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Differential regulation of cyclooxygenase isozymes by cAMP-elevating agents

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Abstract

Bovine aortic endothelial cells produce prostacyclin as their major arachidonic acid metabolite. cAMP, in turn, is the second messenger for prostacyclin. In the present study, we investigated the effects of cAMP-elevating agents on prostacyclin production by bovine aortic endothelial cells. Treatment of resting bovine aortic endothelial cells with cAMP-elevating agents inhibited prostacyclin production and cyclooxygenase activity, without affecting arachidonic acid release. No change was detected in cyclooxygenase-1 protein expression. The specific inhibitor of protein kinase A, Rp-cAMPS (adenosine 3′,5′-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt), and the phosphatase inhibitor, okadaic acid, both suppressed cAMP-induced inhibition, suggesting that this inhibition is mediated by a phosphorylation–dephosphorylation cascade, which is possibly protein kinase A-dependent. In lipopolysaccharide-treated cyclooxygenase-2 expressing bovine aortic endothelial cells, where cyclooxygenase-1 activity was selectively inhibited, dibutyryl cAMP failed to inhibit cyclooxygenase-2 activity. Cyclooxygenase-2 protein was induced upon treatment with dibutyryl cAMP and further induction of cyclooxygenase-2 protein was effected by IBMX (3-isobutyl-1-methyl-xanthine) and dibutyryl cAMP in bacterial lipopolysaccharide-stimulated cells. These results suggest that increased cellular cAMP selectively inhibits cyclooxygenase-1 activity without altering cyclooxygenase-1 protein expression, and at the same time, up-regulates cyclooxygenase-2 protein. This complex regulation of cyclooxygenase activity and protein expression by cAMP may represent a prostacyclin-induced autoregulatory mechanism in bovine aortic endothelial cells.

Keywords: cAMP; Cyclooxygenase; Prostacyclin; Lipopolysaccharide; Endothelium

1. Introduction

Bovine aortic endothelial cells synthesize prostacyclin as their major arachidonic acid product (Eldor et al., 1983). Prostacyclin is a potent vasodilator and antithrombotic agent, the synthesis of which is regulated by various inflammatory and mitogenic stimuli (e.g., phorbol esters, cytokines, lipopolysaccharide) (Akarasereenont et al., 1995). Previously, stimulation by these agents was attributed to actions at the phospholipase level, since the only regulatable and rate-limiting step in prostaglandin production was thought to be the availability of arachidonic acid (Smith et al., 1991). However, more recently, another regulatable step in the control of prostaglandin synthesis has been characterized at the cyclooxygenase level (Reddy and Herschman, 1994). Cyclooxygenase catalyzes the conversion of arachidonic acid to prostaglandin H_2, which is further metabolized to other prostaglandins and thromboxane. At present, two isoforms of cyclooxygenase have been identified, cyclooxygenase-1 and cyclooxygenase-2 (Kujubu et al., 1991; Xie et al., 1991). The former is mostly constitutively expressed in prostanoid-producing cells, while the latter is inducible (Smith et al., 1996). Differences in protein expression, subcellular localization and arachidonic acid utilization between cyclooxygenase-1 and cyclooxygenase-2 have been reported in various cell systems (Morita et al., 1995; Liu and Rose, 1996; Thore et al., 1996; Reddy and Herschman, 1997). In human umbilical vein endothelial cells, upon stimulation with interleukin-1 or phorbol ester, cyclooxygenase-2 expression and activity were enhanced, whereas cyclooxygenase-1 protein levels remained practically unchanged (Habib et al., 1993).

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Prostacyclin is known to exert its effects through receptors coupled to adenylate cyclase (Coleman et al., 1994). Studies with endothelial cells have shown that prostacyclin or its analogs increases intracellular cAMP levels (Langeler and van Hinsbergh, 1991; Schröder and Schrör, 1993). Earlier work on endothelial cells indicated that increased cAMP levels inhibit prostacyclin production (Adler et al., 1981), but whether this occurred at the level of protein expression or involved posttranslational regulation of cyclooxygenase activity was not investigated. More conflicting data have been reported in other studies. Thus, in Madin–Darby canine kidney (MDCK) cells and in platelets, elevation of intracellular cAMP levels inhibited prostaglandin production via inhibition of cyclooxygenase activity (Schafer et al., 1980; Hassid, 1983), whereas in osteoblasts (Oshima et al., 1991) and granulosa cells (Sirois and Richards, 1993), cAMP stimulated cyclooxygenase activity and cyclooxygenase-2 protein expression. We undertook this study to further clarify the effects of elevated cAMP levels and of phosphatase inhibition on prostacyclin production in bovine aortic endothelial cells. Also, in view of the recent characterization of cyclooxygenase-2, we examined the possibility of differential regulation of basal (cyclooxygenase-1) and stimulated (cyclooxygenase-2) cyclooxygenase activities and protein expression by agents that elevate intracellular levels of cAMP in bovine aortic endothelial cells.

2. Materials and methods

2.1. Materials

The following materials were used. Arachidonic acid, lipopolysaccharide (serotype 0127:B8), dibutyryl-cAMP (DBcAMP), *erythro*-9-(2-hydroxy-3-nonyl) adenine (EHNA), bovine serum albumin, benzamidine, trypsin inhibitor type II-S, leupeptin, deoxycholate, indomethacin and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma (St. Louis, MO, USA). Forskolin and Rp-cAMPS (adenosine 3′,5′-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt) were from RBI (Natick, MA, USA). Ro-20-1724 (4-[(3-butoxy-4-methoxy phenyl)methyl]-2-imidazolidinone) and okadaic acid were purchased from Calbiochem (La Jolla, CA, USA). Rabbit polyclonal antibody to cyclooxygenase-2 and mouse monoclonal antibody to cyclooxygenase-1 were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Peroxidase-conjugated goat antimouse and antirabbit antibodies were from Jackson Immunoresearch Laboratories (West Baltimore, USA).

2.2. Cell cultures

Bovine aortic endothelial cells were kindly provided by Dr. I. Vlodavski (The Hebrew University, Jerusalem, Israel) and were used between passages 10 and 16. Bovine aortic endothelial cells were seeded in 24-well tissue culture plates (Costar, Cambridge, MA) with Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Beth Haemek, Israel) containing 10% calf serum (Sigma), 2 mM L-glutamine, and penicillin–streptomycin (Biological Industries) at final concentrations of 100 U/ml and 100 Fg/ml, respectively. The cells were grown at 37°C in a humidified 8% CO_2–92% air atmosphere. Basic fibroblast growth factor (basic FGF) from bovine pituitary glands (2 ng/ml, Sigma) was added every other day until the cultures reached confluency. Cultures were used at least 10 days after reaching confluency. Experiments were performed in the presence of 0.1% serum, unless noted otherwise.

2.3. Determination of 6-keto-prostaglandin $F_{1\alpha}$ and cyclooxygenase assay

Bovine aortic endothelial cells were incubated with the various compounds for the lengths of time indicated for each experiment. At the end of each experiment, the supernatants were collected and used for the determination of 6-keto-prostaglandin $F_{1\alpha}$, the stable hydration product of prostacyclin. To measure cyclooxygenase activity, a saturating concentration of arachidonic acid (50 μM) was added after incubation with the test agent and the cells were incubated for an additional 15 min. The medium was then collected and 6-keto- prostaglandin $F_{1\alpha}$ was measured by single-antibody radioimmunoassay with dextran-coated charcoal precipitation. The radioimmunoasay was performed in duplicate for each sample. Rabbit antiserum to 6-keto-prostaglandin $F_{1\alpha}$ was purchased from Sigma, and tritium-labeled 6-keto-prostaglandin $F_{1\alpha}$ (175 Ci/mmol) was obtained from the Radiochemical Center (Amersham, UK). The sensitivity of the assay was 15 pg/tube. The 6-keto-prostaglandin $F_{1\alpha}$ antiserum cross-reacted with other prostaglandins (at 50% displacement) as follows: prostaglandin E_1, 22%; prostaglandin E_2, 10%; prostaglandin $F_{1\alpha}$, 16%; prostaglandin $F_{2\alpha}$, 10%; other prostaglandins, $< 1\%$.

2.4. Arachidonic acid release

Bovine aortic endothelial cells were preincubated for 24 h in medium containing 1 μCi/ml [^{3}H]arachidonic acid (200 Ci/mmol, DuPont NEN) to label cellular lipids. Unincorporated [^{3}H]arachidonic acid was removed by washing the cells four times and test agents were then added to the medium containing 1% bovine serum albumin. The radioactivity released into the medium was determined by liquid scintillation counting (1214 RackBeta, LKB Wallac, Turku, Finland).

2.5. Protein determination

The amount of protein in each well was determined by the Bradford method (acidic solution of Coomassie brilliant blue G-250) using the Bio-Rad protein assay. Bovine serum albumin was used as standard.

2.6. Western blots

Bovine aortic endothelial cells were plated in six-well plates and were exposed to the various compounds. At the end of each experiment, cells were lysed in 100 μl lysis buffer (containing 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 150 mM sodium chloride, 20 mM Tris–HCl, 1 mM benzamidine, 15.5 μg/ml trypsin inhibitor, 100 μg/ml leupeptin) per well. Then cells were scraped off and centrifuged for 2 min at $1000 \times g$. The solubilized enzyme was mixed with sodium dodecyl sulfate (SDS) Laemmli loading buffer (500 mM Tris–HCl, pH 6.8, 2% w/v SDS, 0.05% w/v bromophenol blue, 10% glycerol) reduced with 5% v/v β-mercaptoethanol and boiled for 5 min. Ten micrograms (cyclooxygenase-1) or 40 micrograms (cyclooxygenase-2) of total protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean II electrophoresis cell (Bio-Rad, CA, USA). Proteins were transferred onto nitrocellulose using the Mini Transfer Blot electrophoretic cell (Bio-Rad). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with Tween 20 (20 mM Tris–HCl, 500 mM NaCl, 0.1% Tween 20) (TBS-T) for 1 h. After being washed with TBS-T, the blots were incubated overnight with either mouse monoclonal antibody to cyclooxygenase-1 (1:1000 dilution) or rabbit polyclonal antibody to cyclooxygenase-2 (1:1000 dilution). Following TBS-T washing, blots were incubated for 1 h with either antimouse or antirabbit immunoglobulin G (IgG) horseradish peroxidase-linked secondary antibodies (1:10,000 dilution), respectively. Signals were visualized on X-ray films by the use of enhanced chemiluminescence reagents (Cayman Chemicals).

3. Results

3.1. Effect of DBcAMP, IBMX and forskolin on endothelial prostacyclin production and arachidonic acid release

Three agents known to elevate intracellular cAMP levels by different mechanisms, namely forskolin, an activator of adenylyl cyclase; DBcAMP, a permeable cAMP analog; and IBMX, an inhibitor of phosphodiesterase, were used. As shown in Table 1, elevation of intracellular cAMP levels by either IBMX or DBcAMP markedly inhibited prostacyclin production without significantly altering arachidonic acid release, although there was a trend (insignificant) towards increased arachidonic acid release. Forskolin (0.05 mM) did not significantly alter prostacyclin production.

3.2. Effect of DBcAMP, phosphodiesterase inhibitors and forskolin on endothelial cyclooxygenase activity

In bovine aortic endothelial cells, two major forms of phosphodiesterase have been characterized: a cGMP-stimulated form (phosphodiesterase-2) and a cAMP-specific form (phosphodiesterase-4) (Lugnier and Schin, 1990). Accordingly, a nonspecific phosphodiesterase inhibitor, IBMX, as well as selective inhibitors of phosphodiesterase-2 and phosphodiesterase-4, EHNA and Ro-20-1724, respectively (Beavo, 1995), were used in these experiments. Table 2 shows that cyclooxygenase activity was inhibited in a dose-dependent manner by DBcAMP and by the phosphodiesterase inhibitors. Forskolin, as well as the phosphodiesterase inhibitors, produced significant decreases in cyclooxygenase activity, and the combination of forskolin with the lower concentrations of the phosphodiesterase inhibitors resulted in potentiation of this action. Since resting bovine aortic endothelial cells have been shown to express only cyclooxygenase-1 activity (Mitchell et al., 1994; Rosenstock et al., 1997), the results shown in Table 2 can probably be ascribed to cyclooxygenase-1.

To determine the time dependence of the changes in cyclooxygenase activity that were induced by elevated

Table 1
Effect of forskolin, IBMX and DBcAMP on prostacyclin production and arachidonic acid release
Bovine aortic endothelial cells were incubated for 3 h in the absence or presence of forskolin, IBMX or DBcAMP. Supernatants were collected and prostacyclin was determined as 6-keto-prostaglandin $F_{1\alpha}$. Results are presented as percentage of control. The mean control value (100%) was 25.7 ± 3.1 pg 6-keto-prostaglandin $F_{1\alpha}$/μg protein. For arachidonic acid (AA) release, bovine aortic endothelial cells were prelabeled for 24 h with [^{3}H]arachidonic acid. Cells were washed three times and test agents were added to media containing 1% bovine serum albumin. After a 3-h incubation, arachidonic acid release was measured by liquid scintillation counting. Results are presented as percentage of control. The mean control value (100%) was 1706.9 ± 105.8 dpm. Data are means ± SEM of triplicate determinations. The results are representative of three separate experiments.

cAMP-elevating agents	Concentration (mM)	6-Keto-prostaglandin $F_{1\alpha}$ (percentage of control)	Arachidonic acid release (percentage of control)
Control	0	100.0	100.0
Forskolin	0.05	89.9 ± 0.7	120.9 ± 6.6
IBMX	1	40.2 ± 5.3[a]	121.4 ± 7.7
DBcAMP	1	32.2 ± 4.6[b]	109.3 ± 8.1

[a] $P < 0.01$ vs. control.
[b] $P < 0.002$ vs. control.

Table 2
Effect of forskolin, phosphodiesterase inhibitors and DBcAMP on cyclooxygenase activity
Bovine aortic endothelial cells were incubated for 3 h in the absence or presence of forskolin, IBMX, EHNA, Ro 20-1724 or DBcAMP. To determine cyclooxygenase (COX) activity, a saturating concentration of arachidonic acid (50 μM) was added in the last 15 min of incubation, and 6-keto-prostaglandin $F_{1\alpha}$ in the supernatants, representing cyclooxygenase activity, was determined by radioimmunoassay. Results are presented as percentage of control. The mean control value (100%) was 1.4 ± 0.4 ng 6-keto-prostaglandin $F_{1\alpha}$ / μg protein. Data are means ± SEM of triplicate determinations. The results are representative of three separate experiments.

cAMP-elevating agents	COX activity (percentage of control)
Control	100.0
Forskolin (0.05 mM)	67.3 ± 2.3^a
IBMX (0.1 mM)	34.3 ± 4.8^b
IBMX (0.1 mM) + forskolin (0.05 mM)	20.5 ± 2.4^c
IBMX (1 mM)	7.9 ± 1.9^c
IBMX (1 mM) + forskolin (0.05 mM)	8.8 ± 1.0^c
EHNA (0.1 mM)	62.8 ± 0.9^a
EHNA (0.1 mM) + forskolin (0.05 mM)	50.5 ± 1.5^b
EHNA (1 mM)	34.4 ± 2.8^b
EHNA (1 mM) + forskolin (0.05 mM)	38.8 ± 2.6^b
Ro 20-1724 (0.1 mM)	79.7 ± 5.9
Ro 20-1724 (0.1 mM) + forskolin (0.05 mM)	54.4 ± 8.1^a
Ro 20-1724 (1 mM)	57.7 ± 4.7^a
Ro 20-1724 (1 mM) + forskolin (0.05 mM)	64.5 ± 4.3^a
DBcAMP (0.1 mM)	62.9 ± 3.0^a
DBcAMP (1 mM)	11.8 ± 2.3^c

[a] $P < 0.05$ vs. control.
[b] $P < 0.01$ vs. control.
[c] $P < 0.002$ vs. control.

cAMP levels, bovine aortic endothelial cells were incubated with DBcAMP or IBMX for variable periods of time (Fig. 1A). With both agents, strong inhibition of cyclooxygenase activity was evident at 15 min, peaked at 60 min and persisted for 24 h. In order to determine whether this inhibition is dependent on the constant presence of cAMP-elevating agents, the reversibility of the inhibition was investigated. Bovine aortic endothelial cells were preincubated for 1 h in the presence of DBcAMP or IBMX, which were then washed away. Fig. 1B shows that the recovery of cyclooxygenase activity after incubation in media free of DBcAMP and IBMX was slow and took at least 12 h (Fig. 1B).

3.3. Effect of DBcAMP on endothelial cyclooxygenase-1 protein expression

To determine whether inhibition of cyclooxygenase activity by cAMP involves an alteration in cyclooxygenase-1 protein, Western blot analyses were performed. As depicted in Fig. 2, the amounts of cyclooxygenase-1 protein were similar in control cells and cells treated with DBcAMP for different periods of time.

3.4. Involvement of phosphorylation in the suppression of cyclooxygenase activity by DBcAMP and IBMX

As cAMP activates protein kinase A, we examine the actions of Rp-cAMPS, a specific protein kinase A inhibitor, and of okadaic acid, an inhibitor of protein phosphatase type 2A. As shown in Fig. 3, the inhibitory effects

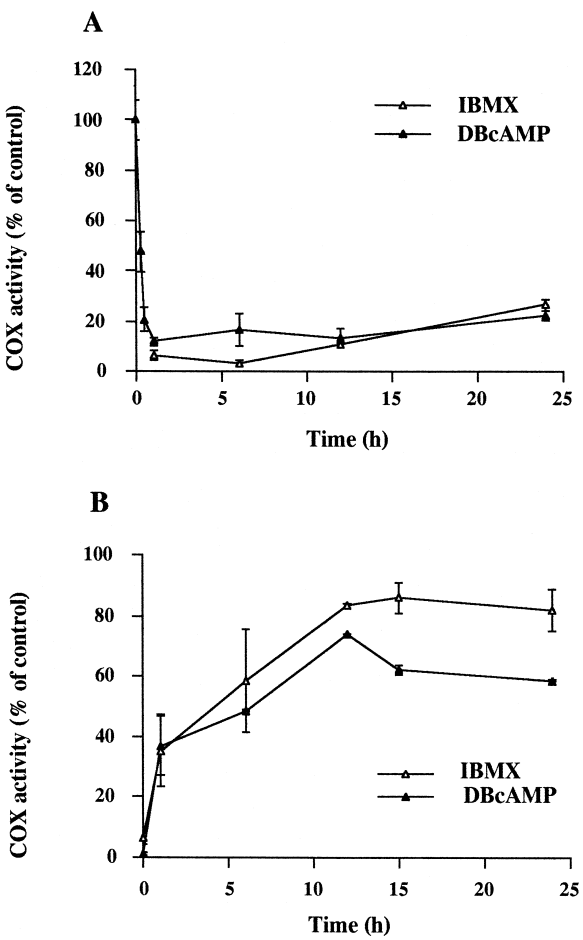

Fig. 1. Time-dependent changes in cyclooxygenase activity induced by DBcAMP and IBMX. Panel A: bovine aortic endothelial cells were incubated with DBcAMP (1 mM) or IBMX (1 mM) for the indicated periods of time. Panel B: bovine aortic endothelial cells were preincubated for 1 h with DBcAMP (1 mM) or IBMX (1 mM). Then the cells were washed three times and further incubated in fresh medium for the indicated periods of time. To determine cyclooxygenase activity, a saturating concentration of arachidonic acid (50 μM) was added in the last 15 min of incubation, and 6-keto-prostaglandin $F_{1\alpha}$ in the supernatant, representing cyclooxygenase activity, was determined by radioimmunoassay. Results are presented as percentage of control. Mean control values (100%) for panels A and B were 1.2 ± 0.2 and 0.8 ± 0.3 ng 6-keto-prostaglandin $F_{1\alpha}$ / μg protein, respectively. Data are means ± SEM of triplicate determinations and are representative of three separate experiments.

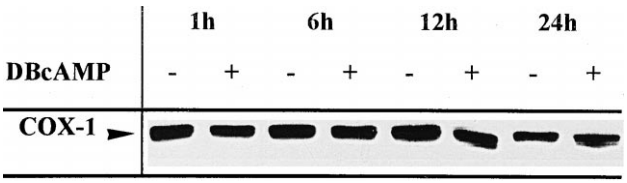

Fig. 2. Western blot of cyclooxygenase-1 protein in DBcAMP-treated cells. Bovine aortic endothelial cells were incubated with or without DBcAMP (1 mM) for the indicated periods of time. Cells were lysed and aliquots of 10 μg protein were loaded in each lane, transferred to nitrocellulose, and incubated with anticyclooxygenase-1 antibody.

of DBcAMP and of IBMX on cyclooxygenase activity were reversed both by Rp-cAMPS and by okadaic acid.

3.5. Effect of DBcAMP on endothelial cyclooxygenase-2 protein expression

Bovine aortic endothelial cells can be induced by lipopolysaccharide to express cyclooxygenase-2 (Akarasereenont et al., 1995). We therefore examined the effect of lipopolysaccharide on cyclooxygenase-2 protein expression by Western blot analysis. As shown in Fig. 4A, cyclooxygenase-2 protein was undetectable in control bovine aortic endothelial cells, whereas lipopolysaccharide time-dependently induced the expression of significant amounts of cyclooxygenase-2 protein. The protein appeared at 3 h, peaked at 6 h and decreased gradually over the next 18 h of exposure to lipopolysaccharide. As shown in Fig. 4B, DBcAMP slightly enhanced cyclooxygenase-2 protein expression. Maximal stimulation of cyclooxygenase-2 was observed after 6 h of incubation with DBcAMP and the protein was again undetectable after 24 h. Fig. 4C shows that a 24-h incubation with lipopolysaccharide in the presence of IBMX or DBcAMP further enhanced lipopolysaccharide-induced cyclooxygenase-2 expression. In control experiments with cells treated with IBMX for 24 h in the absence of lipopolysaccharide, no alterations in cyclooxygenase-2 protein were observed (data not shown).

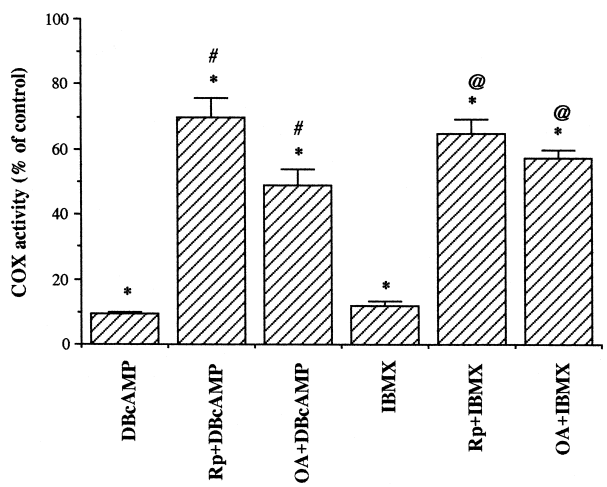

Fig. 3. Inhibitors of phosphorylation and dephosphorylation antagonize DBcAMP- and IBMX-induced inhibition of cyclooxygenase activity. Bovine aortic endothelial cells were incubated for 3 h in the absence or presence of DBcAMP (1 mM) or IBMX (1 mM). In some cells, Rp-cAMP (Rp; 50 μM) or okadaic acid (OA; 1 nM) was added simultaneously with DBcAMP or IBMX. To determine cyclooxygenase activity, a saturating concentration of arachidonic acid (50 μM) was added in the last 15 min of incubation, and 6-keto-prostaglandin $F_{1\alpha}$ in the supernatant, representing cyclooxygenase activity, was determined by radioimmunoassay. Results are presented as percentage of control. The mean control value (100%) was 1.2 ± 0.1 ng 6-keto-prostaglandin $F_{1\alpha}$ / μg protein. Data are means ± SEM of triplicate determinations and are representative of three separate experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. DBcAMP; $^{@}P < 0.05$ vs. IBMX.

3.6. Effect of DBcAMP on cyclooxygenase-2 activity

To determine the effect of elevated cAMP levels on cyclooxygenase-2 activity, bovine aortic endothelial cells were first stimulated with lipopolysaccharide for 6 h to achieve maximal induction of cyclooxygenase-2 protein (Fig. 4A). Cells were then incubated for 30 min with or without 0.45 mM valeryl salicylate, a specific cyclooxy-

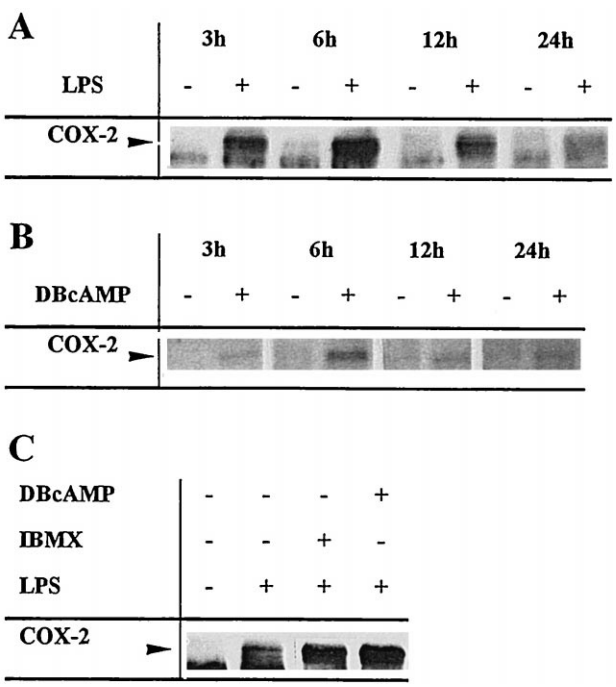

Fig. 4. Western blots of cyclooxygenase-2 protein in cells treated with lipopolysaccharide, DBcAMP, IBMX and forskolin. Panel A: bovine aortic endothelial cells were incubated with or without lipopolysaccharide (1 μg/ml) for the periods of time indicated. Panel B: bovine aortic endothelial cells were incubated with or without DBcAMP (1 mM). Panel C: bovine aortic endothelial cells were incubated for 24 h with lipopolysaccharide (1 μg/ml) alone or in the presence of DBcAMP or IBMX. Cells were lysed, and 40 μg protein aliquots were loaded in each lane, transferred to nitrocellulose, and incubated with anticyclooxygenase-2 antibody.

genase-1 inhibitor (Bhattacharyya et al., 1995), or 10 nM indomethacin, a concentration at which indomethacin has been shown to inhibit cyclooxygenase-1 activity preferentially (Mitchell et al., 1994). Thus, in valeryl salicylate- or low-concentration indomethacin-treated lipopolysaccharide-induced cells, the measured residual enzyme activity probably reflects mainly cyclooxygenase-2 activity. In the last 30 min of the experiment, DBcAMP was also added to the medium. As shown in Fig. 5A and B, in the absence of indomethacin and valeryl salicylate, DBcAMP inhibited cyclooxygenase activity in both control and lipopolysaccharide-treated cells. However, in the presence of indomethacin (Fig. 5A) or valeryl salicylate (Fig. 5B), where presumably mainly cyclooxygenase-2 activity was measured, DBcAMP did not alter lipopolysaccharide-stimulated activity. Similar results were obtained with low concentrations of aspirin (10 μM), which also inhibited cyclooxygenase-1 preferentially (data not shown).

4. Discussion

The present data indicate that elevated intracellular cAMP concentrations promptly inhibit prostacyclin pro-

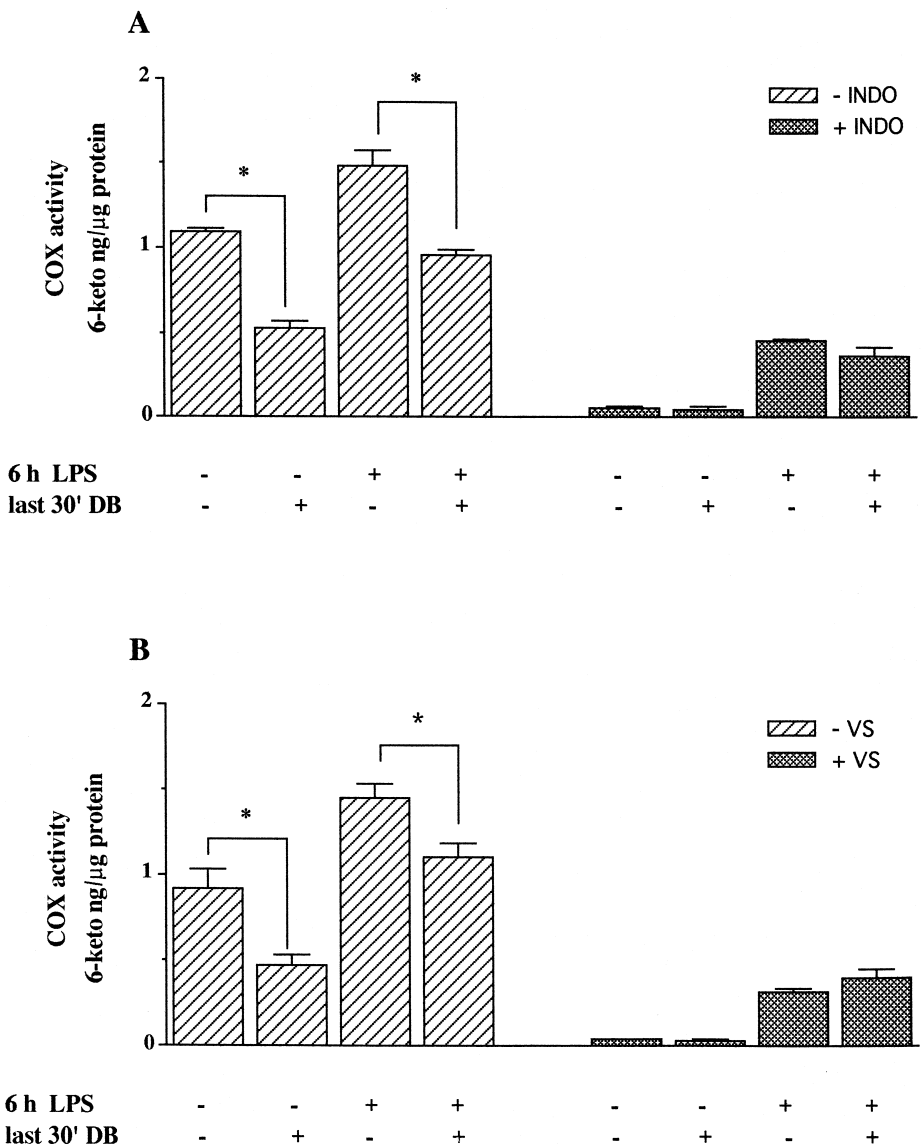

Fig. 5. Effects of indomethacin (INDO) and of valeryl salicylate (VS) on DBcAMP induced inhibition of resting and lipopolysaccharide-stimulated cyclooxygenase activity. Bovine aortic endothelial cells were preincubated with or without lipopolysaccharide (1 μg/ml) for 6 h. Panel A: cells were incubated for 30 min with or without 10 nM INDO. Where indicated, DBcAMP was added for 30 min concomitantly with INDO. Panel B: cells were incubated for 30 min with or without 0.45 mM VS. Where indicated, DBcAMP was added for 30 min concomitantly with VS. To determine cyclooxygenase activity, a saturating concentration of arachidonic acid (50 μM) was added in the last 15 min of incubation, and 6-keto-prostaglandin $F_{1\alpha}$ in the supernatant, representing cyclooxygenase activity, was determined by radioimmunoassay. Data are means ± SEM of triplicate determinations and are representative of three separate experiments. $^{*}P < 0.05$.

duction in bovine aortic endothelial cells, probably by posttranslational suppression of cyclooxygenase-1 activity. Elevated cAMP concentrations also induced cyclooxygenase-2 protein expression, but this action was not reflected by an increased production of prostacyclin. Thus, of the two apparently conflicting actions of cAMP in bovine aortic endothelial cells, inhibition of cyclooxygenase-1 activity predominates. Our results may thus resolve the apparently controversial reports on the action of cAMP on prostaglandin synthesis in different cell types. In cells expressing cyclooxygenase-2, such as monocytes, granulosa cells and osteoblastic cells, increased cAMP enhances cyclooxygenase-2 protein expression and subsequently, cyclooxygenase activity and prostaglandin production (Oshima et al., 1991; Sirois and Richards, 1993; Mertz et al., 1994). By contrast, wherever cyclooxygenase-1 predominates, as in platelets (Schafer et al., 1980), renal MDCK cells (Hassid, 1983), and bovine aortic endothelial cells (Adler et al., 1981), cAMP drastically inhibits prostaglandin synthesis.

The inhibition of cyclooxygenase activity was substantial and long-lasting. It occurred as early as 15 min after addition of the cAMP-elevating agent and lasted for 24 h with the continuous presence of the stimulus. An unusually long recovery of cyclooxygenase activity from this inhibition was also observed (Fig. 1), which could be attributed either to de novo protein synthesis or to a slow process of dephosphorylation. In contrast to other studies, where cAMP was shown to inhibit arachidonic acid release along with prostaglandin production (Adler et al., 1981; Hassid, 1983), our results, obtained in nonstimulated cells, excluded phospholipase inhibition from the cAMP-mediated effect. The possibility, that cAMP could influence the availability of arachidonic acid by affecting arachidonic acid incorporation into and distribution within phospholipid classes (Fonteh and Chilton, 1992), has not been evaluated. This could be of interest since different phospholipid pools are mobilized for the synthesis of lipoxygenase and cyclooxygenase products (Patton et al., 1997). Further studies will be necessary to test this intriguing possibility.

As cAMP is known to activate protein kinase A, the potential role of phosphorylation and dephosphorylation in the regulation of cyclooxygenase activity was considered. Indeed, the Rp-thiophosphate analog of cAMP, which acts as an antagonist of cAMP binding to the regulatory subunit of protein kinase A, largely overcame the effect of cAMP on cyclooxygenase activity (Fig. 3). This finding, together with the inhibitory effects of cAMP-elevating agents that activate protein kinase A by receptor-independent mechanisms (forskolin, phosphodiesterase inhibitors and DB-cAMP), suggests that a protein kinase A-induced phosphorylation event is a possible mechanism whereby inhibition of cyclooxygenase activity by cAMP takes place. The antagonistic effect of okadaic acid, a phosphatase inhibitor, on cAMP-mediated inhibition points to the probable involvement of a phosphorylation–dephosphorylation cascade in the regulation of cyclooxygenase activity. This cascade may involve protein kinase A itself or possibly cross-talk with tyrosine phosphorylation mechanisms. Clearly, further studies are required to unravel the precise mechanism of cAMP-induced inhibition of cyclooxygenase activity.

We showed that cAMP-mediated inhibition of cyclooxygenase activity, probably cyclooxygenase-1, was not associated with decreased cyclooxygenase-1 protein expression, as revealed by Western blot analysis (Fig. 2). Thus, cAMP probably exerts its inhibitory effect at a posttranslational level. Similarly, negative regulation of cyclooxygenase activity by G binding protein activators and positive regulation by NaF and basic FGF without involvement of de novo cyclooxygenase protein synthesis were recently reported by Rosenstock et al. (1997). Furthermore, in cerebral endothelial cells, cyclooxygenase activity was shown to be posttranslationally regulated by tyrosine phosphorylation (Parfenova et al., 1998).

In the present study, elevated cAMP levels induced cyclooxygenase-2 protein expression in a time-dependent manner. Likewise, in preovulatory follicles, elevation of intracellular levels of cAMP by luteinizing hormone (LH) was shown to induce cyclooxygenase-2 production (Morris and Richards, 1995). Also, pharmacological agents that elevate cAMP, such as iloprost, a prostacyclin analog, prostaglandin E_1, and dibutyryl cAMP or 8-bromo-cAMP, stimulated cyclooxygenase-2 synthesis in mouse osteoblastic cells (Takahashi et al., 1994). We show here that in bovine aortic endothelial cells, cAMP-elevating agents considerably potentiate the expression of lipopolysaccharide-induced cyclooxygenase-2 protein. These observations are compatible with early regulation of the cyclooxygenase-2 gene via the cAMP-responsive element in the promoter sequence, possibly through cooperation of more than one transcription factor, as suggested in a study of endothelial cells where cyclooxygenase-2 mRNA was synergistically induced by lipopolysaccharide and phorbol-12-myristate-13-acetate (TPA) (Inoue et al., 1995). Similarly, Fournier et al. (1997) showed a synergistic increase in cyclooxygenase-2 with DBcAMP and tumor necrosis factor-α (TNF-α) in murine macrophages. They observed that TNF-α markedly decreased prostaglandin D_2 production and at the same time, caused a reciprocal increase in prostaglandin E_2 synthesis, which was potentiated by DB-cAMP or prostaglandin E_2 itself. Bovine aortic endothelial cells are known to produce predominantly prostacyclin and only small amounts of prostaglandin E_2 (Eldor et al., 1983), which was also confirmed in our laboratory (unpublished data). Nevertheless, to exclude an action on prostaglandin isomerases, we also examined prostaglandin E_2 production in addition to prostacyclin. The results indicate that following treatment with DBcAMP, prostaglandin E_2 release decreased parallel to prostacyclin (results not shown).

An increasing amount of evidence indicates that cyclooxygenase-1 and cyclooxygenase-2 may respond to different stimuli and probably utilize different pools of arachidonic acid (Murakami et al., 1994; Chulada et al., 1996). Thus, cyclooxygenase-1 and -2 have been proposed to represent independent prostanoid biosynthetic systems. This concept gains further support in our study, where we found that cAMP regulated cyclooxygenase-1 and -2 activities differently and, in fact, in opposite directions. cAMP had no effect on cyclooxygenase-2 activity (Fig. 5), while, as already discussed, cyclooxygenase-1 activity was strongly inhibited by cAMP (Table 2). We observed that cyclooxygenase-2 expression was induced by cAMP without an accompanying increase in the total production of prostacyclin. It may be concluded that the two cyclooxygenase isozymes are targets of different and independent regulatory mechanisms activated by agents that elevate intracellular cAMP. The possibility, that increased cyclooxygenase-2 protein synthesis is the result of positive feedback in response to the initial decrease in prostaglandin production through suppression of cyclooxygenase-1 activity, may be considered. In this context, it may be relevant to note that aspirin, a cyclooxygenase inhibitor, was found to induce cyclooxygenase-2 mRNA and protein (Davies et al., 1997; Johnson et al., 1997).

IP receptors, which mediate the effects of prostacyclin, utilize cAMP as their signal transduction system (Coleman et al., 1994). It is therefore tempting to speculate that the cAMP-dependent effects seen in the present study may represent autocrine regulation of prostacyclin production. Thus, prostacyclin released by bovine aortic endothelial cells elevates intracellular cAMP production, which in turn inhibits further prostacyclin production by suppressing cyclooxygenase-1 activity. In the long-term, the increased intracellular levels of cAMP may lead to induction of cyclooxygenase-2 protein.

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