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Angiotensin I -Converting Enzyme Inhibitors Block PKCε by Activating Bradykinin B_1 Receptors in Human Endothelial Cells*

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ACE inhibitors block PKCε via NO

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Abbreviations: ACE, angiotensin I-converting enzyme; BK, bradykinin, DAKD, des-Arg^{10}-kallidin; DBK, des-Arg^{9}-bradykinin; DETA-NONOate, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate; EPT, enalaprilat; eNOS, endothelial nitric oxide synthase; HLMVE, human lung microvacular endothelial; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; L-NNA, Nω-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-5*H*-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 B_1 receptor at the canonical Zn^{2+} 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, B_1 receptor activation by ACE inhibitors (enalaprilat, quinaprilat) or peptide ligands (des-Arg^{10}-Lys^1-bradykinin, des-Arg^9-bradykinin) inhibited PKCε with an $IC_{50} = 7 \times 10^{-9}$ M. Despite the reported differences in binding affinity to receptor, the two peptide ligands were equally active, even when inhibitor blocked the cleavage of Lys^1, thus the conversion by aminopeptidase. The synthetic undecapeptide (LLPHEAWHFAR) representing the binding site for ACE inhibitors on human B_1 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 B_1 ligands did. In conclusion, NO generated by B_1 receptor activation inhibits PKCε.

INTRODUCTION

The bradykinin B_1 and B_2 receptors are G-protein coupled receptors that mediate the actions of kinins. The B_2 receptors are activated by bradykinin and kallidin (Lys-bradykinin) and are constitutively expressed in normal tissues (Bhoola et al., 1992), whereas the B_1 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 B_1 receptors, des-Arg^9-bradykinin (DBK) and des-Arg^{10}-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 B_2 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 B_1 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 B_1 receptor at a Zn^{2+} binding pentapeptide sequence (Ignjatovic et al., 2002), similar to the active sites in ACE (Corvol et al., 2004).

ACE inhibitors improve endothelial dysfunction even in normotensive patients with coronary artery disease (Mancini et al., 1996). PKCε 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 PKCε after infarction (Simonis et al., 2003; Wang et al., 2003), indicating that PKCε may play a role in the development of cardiac dysfunction, and that ACE inhibitors can influence PKCε expression. We wanted to determine whether direct activation of the B_1 receptors by ACE inhibitors affects PKCε activity and whether two endogenous B_1 receptor agonists, DAKD and DBK are equally active in spite of big differences in their affinity for the B_1 receptors (Hess et al., 1996). We have found that B_1 receptor activation by ACE inhibitors (enalaprilat, quinaprilat) or peptide agonists blocks PKCε activity in human endothelial cells, and that the PKCε 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 Lys^1 (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-1β (IL-1β) 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). PKCε assay

kit, PKCαβγ assay kit, and PKCε 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 ug/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-1β and 50 ng/ml IFN-γ for 18 h prior to the experiments to induce B_1 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 B_1 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 PKCε activity following the manufacturers' instructions for the phosphorylation of the specific substrate peptide (ERMRPRKRQGSVRRRV) in the presence of [γ-^{32}P] ATP. The phosphorylated substrate is separated from residual [γ-^{32}P] 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

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 PKCα 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^{o}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 PKCε expression was performed with HLMVE cell lysates and as a positive control, with Jurkat cell lysate employing polyclonal anti-PKCε antibody. Cell lysate proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to

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

PKCε immunoprecipitation. PKCε was immunoprecipitated by primary PKCε 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-PKCε antibody or non-immune rabbit IgG at 4°C. After mixing with protein A-sepharose for 2 h at 4°C and centrifugation, PKCε activity was measured in Ca^{2+}-free buffer in the resuspended pellet and supernatant.

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

RESULTS

PKCε expression in HLMVE cells. To investigate the effect of the B_1 receptor activation on PKCε activity, we stimulated HLMVE cells with proinflammatory cytokines, IL-1β and IFNγ, for 18 h at 37°C to induce B_1 receptors. Western blotting demonstrated that PKCε was indeed expressed in HLMVE cells (Fig. 1).

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

Inhibition of PKCε. The B_1 receptor agonists, DAKD (10 nM) and enalaprilat (10 nM), inhibited 65% and 70% of the PKCε activity measured in HLMVE cells (Fig. 2A). The B_1 receptor antagonist, des-Arg^{10}-Leu^9-kallidin (100 nM) inhibited the response to both DAKD and enalaprilat, confirming this conclusion. Enalaprilat and the two peptide agonists of the B_1 receptor, had about the same low IC_{50} value (7×10^{-9} M) (Fig. 2B).

Effect of the undecapeptide. The human B_1 receptor contains in its second extracellular loop (residues 195-199) the HEAWH sequence, a canonical Zn^{2+} 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 B_1 receptor) which includes this pentamer, blocked B_1 receptor activation by enalaprilat, but not by DAKD (Ignjatovic et al., 2002). To confirm that enalaprilat inhibits PKCε due to B_1 receptor activation, we pretreated HLMVE cells with 100 μM of peptide, prior to adding either DAKD or enalaprilat (Fig. 2C). The undecapeptide reduced the effect of enalaprilat on PKCε inhibition (26% inhibition versus 63% in the absence of the undecapetide), whereas DAKD was not significantly affected, distinguishing the actions of the B_1 receptor agonists.

The time course of PKCε inhibition. We treated the cells with 100 nM of either DAKD, enalaprilat or quinaprilat for 1, 10, or 20 min. PKCε activity did not change after 1 min; however, after 10 or 20 minutes, the peptide and both ACE inhibitors similarly inhibited PKCε (Fig 3A). This result indicates that B_1 receptor-mediated PKCε inhibition is slower than the increase in intracellular $[Ca^{2+}]_i$ that is almost immediate in response to the B_1 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 PKCε could be NO-dependant (Fig. 3B). HLMVE cells were pretreated for 10 min with both iNOS and eNOS inhibitors (2 μM 1400W and 2 μM 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 B_1 receptor agonists on PKCε inhibition (Fig 3B). The NO donor DETA-NONOate (100 μM) alone inhibited PKCε activity similar to the effects of DAKD and enalaprilat, indicating that the B_1 receptor activation reduces PKCε 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 μM), a peroxynitrate scavenger. In these experiments, 100 nM DAKD or enalaprilat reduced PKCε 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 ± 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 PKCε.

PKCα activity. We further investigated whether the B_1 receptor agonists would affect another PKC isoform, PKCα, 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. PKCα activity in the presence of 100 nM DAKD or enalaprilat was 100 ± 2 % or 104 ± 3 % of control (mean values ± S.E.; n = 3). Because the standard PKCε assay buffer contained calcium, we wondered whether the residual activity remaining after B_1 receptor inhibition of PKCε might be due to PKCα. Indeed, when cells were preincubated with the specific PKCα/ß1 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 PKCε substrate. Furthermore, when the PKCε assay was done in the absence of Ca^{2+}, 100 nM DAKD inhibited PKCε activity by 93% (mean value for n = 2, assayed in triplicate). These data indicate that B_1 receptor activation almost completely (93%-95%) inhibits PKCε 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 PKCε substrate about 25%.

NO release. B_1 agonists inhibited PKCε, which was mediated by NO with a similar IC_{50}. As a follow up, we measured NO release from cytokine-treated HLMVE cells to determine whether the three B_1 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 B_1

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 Lys^1-Arg^2 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/$2x10^5$ cells at 37°C. In the presence of bestatin (150 μM), an aminopeptidase inhibitor, only 3.4 ± 0.72 nmol DBK/$2x10^5$ 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 B_1 agonist peptides are equally active in stimulating NO release and inhibiting PKCε in HLMVE cells.

DISCUSSION

When peptides or ACE inhibitors activates the B_1 receptor, the resulting NO release inhibits PKCε 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 PKCε, as shown in Western blot (Fig.1) and 75% of PKCε activity was immunoprecipitated from lysed cells by primary PKCε antibody.

In endothelial cells, NO suppresses shear stress-induced activation of PKCε and ERK1/2 (Ni et al., 2003). In our study, NOS inhibitors blocked the inhibition of PKCε by DAKD and enalaprilat. The NO donor DETA-NONOate induced a response similar to those of DAKD and enalaprilat, suggesting that B_1 receptor activation inhibits PKCε 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 PKCε inhibition, suggesting that tyrosine nitration and cysteine S-nitrosylation are not involved in the PKCε inhibition.

Macrophages from PKCε knockout mice release far less cytokines (Castrillo et al., 2001) and in rabbit cardiomyocytes, PKCε activates NF-kB (Li et al., 2000), leading to production of cytokines and adhesion molecules (Matsumori, 2004). Here, we found that B_1 receptor activation decreases PKCε activity in human endothelial cells. The B_1 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 PKCε activity, ACE inhibitors can indirectly decrease NF-κB activity, lowering the

production of proinflammatory cytokines and adhesion molecules. Since inflammation induces B_1 receptor expression (Bhoola et al., 1992), inhibition of PKCε by B_1 agonists may represent a negative feedback of NF-κB 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 PKCε, which subsequently phosphorylates and activates ß1 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 B_1 receptors to inhibit PKCε activity.

Overexpression of constitutively active PKCε in the hearts of transgenic mice causes development of concentric cardiac hypertrophy (Takeishi et al., 2000). In heart failure, over-expression of PKCε alters myofilaments (Goldspink et al., 2004). These results indicate that PKCε 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 B_1 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 PKCε and released NO. In published reports DBK or DAKD were shown to have both similar and different efficacies as B_1 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 B_1 receptors, whereas in rabbit

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 B_1 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 Lys^1 greatly enhances the affinity of DAKD for the human B_1 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 B_1 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.

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 B_2 receptor expression is decreased, while B_1 receptor expression is increased, indicating a possible compensatory reaction (Kintsurashvili et al., 2005). B_1 receptor activation can result in noxious consequences, e.g. pain and inflammation, which warrant the interest in development of therapeutically useful B_1 receptor blockers (Marceau and Regoli, 2004). However, ACE inhibitors are beneficial in many pathological conditions where B_1 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 B_1 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 B_1 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 B_1 receptors in human cells and as a consequence, inhibit PKCε via a NO-dependent mechanism. The two endogenous peptide agonists of the B_1 receptors are equally active in stimulating this response. This inhibition of PKCε, 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 PKCε in cytokine-treated HLMVE cell lysates. Jurkat cell lysate was the positive control.

Fig. 2. (A) HLMVE cells were stimulated with either des-Arg^{10}-kallidin (DAKD; 10 nM) or the ACE inhibitor enalaprilat (EPT; 10 nM) for 20 min or pretreated with the B_1 receptor antagonist des-Arg^{10}-Leu^{9}-kallidin (DALKD; 100nM). Both DAKD and enalaprilat inhibited PKCε; inhibition was abolished by a B_1 receptor antagonist. Ordinate: PKCε relative activity. Data are expressed as mean ± SE (n = 3; done in triplicate). (B) Concentration-dependent inhibition of PKCε activity by B_1 agonists. DAKD, des-Arg^{9}-bradykinin (DBK) or EPT, respectively. Data are mean ± SE (n = 3; done in duplicate). (C) The undecapeptide (LLPHEAWHFAR; 100 μM), representing the ACE inhibitor-binding site on the B_1 receptor, reversed the effect of EPT (100 nM) only, but not that of DAKD (100 nM). Data are mean ± SE (n = 3; done in triplicate). Endogenous peptide ligand and enalaprilat are about equally active.

Fig. 3. (A) The time course of B_1 receptor-mediated PKCε 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 ± SE (n = 3; done in duplicate). B_1 agonists decreased PKCε 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 B_1 receptor agonists on PKCε activity. The eNOS and iNOS inhibitors combined (2 μM 1400W + 2 μM LNNA) block the inhibition of PKCε activity by DAKD or EPT. NO donor DETA-

NONOate (100 μM) inhibits PKCε. Results indicate that B_1 receptor agonists inhibit PKCε 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 ± SE (n = 3). (B) The aminopeptidase inhibitor bestatin (10 μM) does not affect DAKD-induced PKCε inhibition. Cells were pretreated with bestatin (10 μM, 10min), then exposed to 100 nM DAKD, DBK or EPT, followed by assay of PKCε 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 uM), then incubated with DAKD (50 uM). Ordinate: DBK released in 20 min. Data are mean ± SE (n =5; done in triplicate). Bestatin inhibited the release of Lys^1 from DAKD, blocking production of DBK.

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Figure 1

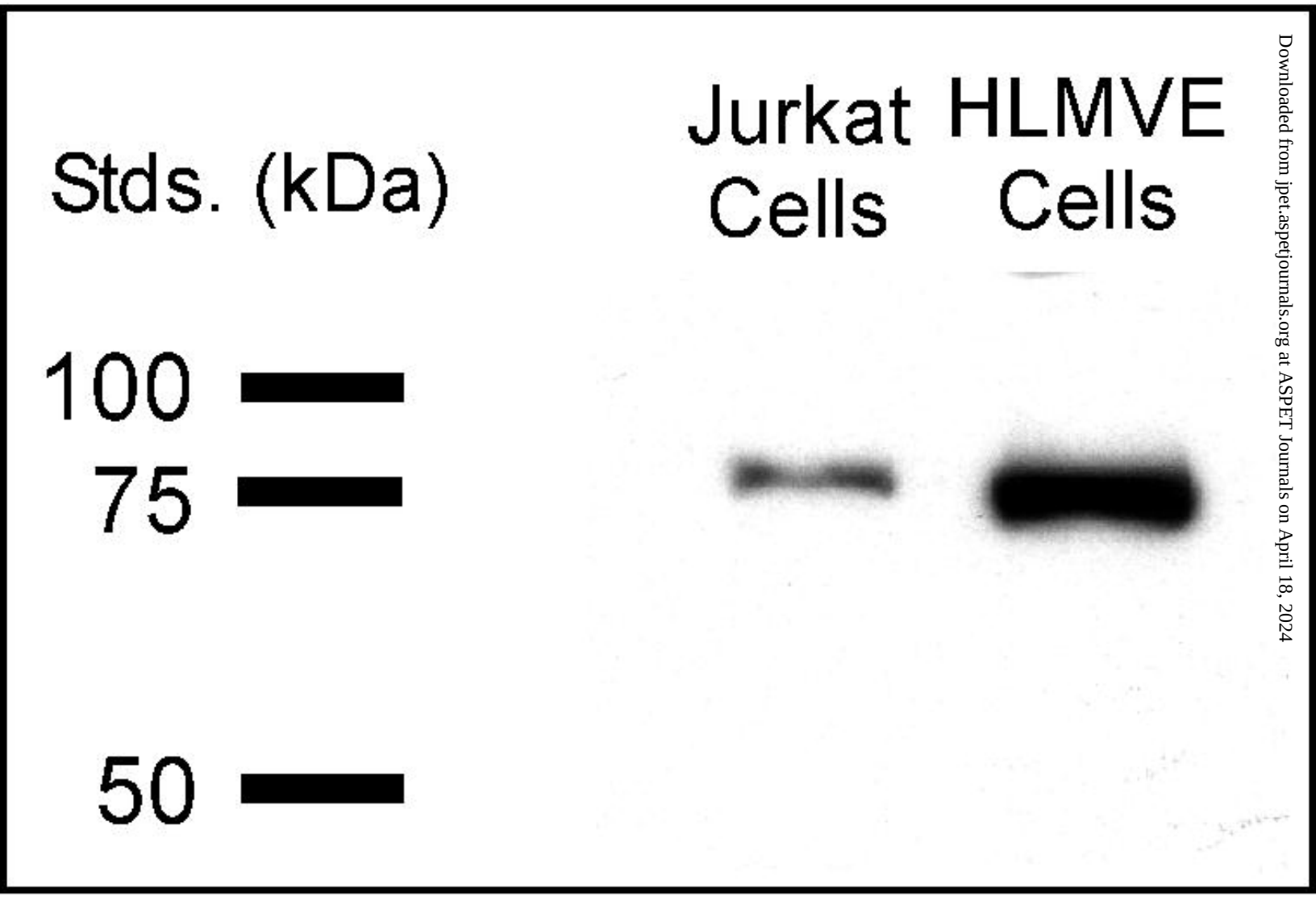

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Figure 2

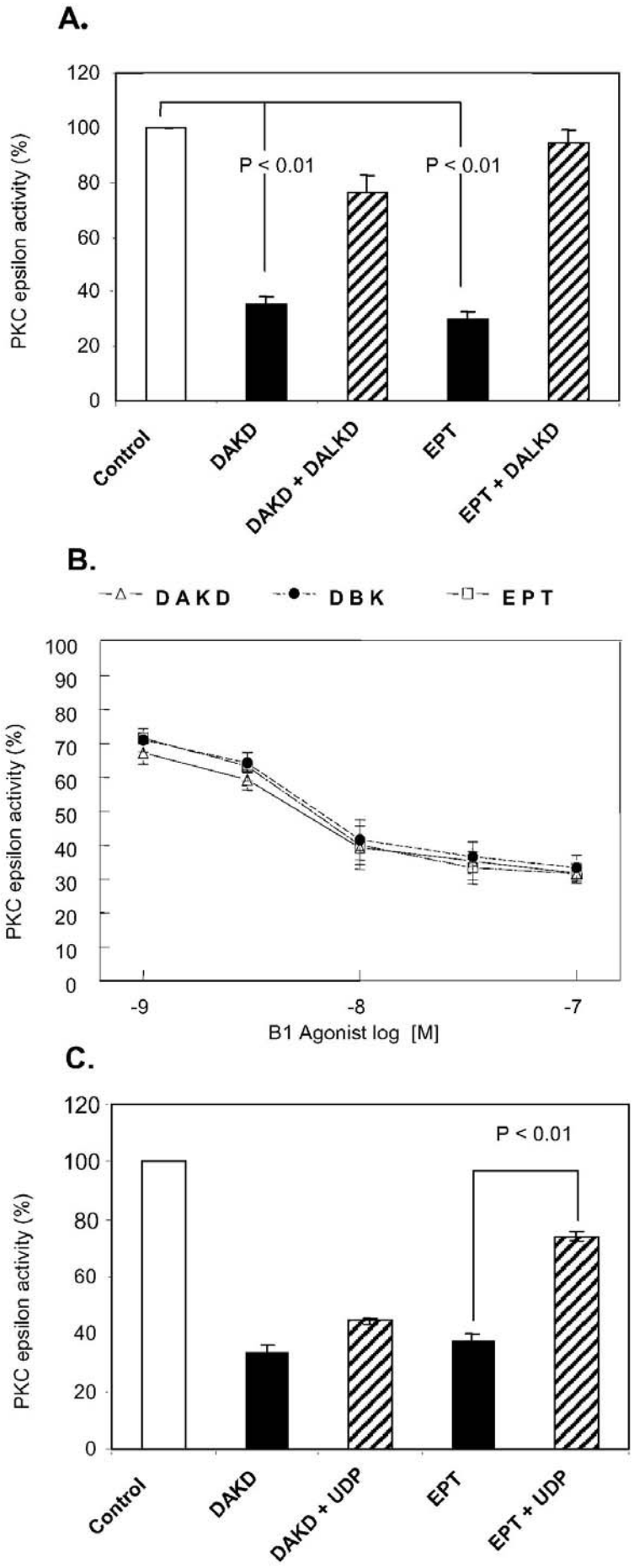

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Figure 3

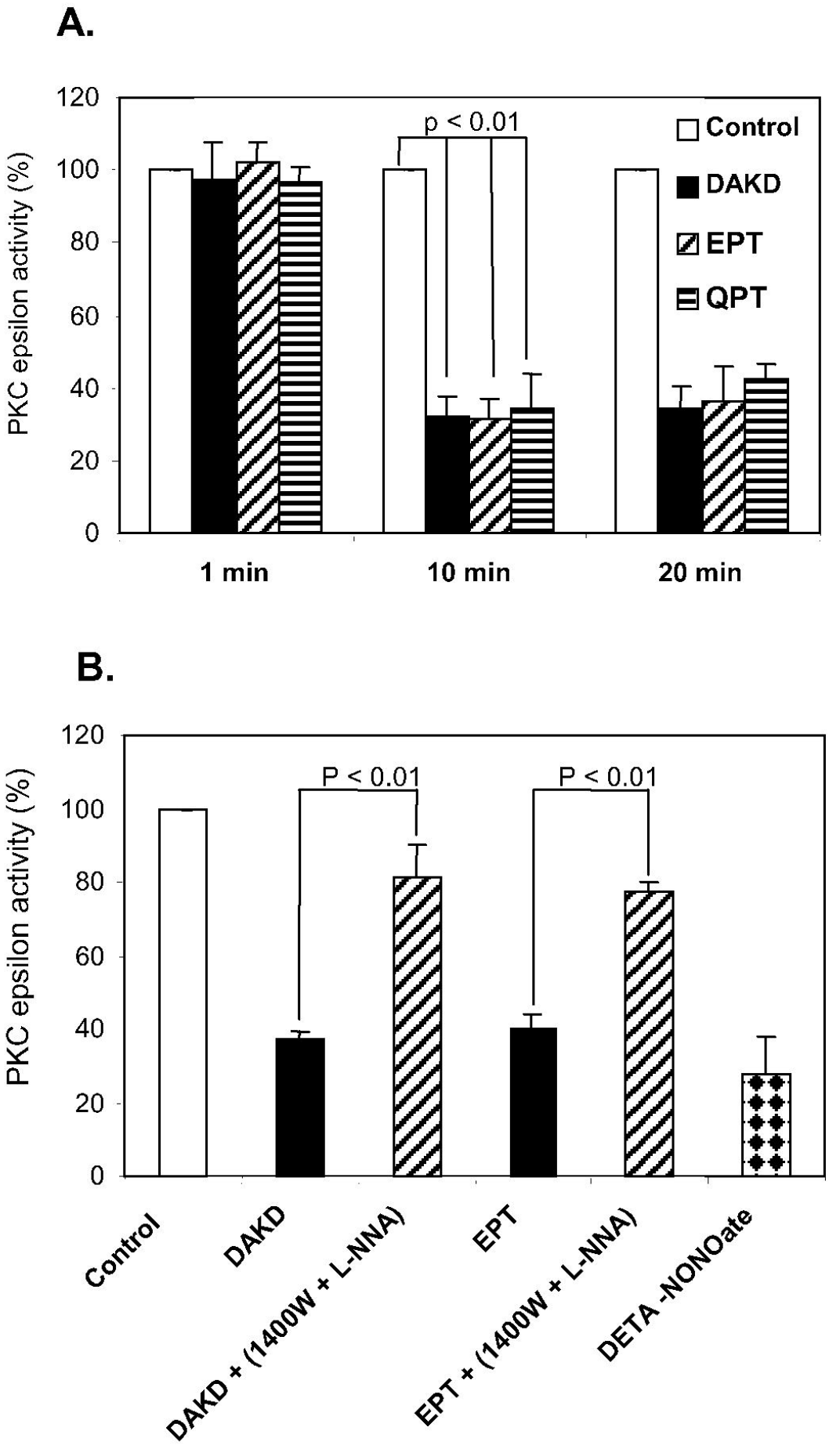

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Figure 4

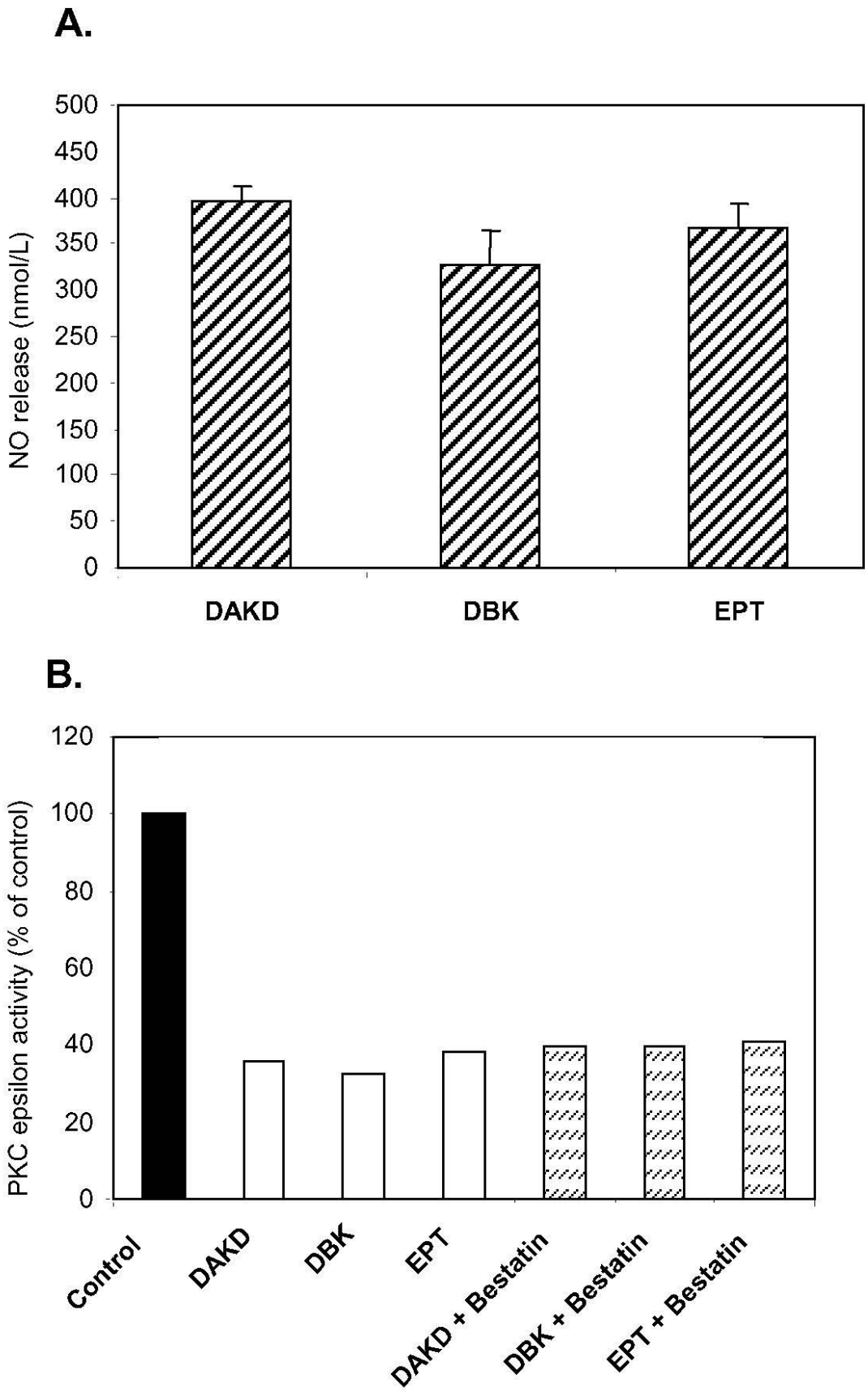

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Figure 5