Involvement of tyrosine kinases in the induction of cyclo-oxygenase-2 in human endothelial cells

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In addition to a constitutive cyclo-oxygenase (Cox-1), human endothelial cells also possess an inducible cyclo-oxygenase (Cox-2) which plays an important role in the regulation of the synthesis of prostacyclin (prostaglandin I_2). Cox-2 is regulated and expressed in large quantities upon activation of the cells by inducers such as phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), or interleukin-1 α . We have investigated the involvement of protein tyrosine kinases in Cox-2 expression by human endothelial cells upon activation by these inducers. PMA or interleukin-1 α provoke an increase in the phosphorylation of substrates of 110 and 120 kDa and additional phosphorylations for a broad band of multiple substrates in the 70 kDa range. This stimulation was accompanied by the induction of Cox-2 protein,

detectable after stimulation for 1 h, which is consistent with an increase in activity reflected by prostacyclin synthesis; no variation in the expression of Cox-1 could be observed. Three distinct inhibitors of protein tyrosine kinases, genistein, herbimycin or AG-213, reduced tyrosine phosphorylation of cell substrates, consistently with their pharmacological effects. Under these conditions, there was selective reduction of Cox-2 expression without modification of Cox-1. Regulation of Cox-2 induction is also dependent on the activation of PKC since Ro 31-8220 or PKC depletion by PMA prevented its induction. Our results suggest that within the time-frame of our experiments these effects on kinases are specific for Cox-2 rather than Cox-1.

INTRODUCTION

Endothelial cells participate in maintenance of the functional integrity of the blood vessel by providing a non-thrombogenic surface between blood constituents and the tissues. Some of the mechanisms that help to maintain this property have been ascribed to the capacity of these cells to synthesize prostacyclin [prostaglandin I_2 (PGI $_2$)], a very potent vasodilator and anti-aggregant substance [1]. The capacity of endothelial cells to produce this autacoid provides the endothelium with a biochemical potential to regulate the vascular tone as well as limiting the extension of thrombotic events known to intervene in pathological processes such as atherosclerosis [2].

Recent studies have shown that endothelial cells, like other cells, contain two distinct cyclo-oxygenases which are the products of different genes with differential regulation [3,4]. Although the two enzymes are relatively homologous for most of their structure and catalytic properties, the second cyclo-oxygenase (Cox-2) is the product of a primary response gene and contains a 19-amino-acid sequence specific to its C-terminus [5,6]. Several studies have demonstrated that Cox-2 is only expressed after stimulation of various growth factors or cytokines, or after stimulation by protein kinase C (PKC) activators such as phorbol esters [4,5,7]. We have recently raised polyclonal and monoclonal antibodies specific to Cox-1 and to Cox-2 and have shown that, in human endothelial cells, only the inducible Cox-2 can be regulated in response to interleukin (IL)-1 α or phorbol myristate acetate (PMA), whereas the constitutive Cox-1 is not modified [7].

Support for the role of kinases in mediating the expression of cyclo-oxygenase by endothelial cells has come mainly from studies with PKC [8,9]. These reports should be reassessed in light of the discovery of Cox-2 and also because tyrosine kinases

have been implicated in various biochemical events linked to cell activation and in the formation of eicosanoids [10,11]. We have studied the tyrosine phosphorylation of proteins upon activation of endothelial cells by PMA or IL-1 α and the expression of Cox-2 and Cox-1 proteins. Our results confirm the importance of PKC but also suggest that protein-tyrosine phosphorylation plays a role in the regulation of Cox-2 expression in endothelial cells.

MATERIALS AND METHODS

Materials

Recombinant human IL-1 α was from Genzyme (Cambridge, MA, U.S.A.). PMA, CHAPS, endotoxin-free BSA, benzamidine hydrochloride, leupeptin, soybean trypsin inhibitor, sodium orthovanadate, NaF, pig skin gelatin, heparin, DMSO, arachidonic acid, anti-(β -actin), were from Sigma, St. Louis, MO, U.S.A. Sheep polyclonal anti-(mouse IgG), and enhanced chemiluminescence substrates (ECL Western blotting detection reagents) were from Amersham Corp. (Les Ulis, France). Electrophoresis reagents were from Bio-Rad and chemical products from Prolabo (Paris, France) and Carlo Erba/Farmitalia (Milan, Italy). Genistein, daidzein and herbimycin were from Biomol Research Labs (Plymouth Meeting, PA, U.S.A.). Ro 31-8220 was a gift from Dr. Bradshaw (Roche Products, Basel, Switzerland).

Cell culture and stimulation

Human umbilical vein endothelial cells (HUVECs) were prepared according to the method of Jaffe et al. [12] with some modifi-

Abbreviations used: Cox-1, constitutive cyclo-oxygenase; Cox-2, inducible cyclo-oxygenase; HUVECs, human umbilical vein endothelial cells; IL, interleukin; PGI $_2$, prostaglandin I_2 (prostacyclin); PKC, protein kinase C; PMA, phorbol myristate acetate.

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cations [7]. Passage cells were subcultured in gelatin-coated 6-well plates (Costar). Heparin and endothelial-cell growth supplement were removed 48 h before stimulation and cells were used at the first passage at subconfluence and incubated in culture medium containing 5% normal human serum. Cells were stimulated with PMA or IL-1 α in culture medium containing 0.75% endotoxin-free BSA. After activation, cells were washed in Hanks buffer, pH 7.4, containing 1 mg/ml BSA and incubated for 30 min in the same buffer with 10 μ M arachidonic acid to evaluate cyclo-oxygenase activity. The stable metabolite of prostacyclin, 6-ketoprostaglandin F $_{1\alpha}$ (6-keto-PGF $_{1\alpha}$), was determined in cell supernatants using enzyme immunoassay [13]. Results were expressed as ng/10⁵ cells, since verification of the cell counts showed no major variation in cell number during the experiments. All incubations were done in triplicate. When inhibitors of tyrosine kinases were used (genistein, herbimycin or AG-213, dissolved in DMSO), they were preincubated with the cells 2 h prior to the addition of the inducers. Ro 31-8220 was co-incubated with PMA or IL-1 α . The DMSO was used at concentrations having no effect on the cells. Absence of cell toxicity of these compounds was evaluated by Neutral Red incorporation by viable cells [14].

Western blot analysis

Monolayers of HUVECs in 6-well plates [(0.3–0.4) $\times$ 10⁶ cells/well] were lysed in 150 μ l of ice-cold buffer (0.02 M Tris/HCl, pH 7.5, 0.016 M CHAPS, 0.001 M EDTA, 0.002 M sodium orthovanadate, 0.001 M benzamidine/HCl, 0.001 M NaF, 1 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor), scraped with a rubber policeman and centrifuged at 4 °C for 10 min at 10000 g. Protein content was determined in the supernatant using a micro-bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) with BSA as standard. Cell lysate (30 μ g of protein) was mixed with Laemmli reagent [final concentration 2% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) Pyronin Red] under reducing conditions [4% (v/v) 2-mercaptoethanol] and heated for 10 min at 85 °C [15]. SDS/PAGE was performed using 7% and 3% acrylamide for the separating gel and stacking gel respectively. Protein transfer on to nitrocellulose membranes (Schleicher and Schuell, Göttingen, Germany) was done as described previously [7]. Blots were incubated with 0.06 μ g/ml (0.1 ml/cm²) monoclonal anti-phosphotyrosine antibodies (UBI, New York) for 1 h at room temperature, washed five times in Tris-buffered saline containing 5% fat-free dry milk and twice, for 10 min each time, in Tris buffer without milk. Membranes were further incubated with an anti-(mouse IgG) antibody conjugated with horseradish peroxidase (Amersham) at a dilution of 1/4000 (0.1 ml/cm²) for 1 h at room temperature. Excess secondary antibody was eliminated by washing in Tris buffer (2 $\times$ 20 min followed by 2 $\times$ 10 min). Chemiluminescence substrates were used to reveal positive bands according to the manufacturer's instructions and bands were visualized after a fixed-time exposure (2 min) to Hyperfilm ECL (Amersham). Stripping of the membranes was done as described and membranes were then incubated with monoclonal antibodies specific to either Cox-1 or Cox-2 [7,16]. Mouse monoclonal antibodies were obtained against Cox-1 purified from ram seminal vesicles, or against the eicosapeptide (C)-NASSRSGLDDINPTVLLK (Cox-2 peptide), which is only present in the C-terminus (amino acid sequence 580–598) of human Cox-2 [3,4]. After detection of Cox-2, the membranes were stripped again and incubated with an antibody against Cox-1.

Protein bands were quantified on the films by densitometry using an LKB Ultrascan XL laser densitometer (Pharmacia).

Statistics

Comparison of the different parameters was performed using Student–Newman–Keuls one-way analysis of variance on ranks (Sigma Stat, Jandel Corporation, San Rafael, CA, U.S.A.).

RESULTS

Tyrosine phosphorylation of HUVECs, cyclo-oxygenase activity and expression of protein after stimulation by PMA and IL-1 α

Analysis of phosphorylation of tyrosine residues was done by Western blot. Control HUVECs exhibited a 116 kDa band containing strongly phosphorylated tyrosine residues. This band may represent either constitutively expressed substrate or the activation elicited by the unavoidable use of serum to maintain cell viability. Stimulation by 20 nM PMA or 25 units/ml IL-1 α was followed by protein tyrosine phosphorylation (Figure 1a). Particularly prominent in all experiments were bands at 110 and 120 kDa. The response was time-dependent (beginning at 15 min up to 2 h) and dose-dependent from 4–100 nM PMA and 5–125 units/ml IL-1 α , with a maximum at 20 nM PMA and 50 units/ml IL-1 (results not shown). In the presence of PMA, additional phosphorylations were seen for a broad band of multiple substrates in the 70 kDa range. Under our conditions no major low-molecular-mass bands were observed even when SDS/12%-PAGE was performed (results not shown). An antibody directed against β -actin was used to confirm that there was homogeneity of protein load between the different lanes (Figure 1a). Because the induction of Cox-2 occurs rapidly, we did not investigate times beyond 2 h. We used 20 nM PMA and 25 units/ml IL-1 α at 2 h of stimulation since under these conditions we could detect reproducible results for all Western blots as well as metabolic measurements.

Under these conditions, there was an increase in cyclo-oxygenase activity evaluated by the addition of exogenous arachidonic acid to the cells at the end of the incubation (Figure 1b). Such results were in agreement with previous data [7]. Analysis of Cox-2 and Cox-1 expression by Western blot on the same membranes as for tyrosine phosphorylation showed a rapid increase in Cox-2 immunoreactivity (in the form of a triplet band

Figure 1 Effect of PMA (20 nM) and IL-1 α (25 units/ml) on the activation of HUVECs

(a) Tyrosine phosphorylation of proteins; (b) cyclo-oxygenase activity; (c) expression of cyclo-oxygenases in HUVECs. After a 2 h incubation with either inducer, cyclo-oxygenase activity was evaluated after removal of supernatants and 30 min incubation with 10 μ M arachidonic acid in Hanks' buffer containing 1 mg/ml albumin. These data (mean $\pm$ S.E.M.) are from three different experiments (* $P < 0.05$). SDS/PAGE, protein transfer and detection were performed as described in the Materials and methods section. Lanes 1, control; lanes 2, IL-1 α activation; lanes 3, PMA activation.

Figure 2 Effect of 1.5 μ M herbimycin (H) and 100 μ M AG-213 (AG) on tyrosine phosphorylation of proteins after a 2 h stimulation of HUVECs by PMA or by IL-1 α

'Control', no activation; 0, no drug.

Figure 3 Effect of genistein on cyclo-oxygenase activity after stimulation of HUVECs by PMA or IL-1 α

Cells were activated in the absence or presence of genistein and cyclo-oxygenase activity was evaluated as described in Figure 1. This Figure is representative of three experiments (mean $\pm$ S.E.M.); * $P < 0.05$.

which corresponds to differences in glycosylation [7]). At 2 h, the signal obtained for Cox-2 was more intense with PMA than with IL-1 α (Figure 1c). The monoclonal antibody directed against Cox-1 recognized a band of approx. 70 kDa corresponding to the migration of authentic Cox-1 from sheep seminal vesicles. However, no significant change in Cox-1 immunoreactivity could be detected.

Effect of tyrosine kinase inhibitors genistein, herbimycin and AG-213 on IL-1 α - and PMA-induced tyrosine phosphorylations, Cox activity and expression

Pretreatment of HUVECs for 2 h with genistein before stimulation with IL-1 α or PMA blocked the tyrosine phosphorylation of the 110 kDa substrate at high concentrations of inhibitor (> 300 μ M); however, at the same concentration, daidzein, which is an inactive negative control for genistein, also decreased the phosphorylation (results not shown). Whereas genistein is a competitive inhibitor with respect to ATP which also inhibits other kinases [17], other molecules are thought to be more efficient: e.g. herbimycin, which inhibits protein tyrosine kinases irreversibly through benzoquinone interactions with protein sulphhydryl groups [18], and a tyrphostine such as AG-213 [18]. Both herbimycin (1.5 μ M) and AG-213 (100 μ M) strongly reduced the phosphorylation of substrates induced either by IL-1 α or PMA (Figure 2). Densitometric analysis of the 110 kDa substrate induced by IL-1 α showed a reduction of $60 \pm 15\%$ with herbimycin and $58 \pm 17\%$ with AG-213; PMA induction of the

Figure 4 Effect of genistein, daidzein, herbimycin and AG-213 on Cox-2 expression after stimulation by 25 units/ml IL-1 α or 20 nM PMA

Lower panel: Effect of AG-213 on the expression of Cox-1 by the two inducers. This Figure is representative of three experiments performed under the same conditions.

same substrate was reduced by $60 \pm 15\%$ and $85 \pm 12\%$ for herbimycin and AG213 respectively.

The effect of these inhibitors was tested on Cox activity and expression. At a lower concentration than that mentioned above (180 μ M), genistein induced a 40% reduction in cyclo-oxygenase activity (see the Materials and methods section) after 2 h stimulation of HUVECs with both inducers (Figure 3). The expression of Cox-2 provoked by the two inducers was also reduced by 180 μ M genistein ($72.8 \pm 8\%$ reduction, mean $\pm$ S.D., $n = 4$) (Figure 4), whereas daidzein was inactive ($10 \pm 4\%$, $n = 4$). Both herbimycin at 1.5 μ M and AG-213 (100 μ M) also strongly reduced the induction of Cox-2 by either PMA (66 ± 15 and $79 \pm 4\%$ respectively, $n = 3$) or IL-1 α (68 ± 11 and $75 \pm 10\%$ respectively, $n = 3$) (Figure 4). The expression of Cox-1 was not affected by the inhibitors as exemplified with AG-213 (Figure 4, bottom). The inhibitory effects of all molecules on the induction of Cox-2 protein, but not Cox-1, was consistent with the residual activity found in the cells after treatment with the drugs even at the highest concentrations (Figure 3). Tyrosine kinase inhibitors appear to exert a selective effect on the expression of inducible Cox-2 compared with the constitutive Cox-1.

Involvement of PKC in tyrosine phosphorylation and in Cox-2 expression

Because a growing number of reports have shown that signal transduction results in cross-talk between different kinases rather than a single pathway, we tested the role of PKC in the phosphorylation of proteins on tyrosine residues. Although it has been shown that PKC is involved in the signal transduction pathway(s) leading to the induction of cyclo-oxygenase in human endothelial cells [8,9], it is not clear which of the enzymes is sensitive. We used Ro 31-8220, which is an inhibitor of PKC [19]. This agent strongly reduced the phosphorylation of tyrosine residues induced by IL-1 α or PMA (Figure 5a). The induction of Cox-2 was also greatly attenuated (> 90%) in the presence of both inducers (Figure 5b). To assess further the PKC requirement in the induction of Cox-2, we depleted PKC by a 20-h treatment with PMA (20 nM). Such pretreatment resulted in a reduction of the capacity of HUVECs to synthesize Cox-2 in response to PMA or IL-1 α (Figure 5c).

Figure 5 (a) Effect of Ro 31-8220 (1 μ M) on tyrosine phosphorylation of proteins and Cox-2 expression (bottom) after stimulation of HUVECs by 25 units/ml IL-1 α or 20 nM PMA, and (b) effect of PKC depletion on the induction of Cox-2 by PMA or IL-1 α .

Cells were incubated for 20 h in the absence or presence of PMA. The supernatant was washed and cells activated by IL-1 α or PMA. This Figure is representative of three experiments.

DISCUSSION

Vascular cells represent an important target of IL-1 α in inflammatory processes such as atherosclerosis [20]. Among various effects resulting from its interactions with its receptor(s) on HUVECs, the formation of PGI₂ induced by IL-1 constitutes an established end-result [21]. This cytokine is an important inducer of cyclo-oxygenase, the enzyme responsible for the synthesis of prostaglandins [22–24]. We have recently developed antibodies specific for the two forms of cyclo-oxygenase and shown an up-regulation of the inducible Cox-2 but not the constitutive Cox-1 after stimulation of HUVECs by PMA or IL-1 α [7]. Induction of *de novo* synthesis of Cox-2 by PMA supports the involvement of PKC. Recent results indicate that in mesangial cells only tyrosine phosphorylation is involved in the induction of Cox-2 by endothelin-1 [10]. However, studies [25] on the same cells stimulated by serotonin indicate that PKC and protein tyrosine kinases are involved in the synthesis of Cox-2. These results confirm the existence of cell- and inducer-specific mechanisms regulating the expression of Cox-2. In the present work we have shown that stimulation of endothelial cells by IL-1 and PMA results in the activation of tyrosine kinases as reflected by the phosphorylation of various proteins on tyrosine residues. The rapid obtention of protein-tyrosine kinase activity after stimulation by both inducers (i.e. before the detection of any change in activity and increase in Cox-2) suggests that the kinases play a role at early stages of cell activation, although we cannot relate directly the phosphorylation of substrate(s) to the increase of Cox-2 expression. A recent work has reported the detection of the mRNA for Cox-2 15 min after stimulation with IL-1 α [26]. A role for protein-tyrosine kinase activity in the induction of Cox-2 was suggested by experiments in which three chemically and mechanistically distinct inhibitors of tyrosine kinases (genistein, herbimycin and AG-213), at different concentrations, inhibited its induction without affecting Cox-1. The residual cyclo-oxygenase activity is probably related to their lack of effect on Cox-1 expression, suggesting that this enzyme does not follow the same regulation and/or kinetics of synthesis as Cox-2. Although genistein has been associated with other undesirable effects, it blocked the increase in Cox-2 induced by IL-1 or PMA, with no effect on Cox-1. The failure of this compound to affect significantly the phosphorylation of proteins on tyrosine residues at the same concentrations as those affecting the induction of Cox-2 may be due to our inability to correlate an individual substrate

with the induction of Cox-2. It is noteworthy to observe that daidzein, an inactive analogue of genistein, did not inhibit Cox-2 expression induced by IL-1 α or PMA. A recent report showing that vanadate, an inhibitor of protein-tyrosine phosphatases, provokes an increase in PGI₂ production as well as protein-tyrosine phosphorylation in HUVECs emphasizes the role of tyrosine kinases in this production of PGI₂ [11].

Inhibition of PKC by Ro 31-8220 greatly reduces protein-tyrosine phosphorylation and the expression of Cox-2 obtained with both inducers in stimulated cells. As additional evidence, we also used long-term incubation with PMA to deplete the pool of PKC sensitive to this agent. This treatment significantly attenuated subsequent stimulation by PMA or induction by IL-1 α . Maier and Ragnotti [9] recently used H-7 and an antisense oligomer which inhibits PKC α translation to establish the role of this kinase in IL-1-induced expression of cyclo-oxygenase in HUVECs. However, in spite of the use of Western and Northern blots, it is not clear which of Cox-1 or Cox-2 was affected. Our results show unequivocally that, in the 2-h time-frame considered here, induction of Cox-2, but not Cox-1, by PMA and IL-1 is regulated by serine/threonine kinases and also by tyrosine kinases. Interestingly, protein-tyrosine phosphorylation is also greatly reduced by Ro 31-8220, suggesting that PKC signalling may either precede or play an important role in loops between these two kinase systems. The signalling pathway activated by IL-1 α is still not fully established, although considerable information can be derived from the functional pleiotropy and redundancy of cytokine signal transduction. Although it is unlikely that the IL-1 α receptor functions as a tyrosine protein kinase, binding of the ligand could activate a separate tyrosine kinase. Indeed, Bird et al. [27] have demonstrated that IL-1 modulates the activity of 43 and 45 kDa mitogen-activated protein (MAP) kinases by altering their degree of phosphorylation. Binding of IL-2 to its receptor has been shown to induce a rapid increase in tyrosine phosphorylation due to the interaction of Src protein-tyrosine kinases with the IL-2 receptor [28] and it was suggested that the non-receptor tyrosine kinase JAK 2 could be activated by IL-3 [29]. Similar events may occur with IL-1.

Finally, since the submission of this manuscript, several articles have appeared showing the involvement of tyrosine kinases in the expression of Cox-2 in different cellular systems [30,31]. The capacity of endothelial cells to synthesize rapidly Cox-2 and generate PGI₂ may contribute to modification of the phenotype of these cells under pathological conditions, such as the atherosclerotic process, or in the septic shock mimicked by bacterial toxins [32]. The implication of our findings is that, by regulating kinases, it would be possible in theory to limit the capacity of vascular cells to generate excessive amounts of PGI₂ by modulating an important enzyme of its metabolism.

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