

Angiotensin I -Converting Enzyme Inhibitors Block PKC ϵ by Activating Bradykinin B₁ Receptors in Human Endothelial Cells*

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ACE inhibitors block PKC ϵ via NO

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Abbreviations: ACE, angiotensin I-converting enzyme; BK, bradykinin, DAKD, des-Arg¹⁰-kallidin; DBK, des-Arg⁹-bradykinin; DETA-NONOate, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazene-1,1,2-diolate; EPT, enalaprilat; eNOS, endothelial nitric oxide synthase; HLMVE, human lung microvascular endothelial; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; IFN- γ , interferon- γ ; L-NNA, N ω -nitro-L-arginine; NO, nitric oxide; PBS, phosphate-buffered saline; PKC, protein kinase C; QPT, quinaprilat; UDP, undecapeptide (LLPHEAWHFAR); 1400W, N-(3(aminomethyl) benzyl) acetamidine dihydrochloride; Gö6976, [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole].

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ABSTRACT

Angiotensin I-converting enzyme (ACE) inhibitors are widely used to treat patients with cardiovascular and kidney diseases, but inhibition of ACE alone does not fully explain the beneficial effects. We reported that ACE inhibitors directly activate bradykinin B₁ receptor at the canonical Zn²⁺ binding site, leading to prolonged NO production in endothelial cells. PKCε, a novel PKC isoform is upregulated in myocardium after infarction, suggesting a role in the development of cardiac dysfunction. In cytokine-treated human lung microvascular endothelial cells, B₁ receptor activation by ACE inhibitors (enalaprilat, quinaprilat) or peptide ligands (des-Arg¹⁰-Lys¹-bradykinin, des-Arg⁹-bradykinin) inhibited PKCε with an IC₅₀ = 7 x 10⁻⁹ M. Despite the reported differences in binding affinity to receptor, the two peptide ligands were equally active, even when inhibitor blocked the cleavage of Lys¹, thus the conversion by aminopeptidase. The synthetic undecapeptide (LLPHEAWHFAR) representing the binding site for ACE inhibitors on human B₁ receptors reduced PKCε inhibition by enalaprilat, but not by peptide agonist. A combination of inducible and endothelial NO synthase inhibitors (1400W and L-NNA, 2 μM) significantly reduced inhibition by enalaprilat (100 nM), whereas the NO donor DETA-NONOate (100 μM) inhibited PKCε activity just as the B₁ ligands did. In conclusion, NO generated by B₁ receptor activation inhibits PKCε.

INTRODUCTION

The bradykinin B₁ and B₂ receptors are G-protein coupled receptors that mediate the actions of kinins. The B₂ receptors are activated by bradykinin and kallidin (Lys-bradykinin) and are constitutively expressed in normal tissues (Bhoola et al., 1992), whereas the B₁ receptors are highly induced during inflammatory conditions (McLean et al., 2000). Plasma carboxypeptidase N and tissue carboxypeptidase M cleave the C-terminal Arg of bradykinin and kallidin and generate the endogenous agonists of the B₁ receptors, des-Arg⁹-bradykinin (DBK) and des-Arg¹⁰-kallidin (DAKD) (Erdös and Skidgel, 1997).

Angiotensin I-converting enzyme (ACE) inhibitors are beneficial in treating patients with hypertension, myocardial infarction, congestive heart failure and diabetic nephropathy (Gavras et al., 1974; Yusuf et al., 2000; Gavras and Gavras, 2001). However, inhibition of ACE alone, thus blocking the release of angiotensin II or inactivation of bradykinin, does not fully explain the effects of ACE inhibitors (Erdös and Skidgel, 1997; Corvol et al., 2004). ACE inhibitors potentiate the effect of bradykinin on the B₂ receptor and resensitize the receptor previously desensitized by the ligand, apart from blocking peptide hydrolysis (Minshall et al., 1997; Erdös et al., 1999; Marcic et al., 1999). ACE inhibitors also directly activate the B₁ receptors, independent of endogenous kinins and ACE (Ignjatovic et al., 2002). In endothelial cells, this activation leads to prolonged nitric oxide (NO) release and increases the uptake of its precursor, L-arginine (Ignjatovic et al., 2002; Ignjatovic et al., 2004). ACE inhibitors activate the B₁ receptor at a Zn²⁺ binding pentapeptide sequence (Ignjatovic et al., 2002), similar to the active sites in ACE (Corvol et al., 2004).

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ACE inhibitors improve endothelial dysfunction even in normotensive patients with coronary artery disease (Mancini et al., 1996). PKC ϵ is a novel PKC isoform that is upregulated in the myocardium after infarction. In a rat model of myocardial infarction, treatment with ACE inhibitors reduces the expression of PKC ϵ after infarction (Simonis et al., 2003; Wang et al., 2003), indicating that PKC ϵ may play a role in the development of cardiac dysfunction, and that ACE inhibitors can influence PKC ϵ expression. We wanted to determine whether direct activation of the B₁ receptors by ACE inhibitors affects PKC ϵ activity and whether two endogenous B₁ receptor agonists, DAKD and DBK are equally active in spite of big differences in their affinity for the B₁ receptors (Hess et al., 1996). We have found that B₁ receptor activation by ACE inhibitors (enalaprilat, quinaprilat) or peptide agonists blocks PKC ϵ activity in human endothelial cells, and that the PKC ϵ inhibition is mediated by NO release. The two endogenous ligands, DAKD and DBK, are almost equally active, even in the presence of an aminopeptidase inhibitor that blocks the conversion of DAKD to DBK by removal of its N-terminal Lys¹ (Erdös et al., 1963; Webster and Pierce, 1963).

METHODS

Materials. Enalaprilat, the active form of the ACE inhibitor enalapril, was from Toronto Research Chemicals, Inc. (North York, ON, Canada); quinaprilat was from Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Interleukin-1 β (IL-1 β) and interferon- γ (IFN- γ) were purchased from Calbiochem (San Diego, CA) and Invitrogen (Carlsbad, CA). The NO donor DETA-NONOate ($t_{1/2}$ = 20 hours at 37°C) was from Cayman Chemical (Ann Arbor, MI). PKC ϵ assay

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kit, PKC $\alpha\beta\gamma$ assay kit, and PKC ϵ polyclonal antibody directed against residues 726-737 (KGFSYFGEDLMP), along with Jurkat cell lysate (a positive control) were obtained from Upstate Biotechnology (Lake Placid, NY). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Culture. Human lung microvascular endothelial (HLMVE) cells (Cell Applications, San Diego, CA and Cambrex Bio Science Baltimore, Inc. Baltimore, MD) were cultured in EBM-2 medium (Cambrex, Walkersville, MD) containing 10 ng/ml human epidermal growth factor, 5 ng/ml vascular endothelial growth factor, 2 ng/ml human fibroblast growth factor, 2 ng/ml insulin-like growth factor, 0.2 μ g/ml ascorbic acid, 50 ng/ml gentamicin-amphotericin B, and 10% fetal bovine serum. HLMVE cells were used in passages 6-8, and routinely treated with 5 ng/ml IL-1 β and 50 ng/ml IFN- γ for 18 h prior to the experiments to induce B₁ receptors (11,12). The experiments were performed using phenol-red free D-MEM/F12 medium (Invitrogen, Carlsbad, CA) containing no fetal bovine serum.

PKC assay. HLMVE cells were treated for 20 min with medium alone (control) or medium containing B₁ receptor agonists. Cells were then scraped into ice-cold PBS, washed twice with PBS and then lysed by sonication (2 times 10 s on ice). Cell lysates were assayed for PKC ϵ activity following the manufacturers' instructions for the phosphorylation of the specific substrate peptide (ERM RPRKRQGSVRRRV) in the presence of [γ -³²P] ATP. The phosphorylated substrate is separated from residual [γ -³²P] ATP on phosphocellulose paper and quantified in a scintillation counter. Other serine/threonine kinases such as protein kinase A, and calmodulin dependant kinases were blocked during assay by an inhibitor cocktail. The kit assay

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buffer contains 1 mM Ca^{2+} , but in selected control experiments, Ca^{2+} -free buffer containing 1 mM EDTA was used to eliminate any contribution of classical PKC isoforms. The same procedure was used to measure PKC α activity using buffer containing 1 mM Ca^{2+} except the peptide substrate for classical PKC isoforms (QKRPSQRSKYL) was used.

DAKD conversion to DBK. The hydrolysis of DAKD by HLMVE cells in culture was assayed by high performance liquid chromatography (HPLC). HLMVE cells were incubated with DAKD (50 μM) for 20 min at 37°C (pH = 7.2). Two groups of the cells were pretreated for 10 min with bestatin (150 μM), an aminopeptidase inhibitor, and then exposed to DAKD (50 μM) for 20 min. The reactions were stopped with 5% trifluoroacetic acid, centrifuged at 14,000 g for 10 min, and peptide products were separated and quantitated by HPLC.

NO release. NO was measured electrochemically with porphyrinic microsensor (Ignjatovic et al., 2002; Ignjatovic et al., 2004). Cells were plated in 12-well plates and used upon reaching confluence in 2 days. Cells were preincubated for a few minutes at 37°C until a stable baseline was established. Ligands were added, and the responses (current versus time) were recorded continuously. The current generated on the porphyrinic electrode was proportional to the NO released and was quantitated with a standard NO solution.

Western blot. Western blot of PKC ϵ expression was performed with HLMVE cell lysates and as a positive control, with Jurkat cell lysate employing polyclonal anti-PKC ϵ antibody. Cell lysate proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to

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PVDF membranes, incubated overnight with polyclonal PKC ϵ primary antibody (1:1000 v/v) at 4°C then with secondary anti-rabbit goat serum (1:300,000).

PKC ϵ immunoprecipitation. PKC ϵ was immunoprecipitated by primary PKC ϵ antibody produced in rabbits. Briefly, cytokine-treated HLMVE cells were washed twice with ice-cold PBS, and then scraped into ice-cold homogenization buffer (pH 7.5, 20 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, 1mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1% protease cocktail inhibitor, 0.1% Triton X-100). Cells were subsequently sonicated twice for 15 s, and then centrifuged for 15 min at 14000 g. Supernatants were collected and incubated overnight or for 2 h with polyclonal anti-PKC ϵ antibody or non-immune rabbit IgG at 4°C. After mixing with protein A-sepharose for 2 h at 4°C and centrifugation, PKC ϵ activity was measured in Ca²⁺-free buffer in the resuspended pellet and supernatant.

Statistics. Data in the figures are expressed as mean \pm SE of n observations. Statistical evaluation was performed by one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

RESULTS

PKC ϵ expression in HLMVE cells. To investigate the effect of the B₁ receptor activation on PKC ϵ activity, we stimulated HLMVE cells with proinflammatory cytokines, IL-1 β and IFN γ , for 18 h at 37°C to induce B₁ receptors. Western blotting demonstrated that PKC ϵ was indeed expressed in HLMVE cells (Fig. 1).

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PKC ϵ immunoprecipitation from HLMVE cells. To determine PKC ϵ activity, we measured phosphorylation of a PKC ϵ peptide substrate. To confirm the specificity of the assay in HLMVE cells, PKC ϵ was immunoprecipitated from HLMVE cell supernatants with polyclonal PKC ϵ antibody and as a control, with non-immune rabbit IgG. As a result, $69.3 \pm 1.5\%$ of the total PKC activity was immunoprecipitated by primary PKC ϵ antibody versus $8.2 \pm 0.8\%$ by non-immune rabbit IgG (n= 4).

Inhibition of PKC ϵ . The B₁ receptor agonists, DAKD (10 nM) and enalaprilat (10 nM), inhibited 65% and 70% of the PKC ϵ activity measured in HLMVE cells (Fig. 2A). The B₁ receptor antagonist, des-Arg¹⁰-Leu⁹-kallidin (100 nM) inhibited the response to both DAKD and enalaprilat, confirming this conclusion. Enalaprilat and the two peptide agonists of the B₁ receptor, had about the same low IC₅₀ value (7×10^{-9} M) (Fig. 2B).

Effect of the undecapeptide. The human B₁ receptor contains in its second extracellular loop (residues 195-199) the HEAWH sequence, a canonical Zn²⁺ binding pentamer, which represents the suggested site of the activation by ACE inhibitors (Ignjatovic et al., 2002). A synthetic undecapeptide (LLPHEAWHFAR) (residues 192-202 of the human B₁ receptor) which includes this pentamer, blocked B₁ receptor activation by enalaprilat, but not by DAKD (Ignjatovic et al., 2002). To confirm that enalaprilat inhibits PKC ϵ due to B₁ receptor activation, we pretreated HLMVE cells with 100 μ M of peptide, prior to adding either DAKD or enalaprilat (Fig. 2C). The undecapeptide reduced the effect of enalaprilat on PKC ϵ inhibition (26% inhibition versus 63% in the absence of the undecapeptide), whereas DAKD was not significantly affected, distinguishing the actions of the B₁ receptor agonists.

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The time course of PKC ϵ inhibition. We treated the cells with 100 nM of either DAKD, enalaprilat or quinaprilat for 1, 10, or 20 min. PKC ϵ activity did not change after 1 min; however, after 10 or 20 minutes, the peptide and both ACE inhibitors similarly inhibited PKC ϵ (Fig 3A). This result indicates that B₁ receptor-mediated PKC ϵ inhibition is slower than the increase in intracellular [Ca²⁺]_i that is almost immediate in response to the B₁ agonists (Ignjatovic et al., 2002).

Effect of NOS inhibitors and NO donor. Both DAKD and enalaprilat release NO from human endothelial cells (Ignjatovic et al., 2004). We explored whether the inhibition PKC ϵ could be NO-dependant (Fig. 3B). HLMVE cells were pretreated for 10 min with both iNOS and eNOS inhibitors (2 μ M 1400W and 2 μ M L-NNA), and then stimulated for 20 min with either DAKD (100 nM) or enalaprilat (100 nM). The NOS inhibitors significantly reduced the effect of the B₁ receptor agonists on PKC ϵ inhibition (Fig 3B). The NO donor DETA-NONOate (100 μ M) alone inhibited PKC ϵ activity similar to the effects of DAKD and enalaprilat, indicating that the B₁ receptor activation reduces PKC ϵ activity via a NO-dependent mechanism. To determine if direct protein modification by NO or peroxynitrite is involved, cells were pretreated for 20 min with dithiothreitol (20 mM), a mercapto compound or ebselen (10 μ M), a peroxynitrate scavenger. In these experiments, 100 nM DAKD or enalaprilat reduced PKC ϵ activity to 36 ± 2 % or 34 ± 3 % of control (respectively) when used alone, to 39 ± 3 % or 37 ± 2 % in the presence of 1 mM dithiothreitol and to 38 ± 1 % or 34 ± 2 % in the presence of ebselen (mean values \pm S.E. for n = 3). The lack of effect of dithiothreitol or ebselen suggest that tyrosine nitration or cysteine S-nitrosylation did not cause the inhibition of PKC ϵ .

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PKC α activity. We further investigated whether the B₁ receptor agonists would affect another PKC isoform, PKC α , a classical PKC (Wang et al., 2003). DAKD (100 nM) or enalaprilat (100 nM) were incubated with HLMVE cells for 20 min and the phosphorylation of the PKC substrate for classical PKC isoforms (α , β , and γ) was measured. PKC α activity in the presence of 100 nM DAKD or enalaprilat was $100 \pm 2\%$ or $104 \pm 3\%$ of control (mean values \pm S.E.; n = 3). Because the standard PKC ϵ assay buffer contained calcium, we wondered whether the residual activity remaining after B₁ receptor inhibition of PKC ϵ might be due to PKC α . Indeed, when cells were preincubated with the specific PKC α / β 1 inhibitor, Gö6976 (100 nM, 20 min), 100 nM DAKD inhibited the remaining PKC activity by 92% (mean value for n = 2, assayed in triplicate). By itself, 100 nM Gö6976 inhibited 33% of the activity (mean value for n = 2, assayed in triplicate) measured with the PKC ϵ substrate. Furthermore, when the PKC ϵ assay was done in the absence of Ca²⁺, 100 nM DAKD inhibited PKC ϵ activity by 93% (mean value for n = 2, assayed in triplicate). These data indicate that B₁ receptor activation almost completely (93%-95%) inhibits PKC ϵ and that the lack of complete inhibition in the studies above was due to the residual activity of a classical PKC (most likely α) that can phosphorylate the PKC ϵ substrate about 25%.

NO release. B₁ agonists inhibited PKC ϵ , which was mediated by NO with a similar IC₅₀. As a follow up, we measured NO release from cytokine-treated HLMVE cells to determine whether the three B₁ agonists would be equally effective. Indeed, this is so (Fig. 4A); DAKD and DBK (100 nM) released NO similarly, in spite of the large differences in binding affinity to B₁

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receptors (16). The ACE inhibitor enalaprilat (100 nM) also stimulated the release of NO, at the levels produced by the peptides.

DAKD hydrolysis. Kallidin (Lys-bradykinin) is converted to bradykinin when an aminopeptidase of plasma (Erdös et al., 1963; Webster and Pierce, 1963) or cell membrane (Bhoola et al., 1992; Erdös and Skidgel, 1997) cleaves its Lys¹-Arg² bond, which applies to the conversion of DAKD to DBK. This could potentially explain the equal potency of DAKD and DBK in our experiments (Fig. 2B). To investigate that, cells were pretreated with aminopeptidase inhibitor bestatin (10 μM, 10 min), but DAKD and DBK still similarly inhibited PKCε (Fig. 4B). To confirm whether DAKD can be converted to DBK by endothelial aminopeptidases, HLMVE cells were incubated with 50 μM DAKD for 20 min, and then the reaction products were analyzed by HPLC using standards to identify and quantify product peaks (Fig. 5). Cytokine-treated HLMVE cells, within 20 min, generated 11.6 ± 0.95 nmol DBK/ 2×10^5 cells at 37°C. In the presence of bestatin (150 μM), an aminopeptidase inhibitor, only 3.4 ± 0.72 nmol DBK/ 2×10^5 cells was generated for the same incubation time (n = 5). Similar results were obtained in control HLMVE cells, indicating that endothelial aminopeptidase was not induced by IL-1β and IFNγ. Thus, the two B₁ agonist peptides are equally active in stimulating NO release and inhibiting PKCε in HLMVE cells.

DISCUSSION

When peptides or ACE inhibitors activates the B₁ receptor, the resulting NO release inhibits PKC ϵ in human endothelial cells. The two endogenous ligands, DAKD and DBK were almost equally active even without the conversion of DAKD to DBK by aminopeptidase. HLMVE cells clearly expressed PKC ϵ , as shown in Western blot (Fig.1) and 75% of PKC ϵ activity was immunoprecipitated from lysed cells by primary PKC ϵ antibody.

In endothelial cells, NO suppresses shear stress-induced activation of PKC ϵ and ERK1/2 (Ni et al., 2003). In our study, NOS inhibitors blocked the inhibition of PKC ϵ by DAKD and enalaprilat. The NO donor DETA-NONOate induced a response similar to those of DAKD and enalaprilat, suggesting that B₁ receptor activation inhibits PKC ϵ by enhancing NO synthesis. Although the inhibition could depend on posttranslational modification of the enzyme by nitration of tyrosine or S-nitrosylation of cysteine residues, the mercapto compound dithiothreitol, and the peroxynitrate scavenger ebselen, did not affect PKC ϵ inhibition, suggesting that tyrosine nitration and cysteine S-nitrosylation are not involved in the PKC ϵ inhibition.

Macrophages from PKC ϵ knockout mice release far less cytokines (Castrillo et al., 2001) and in rabbit cardiomyocytes, PKC ϵ activates NF- κ B (Li et al., 2000), leading to production of cytokines and adhesion molecules (Matsumori, 2004). Here, we found that B₁ receptor activation decreases PKC ϵ activity in human endothelial cells. The B₁ receptors are highly expressed under inflammatory conditions, including atheromatous disease, in endothelial cells or smooth muscle cells (Raidoo et al., 1997) and in rat glomeruli in diabetes (Christopher and Jaffa, 2002). Thus, by reducing PKC ϵ activity, ACE inhibitors can indirectly decrease NF- κ B activity, lowering the

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production of proinflammatory cytokines and adhesion molecules. Since inflammation induces B₁ receptor expression (Bhoola et al., 1992), inhibition of PKC ϵ by B₁ agonists may represent a negative feedback of NF- κ B activation, contributing to beneficial effects of ACE inhibitors in atheromatous disease, with endothelial dysfunction.

Angiotensin II plays an important role in cardiac remodeling and fibrosis and increases the adhesion of cardiac fibroblasts to collagen via activation of PKC ϵ , which subsequently phosphorylates and activates β 1 integrins (Stawowy et al., 2005). Thus, ACE inhibitors could have a dual beneficial effect in this pathway by reducing generation of angiotensin II by ACE as well as by activating B₁ receptors to inhibit PKC ϵ activity.

Overexpression of constitutively active PKC ϵ in the hearts of transgenic mice causes development of concentric cardiac hypertrophy (Takeishi et al., 2000). In heart failure, overexpression of PKC ϵ alters myofilaments (Goldspink et al., 2004). These results indicate that PKC ϵ may play a role in the development of cardiac dysfunction and may provide a basis for ACE inhibitor actions.

DAKD has three orders of magnitude higher affinity for the human B₁ receptor than DBK, in binding studies (Hess et al., 1996). However, in our experiments, both peptides were almost equally active. When HLMVE cells were pretreated with the aminopeptidase inhibitor bestatin to block the conversion of DAKD to DBK, both peptides still similarly inhibited PKC ϵ and released NO. In published reports DBK or DAKD were shown to have both similar and different efficacies as B₁ agonists, depending on the species and/or tissue source used. For example on the rat ileum (Ueno et al., 2002) or in RAW 264.7 macrophages (Burch and Kyle, 1992) the two peptides had the same affinity or activity on B₁ receptors, whereas in rabbit

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vascular smooth muscle cells the two agonists exhibited a 100-fold difference in potency for stimulation of phosphoinositide hydrolysis (Schneck et al., 1994).

The reason for the apparent discrepancy between the reported affinity and the activity we found in our studies is not clear, but it is quite possible that the two peptides have different efficacies (i.e., effectiveness of the peptide-receptor complex). Early studies on receptors and theories of drug-receptor interactions already revealed the fact that equal biologic responses did not necessarily mean equal degrees of receptor occupancy [see, for example (Goldstein et al., 1974; Limbird, 2004)]. Thus, a peptide such as DBK may have a higher efficacy than DAKD, leading to a similar response at a lower level of receptor occupancy. Alternatively, there may exist in HLMVE cells complexes of B₁ receptors with another protein or proteins that could alter receptor binding and equalize the affinities of the two peptides. It should also be noted that binding studies are usually done with cellular plasma membrane preparations, frequently at 4°C to minimize peptide degradation and dissociation after equilibrium binding. This may not accurately reflect the affinity to and efficacy on intact cells in a more physiological environment at 37°C. Consequently, although Lys¹ greatly enhances the affinity of DAKD for the human B₁ receptors over DBK in binding assays, they activate the receptors in the human endothelial cells used similarly. That they are similarly effective may be of significance in cardiovascular functions and extends the potential importance of the B₁ receptor by having one more effective agonist, a stable ligand (DABK), instead of only a metabolically less stable one (DAKD) that is rapidly converted to a purportedly inactive metabolite.

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ACE and its inhibitors are in a central position to regulate both the kallikrein-kinin and renin-angiotensin systems (Bhoola et al., 1992; Erdös and Skidgel, 1997; Corvol et al., 2004). A working kallikrein-kinin system is necessary for normal cardiac and arterial function to counterbalance the renin-angiotensin system (Meneton et al., 2001; Schmaier, 2003), and may protect the heart against remodeling after myocardial infarction (Xu et al., 2005). In aging rat hearts, the B₂ receptor expression is decreased, while B₁ receptor expression is increased, indicating a possible compensatory reaction (Kintsurashvili et al., 2005). B₁ receptor activation can result in noxious consequences, e.g. pain and inflammation, which warrant the interest in development of therapeutically useful B₁ receptor blockers (Marceau and Regoli, 2004). However, ACE inhibitors are beneficial in many pathological conditions where B₁ receptors are likely to be induced, such as after myocardial infarction. The release of NO is considered to be one of the favorable effects of ACE inhibitors and besides potentiating bradykinin, it is also achieved by a direct stimulation of the B₁ receptors (Ignjatovic et al., 2002; Ignjatovic et al., 2004). Clearly, not all proteins induced during inflammation have only deleterious functions as the recent problems with COX2 inhibitors have shown. These considerations suggest caution in applying B₁ receptor blockers to the treatment of patients with chronic cardiovascular diseases, such as hypertension and congestive heart failure.

In conclusion, ACE inhibitors and peptide ligands directly activate the bradykinin B₁ receptors in human cells and as a consequence, inhibit PKC ϵ via a NO-dependent mechanism. The two endogenous peptide agonists of the B₁ receptors are equally active in stimulating this response. This inhibition of PKC ϵ , independent of blocking Ang II generation or bradykinin metabolism by ACE, can contribute to the therapeutic usefulness of these compounds.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Western blot of PKC ϵ in cytokine-treated HLMVE cell lysates. Jurkat cell lysate was the positive control.

Fig. 2. (A) HLMVE cells were stimulated with either des-Arg¹⁰-kallidin (DAKD; 10 nM) or the ACE inhibitor enalaprilat (EPT; 10 nM) for 20 min or pretreated with the B₁ receptor antagonist des-Arg¹⁰-Leu⁹-kallidin (DALKD; 100nM). Both DAKD and enalaprilat inhibited PKC ϵ ; inhibition was abolished by a B₁ receptor antagonist. Ordinate: PKC ϵ relative activity. Data are expressed as mean \pm SE (n = 3; done in triplicate). (B) Concentration-dependent inhibition of PKC ϵ activity by B₁ agonists. DAKD, des-Arg⁹-bradykinin (DBK) or EPT, respectively. Data are mean \pm SE (n = 3; done in duplicate). (C) The undecapeptide (LLPHEAWHFAR; 100 μ M), representing the ACE inhibitor-binding site on the B₁ receptor, reversed the effect of EPT (100 nM) only, but not that of DAKD (100 nM). Data are mean \pm SE (n = 3; done in triplicate). Endogenous peptide ligand and enalaprilat are about equally active.

Fig. 3. (A) The time course of B₁ receptor-mediated PKC ϵ inhibition. HLMVE cells were incubated with 100 nM agonist for 1, 10 or 20 min. DAKD (solid bars); EPT, enalaprilat (diagonal-lined bars); QPT, quinaprilat (horizontal-lined bars). Data are mean \pm SE (n = 3; done in duplicate). B₁ agonists decreased PKC ϵ activity after 10 min and quinaprilat was as active as enalaprilat. (B) NO synthase inhibitors block and an NO-donor mimics the effect of the B₁ receptor agonists on PKC ϵ activity. The eNOS and iNOS inhibitors combined (2 μ M 1400W + 2 μ M LNNA) block the inhibition of PKC ϵ activity by DAKD or EPT. NO donor DETA-

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NONOate (100 μ M) inhibits PKC ϵ . Results indicate that B₁ receptor agonists inhibit PKC ϵ via an NO-dependent mechanism.

Fig. 4. (A) DAKD, DBK and EPT stimulate NO synthesis similarly at 100 nM concentrations, indicating that the two peptides are almost equally active. The ACE inhibitor EPT (100 nM) generated a response similar to that mediated by 100 nM DAKD or DBK. Data are mean \pm SE (n = 3). (B) The aminopeptidase inhibitor bestatin (10 μ M) does not affect DAKD-induced PKC ϵ inhibition. Cells were pretreated with bestatin (10 μ M, 10min), then exposed to 100 nM DAKD, DBK or EPT, followed by assay of PKC ϵ activity. Data are mean values of two experiments done in triplicate. DAKD and DBK were equally active even when conversion of DAKD was blocked.

Fig. 5. Conversion of DAKD to DBK by endothelial aminopeptidase. HLMVE cells, stimulated with cytokines or controls, were pretreated for 10 min with bestatin (150 μ M), then incubated with DAKD (50 μ M). Ordinate: DBK released in 20 min. Data are mean \pm SE (n =5; done in triplicate). Bestatin inhibited the release of Lys¹ from DAKD, blocking production of DBK.

Figure 1

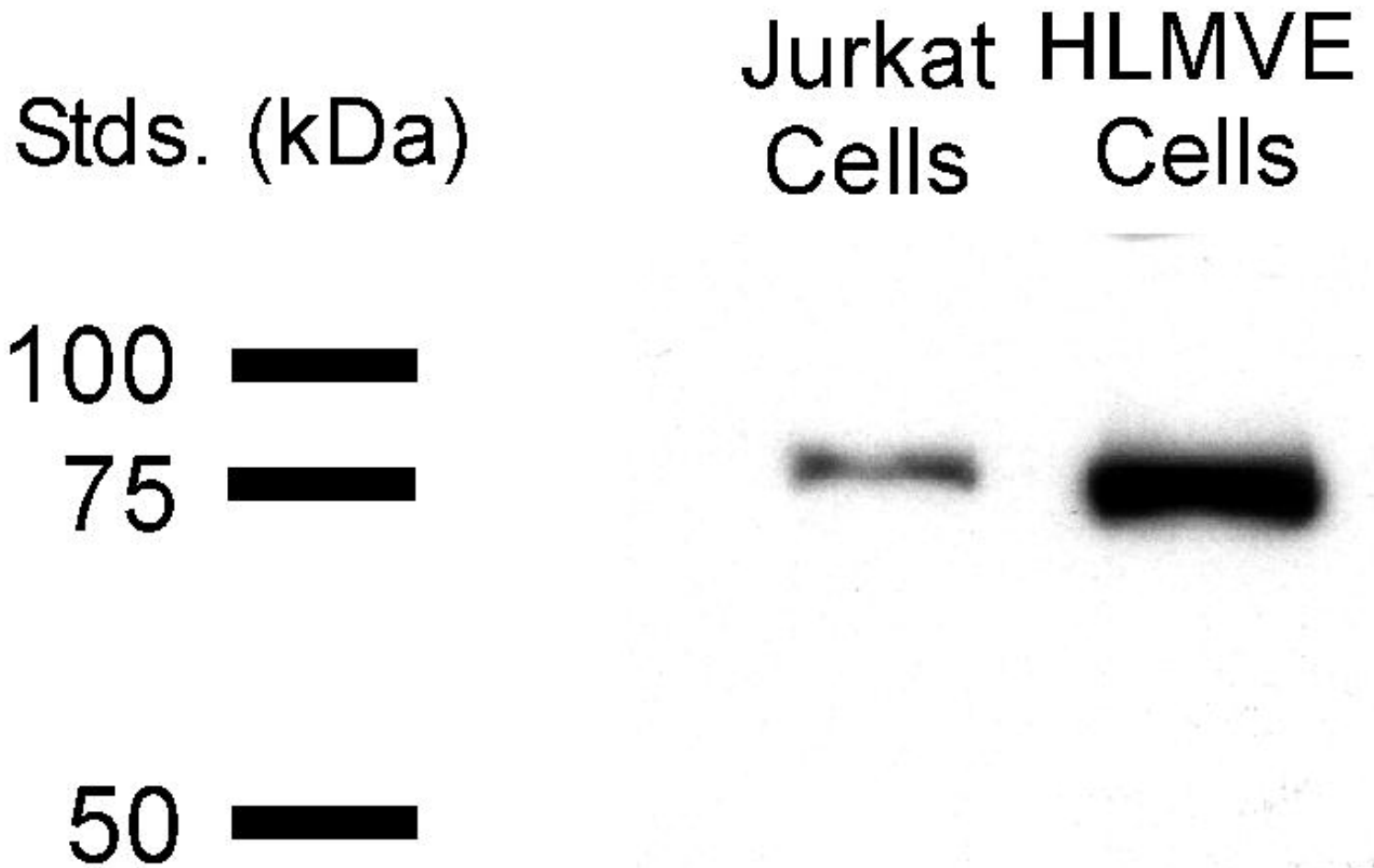


Figure 2

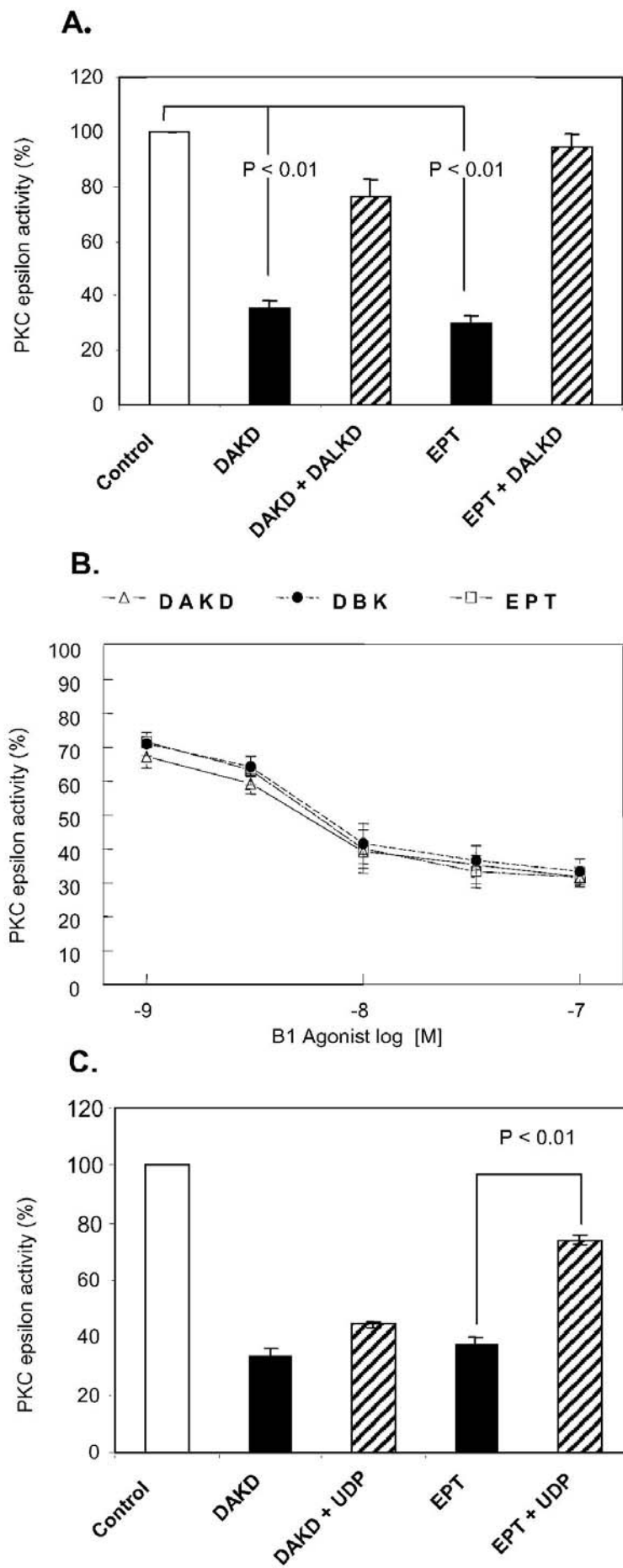


Figure 3

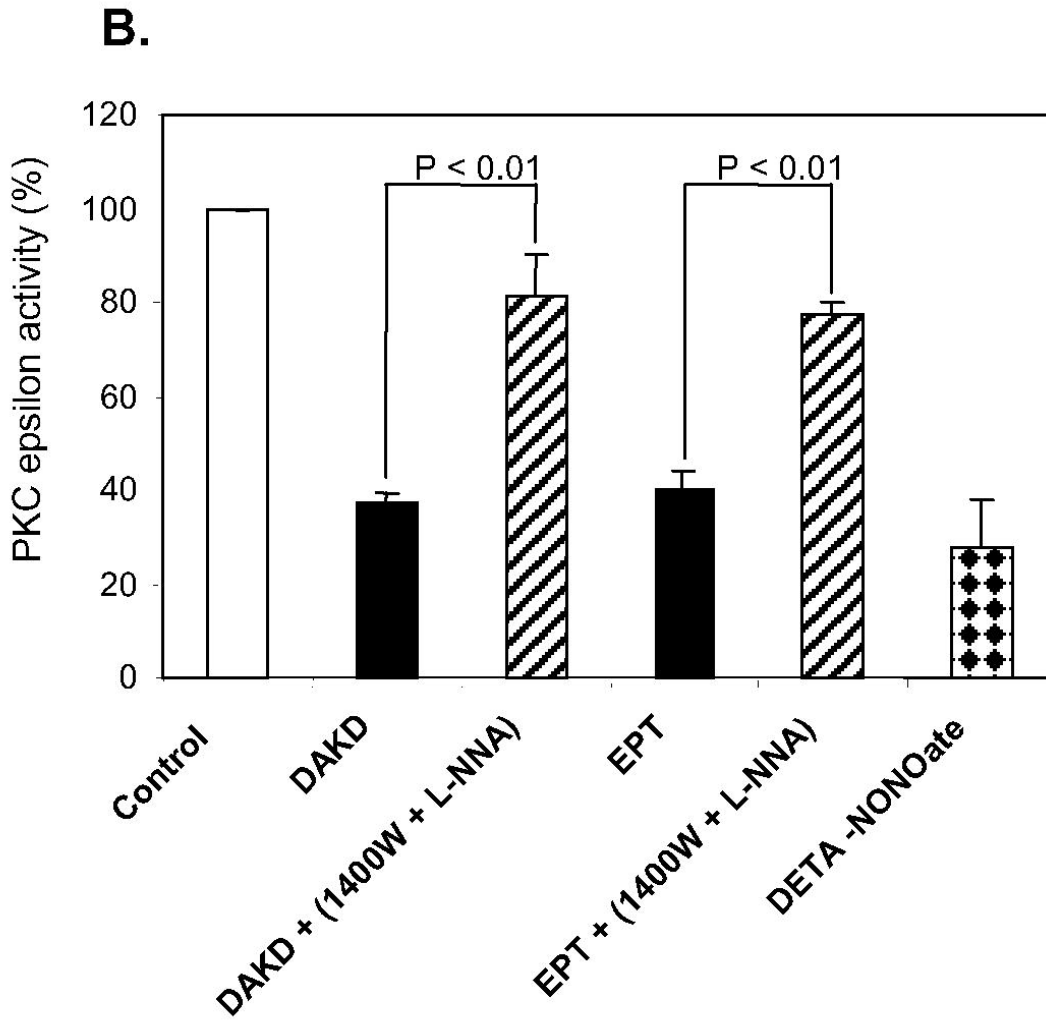
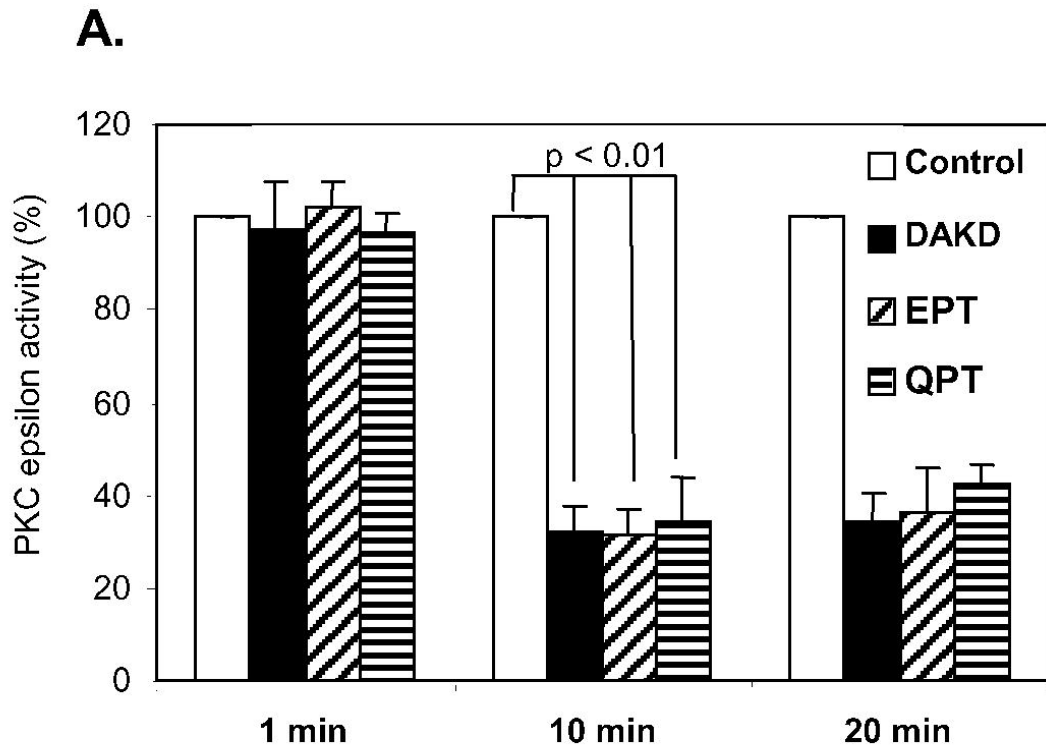


Figure 4

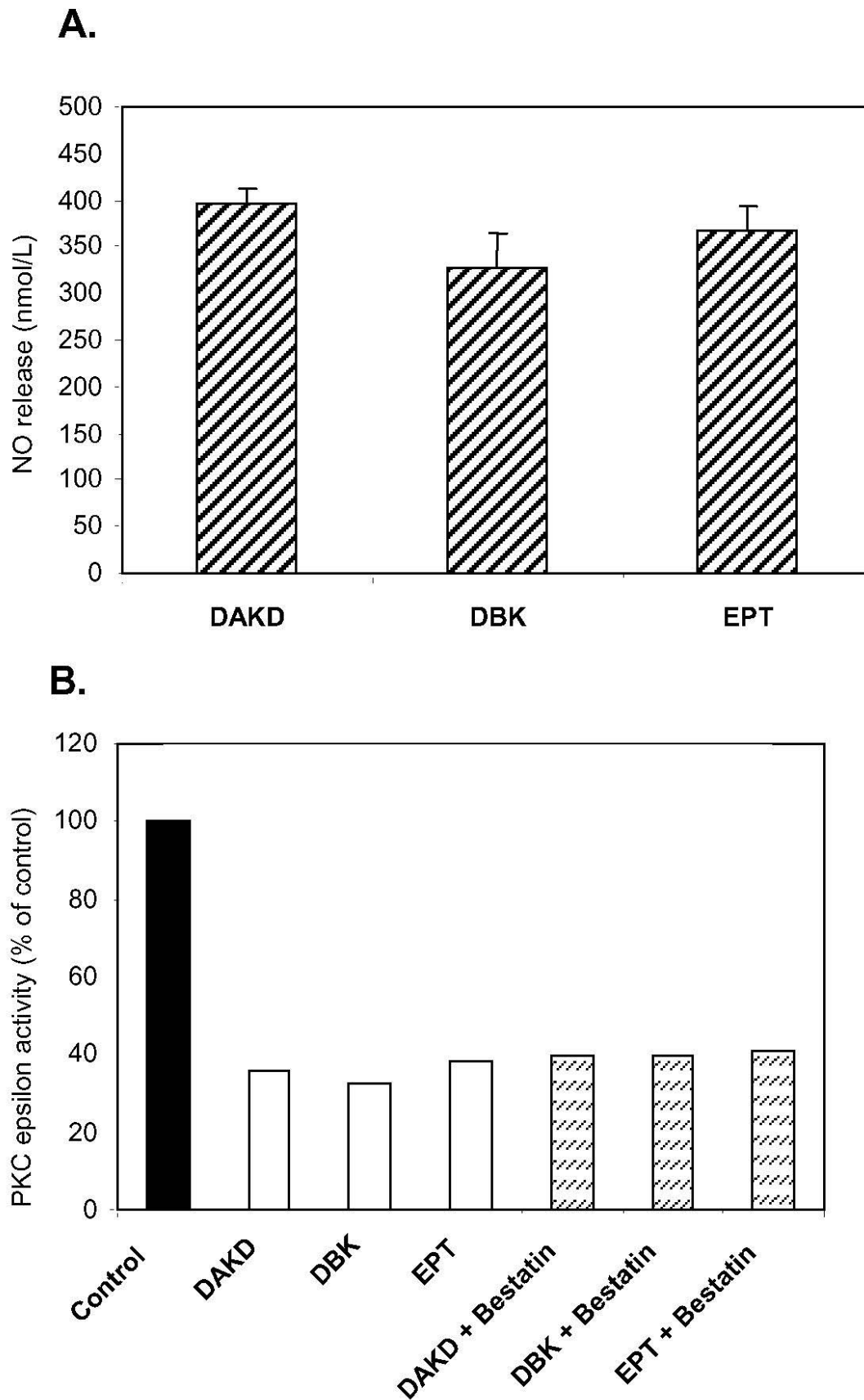


Figure 5

