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Interleukin-1β inhibits ATP-induced protein kinase B activation in renal mesangial cells by two different mechanisms: the involvement of nitric oxide and ceramide

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1 Extracellular nucleotides, like ATP and UTP, have been shown to activate the protein kinase B (PKB) pathway in mesangial cells. In this study we report that the pro-inflammatory cytokine interleukin-1β (IL-1β) inhibits ATP-induced PKB activation.

2 Pretreatment of mesangial cells with IL-1β leads to a time-dependent decrease of ATP-induced PKB phosphorylation. Maximal inhibition is seen after 6 h of pretreatment. Incubating cells with IL-1β in the presence of the NO synthase inhibitor, *N*-monomethyl-L-arginine (L-NMMA), reversed the IL-1β inhibition of PKB activity. A similar decrease in ATP-evoked PKB activation is obtained when cells were pretreated with the nitric oxide (NO) donor, (*Z*)-1-[2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (Deta-NO), but not with the cell-permeable cGMP analogue, 8-bromo-cGMP.

3 The NO- and IL-1β-mediated delayed inhibition of PKB activity is completely reversed by the phosphatase inhibitor calyculin A, but not by ocadaic acid, suggesting that NO upregulates a protein phosphatase activity, which most probably belongs to the group of protein phosphatases type 1.

4 In addition, IL-1β also triggers a short-term and transient inhibitory effect on ATP-induced PKB activation which is maximal after 2–5 min of pre-incubation with IL-1β. This effect occurs independently of NO generation, because no NO synthase is expressed at that time, and consequently, L-NMMA does not reverse the effect. Rather an involvement of the sphingolipid ceramide is likely, since IL-1β triggers rapid ceramide formation and incubation of cells with the cell-permeable C6-ceramide blocked ATP-induced PKB phosphorylation.

5 In summary, our data show that IL-1β exerts both short-term and long-term inhibitory effects on ATP-stimulated PKB activation, the short-term effect probably involves ceramide formation, whereas the long-term effect is due to inducible NO synthase induction and subsequent NO formation. These results reveal a further facet in the mechanisms of ceramide- and NO-induced cell death, i.e. by turning off the survival signal elicited by PKB.

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Abbreviations: BSA, bovine serum albumin; Deta-NO, (Z)-1-[2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ILK, integrin-linked kinase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-NMMA, *N*-monomethyl-L-arginine; MKP, mitogen-activated protein kinase phosphatase; NO, nitric oxide; PDK, phosphoinositide-dependent kinase; PKB, protein kinase B; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A

Introduction

Mesangial cells are a major determinant in the regulation of the glomerular filtration rate. Morphologically, they resemble smooth muscle cells, able to contract upon stimulation by vasoactive hormones like angiotensin II or arginine vasopressin (Pfeilschifter, 1989). In addition, mesangial cells are crucially involved in most pathological processes of the renal glomerulus (Pfeilschifter, 1989; 1994; Kashgarian & Sterzel, 1992). Interleukin-1β (IL-1β) is the prototype of an inflammatory cytokine that induces the expression of a variety of protein factors that, in turn, trigger acute and chronic inflammatory processes (Dinarello, 1998). The local release of IL-1β or tumour necrosis factor-α from monocytes is an early event associated with inflammatory reactions, and may be an important pathogenic determinant of structural and functional alterations accompanying immune injury in diseases such as rheumatoid arthritis, inflammatory bowel disease, septic shock and several auto-immune reactions (Dinarello, 1998).

Extracellular nucleotides, such as ATP and UTP, exert diverse effects on intact cells by activation of plasma membrane receptors, termed P2 purinoceptors. These receptors either couple through G-proteins to their intracel-

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lular effector enzymes (P2Y subtypes) or are themselves part of ligand-gated ion channels (P2X subtypes) (Boarder & Hourani, 1998; Ralevic & Burnstock, 1998).

In renal mesangial cells, ATP acts on a nucleotide receptor ($P2Y_2$ receptor) and mediates phosphoinositide hydrolysis with generation of 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Pfeilschifter, 1990a, b) leading to mobilization of intracellular calcium (Pavenstädt *et al*., 1993) and activation of protein kinase C (Pfeilschifter & Huwiler, 1996a). Subsequently, various cascades are activated, including the classical mitogen-activated protein kinase cascade (Huwiler & Pfeilschifter, 1994) which leads to activation of the cytosolic phospholipase A_2 and stimulation of prostaglandin E_2 synthesis (Pfeilschifter, 1990a), the stress-activated protein kinase cascade (Huwiler *et al*., 1997) as well as the p38-MAPK cascade (Huwiler *et al*., 2000a). Stimulation of mesangial cells with ATP or UTP not only leads to an increased proliferation of the cells (Huwiler & Pfeilschifter, 1994; Schulze-Lohoff *et al*., 1992), but also protects the cells from stress-induced apoptosis. This latter effect is due to an activation of the protein kinase B/Akt (PKB) pathway (Huwiler *et al*., 2002). PKB was first identified as the human homologue of a transforming oncogene (Staal, 1987; Coffer & Woodgett, 1991), and has gained interest because of its possible function in cell survival (for review, see Chan *et al*., 1999; Brazil & Hemmings, 2001). Classical activators of PKB include growth factors, like platelet-derived growth factor or insulin-like growth factor, which act through tyrosine kinase receptors and involve the $p21^{ras}$ and phosphatidylinositol 3-kinase (PI 3-kinase) (Burgering & Coffer, 1995; Franke *et al*., 1995; Alessi *et al*., 1996; Andjelkovic *et al*., 1996). The lipid product of PI 3-kinase, phosphatidylinositol trisphosphate (PIP_3), is an important cofactor for PKB activation. Additionally, PKB requires phosphorylation at two sites for full activation, namely Thr^{308} in the activation loop, and at Ser^{473} in a hydrophobic part at the C-terminus (Alessi *et al*., 1996). Thr^{308} is phosphorylated by the phosphoinositide-dependent kinase-1 (PDK-1) whereas the kinase phosphorylating Ser^{473}, tentatively named PDK-2, is not yet identified. Recent reports suggest that Ser^{473} is an autophosphorylation site (Toker & Newton, 2000) or is phosphorylated by the integrin-linked kinase (ILK) (Persad *et al*., 2001).

In the present study, we show that the pro-inflammatory cytokine interleukin-1β blocks ATP-induced PKB activation, and that this effect is mechanistically linked to the generation of ceramide under short-term conditions, and to the induction of iNOS and production of NO under long-term conditions.

Methods

Chemicals

ATP was obtained from Fluka Chemie GmbH, Buchs, Switzerland; Deta-NO was from Alexis Biochemicals, Läufelfingen, Switzerland; ceramides were purchased from Biotrend Chemikalien GmbH, Cologne, Germany; IL-1β was kindly donated by Novartis Pharma Ltd, Basel, Switzerland; *N*-monomethyl-L-arginine (L-NMMA), ocadaic acid and calyculin were obtained from Calbiochem-Novabiochem, Schwalbach, Germany; anti-rabbit and anti-mouse horseradish peroxidase-linked IgGs and Hyperfilm were purchased from Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany; the PKBα/Akt1-specific antibody was from Upstate Biotechnology, Lake Placid, NY, U.S.A.; the phospho-PKB antibodies were from New England Biolabs, Schwalbach, Germany; all cell culture nutrients were from Life Technologies, Karlsruhe, Germany.

Cell-culture

Rat renal mesangial cells were cultivated and characterized as previously described (Pfeilschifter, 1990a, b). In a second step, single cells were cloned by limited dilution on 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells (Travo *et al*., 1982), positive staining for Thy1.1 antigen, and negative staining for factor-VIII-related antigen and cytokeratin, which excludes endothelial and epithelial contaminations. Generation of inositol trisphosphate upon activation of angiotensin II type I receptor was used as a functional criterion for characterizing the cloned cell line. Passages 7–18 were used for the experiments in this study.

Cell stimulation and Western blot analysis

Confluent mesangial cells in 60 mm-diameter dishes were stimulated with the indicated substances in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg ml^{-1} of fatty acid-free bovine serum albumin. Thereafter the medium was withdrawn and the cells washed once with ice-cold phosphate-buffered saline solution. Cells were scraped into ice-cold lysis buffer (mM: Tris/HCl 50, pH 7.4, NaCl 150, 10% glycerol, 1% Triton X-100, EDTA 2, EGTA 2, β-glycerophosphate 40, sodiumfluoride 50, leupeptin 10 μg ml^{-1}, aprotinin 10 μg ml^{-1}, pepstatin A 1 μM, phenylmethyl sulphonyl fluoride 1 mM) and homogenized by 10 passes through a 26G-needle fitted to a 1 ml syringe. Samples were centrifuged for 10 min at $14{,}000 \times g$ and the supernatant was taken for protein determination. Cell extracts containing 70 μg of protein were prepared in SDS-sample buffer and subjected to SDS–PAGE. Proteins were transferred on to nitrocellulose paper for 1 h at 11 V using a semi-dry blotting apparatus. The blotting buffer used was 25 mM Tris, 190 mM glycine in 20% (vol vol^{-1}) methanol. After the transfer, immunostaining was performed as previously described in detail (Huwiler *et al*., 1995; 2000a). Antibodies were diluted in blocking buffer as indicated in the legends of the figures. Bands were detected by the enhanced chemiluminescence (ECL) method as recommended by the manufacturer.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA).For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni. One-way ANOVA with Bonferroni's post test was performed using GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, California, U.S.A.

Results

IL-1β downregulates ATP-induced PKB activation in mesangial cells

The extracellular nucleotides ATP and UTP are able to activate the PKB pathway in renal mesangial cells *via* the $P2Y_2$ purinoceptor and a pertussis toxin-sensitive G_i protein, as previously shown (Huwiler *et al*., 2002). Pretreatment of the cells with the pro-inflammatory cytokine IL-1β blocks ATP-induced PKB phosphorylation in a time-dependent manner. As shown in Figure 1A, pretreatment of cells with IL-1β time-dependently reduces ATP-induced PKB phosphorylation on Ser^{473}, which is considered to reflect activation of the enzyme (Alessi *et al*., 1996; 1997). Inhibition is first seen after 3 h, and is maximal after 6 h of IL-1β pretreatment and this is sustained for at least 24 h. In parallel to the inhibition of PKB phosphorylation, an induction of the inducible NO synthase (iNOS) protein is detected (Figure 1B) confirming previous studies in mesangial cells which have shown that cytokines trigger iNOS expression and subsequent NO formation (Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter *et al*., 1992; Kunz *et al*., 1994), and this in turn modulates expression and activity of a variety of genes and proteins (Pfeilschifter *et al*., 2001). In order to see whether the long-term effect of IL-1β is mediated by induction of the inducible NO synthase and concomitant generation of NO, N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS activity, was used. Figure 2 shows that pretreatment of cells with IL-1β in the presence of 0.5 mM or 1 mM of L-NMMA completely reverses the inhibitory effect of IL-1β on ATP-induced PKB phosphorylation.

To further corroborate the effect of NO on PKB phosphorylation, the NO donor Deta-NO was applied. As depicted in Figure 3A, the long-term inhibitory effect of IL-1β is indeed mimicked by Deta-NO. Pretreatment for 24 h with different concentrations of Deta-NO causes a dose-dependent decrease of ATP-induced PKB phosphorylation. In contrast, short-term pretreatment for 15 min with Deta-NO has no effect on ATP-induced PKB phosphorylation (Figure 3B). The total PKB protein levels were not changed by NO pretreatment (Figure 3A,B, lower panels) suggesting an indirect effect of NO on PKB. Furthermore, we tested the effect of the cell-permeable cGMP analogue, 8-bromo-cGMP, since it is well established that NO exerts many of its actions *via* guanylate cyclase activation and subsequent cGMP formation. However, as seen in Figure 3C, long-term pretreatment with 8-bromo-cGMP does not inhibit the ATP response. Also short-term stimulation with 8-bromo-cGMP (for 2–30 min) is without effect on PKB phosphorylation (data not shown).

Involvement of phosphatases in the NO-mediated downregulation of PKB activity

Ample evidence has been presented that NO can modulate protein phosphatase activities in a variety of cell systems (Pfeilschifter *et al*., 2001, and references therein). To evaluate whether the NO-triggered inhibition of agonist-induced PKB activity in mesangial cells is due to an activation of a protein phosphatase various phosphatase inhibitors were tested. Cells were pretreated for 24 h with Deta-NO to fully establish the

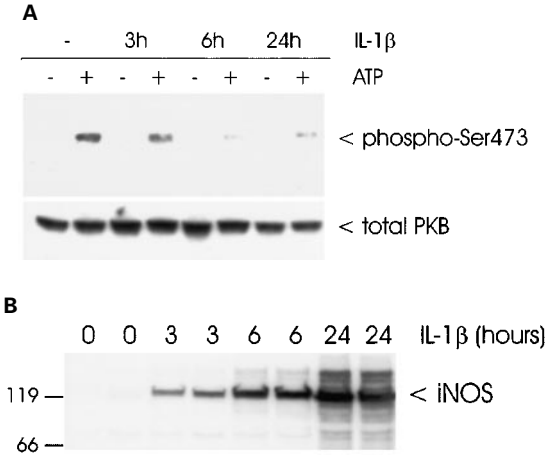

Figure 1 IL-1β induces downregulation of ATP-induced PKB activation which parallels iNOS induction in renal mesangial cells. (A) Quiescent mesangial cells were pretreated for the indicated time periods with IL-1β (1 nM) before stimulation with either vehicle (co) or 100 μM of ATP for 5 min. (B) Mesangial cells were treated for the indicated time periods with IL-1β (1 nM). Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser^{473}-PKB (A, upper panel) and total PKBα (A, lower panel) antibodies at a dilution of 1 : 1000 and 1 : 1600, respectively, or an iNOS-specific antiserum (B) at a dilution of 1 : 2000. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of three independent experiments giving similar results.

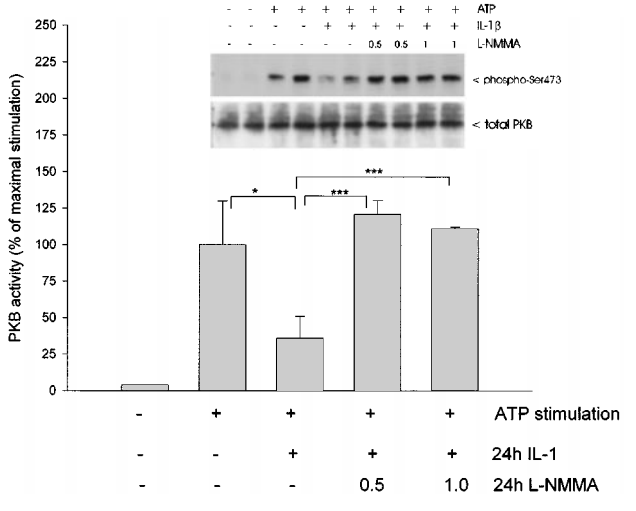

Figure 2 Effect of the iNOS inhibitor L-NMMA on IL-1β-mediated inhibition of PKB activity in mesangial cells. Quiescent mesangial cells were pretreated for 24 h with IL-1β (1 nM) in the absence or presence of the indicated concentrations of *N*-monomethyl-L-arginine (L-NMMA) before stimulation with either vehicle (co) or 100 μM of ATP for 5 min. Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser^{473}-PKB (insert; upper panel) and total PKBα (insert; lower panel) antibodies at a dilution of 1 : 1000 and 1 : 1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation and densitometrically evaluated. Results are expressed as per cent of ATP stimulation and are means ± s.d. ($n = 4$); $^*P < 0.05$, $^{***}P < 0.001$, statistically significant difference compared to the corresponding controls.

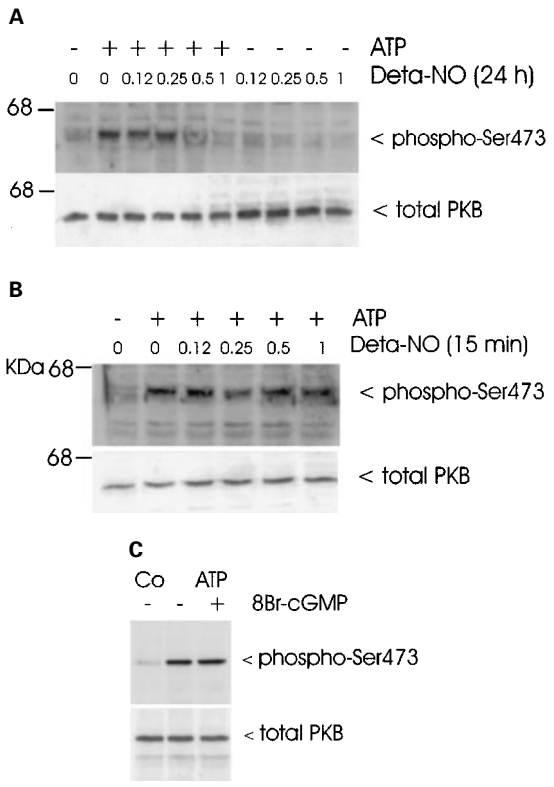

Figure 3 Effect of an NO donor and 8-bromo-cGMP on ATP-induced PKB activation in renal mesangial cells. Quiescent mesangial cells were pretreated for 24 h (A) or 15 min (B) with the indicated concentrations (in mM) of the NO donor Deta-NO, or for 24 h with 8-Br-cGMP (300 μM; C) before stimulation with either vehicle (Co) or 100 μM of ATP for 5 min. Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser^{473}-PKB (upper panel) and total PKBα (lower panel) antibodies at a dilution of 1 : 1000 and 1 : 1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of at least three independent experiments giving similar results.

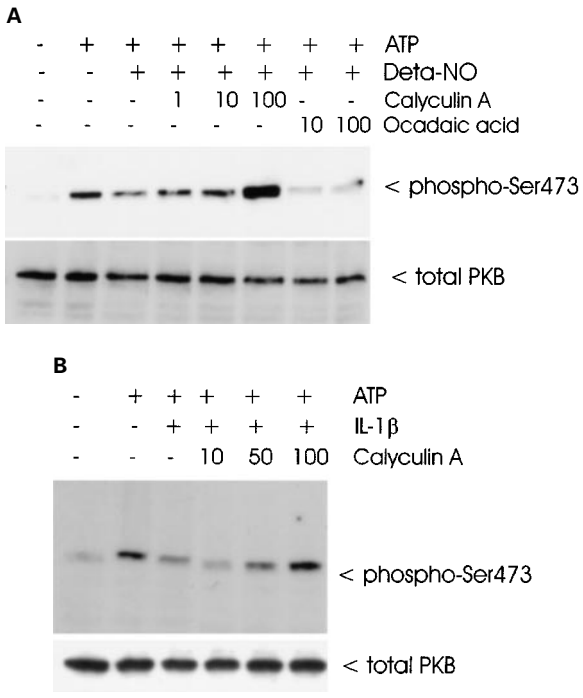

Figure 4 Effect of phosphatase inhibitors on NO- and IL-1β-induced inhibition of PKB phosphorylation in mesangial cells. Mesangial cells were pretreated for 24 h with Deta-NO (0.5 mM) (A) or IL-1β (1 nM) (B), then pre-incubated for 15 min with the indicated concentrations of calyculin A and ocadaic acid (in nM) prior to stimulation for 5 min with ATP (100 μM). Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser^{473}-PKB (upper panel) and total PKBα (lower panel) antibodies at a dilution of 1 : 1000 and 1 : 1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of 3–4 independent experiments giving similar results.

inhibitory action on ATP-induced PKB activation, followed by a 15 min pre-incubation time with increasing concentrations of calyculin A, which potently inhibits PP1 and PP2A (Honkanen *et al*., 1994) and ocadaic acid, a potent inhibitor of protein phosphatase 2A and to a lesser extent PP1 (Honkanen *et al*., 1994). As seen in Figure 4A, calyculin A concentration-dependently reverses the inhibitory action of NO, whereas ocadaic acid is without effect. Neither the PP2B inhibitor cyclosporin A nor the tyrosine phosphatase inhibitor vanadate have a significant effect (data not shown). Moreover, a similar calyculin A-triggered reversal of PKB phosphorylation is obtained for IL-1β-pretreated cells (Figure 4B).

Acute effect of IL-1β on ATP-induced PKB activation is mediated by ceramide

Interestingly, in addition to the delayed effect described above, IL-1β also causes a fast but transient inhibition of ATP-induced PKB activity. A significant reduction is already seen after 2 min of IL-1β pretreatment with a maximal effect occurring at 5–7.5 min which then is reversed after 10 min of pretreatment (Figure 5A). The inhibitory effect occurs in a concentration-dependent manner with a maximal response at 1 nM of IL-1β (Figure 5B). It is obvious that the short-term effect of IL-1β occurs independently of NO, because at these early time points iNOS is not yet expressed (Pfeilschifter & Schwarzenbach, 1990; Kunz *et al*., 1994) and hence no NO is released that could inhibit PKB activation. This is corroborated by the finding that L-NMMA does not reverse the acute inhibitory effect of IL-1β on ATP-stimulated PKB activity (Figure 5C).

One early signalling event that is initiated by IL-1β stimulation in many cell types, including mesangial cells, is the activation of the sphingomyelin/ceramide cycle (Huwiler *et al*., 2000b). IL-1β has been shown to rapidly and transiently activate a neutral sphingomyelinase and thereby increases ceramide levels within minutes in mesangial cells (Huwiler *et al*., 1996; Kaszkin *et al*., 1998). When pretreating mesangial cells with an exogenous cell-permeable C6-ceramide analogue for 15 min, a dose-dependent inhibition of ATP-induced PKB phosphorylation is observed (Figure 6A), suggesting that the short-term effect of IL-1β may be mediated by ceramide. A time course reveals a rapid decrease of ATP-induced PKB phosphorylation within 5–10 min which constantly decreased over the next 8 h (Figure 6B). However, it should be noted that IL-1β only triggers a transient increase of ceramide (Huwiler *et al*., 1996; Kaszkin

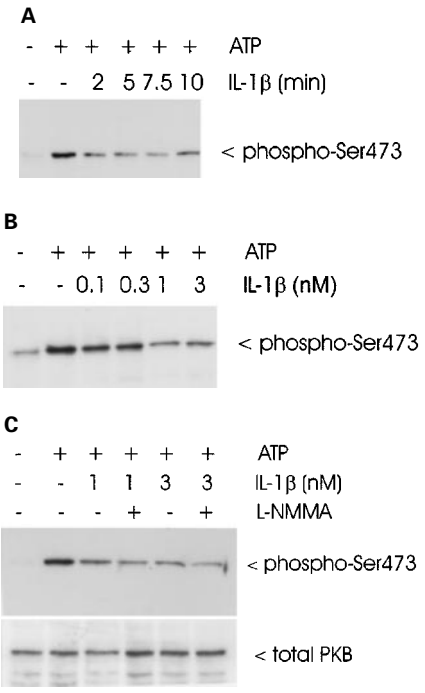

Figure 5 Acute effect of IL-1β on ATP-induced PKB activation in renal mesangial cells. Quiescent mesangial cells were pretreated for the indicated time periods with 2 nM of IL-1β (A) or for 5 min with the indicated concentrations of IL-1β (B), or with the indicated concentration of IL-1β in the presence of 0.5 mM *N*-monomethyl-L-arginine (L-NMMA) (C) before stimulation with 100 μM of ATP for 5 min. Thereafter cells were harvested and Western blot analyses were performed using a specific phospho-Ser^{473}-PKB antibody at a dilution of 1 : 1000. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of two independent experiments giving similar results.

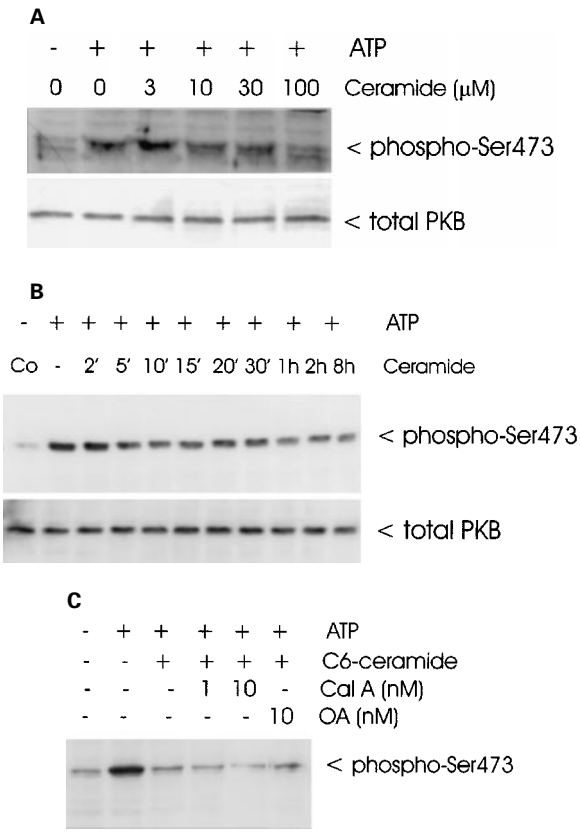

Figure 6 Effect of exogenous ceramide on ATP-induced PKB activation in renal mesangial cells. Quiescent mesangial cells were pretreated for the indicated time periods with C6-ceramide (30 μM; B) or for 15 min with the indicated concentrations of C6-ceramide (A) before stimulation with either vehicle (co) or 100 μM of ATP for 5 min. (C): Mesangial cells were pretreated for 15 min with C6-ceramide (30 μM) in the absence or presence of the phosphatase inhibitors calyculin A and ocadaic acid (both in nM) as indicated before stimulation for 5 min with ATP (100 μM). Thereafter cells were harvested and Western blot analyses were performed using specific phospho-Ser^{473}-PKB (upper panel) and total PKBα (lower panel) antibodies at a dilution of 1 : 1000 and 1 : 1600, respectively. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of at least three independent experiments giving similar results.

et al., 1998) thus explaining the transient inhibitory action of IL-1β.

The short-term inhibition of ATP-induced PKB activation by ceramide is not affected by either calyculin A or ocadaic acid (Figure 6C) thus excluding protein phosphatase of either type 1 or 2A as potential mediators.

Discussion

In this study we show that the pro-inflammatory cytokine IL-1β inhibits extracellular nucleotide-induced PKB activation by a dual action. A short-term inhibitory effect that is probably mediated *via* ceramide generation, and a delayed long-term inhibitory effect that is mediated by NO released by the inducible NO synthase (iNOS), which is potently induced in mesangial cells by IL-1β (Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter *et al*., 1992; Kunz *et al*., 1994). This finding is based on the following findings: (i) L-NMMA, an inhibitor of the iNOS, reverses the inhibitory effect of IL-1β on agonist-induced PKB activation, and (ii) an exogenously added NO donor mimics the long-term inhibitory effect of IL-1β.

Interestingly, and in contrast to the long-term inhibition of PKB by IL-1β, the observed short-term inhibition by IL-1β obviously involves an NO-independent mechanism, since no NO is generated after minutes of IL-1β stimulation. Previous work has clearly demonstrated that a lag period of 4–6 h is needed before IL-1β induces iNOS RNA, protein and activity in mesangial cells (Pfeilschifter & Schwarzenbach, 1990; Pfeilschifter *et al*., 1992; Kunz *et al*., 1994). Since neither the constitutive eNOS nor the neuronal nNOS are expressed in mesangial cells under any condition (Pfeilschifter and Schwarzenbach, 1990; Pfeilschifter *et al*., 1992; Kunz *et al*., 1994), the iNOS is the only NOS isoform that might contribute to increased NO generation in mesangial cells.

An early effect of IL-1 signalling is the activation of a neutral sphingomyelinase (Huwiler *et al*., 1996; Kaszkin *et al*., 1998) and the increased formation of the sphingolipid molecule ceramide. Our present data indeed suggest that ceramide is most likely the mediator of the observed short-term inhibitory effect of IL-1β on PKB activation. This finding is in accordance with several other reports that show an inhibitory effect of ceramide on PKB (Zhou *et al*., 1998;

Zundel & Giaccia, 1998). Comparing the kinetics of PKB inhibition by exogenously-added ceramide and endogenously-generated ceramide seem to be different, which, however, can be explained by the different degradation kinetics. Stimulation of cells with IL-1β not only leads to an activation of neutral sphingomyelinase but also to an induction of neutral ceramidase (Franzen *et al*., 2001) which degrades ceramide and results in an only transient ceramide accumulation. In contrast, exogenously-added ceramide can circumvent this ceramidase activation and therefore allows a higher concentration of ceramide and a longer time period of action. The mechanism of ceramide-triggered inhibition of PKB is still unknown, but it was speculated that ceramide prevents membrane translocation of PKB. Alternatively, it was suggested that ceramide causes activation of a phosphatase and subsequent dephosphorylation of PKB (Salinas *et al*., 2000; Schubert *et al*., 2000; Zinda *et al*., 2001). However, in mesangial cells, ocadaic acid and calyculin A, which potently inhibit protein phosphatases of the type 1 and 2A, have no effect on the inhibitory action of ceramide (Figure 6C), thus excluding the involvement of phosphatases 1 and 2A, at least in our experimental model system.

Recently, Bourbon *et al*. (2002) reported that ceramide-induced inhibition of PKB is mediated through activation of protein kinase C-ζ in smooth muscle cells. In our cell system such an involvement of PKC-ζ in the ceramide-mediated inhibition of PKB can be excluded, since ceramide does neither target nor activate PKC-ζ in mesangial cells (Huwiler *et al*., 1996; 1998).

Concerning the delayed inhibitory action of IL-1β on PKB, which occurs in a NO-dependent manner, the detailed mechanism is still unclear. In view of the finding that short-term NO treatment has no effect on agonist-induced PKB activation, a direct inhibitory effect of NO on one of the PKB cascade members is unlikely. Moreover, chronic treatment with NO does not affect the total protein level of PKB (Figure 3A), thus also excluding an effect on protein synthesis or degradation of PKB.

However, since the protein phosphatase inhibitor calyculin A, but not ocadaic acid, was able to reverse the NO-mediated effect on PKB phosphorylation, the involvement of a calyculin A-sensitive phosphatase is evident. Considering the selectivity pattern of these two phosphatase inhibitors (calyculin A inhibits PP1 and PP2A with an IC50 of 0.5 nM and 2 nM, respectively; ocadaic acid with 15 nM and 0.1 nM, respectively) a protein phosphatase of the type 1 is a likely candidate.

Indeed, NO has been reported to stimulate a membrane-associated protein tyrosine phosphatase activity in T cells (Lander *et al*., 1993) and in rat aortic smooth muscle cells (Dhaunsi *et al*., 1997; Kaur *et al*., 1998). In addition, it was reported that NO can upregulate the mitogen-activated protein kinase phosphatase-1 (MKP-1) expression in human embryonic lung fibroblasts (Marquis & Demple, 1998). In contrast, NO was reported to inactivate a low molecular mass protein tyrosine phosphatase *in vitro* by S-nitrosation (Caselli *et al*., 1994). NO was also observed to inhibit platelet-derived growth factor phosphotyrosine phosphatase in renal mesangial cells (Callsen *et al*., 1999). In a similar manner NO was found to block epidermal growth factor (EGF) tyrosine phosphatase activity in HER14 cells and thus to increase tyrosine phosphorylation of the receptor even in the absence of EGF (Peranovich *et al*., 1995). In contrast, Estrada *et al*. (1997) showed that NO reversibly inhibits EGF receptor tyrosine kinase in fibroblasts and that this phenomenon correlated well with the anti-proliferative effect of NO in the same cells. Despite these apparently discrepant findings it is obvious that protein phosphatases are targets of NO and reactive oxygen species and are modulated by the cellular redox state (for review, see: Pfeilschifter *et al*., 2001).

The identity of the phosphatase involved in the present study is unknown and certainly deserves further investigation. In any case, it is a calyculin A-sensitive enzyme. Unfortunately, not much is known about the specificity of calyculin A for the more recently discovered phosphatases such as the mitogen-activated protein kinase phosphatases (MKPs), the integrin-linked kinase-associated phosphatase (ILKAP) (Leung-Hagesteijn *et al*., 2001), and the tumour suppressor gene product PTEN (Simpson & Parsons, 2001), which all represent attractive candidates for inhibiting the PKB pathway.

What the cellular consequence of this IL-1β-triggered inhibition of PKB is, is difficult to analyse, as ceramide- and NO-mediated inhibition of PKB is part of a fine-tuned system of check and balances that regulate mesangial cell fidelity. Inhibition of apoptosis is a biological response, commonly thought to involve PKB activation. However, mesangial cells need to be exposed to IL-1β or TNFα plus cycloheximide in order to evoke apoptosis, and indeed NO as well as ceramide have been suggested to contribute to mesangial cell apoptosis (Pfeilschifter & Huwiler, 1996b; Mühl *et al*., 1996; Huwiler *et al*., 1999). However, whether, and to what extent, this is due to inhibition of PKB is difficult to prove.

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