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PI 3-kinase and MAP kinase regulate bradykinin induced prostaglandin E_2 release in human pulmonary artery by modulating COX-2 activity

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Abstract Here we studied the role of phosphoinositide 3-kinase (PI 3-kinase) and mitogen activated protein (MAP) kinase in regulating bradykinin (BK) induced prostaglandin E_2 (PGE_2) production in human pulmonary artery smooth muscle cells (HPASMC). BK increased PGE_2 in a three step process involving phospholipase A_2 (PLA_2), cyclooxygenase (COX) and PGE synthase (PGES). BK stimulated PGE_2 release in cultured HPASMC was inhibited by the PI 3-kinase inhibitor LY294002 and the p38 MAP kinase inhibitor SB202190. The inhibitory mechanism used by LY294002 did not involve cytosolic PLA_2 activation or COX-1, COX-2 and PGES protein expression but rather a novel effect on COX enzymatic activity. SB202190 also inhibited COX activity.

Key words: Bradykinin; Cyclooxygenase; Prostaglandin E_2; Phosphoinositide 3-kinase; Mitogen-activated protein kinase

1. Introduction

Bradykinin (BK) is a nonapeptide formed from the proteolytic cleavage of high molecular weight kininogen by plasma kallikrein and is an important inflammatory mediator concerned with the maintenance of normal cardiovascular function [1]. BK produces many of its effects through the secondary generation of prostanoids. For example BK induces increased prostaglandin E_2 (PGE_2) release in both human and bovine cultured airway smooth muscle (ASM) cells and human pulmonary artery smooth muscle cells (HPASMC) [2–4]. PGE_2 has important functional effects including inhibiting smooth muscle cell proliferation and inducing vasodilation and may act as a negative feedback mechanism in the pathogenesis of pulmonary hypertension [5,6].

Prostaglandins are produced from arachidonic acid (AA) by the combined actions of phospholipase A_2 (PLA_2), cyclooxygenase (COX) and specific terminal prostaglandin synthases. PLA_2 mobilises AA from membrane phospholipids and exists in several different forms that are categorised according to molecular weight, calcium dependence and cellular localisation [7,8]. The intracellular, cytosolic PLA_2 ($cPLA_2$) isoforms are activated by increases in intracellular calcium concentrations induced by inflammatory mediators such as BK [9]. AA is then converted to PGH_2 by COX. There are three isoforms of cyclooxygenase, COX-1, COX-2 and COX-3. COX-1, which is constitutively expressed, is associated with the immediate response and basal levels of prostanoid production. COX-2 is induced in response to inflammatory cytokines and mediators and results in increased and sustained prostanoid release [10]. The recently characterised COX-3 is constitutively expressed in specific tissue with highest levels in the brain and heart [11]. Enzymes downstream of COX isomerases and synthases are responsible for the production of terminal prostaglandins. PGH_2 is converted to PGE_2 by prostaglandin E synthase (PGES) of which there are two different forms [12]. Cytosolic PGES (cPGES) is expressed constitutively in many cell types and is associated with COX-1 derived PGH_2 [13]. Microsomal PGES (mPGES) is induced by inflammatory cytokines such as interleukin (IL)-1β and is associated with COX-2 derived PGH_2 [14].

Phosphoinositide 3-kinases (PI 3-K) are a family of kinases that regulate a diverse array of cellular responses such as differentiation, proliferation, activation and survival by generating lipid second messengers [15]. Using the specific PI 3-K inhibitor LY294002 [16], mitogen stimulated proliferation in human airway smooth cells was shown to be mediated by PI 3-Ks [17]. Mitogen activated protein (MAP) kinases are activated by inflammatory mediators [18]. As hypoxic induction of COX-2 in distal HPASMC is dependent on p38 MAP kinase [19] we also studied the effect of MAP kinase inhibitors. The role of PI 3-K in regulating prostanoid synthesis in HPASMC has not been studied. Here we investigate the mechanisms involved in BK induced PGE_2 release in HPASMC focussing on the role of PI 3-K. We found that BK induced PGE_2 was mediated via $cPLA_2$, COX and PGES. We found that LY294002 inhibited PGE_2 production due to a novel effect on COX enzymatic activity.

2. Materials and methods

2.1. Cell culture

Proximal HPASMC were purchased at passage 3 from Clonetics® (BioWhittaker UK, Wokingham, Berkshire, UK). The PASMC were cultured to passage 6 in smooth muscle cell growth medium-2 BulletKit® (Clonetics® BioWhittaker UK).

All experiments were set up in triplicate or quadruplicate using passage 6 cells cultured in 6 or 24 well plates at 37°C in a 5% CO_2,

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Abbreviations: AA, arachidonic acid; BK, bradykinin; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DMSO, dimethyl sulphoxide; HPASMC, human pulmonary artery smooth muscle cells; LY294002, [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one]; MAP kinase, mitogen activated protein kinase; PI 3-K, phosphoinositide 3-kinase; PG, prostaglandin; SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole

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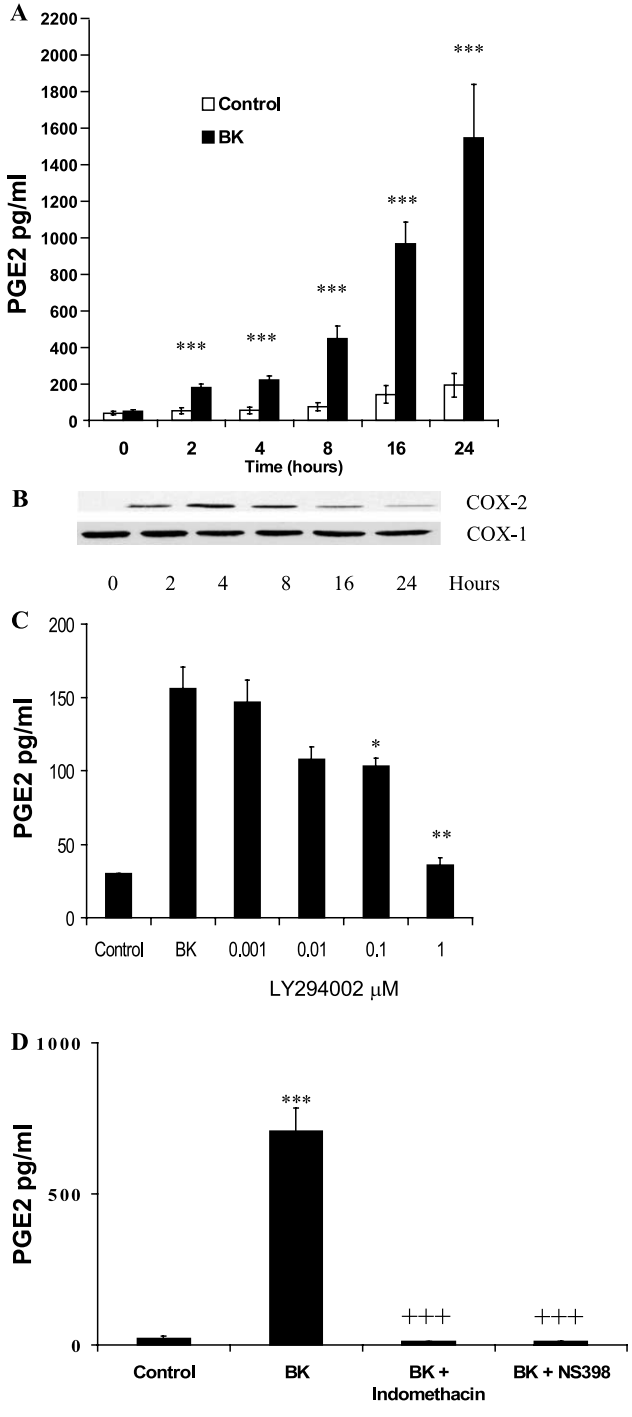
humidified incubator (Leec, Colwick, Nottingham, UK). The confluent cells were growth arrested by serum withdrawal for 24 h. The medium was replaced with fresh serum free medium containing the cytokines or peptides under investigation. The cells were pre-incubated for 1 h with the inhibitors. At the end of the incubation time the culture supernatants were harvested and the protein was extracted from the cells.

2.2. Assessment of cell viability

One well of the 24 well plate in each condition was detached using 0.025% trypsin and 0.01% EDTA. The cell number and percentage viability were assessed using a haemocytometer and 0.4% trypan blue (Sigma, Poole, Dorset, UK).



2.3. PGE_2 assay

PGE_2 levels were assessed by radioimmunoassay (RIA) as described previously [3,20]. The bound [^{3}H]PGE_2 (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK) was measured using the Tri-Carb 2100TR liquid scintillation analyser (Packard Bioscience, Pangbourne, Berkshire, UK). The PGE_2 levels were calculated with Riasmart software (Packard Bioscience). PGE_2 was chosen because it is the main prostaglandin produced by PASMC [6].

2.4. [^{3}H]AA release

Confluent PASMC were growth arrested by withdrawal of serum for 24 h. The medium was then replaced with serum-free medium containing 18.5 kBq/ml of [^{3}H]AA (Amersham Pharmacia Biotech) and the cells incubated for 16 h. The culture supernatant was removed and the cells washed three times in sterile serum free medium. The cells were then treated with or without 10 μM BK and 1 μM LY294002 for 0.25, 0.5, 1, 2, 3 and 4 h. The antibiotic and calcium ionophore calcimycin A23187 was used as a positive control. After the incubation times the supernatants were removed and the released [^{3}H]AA was counted using Tri-Carb 2100TR liquid scintillation analyser and Emulsifier Safe liquid scintillation cocktail (Packard Bioscience). The remaining cells were lysed with 0.1% Triton X-100 (Sigma) and counted as above. The percentage [^{3}H]AA release was calculated as follows:

$$\%\ [^3\mathrm{H}]\mathrm{AA\ release} = \frac{\textit{radioactivity in medium}}{\textit{radioactivity in medium} + \textit{radioactivity in cell lysate}} \times 100$$

2.5. Western blot analysis

Western blotting for COX-1 and COX-2 was performed as described previously [4]. Western blotting for cPGES and mPGES was performed in the same manner but used sheep anti-human polyclonal primary antibodies (Cayman Chemical, Bingham, Nottinghamshire, UK). The goat anti-sheep and anti-mouse horseradish peroxidase conjugated secondary antibody was purchased from BD Biosciences (Cowley, Oxford, UK). Staining intensity of the bands was measured using a densitometer (Syngene, Braintree, Essex, UK) together with Genesnap and Genetools software (Syngene). The figures depicting Western blotting are representative of four blots.

2.6. Assay of COX activity using exogenous AA

Using confluent growth arrested HPASMC, the medium was replaced with medium containing either 1 μM LY294002 or 0.2% dimethyl sulphoxide (DMSO) as a vehicle control and incubated at 37°C for 30 min. The cells were incubated for a further 30, 60, 90 or 120 min with or without 10 μM BK. At the end of the incubation times the supernatants were removed and assayed for PGE_2. The cells were washed twice in sterile phosphate-buffered saline and incubated for a further 30 min with medium containing 1 μM AA. The supernatants were removed and assayed for PGE_2.

2.7. Materials

BK, LY294002 ([2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-

←

Fig. 1. A: Time course of PGE_2 production in PASMC treated with 10 μM BK for 0, 2, 4, 6, 8, 16 and 24 h and unstimulated cells. The PGE_2 released into the culture medium was measured by RIA. Each point represents the mean ± S.E.M. of quadruple determinations from three independent experiments. $^{***}P < 0.001$ by ANOVA. B: Western blotting showing time dependent COX-2 protein induction by BK. PASMC were incubated for 0, 2, 4, 6, 8, 16 and 24 h with 10 μM BK. COX-2 protein expression was maximal at 4 h whereas COX-1 protein expression was constitutive. The figure is representative of four blots from independent experiments. C: Concentration related inhibition of BK induced PGE_2 release by the PI 3-K inhibitor LY294002. Confluent growth arrested HPASMC were cultured with concentrations of LY294002 ranging from 1 nM to 1 μM followed by 4 h incubation with 10 μM BK. $^{**}P < 0.01$, $^{*}P < 0.05$ by ANOVA. Subsequent experiments used 1 μM LY294002. D: The increased BK mediated PGE_2 production ($^{***}P < 0.001$ by ANOVA) is inhibited by 1 μM indomethacin and 1 μM NS398 ($^{+++}P < 0.001$ by ANOVA).

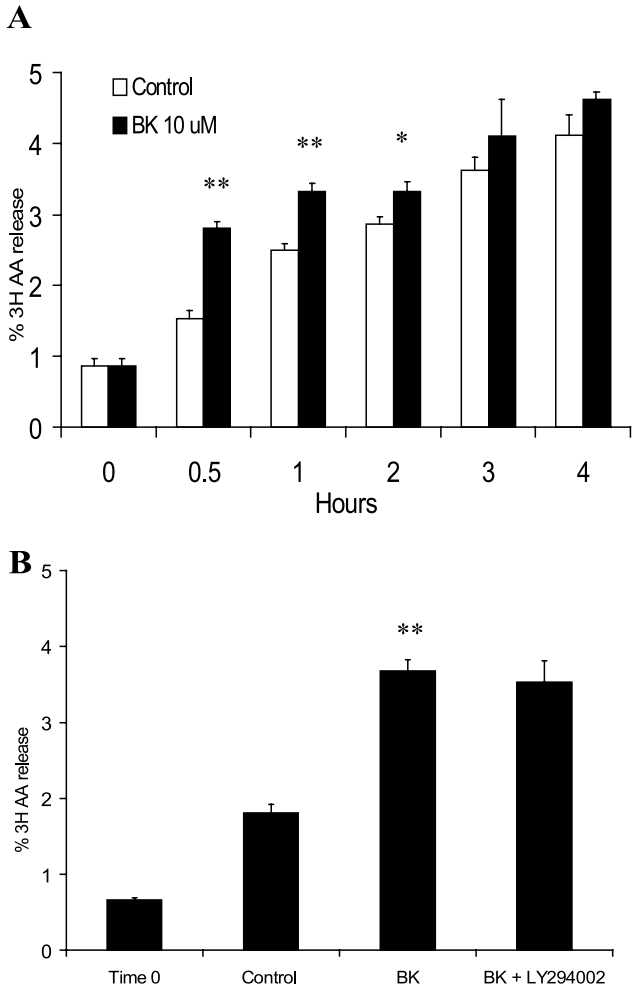


Fig. 2. A: BK stimulation for 30, 60 and 120 min induced a significant increase in [^{3}H]AA release compared with unstimulated cells. Each point represents the mean ± S.E.M. of quadruple determinations from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ by ANOVA. B: Incubation for 1 h with 10 μM BK induced an increase in [^{3}H]AA release which was not inhibited by 1 μM LY294002. Each point represents the mean ± S.E.M. of quadruple determinations from three independent experiments. $^{**}P < 0.01$ by ANOVA.

one]) and DMSO were purchased from Sigma. The MEK inhibitor PD98059 and potent MAP kinase inhibitor SB202190 were purchased from Calbiochem (Merck Biosciences, Nottingham, UK).

2.8. Statistical analysis

The results of the PGE_2 levels and [^{3}H]AA release were expressed as the mean of the triplicate or quadruplicate wells for each experiment which were repeated at least three times. The results shown represent the mean and S.E.M. Analysis of variance (ANOVA) was used to determine significant differences. A P value of < 0.05, two tailed, was considered to be significant.

3. Results

3.1. BK induced PGE_2 production is inhibited by the PI 3-K inhibitor LY294002

We have previously reported that BK induces COX-2 protein and stimulates PGE_2 release in cultured HPASMC [2]. Here we found a significant increase in PGE_2 levels in cells treated with 10 μM BK at 2, 4, 8, 16 and 24 h compared with unstimulated control cells (Fig. 1A). Western blotting showed that BK induced COX-2 protein with bands detected at 2, 4, 8, 16 and 24 h, with the strongest bands at 4 and 8 h, while the constitutively expressed COX-1 protein was constant at all the time points (Fig. 1B). As the COX-2 protein band was

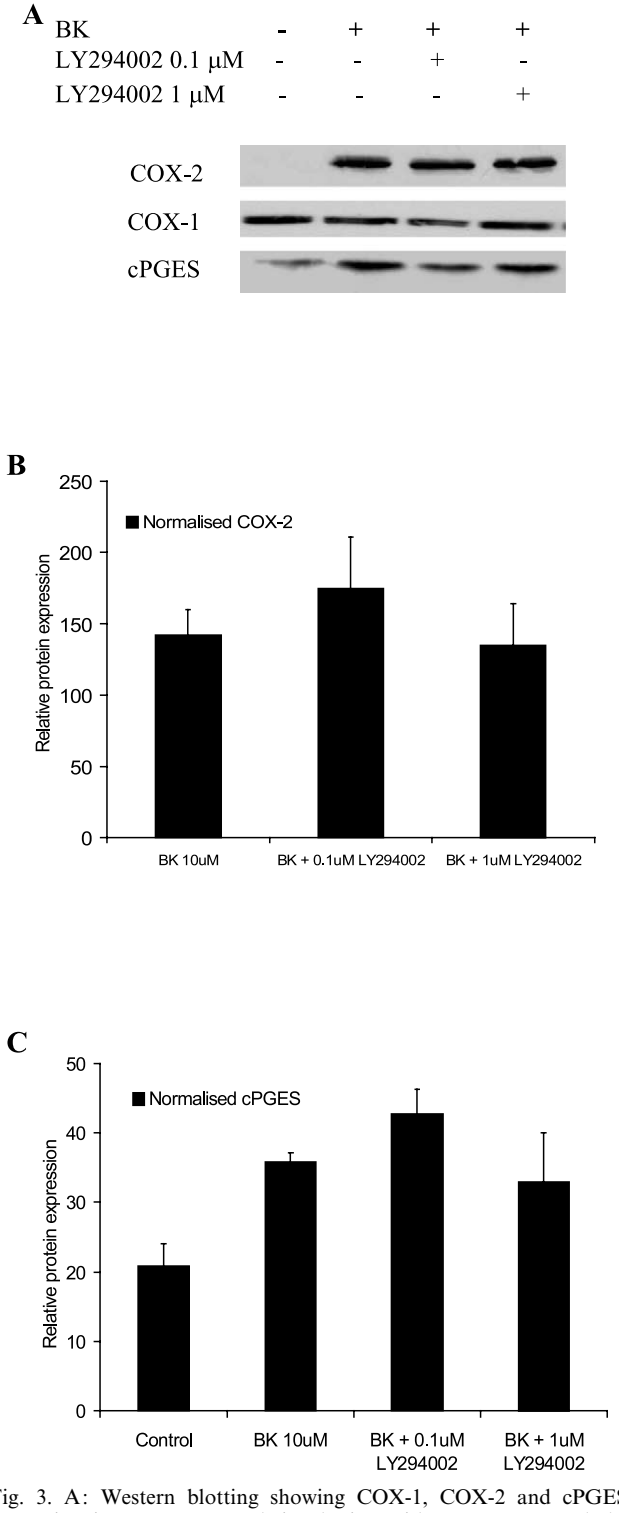


Fig. 3. A: Western blotting showing COX-1, COX-2 and cPGES expression in response to 4 h incubation with 10 μM BK and the affect of LY294002. The blot is representative of four separate experiments. B,C: The relative protein expression measured by densitometry of COX-2 and cPGES expression that has been normalised to COX-1 protein, respectively, $n = 4$.

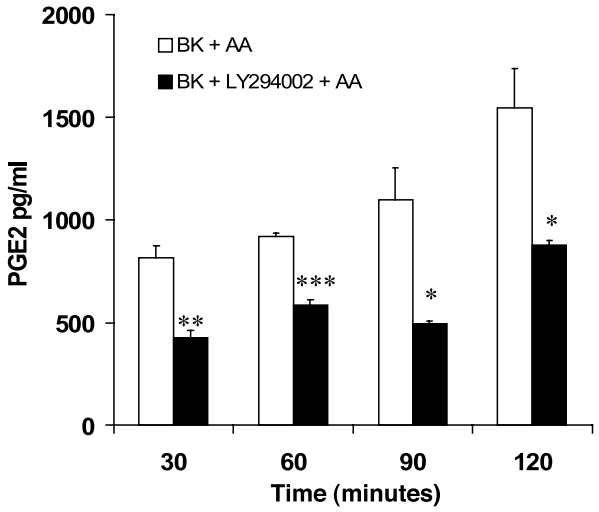


Fig. 4. HPASMC were incubated for 30 min with either 1 μM LY294002 or 0.2% DMSO vehicle control and then cultured for 30, 60, 90 or 120 min with 10 μM BK and the medium removed. Medium containing 1 μM AA was added for a further 30 min. This medium was assayed for PGE_2. The post AA PGE_2 levels reflect the degree of COX activity. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ by ANOVA.

strong at 4 h we chose this time point for most subsequent experiments.

Pre-incubation for 30 min with the specific PI 3-K inhibitor LY294002 before incubation with BK for 4 h resulted in a dose dependent reduction in PGE_2 release which was significant with 0.1 and 1 μM LY294002 (Fig. 1C). 0.2% DMSO was used as a vehicle control. 1 μM LY294002 gave the most significant reduction in PGE_2 without affecting cell viability. This concentration was used in the subsequent experiments. LY294002 had no effect on basal PGE_2 production in unstimulated control cells suggesting that it was not acting on COX-1.

3.2. LY294002 does not inhibit BK increased $cPLA_2$ activity

Confluent, growth arrested HPASMC were incubated with 10 μM BK for 0.5, 1, 2, 3, and 4 h. BK induced increased [^{3}H]AA release at 0.5, 1 and 2 h compared with unstimulated control cells (Fig. 2A). However, there was no reduction in BK mediated [^{3}H]AA release after pre-incubation with 1 μM LY294002 (Fig. 2B).

3.3. LY294002 does not inhibit COX-1, COX-2 or cPGES protein expression

In order to determine if LY294002 affected COX protein or PGES expression, Western blotting was performed using specific monoclonal antibodies to COX-1 or COX-2 and a rabbit polyclonal antibody to cPGES. COX-1 and cPGES were constitutively expressed. Maximal COX-2 expression was induced by incubation for 4 h with BK. The mPGES protein was induced by IL-1β but not BK in HPASMC but only after 48 h incubation (data not shown). There was no detectable reduction in either COX-1, COX-2 or cPGES expression as a result of pre-treatment with LY294002 (Fig. 3).

3.4. LY294002 inhibits COX activity

To determine whether the reduction in PGE_2 by inhibition of PI 3-K was due to a decrease in COX activity we measured COX activity by adding exogenous AA and measuring PGE_2 production. PASMC were cultured for 30 min with medium containing either 0.2% DMSO or 1 μM LY294002 followed by 30, 60, 90 or 120 min with or without 10 μM BK. The supernatants were removed for PGE_2 assay and replaced with medium containing 1 μM AA for a further 30 min. This medium was removed and assayed for PGE_2. There was a reduction in PGE_2 release post AA in cells that had been incubated with LY294002 prior to BK suggesting inhibition of COX activity (Fig. 4).

3.5. SB202190 inhibits BK mediated PGE_2 and COX activity

To investigate whether MAP kinase pathways were involved in BK induced PGE_2 production we performed experiments with the potent p38 MAP kinase inhibitor SB202190 and the MEK inhibitor PD98059. SB202190 significantly reduced BK mediated PGE_2 release (Fig. 5A) and COX activity (Fig. 5B) whereas PD98059 had no effect on COX activity (data not shown).

4. Discussion

We found that BK induced PGE_2 release in a three step process involving mobilisation of AA from $cPLA_2$, induction

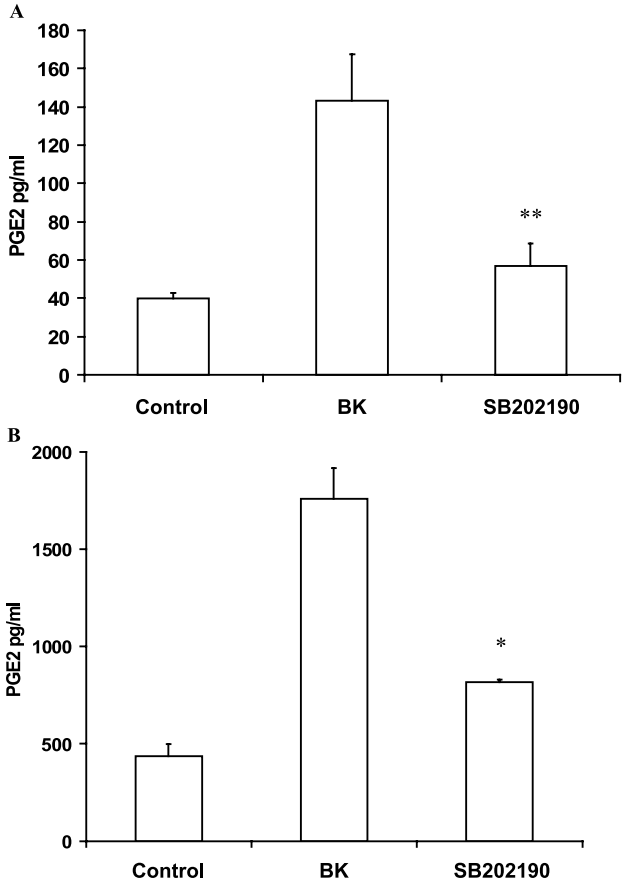


Fig. 5. A: HPASMC were incubated for 30 min with either 1 μM of the potent p38 MAP kinase inhibitor SB202190 or 0.2% DMSO vehicle control and then cultured for 4 h with 10 μM BK and the medium removed and assayed for PGE_2. B: Medium containing 1 μM AA was added for a further 30 min. This medium was assayed for PGE_2. The post AA PGE_2 levels reflect the degree of COX activity. $^{**}P < 0.01$, $^{*}P < 0.05$ by ANOVA.

of COX-2 and action of cPGES. There was no increase in mPGES, the inducible form of PGES. Cytosolic PGES is expressed constitutively whereas the microsomal PGES is inducible in some cells by inflammatory cytokines, such as IL-1β. Western blotting showed that cPGES expression did not change in the presence of LY294002. Culture of HPASMC with IL-1β resulted in induction of mPGES at 48 and 72 h but not at 4 h. BK did not induce mPGES at any time point (data not shown).

The increased PGE_2 release with BK was abrogated by the presence of the specific PI 3-K inhibitor LY294002 suggesting that one or more mechanisms involved in PGE_2 generation is dependent on PI 3-K signalling. We designed a series of experiments to explore the site in the pathway that was involved. We initially studied the effect of LY294002 on $cPLA_2$, the enzyme upstream of COX. We found no effect of LY294002 on $cPLA_2$ activity measured as AA release. We have previously shown that different COX enzymes participate in PGE_2 release at different times after BK stimulation. We found no effect of LY294002 on COX-1 protein expression or basal COX-1 derived PGE_2 levels. Similarly COX-2 protein expression did not change significantly in the presence of LY294002 suggesting that it was not acting to prevent COX-2 induction. However, when we measured COX activity by adding exogenous AA to BK stimulated cells we found that it was inhibited by LY294002. cPGES, the downstream enzyme that converts PGH_2 to PGE_2, was unchanged.

These results contrast with studies in other biological systems. In colonic epithelial cells tumour necrosis factor-α induced COX-2 and increased PGE_2 production which was paradoxically enhanced by PI 3-K inhibition [21]. Both IL-1β mediated COX-2 induction and PGE_2 production in canine tracheal smooth muscle cells was reduced by PI 3-K inhibition, suggesting partial involvement of PI 3-K activation in COX-2 induction [22]. In contrast LY294002 inhibited staurosporine induced PGE_2 generation in rat peritoneal macrophages by inhibiting $cPLA_2$ but did not affect COX-2 induction [23]. Previous studies in HeLa cells have shown that BK activates PI 3-K [24]. Our results with SB202190 suggest that p38 MAP kinases also play a role in regulating COX-2 activity.

Collectively these studies suggest that PI 3-K can regulate PGE_2 production through different mechanisms in a cell and species specific manner. In HPASMC PI 3-K and p38 MAP kinase inhibition results in a reduction in PGE_2 release mediated by inhibition of COX enzymatic activity.

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