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Diversity and similarity in signaling events leading to rapid Cox-2 induction by tumor necrosis factor-α and phorbol ester in human endothelial cells

Sonia Eligini[a], Silvia Stella Barbieri[a], Viviana Cavalca[b], Marina Camera[b], Marta Brambilla[a,b], Michela De Franceschi[b], Elena Tremoli[a,b], Susanna Colli[a,*]

[a]*E. Grossi Paoletti Center, Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy*
[b]*Department of Cardiac Surgery, Centro Cardiologico Fondazione Monzino I.R.C.C.S., University of Milan, Italy*

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Abstract

Objective: This study examines whether cyclooxygenase 2 (Cox-2) synthesis in human endothelial cells involves different signaling pathways when induced by the proinflammatory cytokine tumor necrosis factor-α (TNFα) or by the tumor and angiogenic promoter phorbol ester (PMA). Moreover, the hypothesis that reactive oxygen species (ROS) and an altered redox status within the cell are fundamental steps for Cox-2 synthesis is verified.
Methods: Human endothelial cells isolated from umbilical vein (HUVEC) were exposed to PMA and TNFα and Cox-2 protein and mRNA levels were evaluated by Western blot and Real-Time Quantitative Reverse Transcription–PCR analysis. Prostaglandin E_2 (PGE_2) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) levels were measured in cell medium as an index of Cox-2 activity. Intracellular ROS formation was detected by flow cytometry in HUVEC loaded with the oxidant-sensitive 2′ ,7′ -dichlorofluorescein diacetate (DCFH-DA) and by nitroblue tetrazolium (NBT) reduction. Reduced and oxidized glutathione (GSH and GSSG) were measured by HPLC.
Results: Data show that TNFα and PMA signal for early Cox-2 induction through distinct pathways. PMA-induced Cox-2 expression involves a small GTPase-dependent pathway acting via tyrosine kinase, activation of protein kinase C (PKC) and of the mitogen-activated protein kinase (MAPK) ERK1/2. Conversely, MAPK p38 is critical for Cox-2 induction by TNFα. Of interest, intracellular ROS generation and consequent GSH/GSSG ratio reduction represents a common step through which PMA and TNFα signal for early Cox-2 induction. In addition, we provide evidence that phosphatidylinositol 3 (PI3)-kinase activation plays a regulatory role for Cox-2 synthesis in HUVEC.
Conclusion: Cox-2 represents a critical link among vascular homeostasis, inflammatory response, angiogenesis and tumor growth. The finding that two independent pathways and an overlapping upstream event signal for Cox-2 induction in HUVEC may be of relevance to develop strategies aimed at selectively interfering with Cox-2 regulating pathways.

Keywords: Endothelial function; Cyclooxygenase; Redox signaling; Infection/inflammation; Prostaglandins

1. Introduction

Arachidonic acid metabolites are considered of importance in the modulation of vascular homeostasis, inflammation, tumorigenesis and angiogenesis [1–3]. The major rate-limiting enzymes involved in their synthesis are the cyclooxygenases (Coxs). Two Cox genes have been cloned. Cox-1 is generally expressed constitutively; its expression may be, however, regulated by certain cytokines [4]. Conversely, cyclooxygenase 2 (Cox-2) is rarely expressed constitutively, but it is highly induced by cytokines, growth factors and tumor promoters [5].

* Corresponding author. Tel.: +39 02 50319913; fax: +39 02 50318250.
E-mail address: Susanna.Colli@unimi.it (S. Colli).

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Cox-2 metabolites produced by endothelial cells, namely prostacyclin (PGI_2), thromboxane A_2 (TXA_2) and prostaglandin E_2 (PGE_2), have profound influences on vascular tone, regional blood flow, vascular permeability and remodelling, and angiogenesis. The balance between these autacoids is consequently critical in a variety of pathophysiologic condition [6].

Cox-2 induction is characterized by "redundancy" both at the extranuclear signaling level and at the level of transcriptional activation [7]. Mitogen-activated protein kinase (MAP-kinase), protein kinase C (PKC), tyrosine kinases and Rho proteins are reported as involved in Cox-2 induction in endothelial cells [8–11]. The involvement of these pathways varies, however, according to the stimulus and the source of endothelium [12].

Reactive oxygen species (ROS) are ubiquitous, highly diffusible and reactive molecules produced by the reduction of molecular oxygen. They are normally produced during the respiratory burst of phagocytes as a defence mechanism against pathogens. More recently, it has been appreciated that vascular cells produce ROS [13] and that these molecules are implicated in endothelial dysfunction associated with hypertension [14], atherosclerosis, and ischemia–reperfusion [15]. Endothelial ROS production stimulated by growth factors, cytokines, hypoxia–reoxygenation, pulsatile stretch and ischemia results in the modulation of redox-sensitive pathways as tyrosine kinase and serine/threonine kinase phosphorylation and, ultimately, in changes of gene expression profile [16].

Reduced glutathione (GSH) is an important intracellular redox buffer that exists as a reduced predominant form, as a disulfide form (GSSG), or as mixed disulfide (GSSR) with protein thiols [17]. The redox status within the cell, reflected by the ratio between GSH and GSSG (GSH/GSSG) [18] has been shown to be relevant for the regulation of proinflammatory genes [19].

In this study, we report that rapid Cox-2 induction in HUVEC exposed to the representative inflammatory cytokine TNFα and to the proangiogenic agent PMA is characterized by diversity and similarity in the individual steps that act in transforming events at the cell surface into transcriptional responses.

2. Methods

2.1. Reagents

Reagents and their sources were as follows: phorbol-12-myristate-13-acetate (PMA), human recombinant tumor necrosis factor-α (TNFα), phenazine methosulfate (PMS), *N*-acetyl-cysteine (NAC), nitroblue tetrazolium (NBT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), glutathione reduced form ethyl ester (GSH-Et) and L-buthionine-[*S*,*R*]-sulphoximine (BSO) from Sigma (Sigma-Aldrich, Milano, Italy). Other reagents: PD98059 and SB203580 were from Biomol (Tebu-Bio, Milano, Italy), wortmannin, JNK inhibitor I (L-form, cell permeable) and its negative control, dimethoxy-1,4-napthoquinone (DMNQ) were from Calbiochem (Inalco, Milano, Italy). Mevalonate (as mevalonolactone) was from Aldrich, Milano, Italy. Fluvastatin was from Novartis, Basel, Switzerland. RO 31-8220 was gift from C.H. Hill (Roche, London, UK). Arachidonic acid sodium salt was from Cayman Chemical (Spi-Bio, Massy Cedex, France).

2.2. Cell culture

HUVEC were isolated from freshly and anonymously acquired human umbilical veins, as described [20]. Informed consent was provided according to the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3). Cells were cultured in Medium 199 (BioWhittaker Italia, Bergamo, Italy) supplemented with 10% heat-inactivated human AB serum, L-glutamine, antibiotics, heparin (15 μg/ml) and crude extract of endothelial cell growth factor (50 μg/ml). The cells used for the experiments were at the 1st passage. Heparin and endothelial cell growth supplement were removed 24 h prior to stimulation. Incubations with stimuli were carried out for 6 h in Medium 199 supplemented with 0.75% bovine serum albumin (fatty acid-free and low endotoxin, Sigma-Aldrich) and 1% FCS. Inhibitors were added 1 h before stimulation, with the exception of fluvastatin, mevalonate and BSO that were preincubated for 18 and 24 h, respectively. Cell viability was assessed by neutral red and MTT assays.

2.3. Antibodies

Monoclonal antibodies against Cox-2 (mAb 29) and Cox-1 (mAb 10 and 11) were gift from Aida Habib (American University of Beirut, Lebanon). Antibody against phosphotyrosine was from Upstate Biotechnology (D.B.A., Milano, Italy). Monoclonal antibodies against phosphorylated and total ERK1/2 and p38 were from Cell Signaling (Celbio, Milano, Italy) and Biosource (Prodotti Gianni, Milano, Italy) respectively.

Peroxidase-conjugated anti-mouse IgG antibody was from Jackson ImmunoResearch Labs (Li StarFISH, Milano, Italy). Monoclonal antibody directed against β-actin (Sigma-Aldrich) was used as internal standard for control of protein load.

2.4. Prostanoid measurement

Cox-2 activity was determined in HUVEC exposed to stimuli for 6 h. Cells were washed in Hank's buffer, pH 7.4, containing 1 mg/ml bovine serum albumin and incubated for 30 min with 10 μM arachidonic acid in the same buffer. Supernatants were collected and 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and PGE_2 levels were measured by enzyme immunoassay (EIA, Cayman Chemical, Spi-Bio).

2.5. Intracellular ROS formation

ROS formation was assessed in HUVEC exposed to PMA and TNFα for 90 min by both flow cytometry and NBT reduction. HUVEC were loaded (30 min at 37 °C) with 5 μM DCFH-DA, which becomes fluorescent on oxidation to DCF by hydrogen peroxide (H_2O_2) within the cell [21]. HUVEC were then dislodged with trypsin/EDTA and the number of cells exhibiting increased fluorescence was measured by flow cytometry (FACSCalibur, Becton Dickinson). Excitation and emission wavelengths were 488 and 525 nm, respectively. Tracings were obtained by displaying the log fluorescence of the samples (FL2-H) generated against the background staining of cells. Superoxide anion ($O_2^{\cdot-}$) generation was detected by conversion of NBT to formazan [22]. NBT (1 mg/ml) was added to medium and incubations were carried out at 37 °C for 90 min. HUVEC were lysed with a solution containing 90% dimethylsulfoxide, 0.01 N NaOH and 0.1% SDS. Absorbance of formazan was measured at 715 nm. NBT reduction was correlated with the amount of $O_2^{\cdot-}$ produced by cells. Data are expressed as nmol/ml (molar extinction coefficient 18,000 M^{-1} cm^{-1}).

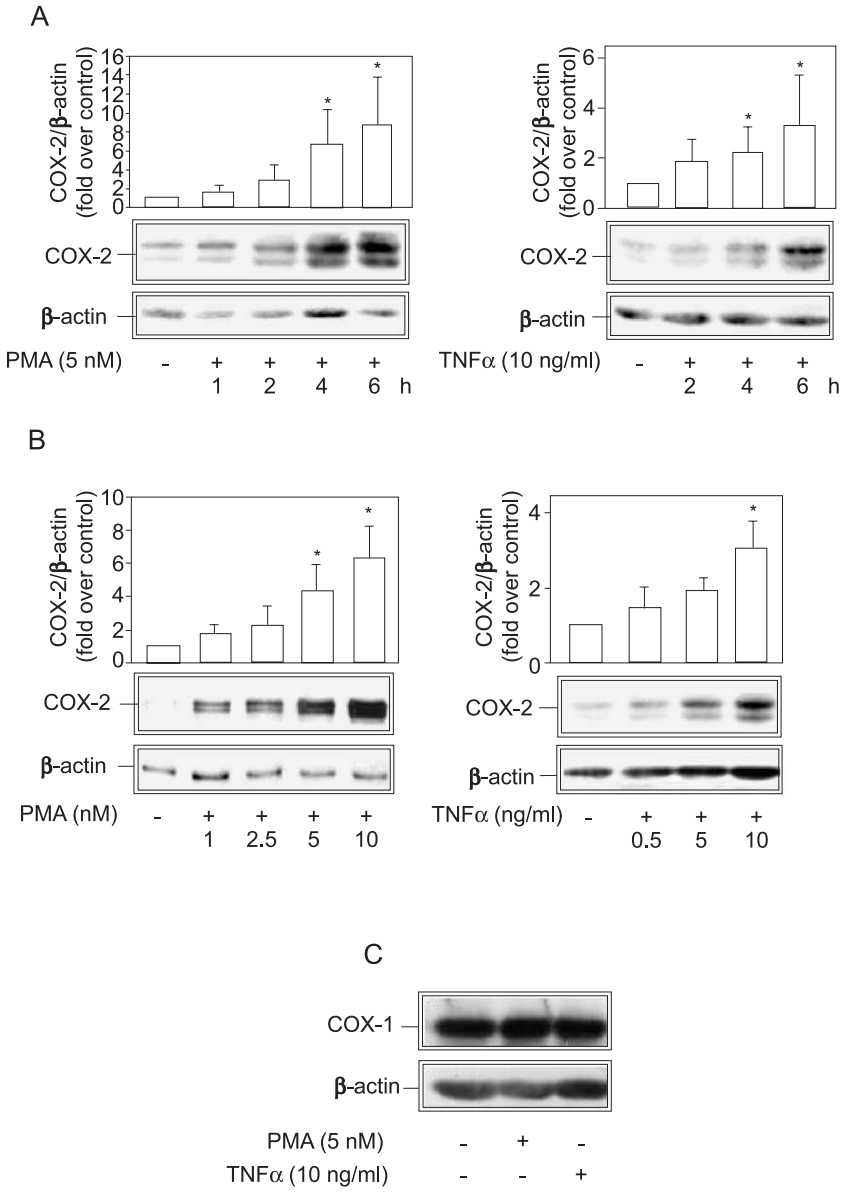

Fig. 1. Cox-2 is induced by PMA and TNFα in HUVEC. Serum-starved HUVEC were incubated with different concentrations of PMA or TNFα for different time points. (A) Time-course of Cox-2 induction. (B) Cox-2 synthesis in HUVEC exposed to increasing concentration of PMA and TNFα for 6 h. (C) Cox-1 expression in HUVEC exposed to PMA or TNFα. Cox-1 and Cox-2 protein was identified by Western analysis. β-Actin was used as internal standard for control of protein load. Blots are representative of four separate experiments performed with different cell cultures. Densitometry (mean±S.D., n=4) is shown in the top of the panels. Signals were quantified relative to β-actin. Statistically significant different (*p<0.05) from unstimulated HUVEC.

Table 1
Synthesis of 6-keto-$PGF_{1\alpha}$ and PGE_2 in HUVEC exposed to PMA and TNF_α

	6-keto-$PGF_{1\alpha}$ (ng/ml)	PGE_2 (ng/ml)
Unstimulated	3.03±1.5	0.34±0.06
PMA (5 nM)	7.86±2.4**	0.76±0.15*
TNF_α (10 ng/ml)	3.88±1.7*	0.52±0.09*

HUVEC were incubated in medium alone or in the presence of PMA or TNF_α for 6 h. Medium was then replaced with Hank's buffer containing arachidonic acid (10 μM) and bovine serum albumin (1 mg/ml). Incubation was continued for 30 min. Prostanoids were measured by EIA. Mean±S.D. of six separate experiments.
Statistically significant different (*p<0.05 and **p<0.01) from unstimulated cells.

2.6. GSH and GSSG measurement

Cellular GSH and GSSG levels were measured by HPLC in HUVEC exposed to stimuli for 90 min. In brief, HUVEC were harvested in lysis buffer (trichloroacetic acid 5%, 0.5 mM EDTA). Cell lysates were centrifuged after three cycles of freezing and thawing. GSH and GSSG were separated by a Discovery® C18 5 mm RP column (4.6×250 mm, Supelco, USA), as described [23].

2.7. Western blot analysis

Cells were harvested in lysis buffer pH 6.8, as described [24]. Cell debris was removed by centrifugation (10,000×g for 5 min) and protein concentration was determined by the micro-bicinchoninic acid assay. Equal amounts of lysates were subjected to SDS–PAGE (7% polyacrylamide) and transblotted onto nitro-cellulose membrane by a semidry transfer unit (Hoefer Scientific Instruments). Membranes were incubated for 1 h with antibodies directed against Cox-1 (5 μg/ml), Cox-2 (1/10,000), phosphorylated and total ERK1/2 (1/2000) and phosphorylated and total p38 (1/1000). Phosphotyrosine detection was performed on membranes stripped and reprobed with an antibody against phosphotyrosine (1/5000). Blots were incubated with peroxidase-conjugated secondary antibody (1/5000) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech.). Optical density was assessed using Multianalyst software (Bio-Rad Labs).

2.8. Real-Time Quantitative Reverse Transcription–PCR

Total RNA was extracted from cells with TRIzol Reagent and reverse-transcribed (42 °C for 60 min) by using 20U reverse transcriptase (RT; Stratascript, Stratagene), 0.5 μg random hexamers primers, 500 nM dNTPs and 40U RNAse inhibitor. 18S rRNA was used for sample normalization. Primers were designed with Primer Express Software (Applied Biosystems) against GeneBank published sequences. cDNA (25 ng) was incubated in 25 μl IQ Supermix containing Cox-2 or 18S primers (300 and 50 nM, respectively), and the fluorescent dye SYBRGreen (Bio-Rad Labs). PCR reaction was carried out in two steps: 50 cycles denaturation at 95 °C for 15 sec and annealing/extension at 60 °C for 60 s. Specificity of amplified products was monitored by performing melting curves at the end of each amplification; melting curves were acquired by stepwise increase of the temperature from 55 to 95 °C. All the amplicons generated a single peak, thus reflecting the specificity of the primers. Experiments were performed on iCycler Optical System (Bio-Rad Labs).

2.9. Statistical analysis

Data, reflecting measurements in HUVEC cultures from different cords, are reported as mean±S.D. Paired observations were compared using paired Student's t test or Wilcoxon signed rank test. Grouped differences were compared with ANOVA (Dunnett's test). $P \leq 0.05$ values

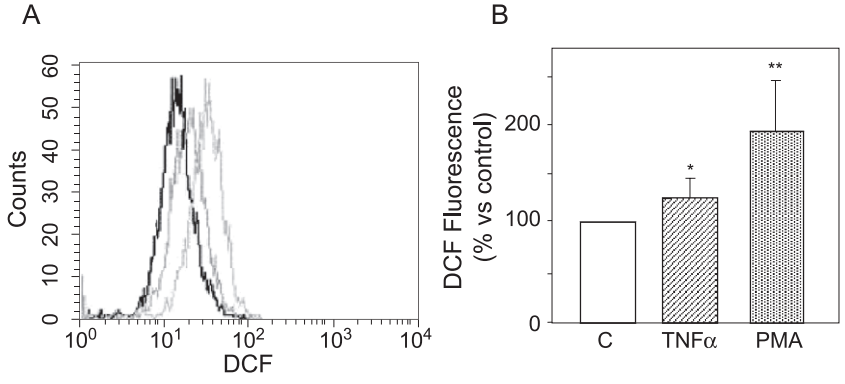

Fig. 2. Intracellular ROS generation by PMA and TNFα in HUVEC. HUVEC were pre-incubated with the cell permeable DCFH-DA (5 μM). Incubation was continued with or without TNFα (10 ng/ml) or PMA (5 nM) for 90 min. (A) Representative flow cytometry tracings of DCFH-DA-loaded HUVEC in resting condition (left) and exposed to TNFα (middle) or to PMA (right). (B) Data analysis from seven experiments performed with different cell cultures and expressed as relative fluorescence (mean±S.D.). *p<0.05, **p<0.01 vs. untreated condition.

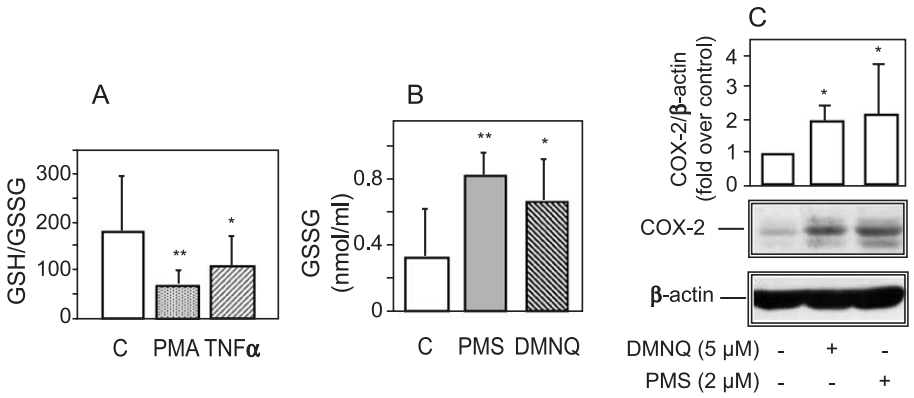

Fig. 3. GSH/GSSG imbalance triggers Cox-2 synthesis in HUVEC. (A, B) HUVEC were incubated with PMA (5 nM), TNFα (10 ng/ml) or ROS generators DMNQ (5 μM) and PMS (2 μM) for 90 min. GSH and GSSG levels were determined by HPLC. Data are expressed as nmol/ml and are mean values±S.D. of five separate experiments. Statistically significant different (*p<0.05, **p<0.01) from unstimulated HUVEC. (C) HUVEC were incubated with DMNQ (5 μM) and PMS (2 μM) for 6 h. Cox-2 protein was evaluated by Western analysis. Blot is representative of three separate experiments performed with different cell cultures. Densitometry (mean±S.D., n=3) is shown in the top of the panel. Statistically significant different (*p<0.05) from unstimulated HUVEC.

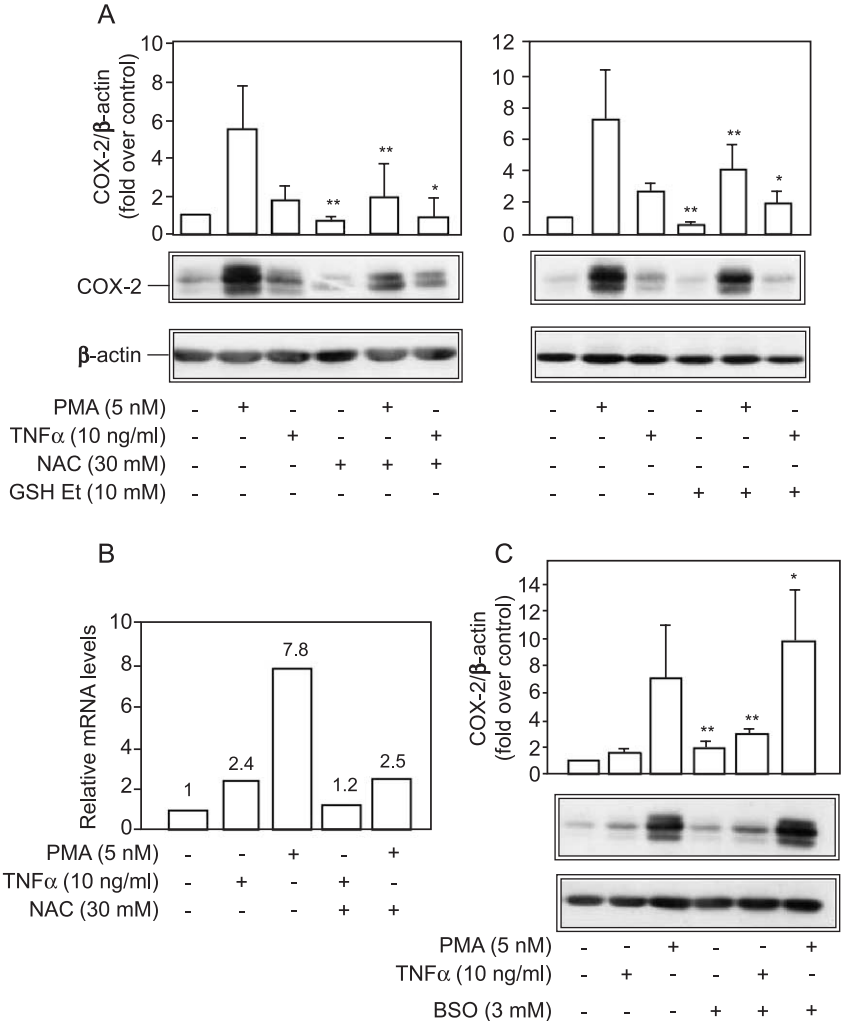

Fig. 4. GSH level influence Cox-2 expression in HUVEC. (A, C) HUVEC were incubated with NAC or GSH-Et (1 h) or BSO (24 h) and then exposed to stimuli for 6 h. Cox-2 protein was determined by Western analysis. Blots are representative of six to eight separate experiments performed with different cell cultures. Densitometry (mean±S.D., n=6–8) is shown in the top of the panels. Statistically significant different (*p<0.05, **p<0.01) from unstimulated and PMA or TNFα-stimulated HUVEC. (B) HUVEC were preincubated with NAC for 1 h and exposed to stimuli for 2 h. mRNA levels were measured by Real-Time Quantitative Reverse Transcription–PCR. Numbers above the bars indicate levels of Cox-2 mRNA relative to controls. Measurement were carried out in triplicate for each sample with RNA preparations from at least four independent experiments.

were considered statistically different. Statistical analysis was performed with SigmaStat.

3. Results

3.1. Induction of Cox-2 by PMA and TNFα in endothelial cells: role of intracellular ROS and GSH/GSSG

Exposure of HUVEC monolayers to PMA or TNFα resulted in time and dose-dependent increase of Cox-2 protein detected by a specific mAb [25] as a doublet at 70–72 kDa (Fig. 1A and B). Cox-1 was detectable in resting cells and its level did not change upon stimulation by both PMA and TNFα for 6 h (Fig. 1C). Cox-2 was functional as assessed by the increase of 6-keto-$PGF_{1\alpha}$ and PGE_2 levels measured in medium of HUVEC exposed to TNFα and PMA in the presence of exogenous arachidonate (Table 1). The occurrence of Cox-2 in HUVEC exposed to PMA and TNFα was consequent to the appearance of Cox-2 mRNA which was quantified by real-time PCR. Cox-2 mRNA, almost undetectable in unstimulated HUVEC, increased by 7.8- and 2.4-fold after PMA and TNFα addition, respectively (n=4 experiments performed in triplicate).

Cell viability, determined by neutral red or MTT reduction assays, showed no evidence of overt cell damage with all agents (data not shown).

HUVEC exposure to PMA and TNFα for 90 min resulted in intracellular H_2O_2 generation (Fig. 2A and B). Intracellular O_2^- levels were also increased (from 10.02±7.6 nmol/ml measured in resting HUVEC to 15.15±11.3 and 12.39±7.1 nmol/ml measured in HUVEC stimulated with PMA and TNFα, respectively, mean±S.D., p<0.01 and p<0.005, n=11).

Intracellular GSH/GSSG was significantly reduced in HUVEC exposed to PMA and TNFα respectively to resting cells (Fig. 3A). GSH/GSSG reduction was attributable to increased GSSG levels (from 0.32±0.26 nmol/ml measured in resting HUVEC to 0.62±0.18 and 0.45±0.26 nmol/ml measured in HUVEC stimulated with PMA and TNFα, respectively, mean±S.D., p<0.01 and p<0.05, n=5). These values were comparable to those measured in HUVEC exposed to the ROS generators PMS and DMNQ (Fig. 3B). Notably, the latter agents were able to induce Cox-2 synthesis in HUVEC within 6 h of incubation (Fig. 3C).

Conversely, HUVEC exposure to agents that replenish GSH levels within the cell as NAC or GSH-Et, reduced Cox-2 protein levels induced by PMA and TNFα (Fig. 4A). The effect was consequent to reduced mRNA levels (Fig. 4B).

To assess whether GSH depletion affects Cox-2 synthesis HUVEC were treated with BSO for 24 h and subsequently exposed to stimuli for 6 h. Results shown in Fig. 4C indicate that BSO treatment augmented Cox-2 expression in HUVEC, be they in resting condition or exposed to stimuli.

Taken together, these results point the redox status within the cell as pivotal in Cox-2 induction in HUVEC.

3.2. PKC is differentially involved in Cox-2 induction by PMA and TNFα in HUVEC

We analyzed next the involvement of PKC in the signaling pathways driven by PMA and TNFα. Preincubation of HUVEC with the PKC inhibitor RO 31-8220 resulted in reduced Cox-2 levels in PMA-challenged cells. In contrast, Cox-2 induced by TNFα was not affected (Fig. 5A). The involvement of PKC was further addressed by

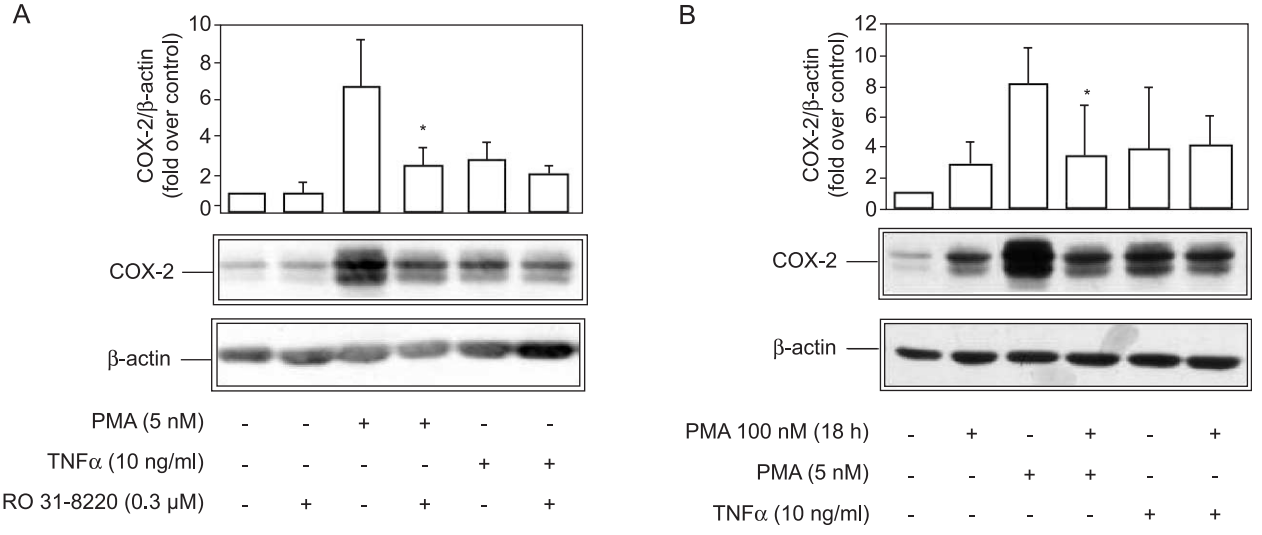

Fig. 5. Involvement of PKC in Cox-2 induction by PMA and TNFα. (A) The PKC inhibitor RO 31-8220 was added to HUVEC 1 h before stimuli. Incubation was continued for 6 h. (B) PKC-depleted HUVEC were exposed to stimuli for 6 h. Cox-2 protein levels were determined by Western analysis. Blots are representative of three separate experiments performed with different cell cultures. Densitometry (mean±S.D., n=3) is shown in the top of the panels. Statistically significant different (*p<0.05) from PMA-stimulated HUVEC.

experiments performed in PKC-depleted HUVEC. PKC depletion was obtained by prolonged (18 h) HUVEC incubation with high PMA concentration (100 nM). In this condition, Cox-2 expression in response to a subsequent PMA exposure was markedly reduced, whereas minor change was observed in HUVEC exposed to TNFα (Fig. 5B). Overall, these results play in favour of a divergent involvement of PKC in Cox-2 expression induced by PMA and TNFα, at least within the incubation time selected in this study (6 h).

3.3. Induction of Cox-2 by PMA and TNFα in endothelial cells: divergent role of small GTPases

The role of small GTPases in Cox-2 expression by PMA and TNFα was explored taking advantage from the use of the HMG-CoA reductase inhibitor fluvastatin, which impairs small GTPase function through inhibition of isoprenylation. Cox-2 induction was markedly reduced in PMA-stimulated HUVEC exposed to fluvastatin and the effect was fully prevented by the addition of mevalonate to the incubation medium. Conversely, fluvastatin did not affect Cox-2 expression induced by TNFα (Fig. 6). These results indicate that isoprenoids positively regulate Cox-2 synthesis only when induced by PMA.

Protein tyrosine phosphorylation (*M*(r) 55–205 kDa) was increased by both PMA and TNFα after 6 h of incubation (Fig. 7). Bands were particularly prominent within 55–66 and 97–116 kDa. The extent of phosphorylation went hand in hand with Cox-2 induction and was prevented by fluvastatin in PMA-stimulated HUVEC only (Fig. 7). This finding indicates that small GTPases are essential for protein tyrosine phosphorylation in PMA-stimulated HUVEC and that tyrosine phosphorylation and Cox-2 expression are causally linked.

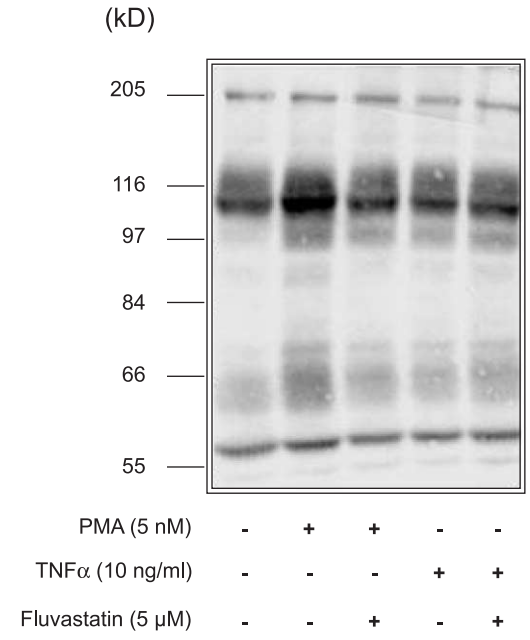

Fig. 7. Fluvastatin prevents protein tyrosine phosphorylation in PMA-stimulated HUVEC. Cells were incubated with fluvastatin for 18 h prior to the addition of PMA or TNFα. Incubation was continued for 6 h. Protein tyrosine phosphorylation was determined by Western analysis. Blot is representative of three independent experiments performed with different cell cultures.

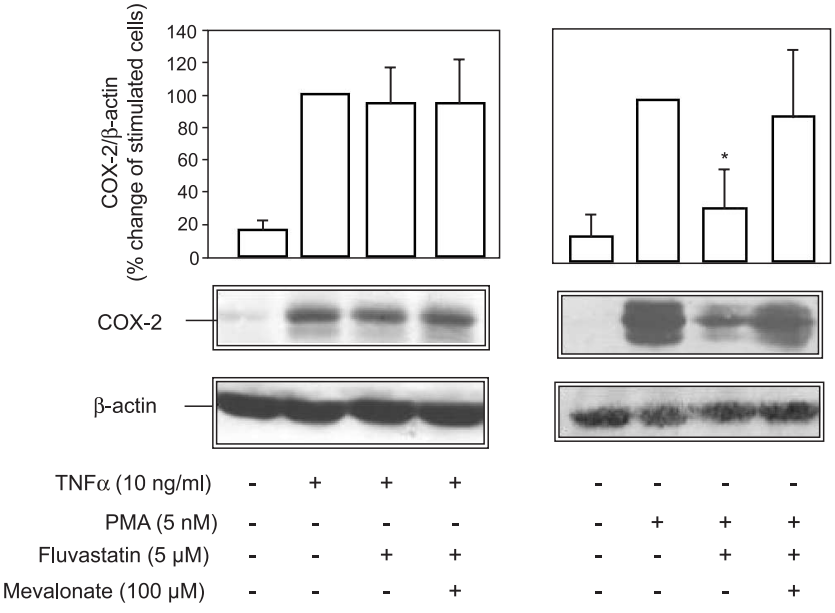

Fig. 6. Role of small GTPases in Cox-2 induction by PMA and TNFα. Cells were incubated with fluvastatin and mevalonate for 18 h prior to the addition of PMA or TNFα. Cox-2 protein was determined by Western analysis. Blots are representative of three independent experiments performed with different cell cultures. Densitometry (mean±S.D., n=3) is shown in the upper part of the panels. Statistically significant different (*p<0.05) from PMA-stimulated HUVEC.

3.4. Divergent role of p38 MAP-kinase in Cox-2 induction by PMA and TNFα

Subsequent experiments were designed to elucidate the role of MAP-kinase activation in Cox-2 induction by PMA and TNFα in HUVEC. Cells were preincubated for 30 min with PD98059 or SB203580 or the JNK inhibitor peptide I that inhibit ERK1/2, p38 and JNK kinase, respectively. HUVEC were then exposed to PMA or TNFα for further 6 h. SB203580 completely abrogated Cox-2 induction by TNFα, being that induced by PMA not affected (Fig. 8A). PD98059 reduced Cox-2 synthesis in PMA-stimulated HUVEC only (Fig. 8A), whereas the JNK inhibitor (1 μM) did not affect Cox-2 levels induced by both stimuli (not shown). TNFα caused a rapid phosphorylation of both p38 and ERK1/2 kinase which was reduced by SB203580 and by PD98059 (Fig. 8B). In contrast, PMA caused phosphorylation of ERK1/2 kinase only, which was abrogated by PD98059 (Fig. 8B).

This finding points to a key role of p38 MAP-kinase cascade in TNFα-induced Cox-2 synthesis, whereas ERK1/2 activation is involved in rapid Cox-2 induction by PMA.

3.5. Negative regulatory role of phosphatidylinositol 3 (PI3)-kinase activation in Cox-2 induction by PMA and TNFα

PI3-kinase inhibition by wortmannin resulted in augmented Cox-2 synthesis either in unstimulated or in PMA- and TNFα-stimulated HUVEC. This finding indicates that PI3-kinase activation exerts a negative control on Cox-2 induction in HUVEC (Fig. 9).

4. Discussion

The relevance of Cox-2 in vascular homeostasis, inflammation, cancer and angiogenesis has prompted the investigation of signaling pathways that mediate its induction in normal physiology and disease. The mechanisms underlying Cox-2 synthesis in endothelial cells are still an

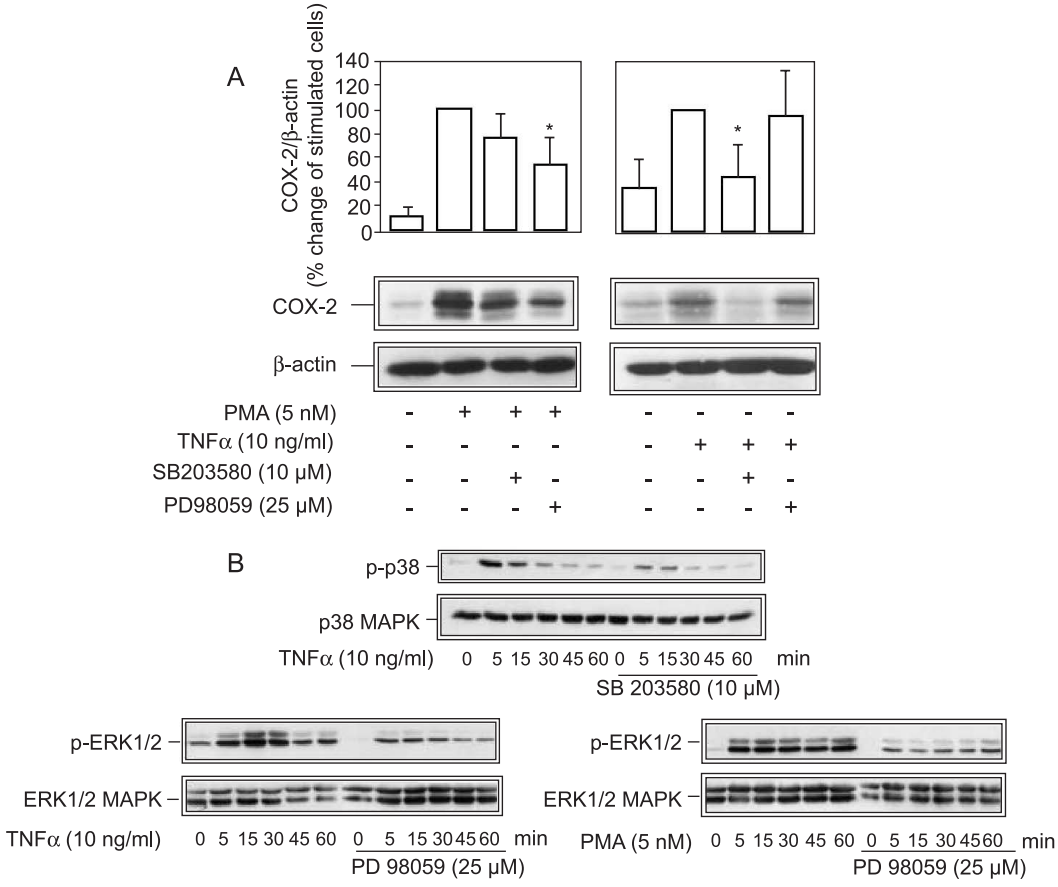

Fig. 8. Involvement of MAP-kinases ERK1/2 and p38 in Cox-2 induction by PMA and TNFα. (A) The MAP-kinase inhibitors SB203580 and PD98059 were added to HUVEC 1 h before stimuli. Incubation was continued for 6 h. Cox-2 protein levels were determined by Western analysis. Blots are representative of three separate experiments performed with different cell cultures. Densitometry data are mean±S.D., n=3. Statistically significant different (*p<0.05) from stimulated HUVEC. (B) Time-dependent induction of phosphorylated p38 and ERK1/2 kinases by TNFα or PMA. Cell lysates were analyzed by Western analysis for phosphorylated and total MAP-kinases. Cells were pretreated with or without SB203580 and PD98059 for 60 min before PMA or TNFα addition. The results shown in panel B are representative of those from three independent experiments.

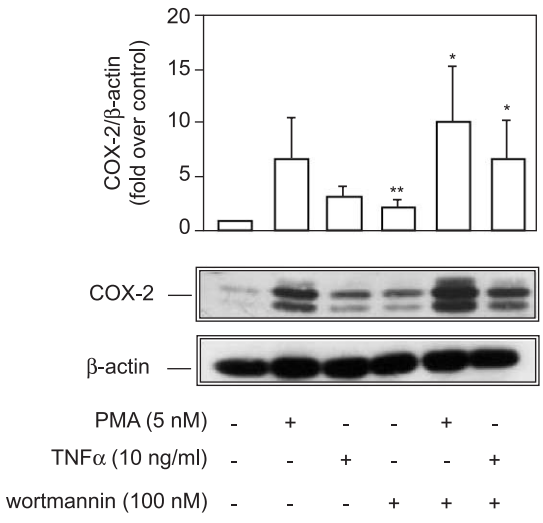

Fig. 9. PI-3 kinase activation controls Cox-2 induction in HUVEC. The PI-3 kinase inhibitor wortmannin was added to HUVEC 1 h before stimuli. Incubation was continued for 6 h. Cox-2 protein levels were determined by Western analysis. Blot is representative of seven separate experiments performed with different cell cultures. Densitometry data are presented as mean±S.D., n=7. Statistically significant different (*p<0.05, **p<0.01) from unstimulated and PMA or TNFα-stimulated HUVEC.

open issue. Data herein described show that signal transduction pathways involved in early Cox-2 induction in HUVEC exposed to the tumor promoter and proangiogenic PMA and to the inflammatory cytokine TNFα are fundamentally divergent. A line of demarcation between these pathways can be therefore marked.

This conclusion is based on the observation that PKC, small GTPases and ERK1/2 are key steps turning on Cox-2 in response to PMA. In contrast, p38 MAP-kinase activation is cardinal when Cox-2 is induced by TNFα. GTPases promote Cox-2 expression transcriptionally, through NF-κB activation [26]. It has been previously shown that PKC activation is required for Rho protein function in endothelial cells [27] and that its activation is critical in the regulation of barrier function [28]. Our data indicate that the activation of both PKC and GTPases are involved when Cox-2 expression is induced by PMA. In contrast, the activity of these enzymes is only marginally involved when Cox-2 is induced by TNFα. This latter finding is in accordance with the observation that MAP-kinase activation in TNFα-treated HUVEC is PKC-independent [29]. Moreover, our data disclose a critical role of p38 MAP-kinase activation in TNFα-induced Cox-2 expression.

Protein tyrosine phosphorylation is essential for Cox-2 induction in HUVEC [9,11]. Our data show that prevention of small GTPase activation results in reduced tyrosine phosphorylation and impaired Cox-2 expression in PMA-stimulated HUVEC. This finding indicates that small GTPase activation represents an upstream event essential for protein tyrosine phosphorylation. The recent observation that HMG-CoA reductase inhibitors block the redox-sensitive tyrosine kinase Pyk2-dependent signaling in PMA-stimulated HUVEC supports our conclusion [30].

The observed differences in signaling pathways initiated by PMA and TNFα are reflected by the different modulation of Cox-2 gene transcription exerted by the two agents. It has been recently appreciated that stimulation of Cox-2 promoter activity by PMA and TNFα involves different transactivators [31] and that p38 MAP-kinase is critical for the activation of the transcription factor CRE-binding protein by TNFα [32]. In addition, p38 plays also an essential role when endothelial Cox-2 expression is triggered by interleukin-1α and by heat shock protein 60, a key endogenous inflammatory mediator generated in response to tissue injury and/or stress [33,34].

The results herein described highlight, for the first time, the involvement of intracellular ROS and GSH/GSSG alteration in PMA- and TNFα-induced Cox-2 synthesis in HUVEC. This finding is based on multiple lines of evidence. Firstly, both PMA and TNFα elicit ROS generation in HUVEC, as already observed by others [35]. Secondly, ROS generators trigger Cox-2 expression “per se”. Thirdly, both PMA and TNFα reduce GSH/GSSG ratio and increase GSSG levels at a similar extent to that observed with ROS generators. Lastly, HUVEC replenishment with GSH prevents Cox-2 expression, whereas GSH depletion augments it. The latter findings indicate that GSH levels, besides the activation of terminal prostaglandin synthase activity [36,37], modulate Cox-2 expression, thus tuning the functional coupling between the enzymes.

The contribution of ROS to Cox-2 induction has been previously disclosed in mesangial cells [38], in the protection of macrophages against apoptosis [39] and in the differentiation of monocytes into macrophages [40]. The finding that both PMA and TNFα reduce GSH/GSSG ratio in HUVEC brings us to conclude that GSH/GSSG decrease represents a basic mechanism through which Cox-2 expression is induced in these cells. An additional point of convergence between PMA- and TNFα-driven Cox-2 expression is represented by the common regulation exerted by PI3-kinase activation. The blockade of PI3-kinase results, indeed, in increased Cox-2 levels, both in resting and stimulated HUVEC, independently from the stimulus. Of interest, a similar negative regulation of Cox-2 expression has been observed in colonic epithelial cells [41]. More recently, a role of PI3-kinase in TNF-induced tumor necrosis “in vivo” has been addressed and the inhibition of this pathway has been proposed as a novel concept in anticancer therapy [42].

Overall, data here presented indicate the existence of at least two independent pathways leading to Cox-2 expression in HUVEC, together with an overlapping of upstream events. Cox-2 represents a critical link for inflammatory response, tumor growth and angiogenesis. These findings, therefore, may be of relevance in view of the development of pharmacological strategies targeted to selectively interfere with regulating pathways.

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