. Thus, in this study, we clarified dose-dependent effects of EPO administered just before reperfusion on infarct size and the incidence of ventricular fibrillation and evaluated the involvement of the phosphatidylinositol-3 (PI3) kinase in the *in vivo* canine model. The canine left anterior descending coronary artery was occluded for 90 min followed by 6 h of reperfusion. A single intravenous administration of EPO just before reperfusion significantly reduced infarct size (high dose (1,000 IU/kg): 7.7 ± 1.6%, low dose (100 IU/kg): 22.1 ± 2.4%, control: 40.0 ± 3.6%) in a dose-dependent manner. Furthermore, the high, but not low, dose of EPO administered as a single injection significantly reduced the incidence of ventricular fibrillation during reperfusion (high dose: 0%, low dose: 40.0%, control: 50.0%). An intracoronary administration of a PI3 kinase inhibitor, wortmannin, blunted the infarct size-limiting and anti-arrhythmic effects of EPO. Low and high doses of EPO equally induced Akt phosphorylation and decreased the equivalent number of TUNEL-positive cells in the ischemic myocardium of dogs. These effects of EPO were abolished by the treatment with wortmannin. In conclusion, EPO administered just before reperfusion reduced infarct size and the incidence of ventricular fibrillation via the PI3 kinase-dependent pathway in canine hearts. EPO administration can be a realistic strategy for the treatment of acute myocardial infarction.**

Key Words. **erythropoietin, myocardial infarction, ventricular arrhythmia, phosphatidylinositol-3 kinase, ischemia-reperfusion injury, apoptosis**

Introduction

Recent studies have extended the traditional role of erythropoietin (EPO) from a mediator of erythroid maturation to one that provides protection against apoptotic cell death [1,2]. Recombinant human EPO (rhEPO) has been shown to exert marked protective effects against ischemia/reperfusion injury in rats and rabbits when rhEPO is administered at different time points [3–8]. Indeed, rhEPO reduced myocardial infarct size, enhanced recovery of left ventricular developed pressure, reduced the number of apoptotic cells, and induced the phosphorylation of Akt [3–8]. In these studies, high (1,000–5,000 IU/kg) doses of rhEPO, nearly 10 times higher than that used in anemic patients with chronic renal failure [9], have been applied. Recently, it was reported that phosphatidylinositol-3 (PI3) kinase

Address for correspondence: Tetsuo Minamino, MD, PhD, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, Tel.: 81-6-6879-3635; Fax: 81-6-6879-3473; E-mail: minamino@medone.med.osaka-u.ac.jp

activity is required for rhEPO to recover contractile dysfunction and to block apoptosis induced by myocardial ischemia-reperfusion in isolated hearts (*ex vivo*) [10]. However, it is not determined whether rhEPO just before reperfusion reduces infarct size via PI3 kinase-dependent pathway in the *in vivo* model.

In addition to myocardial cell death, myocardial ischemia-reperfusion triggers lethal arrhythmias [11]. It is believed that at least half of the deaths due to coronary artery disease are caused by a lethal arrhythmia [12]. Although high doses of rhEPO exert cardioprotective effects against ischemia/reperfusion injury in small animals [3–8], its effects on lethal arrhythmias remain unknown. If rhEPO reduces the incidence of ventricular fibrillation (VF) in the clinical setting, there would be additional advantage to use this drug in the realistic situation of acute myocardial infarction. Thus, in the present study, we examined dose-dependent effects of rhEPO administered just before reperfusion on myocardial infarct size and the incidence of VF in the *in vivo* canine model. We also evaluated whether any such effects were mediated via the PI3 kinase pathway.

Materials and Methods

Materials

Wortmannin was obtained from Sigma (St. Louis, MO) and Phospho-Akt and Akt antibodies were obtained from Cell Signaling Technologies (Beverly, MA). RhEPO was provided by Chugai Pharmaceutical Co., Ltd (Tokyo, Japan).

Instrumentation

Forty-eight beagle dogs (Kitayama Labes, Yoshiki Farm, Gifu, Japan) weighing 8 to 12 kg were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air mixed with oxygen (100% O_2 at flow rate of 1.0 to 1.5 L/min). Thoracotomy was done at the left fifth intercostal space, and the heart was suspended by a pericardial cradle. After intravenous administration of heparin (500 U/kg), the left anterior descending coronary artery (LAD) was cannulated for perfusion with blood from the left carotid artery through an extracorporeal bypass tube. Coronary blood flow was measured with an electromagnetic flow probe attached to the bypass tube. We can selectively infuse drugs into LAD-perfused areas through this bypass tube. The left atrium was catheterized for microsphere injection to measure myocardial collateral blood flow during ischemia. Hydration was maintained by a slow normal saline infusion. The femoral artery was also cannulated to measure the mean systemic blood pressure (SBP). Both SBP and heart rate (HR) were monitored continuously during the study. 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.

Experimental protocols

Protocol 1. Long-term effects of rhEPO on hematometric parameters in dogs. To test the long-term effects of rhEPO on hematometric parameters, 100 IU/kg ($n = 5$) or 1,000 IU/kg ($n = 5$) of rhEPO was intravenously administered as a single injection. Blood was collected under pentobarbital (15 mg/kg) anesthesia before and 7, 14 days after rhEPO treatment. Hematometric parameters including hematocrit, white blood cell, and platelet counts were measured.

Protocol 2; Measurement of infarct size, coronary blood flow and myocardial collateral blood flow. After hemodynamic stabilization, we administered a low (100 IU/kg), or high (1,000 IU/kg) dose of rhEPO, or saline 10 min prior to reperfusion (n = 8–12 each) as a single intravenous injection (Fig. 1). To clarify whether rhEPO reduces myocardial infarct size through a PI3 kinase-dependent pathway, a PI3 kinase inhibitor, wortmannin, was selectively administered into the LAD (1.5 μg/kg/min) for 60 min after the onset of reperfusion. We have previously confirmed that the dose of wortmannin employed in this study is appropriate for blocking the phosphorylation of Akt in myocardium [13]. We measured infarct size and regional myocardial collateral blood flow during 90 min of ischemia as described previously [14]. In brief, infarct size was evaluated at the end of the protocol by Evans blue/TTC staining, while collateral blood flow was assessed by the non-radioactive microsphere method [14]. Coronary blood flow was monitored continuously during the study. To ensure that all of the animals included in the data analysis were healthy and were exposed to a similar extent of ischemia, the exclusion criteria reported previously for excessive myocardial collateral blood flow (>15 mL/100 g/min) and lethal arrhythmia (more than two consecutive attempts required to convert VF with low-energy DC pulses applied directly to the heart) were adopted [14].

Effects of rhEPO on VF during reperfusion period

In Protocol 2, we also evaluated the incidence of VF during the 6 h reperfusion period (Fig. 1). Since myocardial collateral blood flow during ischemia exhibited a negative correlation with the incidence of VF [15,16], the dogs with excessive collateral blood flow (>15 mL/100 g/min) were excluded from VF analysis.

Phosphorylation of Akt

We used 12 dogs for western blot in the control, low EPO, high EPO, and high EPO + WTMN groups ($n = 3$ each) in Protocol 2 (Fig. 1). After 90 min of ischemia followed by 6 h of reperfusion, hearts were excised and

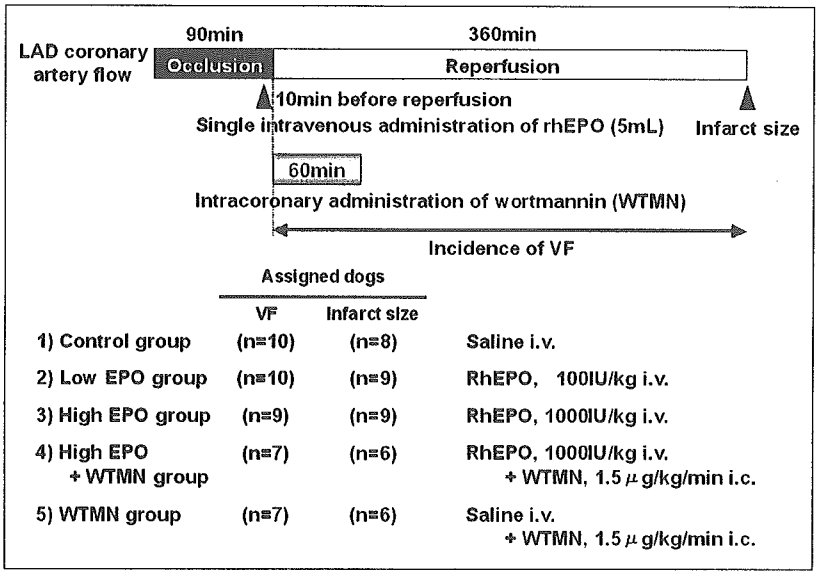

Fig. 1. *Experimental protocol for infarct size and VF.*

the myocardial tissue in the ischemic zone was quickly placed into liquid nitrogen and stored at −80°C. Phosphorylation of Akt and total content of Akt were evaluated as reported previously [13]. The immunoreactive bands were quantified by densitometry (Molecular Dynamics).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

In Protocol 2, the myocardial tissue samples were taken from the ischemic zone of dogs in the control, low EPO, high EPO, and high EPO + WTMN groups ($n = 3$ each). These were fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned in the frontal plane at 5-μm thickness. Analysis by TUNEL method was performed according to the protocol supplied with the in situ apoptosis detection kit, the Apop Tag Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON International, USA). TUNEL-positive cell nuclei and total cell nuclei stained metylgreen were counted in 10–15 random high-power fields (×400), and the percentage of TUNEL-positive cell nuclei to total cell nuclei ($n =$ 1,000) were then calculated.

Statistical analysis

Statistical analysis was performed by one-way factional analysis of variance (ANOVA) with modified Bonferroni's post hoc test when the data were compared among groups. Time courses of the changes were compared by repeated measures ANOVA. The incidence of VF was compared using the χ^2-test and Fisher's exact probability test. Results were expressed as the mean ± SEM, with $p < 0.05$ considered significant.

Results

The long-term effects of rhEPO on hematometric parameters

The single administration of either 100 IU/kg or 1,000 IU/kg of rhEPO did not change any hematometric parameters including hematocrit, white blood cells, and platelet counts 7 or 14 days after rhEPO treatment (Table 1).

Table 1. *Long-term effects of rhEPO on hematometric parameters in dogs*

	EPO 100 IU/kg			EPO 1000 IU/kg		
Parameters	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Ht (%)	50.1 ± 0.8	51.1 ± 0.9	51.3 ± 1.0	51.5 ± 1.3	52.0 ± 1.0	53.1 ± 0.7
WBC ($10^3/\mu$L)	11.7 ± 1.3	12.1 ± 0.7	11.4 ± 0.6	11.5 ± 1.3	11.7 ± 0.7	12.0 ± 1.0
Platelet (10^4/mm^3)	33.7 ± 2.0	33.7 ± 1.8	33.3 ± 1.7	33.2 ± 1.9	34.0 ± 2.4	36.4 ± 3.4

Data are presented as Mean ± SEM. $n = 5$.
Abbreviations: rhEPO = recombinant human erythropoietin, Ht = hematocrit, WBC = white blood cell.

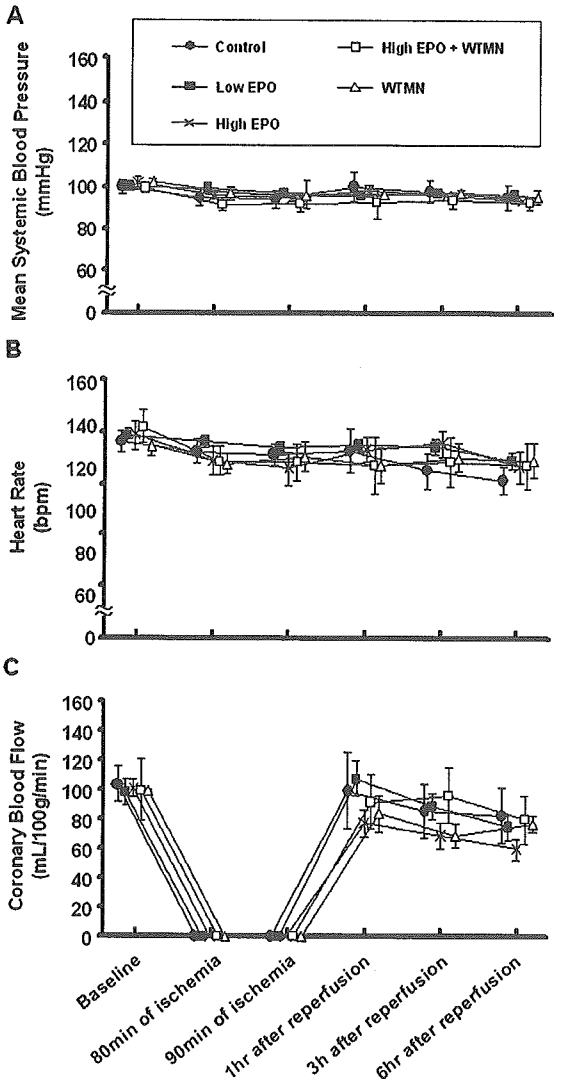

Fig. 2. The changes in mean systemic blood pressure, heart rate and coronary blood flow during the experiment in groups tested.

Effects of rhEPO on infarct size and VF during the reperfusion period

Since 5 of 48 dogs were excluded from analysis because of excessive collateral blood flow (>15 mL/100 g/min) (control: 1, low EPO: 2, high EPO: 1, high EPO + WTMN: 0, WTMN: 1), 43 dogs were evaluated for VF analysis. Among these 43 dogs, we excluded 5 dogs (control: 2, low EPO: 1, high EPO: 0, high EPO + WTMN: 1, WTMN: 1) that matched the exclusion criteria of lethal arrhythmia from infarct size analysis.

Throughout the study, neither SBP (Fig. 2A), nor HR (Fig. 2B), nor coronary blood flow (Fig. 2C) differed among the 5 groups. The area at risk (Fig. 3A) and myocardial collateral blood flow in the LAD region during myocardial ischemia (Fig. 3B) were also comparable in the groups tested.

Table 2. *Effects of rhEPO on the incidence of VF during reperfusion periods*

Group	Incidence of VF	
Control	50.0%	(5/10)
Low EPO	40.0%	(4/10)
High EPO	0%*	(0/9)
High EPO + WTMN	42.9%	(3/7)
WTMN	42.9%	(3/7)

* $p < 0.05$ vs. control group.
Abbreviations: VF = ventricular fibrillation, rhEPO = recombinant human erythropoietin, WTMN = wortmannin.

Figure 4 shows infarct size in the groups tested. A low or high dose of rhEPO significantly ($p < 0.05$) reduced the infarct size compared with that in the control group. Furthermore, a high dose of rhEPO reduced infarct size more than a low dose of rhEPO did. The intracoronary administration of wortmannin for 60 min after the onset of reperfusion abrogated the infarct-limiting effect of rhEPO, although wortmannin alone did not affect infarct size.

The high, but not low, dose of rhEPO significantly ($p < 0.05$) reduced the incidence of VF during the 6 h reperfusion period compared with the control. The anti-arrhythmic effects of rhEPO were abolished by wortmannin (Table 2).

Effects of rhEPO on Akt phosphorylation

After 90 min of ischemia followed by 6 h of reperfusion, the ratio of phosphorylated Akt to total Akt in the low and high EPO groups significantly ($p < 0.05$) increased compared with that in the control group. The increase in this ratio was completely abolished by the treatment with wortmannin (Fig. 5).

Effects of rhEPO on apoptosis

The ratio of TUNEL positive cells to total cells in the low and high EPO groups decreased compared with that in the control group. The reduction of TUNEL-positive cells by rhEPO was completely abolished by the treatment with wortmannin (Fig. 6).

Discussion

In this study, we demonstrated that a single intravenous administration of rhEPO just before reperfusion limited not only infarct size but also the incidence of VF. Moreover, our data suggest that the infarct size-limiting and anti-arrhythmic effects of rhEPO were through the PI3 kinase-dependent pathways in the *in vivo* canine hearts.

Important considerations towards clinical application of rhEPO are the timing and dose of its administration. The previous studies reported that rhEPO administered at the onset of reperfusion [7,8] as well

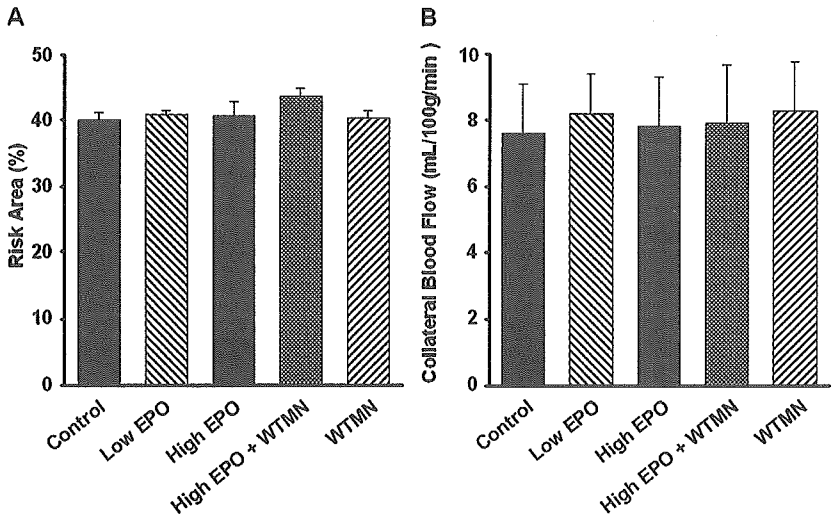

Fig. 3. *Area at risk and myocardial collateral blood flow during ischemia in groups tested.*

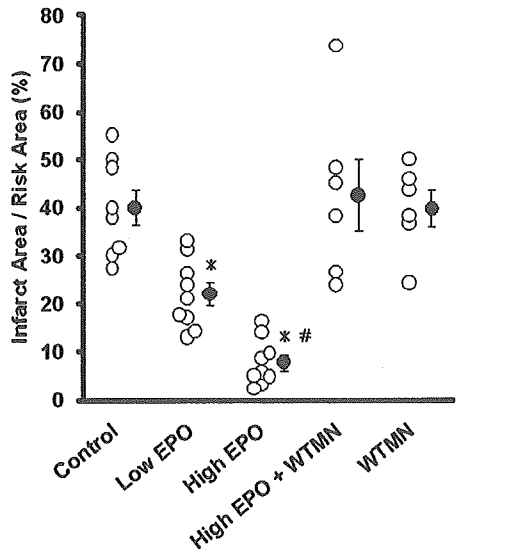

Fig. 4. *Infarct size in groups tested. *p < 0.05 vs. control group. #p < 0.05 vs. low EPO group. Open circles show the infarct size in each individual.*

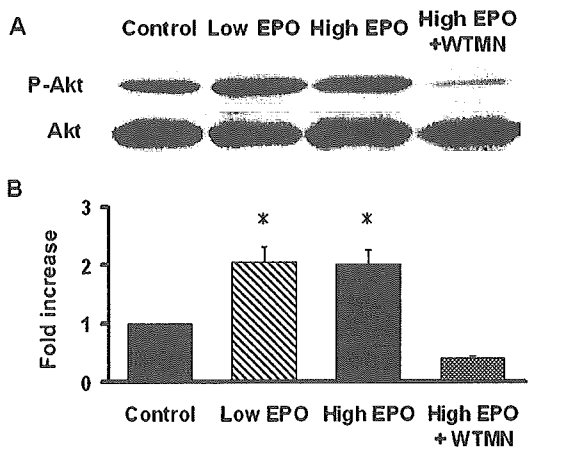

Fig. 5. *Phosphorylation of Akt in canine hearts. (A) Representative Western blot for phosphorylated and total Akt. (B) Densitometry graphs indicating fold expression over control for Akt. n = 3 each. *p < 0.05 vs. control group.*

as ischemia [7,8] reduces infarct size in rabbit and rat hearts. Consistent with these reports, we confirmed that rhEPO administered 10 min before reperfusion reduced myocardial infarct size in dogs. Our findings support the idea that in humans the adjunctive therapy with rhEPO treatment during coronary intervention would reduce myocardial infarct size.

The doses of rhEPO (1,000–5,000 IU/kg) administered in previous experimental studies [3–8] were nearly 10 times higher than those clinically used in anemic patients with chronic renal failure [9]. In the present study, we demonstrated that both 100 IU/kg and 1,000 IU/kg of rhEPO as a single administration significantly reduced myocardial infarct size, although a high dose of rhEPO significantly reduced infarct size more than a low dose of rhEPO did. This finding suggests that the clinically relevant dose of rhEPO used in patients with chronic renal failure can reduce myocardial infarct size. In the previous clinical studies, a high dose (33,000 IU once daily for the first 3 days) of intravenously administered rhEPO was well tolerated in patients with stroke and improved clinical outcome at 1 month [17]. On the other hand, a high dose (40,000–60,000 IU per week) of subcutaneously administered rhEPO, while not as a single injection,

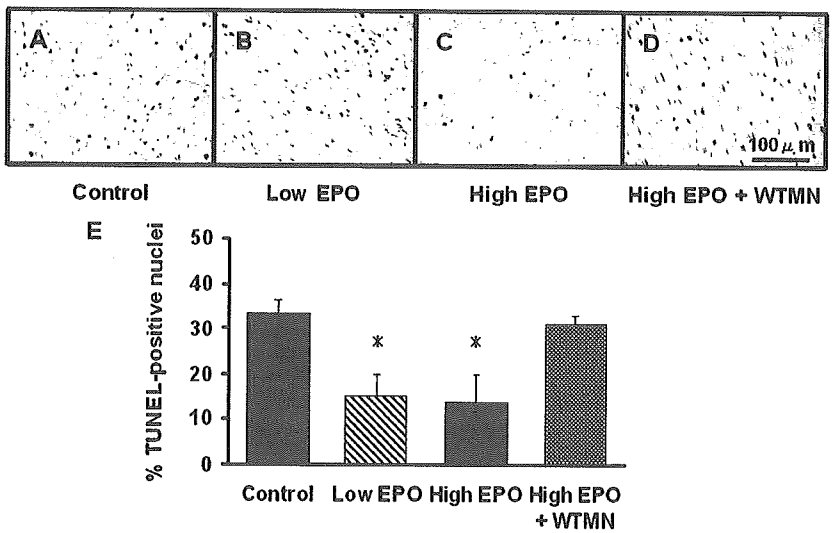

Fig. 6. *TUNEL staining in canine hearts after 90 min ischemia followed by 6 h of reperfusion. Representative examples of TUNEL-staining from canine hearts in the control (A), low EPO (B), high EPO (C), and high EPO + WTMN groups (D). (E) Quantitative data of the percentage of TUNEL-positive nuclei to total cell nuclei. *p < 0.05 vs. control group.*

increased the incidence of thrombotic events such as deep venous thrombosis or pulmonary embolisms in patients with breast cancer [18]. Furthermore, there are some reports that rhEPO increases the number of platelets in normal rats [19] and chronically hemodialyzed patients [20]. In the present study, we didn't find that either low or high dose of rhEPO, as a single injection, changed hematometric parameters. Although these findings suggest that a single administration of 1,000 IU/kg of rhEPO, that induced marked reduction of myocardial infarct size, could be used safely, we must be careful for the use of a high dose of rhEPO for the treatment of myocardial infarction.

Previous reports have shown that both phosphorylation of Akt and inhibition of apoptosis are associated with infarct size-limiting effects due to rhEPO [4,6–8]. Recently, it was reported that PI3 kinase activity is required for rhEPO to recover contractile dysfunction and to block apoptosis induced by myocardial ischemia-reperfusion in isolated hearts (*ex vivo*) [10]. Although the recovery of contractile function could be related to the reduction of infarct size, no evidence was presented that rhEPO reduced infarct size via the PI3 kinase-dependent pathway. In the present study we have demonstrated that the infarct size-limiting effect of rhEPO was blunted by the intracoronary administration of wortmannin in dogs. This is the first evidence showing that the infarct size-limiting effect of rhEPO is dependent on the PI3 kinase pathway in *in vivo* hearts.

In the present study, low and high doses of rhEPO equally increased phosphorylation of Akt and decreased equivalent number of TUNEL-positive cells in the ischemic myocardium of dogs. Either Akt phosphorylation or a decrease in the number of TUNEL-positive cells was prevented by the PI3 kinase inhibitor, wortmannin. This finding suggests that rhEPO prevents apoptotic cell death through PI3 kinase/Akt-dependent pathway in canine hearts. However, since the TUNEL method also detects single strand breaks occurring in the course of necrotic cell death [21], it is likely that rhEPO attenuates apoptotic and necrotic cell death. Indeed, if rhEPO only inhibits the apoptotic cell death, it may be difficult to explain the marked reduction of infarct size by rhEPO. Interestingly, the previous studies reported that the PI3 kinase activates not only Akt but also protein kinase C or mitogen-activated protein kinase in ischemia/reperfusion models [22–24], either of which mediates the cellular protection against necrotic process [25,26]. Furthermore, recent reports suggest that rhEPO can inhibit the release of free radicals from neutrophils [27] and act as a radical scavenger [28], both of which may reduce cardiac cell death after ischemia/reperfusion. Although further investigation will be needed, these characteristics of rhEPO may contribute to the reduction of necrotic as well as apoptotic cell death in ischemia/reperfused myocardium. In addition, since wortmannin inhibits not only PI3 kinase but also PI4 kinase and PI kinase related protein kinase, there is a limitation in using wortmannin as a specific inhibitor of PI3 kinase [29].

In clinical settings, ventricular arrhythmias are often observed in patients following reperfusion therapy and they can be life-threatening [30]. Importantly, the present study demonstrated that a high, but not a low dose of rhEPO prevented VF during reperfusion via the PI3 kinase-dependent pathway. Since low and high doses of rhEPO equally increased phosphorylation of Akt, it is unlikely that Akt is responsible for

the rhEPO-induced anti-arrhythmic effect. There are several possible mechanisms by which rhEPO exerts anti-arrhythmic effects via the PI3 kinase-dependent, but Akt-independent, pathway. First, under conditions of reperfusion, production of inositol-1,4,5-trisphospate (IP3) increases when phospholipase C (PLC) is activated through α-adrenoreceptors on the myocardial cell membrane [11]. This increase in IP3 activates IP3 receptors on the sarcoplasmic reticulum causing the release of Ca^{2+}. The increases in the intracellular Ca^{2+} levels caused by IP3 have been reported to initiate slow Ca^{2+} oscillations, which underlies the delayed afterdepolarizations that trigger many arrhythmias including VF [11,31]. PLC hydrolyzes phosphatidylinositol-4,5-bisphospate (PIP2) to produce IP3. Since PI3 kinase and PLC can act upon the common substrate, PIP2 [32], rhEPO may prevent lethal arrhythmia by activating the PI3 kinase pathway that results in the decrease in PIP2 levels, which will lead to prevent Ca^{2+} overload by IP3. Second, since oxygen-derived free radicals are involved in the generation of reperfusion arrhythmia [30,33,34], rhEPO may decrease reperfusion arrhythmia through the prevention of free radicals release from neutrophils or acting as a radical scavenger [27,28]. Finally, we need to consider that rhEPO exerts anti-arrhythmic effects by the reduction of myocardial infarct size.

In conclusion, our findings, when translated into clinical practice, may support the use of rhEPO as a cardioprotective agent in the treatment of patients with myocardial infarction.

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Amlodipine ameliorates myocardial hypertrophy by inhibiting EGFR phosphorylation

Yulin Liao[a], Masanori Asakura[a], Seiji Takashima[a], Hisakazu Kato[a], Yoshihiro Asano[a], Yasunori Shintani[a], Tetsuo Minamino[a], Hitonobu Tomoike[b], Masatsugu Hori[a], Masafumi Kitakaze[b,*]

[a] *Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0781, Japan*
[b] *Cardiovascular Division of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*

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Abstract

The effects of long-acting calcium channel blockers on pressure overload-induced cardiac hypertrophy have been little studied in experimental animals and the underlying mechanisms are not fully understood. We previously reported that cardiomyocyte hypertrophy could be induced via phosphorylation of the epidermal growth factor receptor (EGFR). In this study, we investigated whether amlodipine attenuates cardiac hypertrophy by inhibiting EGFR phosphorylation. We found that amlodipine dose-dependently inhibited epinephrine-induced protein synthesis and EGFR phosphorylation in cultured neonatal rat cardiomyocytes. Our in vivo study revealed that amlodipine could ameliorate myocardial hypertrophy induced by transverse aortic constriction (TAC) in C57/B6 mice. One week after TAC, amlodipine treatment (3 mg/kg/day) significantly reduced the heart-to-body weight ratio (6.04 ± 0.16 mg/g vs. 6.90 ± 0.45 mg/g in untreated TAC mice, $P < 0.01$). These results indicate that amlodipine ameliorates cardiomyocyte hypertrophy via inhibition of EGFR phosphorylation.

Keywords: Calcium channel blocker; Cardiomyocyte; Hypertrophy; Epidermal growth factor; Phosphorylation; Mouse

Calcium channel blockers (CCBs) are widely used for the treatment of hypertension. Amlodipine is a long-acting dihydropyridine CCB that is effective for lowering the blood pressure, amelioration of cardiac remodeling, and reduction of mortality and morbidity [1]. However, the mechanisms underlying the beneficial effects of CCBs on cardiac remodeling are not fully understood. We have reported that stimulation of the G protein-coupled receptor (GPCR) in cardiomyocytes causes the release of heparin-binding epidermal growth factor (HB-EGF), which subsequently binds to the epidermal growth factor receptor (EGFR) and produces cardiac hypertrophy [2]. There is evidence that calcium channels play an important role in activation of the EGFR [3]. Calcium channels were reported to be involved in endothelin-1-induced activation of the EGFR [3], and calcium channels also induce tyrosine phosphorylation of this receptor to levels that can activate the mitogen-activated protein kinase signaling pathway [4]. In addition, blockade of calcium uptake and mobilization by mammary gland epithelial cells suppress EGF-induced cell proliferation [5]. Considering these findings, we hypothesized that amlodipine may ameliorate cardiomyocyte hypertrophy by inhibiting EGFR phosphorylation. In the present study, we evaluated the effect of amlodipine on EGFR phosphorylation induced by a GPCR agonist in vitro and

* Corresponding author. Fax: +81 6 6836 1120.
E-mail address: kitakaze@zf6.so-net.ne.jp (M. Kitakaze).

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on cardiomyocyte hypertrophy induced by left ventricular pressure overload in vivo.

Materials and methods

Cell culture. Rat neonatal ventricular myocytes were isolated as described previously [2], and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS (Equitech-Bio). The medium was changed to serum-free medium after 72 h and cells were cultured under serum-free conditions for 48 h before addition of agents. Protein synthesis by the cultured cells was evaluated through analysis of [^{3}H]leucine incorporation [2,6]. Cardiomyocytes were exposed to either epinephrine (Epi: 10^{-5} M) or HB-EGF (10^{-8} M) for 24 h in the presence or absence of amlodipine (kindly provided by Sumitomo Pharmaceuticals, Japan), and the increase of [^{3}H]leucine incorporation was examined.

EGFR phosphorylation. Cultured cardiomyocytes were exposed to 10^{-5} M Epi or 10^{-8} M HB-EGF with or without pretreatment by amlodipine (10^{-6} or 10^{-9} M) or HB-EGF neutralizing antibody #19 for 30 min. Cells were lysed by incubation for 20 min at 4 °C in a buffer (50 mM Tris–HCl, pH 7.3; 150 mM NaCl; 2 mM EDTA; 0.5% sodium fluoride; 10 mM sodium pyrophosphate; 0.5 mM Na_3VO_4; 100 μg/ml phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; protease inhibitor cocktail; and 1% Nonidet P-40). Immunoprecipitation with an antibody directed against the EGFR and immunoblotting using phosphorylation antibody (Anti-pY) were performed as described elsewhere [7].

Animal model. All procedures were performed in accordance with the institutional guidelines for animal research. Male C57BL/6 mice (8–9 weeks-old, wt 19–25 g) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg intraperitoneally). The animal model of pressure overload was created as described previously [8]. Briefly, transverse aortic constriction (TAC) was produced by tying a 7-0 suture tied twice around the aorta and a 27-gauge needle, after which the needle was gently removed to yield 60–80% constriction of the aortic arch.

To determine whether amlodipine could attenuate cardiac hypertrophy induced by TAC, we treated the mice with saline (TAC group) or oral amlodipine 3 mg/kg/day. To confirm that the extent of pressure overload was similar between the amlodipine-treated and untreated groups, we measured the pressure in the ascending aorta of 2–3 mice from each group using a 1.4 F Millar catheter on the 2nd day after TAC. The tail-cuff blood pressure and heart rate (BP-98A, Softron, Tokyo, Japan) were examined before sacrifice. One week after the induction of pressure overload, mice were killed to determine organ weights and perform morphometric analysis. The cross-sectional surface area of cardiomyocytes was measured using three hearts in each group with the method described previously [6].

Statistical analysis. Multiple comparisons were performed by one-way ANOVA with the Tukey–Kramer exact probability test. Results are reported as means ± SEM. For all analyses, $P < 0.05$ was considered statistically significant.

Results and discussion

Amlodipine attenuates the induction of cardiomyoctyte protein synthesis by epinephrine

As shown in Fig. 1A, amlodipine markedly inhibited epinephrine-induced neonatal rat cardiomyocyte protein synthesis over a concentration range of 10^{-7}–10^{-5} M. Epinephrine is one of the GPCR agonists and is well known to induce cardiomyocyte hypertrophy. Pignier et al. [9] reported that hypertrophy induced by long-term stimulation of α_1-adrenoceptors is accompanied by an increase in the expression of functional calcium channels in neonatal rat cardiomyocytes, indicating the existence of a novel α_1-mediated pathway for positive regulation of the L-type calcium current. This agrees with our finding that blockade of L-type calcium channels inhibits cardiomyocyte hypertrophy. There is substantial evidence to support the notion that calcium signaling pathways contribute to the progression of cardiac hypertrophy [10,11], so it is likely that blockade of calcium signaling would lead to the regression of hypertrophy.

Amlodipine causes concentration-dependent inhibition of EGFR phosphorylation induced by epinephrine

Based on our earlier demonstration that EGFR activation by GPCR agonists led to the development of cardiac hypertrophy [2] and the present in vitro finding that

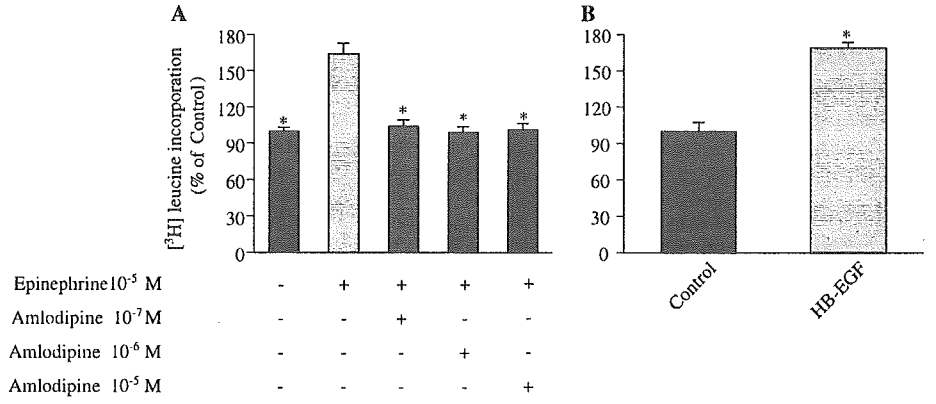

Fig. 1. Effect of amlodipine and HB-EGF on protein synthesis in rat cardiomyocytes. (A) Protein synthesis stimulated by epinephrine (10^{-5} M) was inhibited by amlodipine at concentrations ranging from 10^{-7} to 10^{-5} M. *$P < 0.01$ vs. epinephrine alone. (B) HB-EGF (10^{-8} M) significantly increased myocyte protein synthesis. *$P < 0.01$ vs. Control.

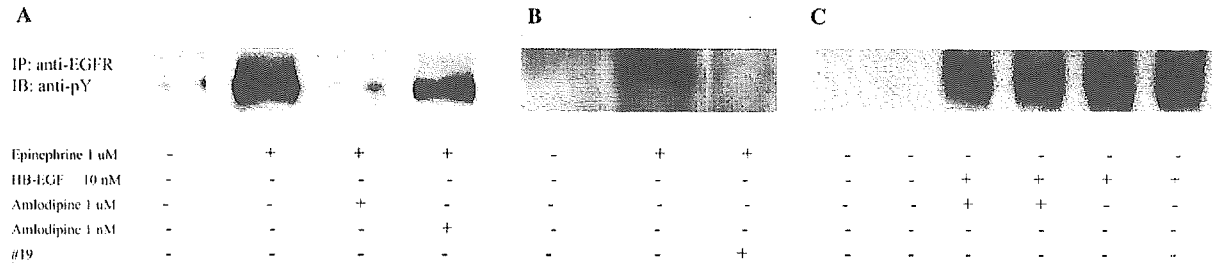

Fig. 2. EGFR phosphorylation and release of HB-EGF. (A) EGFR phosphorylation showed concentration-dependent inhibition by amlodipine. (B) HB-EGF neutralizing antibody #19 blocked epinephrine-induced EGFR phosphorylation. (C) Amlodipine did not influence EGFR phosphorylation induced by HB-EGF. Each experiment was repeated at least three times.

amlodipine inhibits cardiomyocyte protein synthesis stimulated by a GPCR agonist (epinephrine), we hypothesized that amlodipine may also inhibit cardiomyocyte hypertrophy by preventing tyrosine phosphorylation of the EGFR. In the present study, HB-EGF significantly increased protein synthesis by neonatal rat cardiomyocytes (Fig. 1B), a finding that agreed with our previous report [2]. Interestingly, we also demonstrated that amlodipine inhibits EGFR phosphorylation in cardiomyocytes in a concentration-dependent manner (Fig. 2A). In recent years, information about the mechanisms related to Ca^{2+} influx has accumulated. Zwick et al. [12] reported that calcium-dependent EGFR activation led to subsequent activation of the Ras/mitogen-activated protein pathway in neurons. In addition, Kawanabe et al. [3] have shown that Ca^{2+} influx plays an important role in endothelin-1-induced EGFR activation, and endothelin-1 is well known to stimulate cardiomyocyte growth.

Amlodipine inhibits epinephrine-induced release of HB-EGF

We previously reported that phenylephrine induces EGFR activation by increasing the release of the HB-EGF ectodomain [2]. Here we found that amlodipine could inhibit EGFR activation by reducing the epinephrine-induced release of HB-EGF. Since the extracellular level of the ectodomain of HB-EGF (soluble HB-EGF) was generally too low to be detected by Western blotting, we assessed it by an indirect method. If epinephrine induces release of the HB-EGF ectodomain, its depletion was assumed to block epinephrine-induced EGFR activation. As expected, we found that an HB-EGF neutralizing antibody #19 almost completely prevented epinephrine-induced phosphorylation of the EGFR (Fig. 2B), suggesting that epinephrine-induced EGFR activation is mediated by the release of HB-EGF, at least in newborn rat cardiac myocytes. When we investigated whether amlodipine prevents HB-EGF-induced activation of the EGFR, we found that this drug did not have any influence on HB-EGF-mediated EGFR phosphorylation (Fig. 2C), suggesting that it acts upstream of HB-EGF. Finally, we revealed that amlodipine caused marked inhibition of epinephrine-induced phosphorylation of the EGFR (Fig. 2A), a result that supported an inhibitory effect of the drug on EGFR activation by preventing the release of HB-EGF. Further studies are needed to elucidate the exact mechanism by which CCBs inhibit EGFR phosphorylation. Src kinase is reported to contribute to EGFR activation by GPCR agonists [13,14], while a link between calcium release through L-type calcium channels and Src has also been demonstrated [4,15–18], and the release of calcium seems to be necessary for activation of Src [4,18]. Thus, it is likely that amlodipine blocks the signal transduction pathway upstream of Src.

Amlodipine inhibits myocardial hypertrophy in vivo

We used a well-established mouse model of left ventricular pressure overload to further confirm the preventive effect of amlodipine on cardiac hypertrophy. An increase of GPCR agonists, such as catecholamines [6], angiotensin II, and endothelin-1, is known to occur in the myocardium of these mice. Since EGFR activation leads to cardiomyocyte hypertrophy [2] and amlodipine inhibits epinephrine-induced EGFR phosphorylation in cardiomyocytes in vitro, as shown in the present study, it would seem plausible that amlodipine also attenuates cardiac hypertrophy induced by TAC. Indeed, consistent with our in vitro results, we found that oral administration of amlodipine (3 mg/kg/day) for 1 week markedly ameliorated cardiac hypertrophy. Histological examination confirmed that myocyte hypertrophy was less severe (Figs. 3A and B) in mice treated with amlodipine. Compared with sham mice, the heart-to-body weight ratio (HW/BW) increased by about 43% in TAC mice, while the amlodipine-treated mice only showed an increase of about 25% (Fig. 3C). Cardiomyocytes cross-surface area was also significantly decreased in amlodipine-treated mice (Fig. 3D). Hemodynamic parameters are summarized in Table 1; amlodipine did not significantly affect either the tail-cuff systolic blood pressure or the heart rate. Ascending aortic pressure was similar in the TAC and amlodipine-treated TAC