Protein-tyrosine-kinase-dependent expression of cyclo-oxygenase-1 and -2 mRNAs in human endothelial cells

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Endothelial cells possess constitutive or inducible cyclo-oxygenase (COX) isoenzymes for prostacyclin production, but the mechanisms for their expression are largely unknown. We found that vanadate, an inhibitor of protein-tyrosine phosphatases, induced the expression of two COX isoenzyme mRNAs in human umbilical vein endothelial cells (HUVEC) in a time- and dose-dependent manner. Vanadate also stimulated an increase in COX-2 protein levels, but did not affect significantly the levels of constitutively expressed COX-1 protein. Synergistic enhancement

of expression of the two COX isoenzyme mRNAs was observed on stimulation of HUVEC with vanadate plus interleukin-1 α . Tyrphostin-47, which as an inhibitor of protein-tyrosine kinases abolished vanadate-induced protein-tyrosine phosphorylation, inhibited expression of the two COX isoenzyme mRNAs in HUVEC stimulated with vanadate or interleukin-1 α . These data provide conclusive evidence that activation of protein-tyrosine kinases is causally linked to expression of the mRNAs for the two COX isoenzymes in HUVEC.

INTRODUCTION

Endothelial cells are known to produce prostacyclin (PGI₂), a potent inhibitor of platelet aggregation and a powerful vasodilator, upon stimulation by various agonists [1]. One of the control mechanisms for PGI₂ synthesis has been ascribed to *de novo* synthesis of cyclo-oxygenase (COX; prostaglandin-endoperoxide G/H synthase). Studies have shown that several cytokines and tumour promoters cause a late and sustained release of PGI₂ over a prolonged period of time through the *de novo* synthesis of COX [2–4].

It is now evident that two isoforms of the COX enzyme exist which are encoded by separate genes [5–10]. COX-1, with an mRNA transcript of 2.8–3.0 kb, is expressed constitutively in many tissues, but has been shown to be regulated by serum, growth factors and cytokines [4,11–13]. COX-2, with an mRNA transcript size of 4.0–4.5 kb, is not constitutive in most tissues, but can be induced by a variety of mitogens and inflammatory mediators [12]. These two COX isoforms share about 60 % sequence similarity at the amino acid level, while the residues that are critical for enzyme function are highly conserved [10]. Although it has been well established that endothelial cells possess constitutive or inducible COX isoenzymes [10], the regulatory mechanisms for the expression of the two COX isoenzymes in these cells are largely unknown.

Shimizu et al. [14], and others [15,16], have suggested that the activation of protein-tyrosine kinases (PTKs) may be involved in the induction of COX enzymes in endothelial cells. However, many important questions about the involvement of PTKs in the expression of COX isoenzyme mRNAs in endothelial cells have not been answered. Therefore, in the present work, we studied the effects of vanadate, an inhibitor of protein-tyrosine phosphatases (PTPases), on the expression of the two COX isoenzyme mRNAs in human umbilical vein endothelial cells (HUVEC). We also investigated the involvement of PTKs in both vanadate-

and interleukin-1 α (IL-1 α)-induced gene expression of COX isoenzymes using tyrphostin-47, an inhibitor of PTKs.

MATERIALS AND METHODS

Materials

Cloned Moloney murine leukaemia virus reverse transcriptase was purchased from Life Science Inc. (Florida, CA, U.S.A.). *Taq* polymerase and dNTPs were from Takara (Otsu, Japan). RNase inhibitor and random hexamers were from Pharmacia Biotechnology Inc. (Uppsala, Sweden). Hybond-N⁺ nylon membrane, [³²P]dCTP and [³⁵S]methionine were from Amersham Life Science (Amersham, Bucks., U.K.). Denhardt's solution was from Wako Junyaku Co. (Osaka, Japan). cDNA probes for COX isoenzymes and purified ram seminal vesicle prostaglandin synthase were from Oxford Biochemical Research Inc. (Oxford, MI, U.S.A.). A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was from Clontech Laboratories Inc. (Palo Alto, CA, U.S.A.). Polyclonal anti-COX-2 serum was from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). IL-1 α was a gift from Dainippon Pharmaceutical Co. (Osaka, Japan). All other materials were obtained as described previously [14,17,18].

Cell culture

HUVEC were isolated from human umbilical veins, maintained in primary culture and further subcultured as described previously [14,19,20]. Unless otherwise stated, HUVEC were utilized at passage 3. Confluent cultures of HUVEC were starved in M199 containing 5 % (v/v) fetal bovine serum for at least 12 h before the experiments were initiated.

Experiments

Confluent monolayers of HUVEC in M199 containing 5 % fetal bovine serum were stimulated with up to 200 μ M vanadate or

Abbreviations used: COX, cyclo-oxygenase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; IL-1, interleukin-1; PGI₂, prostacyclin; PTK, protein-tyrosine kinase; PTPase, protein-tyrosine phosphatase; RT-PCR, reverse transcriptase-PCR.

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0.1–1.0 ng/ml IL-1 α , or a combination of the two, for the indicated times, and treated for various samplings as described below. In some experiments the monolayers were preincubated either with 20 μ M tyrphostin-47 for 12 h or with 10 μ g/ml actinomycin D for 2 h prior to stimulation. Under these experimental conditions, greater than 95% viability of the cultured HUVEC was confirmed by Trypan Blue dye exclusion.

RNA isolation and reverse transcriptase-PCR (RT-PCR)

After the reactions had been terminated, confluent HUVEC monolayers were washed twice with PBS and lysed in 4 M guanidinium isothiocyanate solution. Total cellular RNA was extracted and purified by the method of Chomczynski and Sacchi [21]. The RNA concentration was determined by measuring the absorbance at 260 nm. Total RNA (1 μ g) was converted into cDNA by incubation for 1 h at 42 °C with reverse transcriptase buffer (50 mM Tris/HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂), 2.5 units of cloned Moloney murine leukaemia virus reverse transcriptase, 0.25 mM dNTPs, 1 unit/ml RNase inhibitor and 2.5 μ M random hexamers, in a final volume of 20 μ l. The reaction was stopped by boiling for 5 min.

PCR was performed using 0.5 μ l of cDNA in 20 μ l of 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, 0.5 mM dNTPs, 1.5 mM MgCl₂, 0.4 μ g each of sense and antisense primers and 0.5 unit of *Taq* polymerase. The sequences of the human COX-1 and COX-2 specific primers, without a region of homology, were: 5'-TGCCCAGCTCCTGGCCCCGCGCTT-3' (sense; bases 516–539)/5'-GTGCATCAACACAGGCGCCTCTTC-3' (antisense; bases 796–819) and 5'-TTCAAATGAGATTGTGGAAAATTGCT-3' (sense; bases 574–600)/5'-AGATCATCTCTGCCTGAGTATCTT-3' (antisense; bases 855–878) respectively. Amplification of the G3PDH primers was performed with sense (5'-TGAAGGTCGGAGTCAACGGATTTGGT-3') and antisense (5'-CATGTGGGCCATGAGGTCCACCAC-3') primers. The amplification conditions were: COX-1, 94 °C for 1 min (denaturation), 55 °C for 2 min (annealing) and 72 °C for 3 min (extension) for 35 cycles; COX-2, 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min for 30 cycles; G3PDH, 94 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min for 35 cycles, followed by 72 °C for 7 min. Aliquots of PCR products were analysed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining.

Southern blot analysis

For quantification of amplified DNA, Southern blot analysis was performed using a Hybond-N⁺ nylon membrane according to the manufacturer's instructions (Amersham). The cDNA probes for COX-1, COX-2 and G3PDH were labelled using an oligolabelling kit (Pharmacia Biotechnology Inc.). The membranes were exposed to an imaging plate for computed radiography (Fuji Photo Film Co.) at room temperature and the signal was quantified using a Fujix BAS 2000 bioimaging analyser. All estimated signals for COX-1 and COX-2 were normalized to the corresponding values for G3PDH.

Northern blot analysis

Poly(A)⁺ RNA was prepared using Fast Track (Invitrogen Co.). Northern blotting and subsequent hybridizations were performed using a Hybond-N⁺ nylon membrane according to the manufacturer's instructions (Amersham). The radiolabelling of cDNA probes and the estimation of signals were performed as described for Southern blots.

Quantitative measurement of COX-1 mRNA by competitive PCR

In addition to performing quantitative RT-PCR involving co-amplification of COX mRNA with G3PDH mRNA as an internal standard and Northern blot analysis, a competitive PCR method was also used to quantify COX-1 mRNA using serial dilutions of a competitor template, based on the principle originally described by Gilliland et al. [22]. Competitive DNA templates (221 bp), which were 83 bp smaller than the PCR products of target sequences, were prepared using the following primers by the method of Celi et al. [23]: 5'-TGCCCAGCTCCTGGCCCCGCGCTT-3', which is the same as the sense primer of COX-1 described above (bases 516–539), and 5'-GTGCATCAACACAGGCGCCTCTTCCCAGATTGTCTCCATAAA-3', in which 3'-end of the COX-1 antisense primer (bases 796–819) was combined with another antisense primer (bases 695–712). For PCR under the same amplification conditions as stated above, 0.5 μ l of a competitive DNA template in a series of four concentrations (6.0×10^{-5} , 6.0×10^{-4} , 6.0×10^{-3} and 6.0×10^{-2} amol) was added simultaneously to the PCR reaction mixture (20 μ l) containing cDNA copies of COX-1 mRNA. Each PCR product was electrophoresed on a 1% agarose gel and stained with ethidium bromide to detect the bands corresponding to competitive DNA templates and cDNA fragments. The intensity of the ethidium bromide luminescence was measured by a CCD image sensor (Densitograph AE-6900-F; Atto, Tokyo, Japan), as reported by Nakayama et al. [24].

Western blot analysis

To prepare the samples for immunoblotting analysis of COX polypeptides, confluent HUVEC were washed with PBS and harvested immediately in 100 μ l of ice-cold buffer [25]. After centrifugation at 10000 *g* for 10 min at 4 °C, the resulting supernatants were mixed with 0.5 vol. of 3 $\times$ Laemmli SDS sample buffer and boiled for 5 min. The samples for phosphotyrosine immunoblotting were prepared as described previously [14].

Proteins in the samples were separated by SDS/PAGE (12% gel). After electrophoresis, the proteins were transferred on to nitrocellulose membranes and analysed by immunoblotting with the antibody of interest, as described previously [18]. The primary antibodies used for immunoblotting were anti-phosphotyrosine monoclonal antibody 4G10, polyclonal anti-COX-2 serum and polyclonal anti-COX-1 serum, which was raised in rabbits against purified ram seminal vesicle prostaglandin synthase.

Metabolic labelling and immunoprecipitation of COX

HUVEC were stimulated with 100 μ M vanadate in the presence of methionine-free M199 containing 100 μ Ci of [³⁵S]methionine at 37 °C for 6 h. HUVEC were washed two times with PBS and lysed in 100 μ l of ice-cold buffer [25]. Immunoprecipitates were prepared from the lysates with anti-COX-1 serum and resolved on SDS/PAGE followed by autoradiography, as described previously [17].

RESULTS

Vanadate induces the expression of the two COX isoenzyme mRNAs in a time- and dose-dependent manner

As shown in Figure 1, vanadate induced COX-1 and COX-2 mRNA expression in HUVEC. In quiescent HUVEC, we detected low levels of COX-1 and COX-2 mRNAs using the RT-PCR assay to amplify mRNA, confirming the findings of Hla and Neilson [10]. After addition of 100 μ M vanadate to HUVEC, the

Figure 1 Vanadate induces expression of the two COX isoenzyme mRNAs in HUVEC in a time-dependent manner

(A) Confluent monolayers of HUVEC were incubated with 100 μ M vanadate for the indicated times at 37 °C. RNA was extracted, converted into cDNA, amplified by PCR and analysed by hybridization with cDNA probes for both COX isoenzymes and G3PDH, as described in the Materials and methods section. PCR was performed for 35 cycles for COX-1 and G3PDH and for 30 cycles for COX-2. COX-1 and COX-2 mRNA levels are expressed relative to the G3PDH mRNA signal (means $\pm$ S.E.M., $n = 3$). (B) Confluent HUVEC were treated as described above for the times indicated. Poly(A)⁺ RNA was prepared and Northern blot analyses were performed as described in the Materials and methods section. A 1 μ g sample of poly(A)⁺ RNA was loaded in each lane. Each Northern blot was hybridized with COX-2 cDNA, stripped and reprobed for COX-1, and then stripped and reprobed for G3PDH. The data are from one of three separate experiments giving similar results.

Figure 2 Time course of changes in protein levels of the two COX isoenzymes in vanadate-stimulated HUVEC

Confluent monolayers of HUVEC were incubated with 100 μ M vanadate for the indicated times. The samples containing COX polypeptides were prepared and analysed by immunoblotting using specific antibodies against COX-1 and COX-2 as described in the Materials and methods section. The data are from one of three separate experiments giving similar results.

kinetics of the vanadate-induced expression of the two COX isoenzyme mRNAs were studied at 4, 12 and 24 h using the quantitative RT-PCR co-amplification of COX mRNA with a reporter G3PDH mRNA as an internal standard (Figure 1A). The most prominent increase in COX-2 mRNA expression was observed at 4 h of vanadate stimulation. This increase was not sustained, but had decreased by 12 and 24 h of stimulation. In contrast, the greatest increase in COX-1 mRNA expression was observed at 12 h of stimulation. The extent of the response of COX-1 mRNA to vanadate was smaller than that of COX-2 mRNA. Thus the response of COX-2 mRNA was greater and faster than that of COX-1 mRNA. Both responses were not sustained at more prolonged incubation times up to 24 h. When HUVEC were preincubated with 10 μ g/ml actinomycin D, an inhibitor of transcription, for 2 h and then stimulated by 100 μ M vanadate for 6 h, the vanadate-induced increases in the mRNA transcripts of both COX isoenzymes were almost completely suppressed (results not shown).

Since the increase in COX-1 mRNA was modest in the present study, and has been reported to occur under some restricted circumstances [26], we also carefully studied COX-1 mRNA expression not only by Northern blot analysis but also by the competitive PCR method described in the Materials and methods

section. As shown in Figure 1(B), the same kinetic patterns of COX-1 and -2 mRNA expression were confirmed by Northern blot analysis. Furthermore, the data for COX-1 mRNA expression obtained using the competitive PCR method showed identical kinetic behaviour to the data in Figure 1(A) (results not shown). Therefore we primarily employed the PCR co-amplification method using a reporter G3PDH mRNA as internal standard to detect COX mRNA expression in the following studies.

Dose-dependent increases in COX-2 mRNA expression were observed with concentrations of vanadate up to 100 μ M, whereas the levels of COX-1 mRNA increased almost linearly on stimulation by vanadate up to 50 μ M, and were maximally stimulated by 50–200 μ M vanadate (results not shown).

Protein levels of the two COX isoenzymes in vanadate-treated HUVEC: time courses

The protein levels of the two COX isoenzymes in vanadate-treated HUVEC were assessed by Western blot analysis using specific polyclonal antibodies (Figure 2). In quiescent HUVEC, COX-1 protein was constitutively expressed, but COX-2 protein was not. After stimulation of HUVEC with 100 μ M vanadate, the expression of COX-2 was induced, becoming prominent at 6–12 h and appearing to level off thereafter up to 24 h. On the other hand, levels of COX-1 protein were apparently unchanged throughout the time course both before and after stimulation with vanadate. In order to confirm the lack of change in COX-1 protein levels during 24 h of treatment with vanadate, HUVEC were treated with 100 μ M vanadate in the presence of [³⁵S]methionine-containing medium, as described in the Materials and methods section, to label newly synthesized proteins. However, the immunoprecipitated COX-1 proteins from the lysates of vanadate-stimulated HUVEC were barely radiolabelled throughout 24 h of stimulation (results not shown).

Synergistic effects of vanadate and IL-1 α on expression of the two COX isoenzyme mRNAs

We next examined whether vanadate acts synergistically with IL-1 α with regard to the induction of expression of COX-1 and COX-2 mRNAs. When HUVEC were treated with either 10 μ M

Figure 3 Synergistic effects of vanadate and IL-1 α on expression of the two COX isoenzyme mRNAs in HUVEC

Confluent monolayers of HUVEC were incubated without (lane 1) or with 10 μ M vanadate (lane 2), 0.1 ng/ml IL-1 α (lane 3), 10 μ M vanadate plus 0.1 ng/ml IL-1 α (lane 4), 100 μ M vanadate (lane 5) or 1.0 ng/ml IL-1 α (lane 6) for 6 h at 37 $^{\circ}$ C. RT-PCR followed by Southern blot analysis for COX-1, COX-2 and G3PDH was performed as described in the legend to Figure 1(A). The data are from one of three separate experiments giving similar results.

vanadate or 0.1 ng/ml IL-1 α for 6 h, the levels of both COX-1 and COX-2 mRNAs were slightly increased compared with the control (Figure 3, lanes 1–3). However, when HUVEC were incubated with 10 μ M vanadate plus 0.1 ng/ml IL-1 α for 6 h, there were remarkable increases in the levels of both COX-1 and COX-2 mRNAs which were almost comparable with the stimulatory effects of 100 μ M vanadate and rather higher than those of 1.0 ng/ml IL-1 α alone (Figure 3, lanes 4–6).

Effects of a PTK inhibitor on the vanadate- and IL-1 α -induced expression of the two COX isoenzyme mRNAs

We studied the inhibitory effects of tyrphostin-47, a known inhibitor of PTKs, on both the vanadate-induced expression of COX isoenzyme mRNAs and protein-tyrosine phosphorylation. HUVEC were treated or not with 20 μ M tyrphostin-47 for 12 h, and then stimulated with 50 μ M vanadate for an additional 6 h, and analysed for both expression of COX isoenzyme mRNAs and protein-tyrosine phosphorylation. As shown in Figure 4, pretreatment of HUVEC with tyrphostin-47 completely inhibited both the vanadate-induced increases in the mRNA transcripts of

the two COX isoenzymes and protein-tyrosine phosphorylation. We further studied the effects of tyrphostin-47 on IL-1 α -induced expression of the two COX isoenzyme mRNAs. Pretreatment of HUVEC with 20 μ M tyrphostin-47 completely inhibited the increases in COX-1 and COX-2 mRNAs induced by 1.0 ng/ml IL-1 α , as with 50 μ M vanadate.

DISCUSSION

We have characterized for the first time the vanadate-induced expression of COX isoenzyme mRNAs in HUVEC, and provided conclusive evidence that the induction of protein-tyrosine phosphorylation is causally linked to expression of mRNAs for the two COX isoenzymes.

Several reports have shown that vanadate inhibits various ATPases involved in transmembrane ion transport and the turnover of short-lived proteins other than PTPases [27–30]. In this regard, our group has previously shown that vanadate-induced protein-tyrosine phosphorylation in HUVEC is persistent [14], supporting the concept that vanadate is acting as an inhibitor of PTPases. Furthermore, the inhibitory effects of tyrphostin-47, a specific inhibitor of PTKs, both on protein-tyrosine phosphorylation and on expression of COX isoenzyme mRNAs suggest that vanadate affects the latter by inducing the former. Therefore possible actions of vanadate other than inhibition of PTPases are negligible with regard to the induction of expression of the two COX isoenzyme mRNAs.

It is generally accepted that COX-2 mRNA expression is inducible by various agents, whereas COX-1 mRNA expression varies depending on the cells and the stimulants [26]. In endothelial cells, four previous reports [4,10,13,31] have shown phorbol ester-, IL-1- or transforming growth factor- β -induced increases in COX-1 mRNA expression by use of Northern blot analysis or a competitive PCR method. In the present study we also found that vanadate and IL-1 α each stimulated an increase in the expression of COX-1 as well as COX-2 mRNAs. This was first observed by the PCR co-amplification of COX mRNA with a reporter G3PDH mRNA using specific primers to each COX isoenzyme. Although the PCR co-amplification method has been

Figure 4 Effects of a PTK inhibitor on COX mRNA expression

(A) Effects of tyrphostin-47 on vanadate- and IL-1 α -induced expression of the two COX isoenzyme mRNAs in HUVEC. After confluent monolayers of HUVEC had been pretreated without (lanes 1–3) or with (lanes 4 and 5) 20 μ M tyrphostin-47 for 12 h, the cells were treated with either 1.0 ng/ml IL-1 α (lanes 2 and 4) or 50 μ M vanadate (lanes 3 and 5) for 6 h at 37 $^{\circ}$ C. RT-PCR followed by Southern blot analysis for COX-1, COX-2 and G3PDH was performed as described in the legend to Figure 1(A). (B) Effects of tyrphostin-47 on vanadate-induced protein-tyrosine phosphorylation in HUVEC. After confluent monolayers of HUVEC had been pretreated without (lanes 1–3) or with (lane 4) 20 μ M tyrphostin-47 for 12 h, the cells were immediately harvested (lane 1) or incubated further in the absence (lane 2) or presence (lanes 3 and 4) of 50 μ M vanadate for 6 h at 37 $^{\circ}$ C. The samples were prepared and analysed for immunoblotting using anti-phosphotyrosine monoclonal antibody 4G10, as described in the Materials and methods section. Molecular masses (kDa) are indicated on the left. The data are from one of three separate experiments giving similar results.

widely used to detect alterations in mRNA transcripts [4,10,32,33], two other methods, i.e. Northern blot analysis and the competitive PCR method, were also used to analyse COX-1 mRNA expression in particular, and gave the same results as obtained with the PCR co-amplification method. Thus we ruled out the possibility that the data obtained with the PCR co-amplification method might not reflect the changes in mRNA transcripts specific to each COX isoenzyme.

It has been suggested, but not conclusively demonstrated, that protein-tyrosine phosphorylation is involved in the expression of COX-2 mRNA in cells other than endothelial cells [34–37]. Chanmugam et al. [37] reported that radicicol, a fungal antibiotic, suppresses lipopolysaccharide-induced COX-2 expression and protein-tyrosine phosphorylation in macrophages, and suggested that the inhibition of COX-2 expression by radicicol occurs mainly at post-transcriptional steps. This differs from our conclusion that protein-tyrosine phosphorylation is involved in the expression of mRNAs for the two COX isoenzymes. Three other groups have reported the possible involvement of PTK-dependent pathways in COX-2 gene expression in rat mesangial cells [34–36]. However, they did not show specific and parallel relationships between protein-tyrosine phosphorylation and COX-2 mRNA expression as we did in the present study. Notably, none of the previous studies have shown the involvement of protein-tyrosine phosphorylation in COX-1 mRNA expression.

There have been three previous reports studying the relationship between protein-tyrosine phosphorylation and COX enzyme expression in endothelial cells [14–16]. Our group [14] has previously shown that vanadate stimulates the recovery of PGI₂-producing ability in aspirin-treated HUVEC, possibly through COX gene expression, at concentrations sufficient to induce protein-tyrosine phosphorylation. Continuing this work, the present study is the first report showing that not only vanadate but also IL-1 α induces the expression of the two COX isoenzyme mRNAs in HUVEC via PTK-dependent pathways. Our efforts are now directed towards the delineation of PTK-containing pathways leading to the expression of the two COX isoenzyme mRNAs.

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